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CONTENTS

CHAPTER 1

Surgical Management of Spinal Ependymomas: Techniques and Advances

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Chapter 1: Introduction

Spinal ependymomas are relatively rare neoplasms that arise from ependymal cells lining the central canal of the spinal cord. These tumors constitute about 7-10% of all spinal cord tumors and are the most common type of intramedullary spinal cord tumors, representing approximately 40-50% of this category[^1]. Though these tumors typically exhibit slow growth and a favorable prognosis when treated with complete surgical resection, they pose significant challenges in terms of management. The proximity of spinal ependymomas to critical neural structures, the possibility of infiltrative growth, and the risk of postoperative neurological deficits complicate treatment and patient recovery.

This chapter provides an in-depth review of the surgical management of spinal ependymomas, including preoperative assessment, pathological characteristics, advanced surgical techniques, intraoperative considerations, postoperative care, and prognostic factors that influence patient recovery. The chapter also incorporates recent advances in surgical strategies, molecular pathology, and genetic understanding of these tumors.

Chapter 2: Pathology of Spinal Ependymomas

2.1 General Overview

Spinal ependymomas originate from ependymal cells, which are responsible for producing cerebrospinal fluid (CSF) and lining the central canal of the spinal cord. These tumors are most commonly found in the cervical, thoracic, and conus medullaris regions of the spinal cord[^2]. The majority of spinal ependymomas are low-grade (World Health Organization [WHO] grade II) tumors, though higher-grade (grade III) variants such as anaplastic ependymomas may also be encountered. The histopathological characteristics of these tumors often include perivascular pseudorosettes or true ependymal rosettes, with a mixture of cellular and fibrillary components[^3].

Histologically, spinal ependymomas exhibit relatively uniform cell morphology with round or oval nuclei and well-defined cytoplasmic borders. Immunohistochemically, these tumors are typically positive for glial fibrillary acidic protein (GFAP), epithelial membrane antigen (EMA), and S100 protein, markers that help differentiate them from other spinal cord tumors[^4].

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2.2 Tumor Classification and Subtypes

Spinal ependymomas are classified primarily by histological grade, which dictates their biological behavior and prognosis:

- **Grade II (Low-Grade Ependymomas):** These tumors are typically well-circumscribed, with slow and indolent growth. They generally exhibit mild cellular atypia and a relatively benign clinical course. Gross total resection (GTR) is often achievable and associated with excellent long-term survival.
- **Grade III (Anaplastic Ependymomas):** These tumors are characterized by increased cellularity, mitotic activity, and nuclear atypia, and they grow more rapidly. Anaplastic ependymomas have a higher propensity for recurrence and poorer prognosis, often requiring adjuvant therapies such as radiation[^5].

2.3 Molecular Pathology

Molecular alterations play an increasingly significant role in understanding the pathogenesis and prognosis of spinal ependymomas. Genetic mutations and chromosomal abnormalities have been identified as key factors in tumor progression.

- **NF2 gene mutations** are frequently associated with spinal ependymomas, particularly those that occur in the setting of neurofibromatosis type 2 (NF2). Loss of function of the NF2 gene, which encodes the protein merlin, is a common feature in these tumors and is associated with higher recurrence rates[^6].
- **Chromosomal deletions** on chromosomes 1p and 19q have been linked to worse prognosis and are often found in higher-grade tumors[^7]. Other genetic alterations, such as in the PI3K/AKT/mTOR pathway, may also play a role in tumor progression and represent potential therapeutic targets for future treatments[^8].

2.4 Diagnostic Imaging

Magnetic resonance imaging (MRI) is the gold standard for diagnosing spinal ependymomas. On MRI, these tumors typically appear as well-circumscribed intramedullary masses that enhance with contrast. T2-weighted MRI images show hyperintensity, while T1-weighted images demonstrate variable hypointensity. However, in cases of infiltrative tumors, the demarcation between tumor and normal spinal cord tissue may be difficult to visualize[^9]. Advanced imaging techniques such as **diffusion tensor imaging (DTI)** and **functional**

MRI (fMRI) can be used to assess tumor proximity to vital neural structures, aiding in surgical planning[^10].

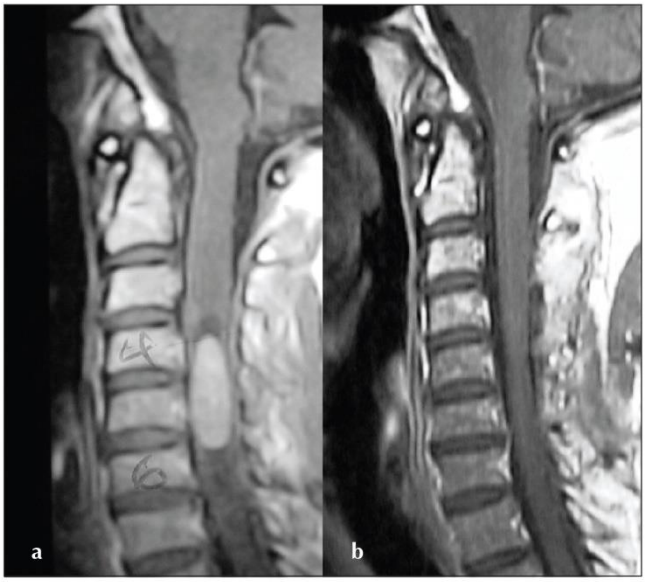

Figure 1: Sagittal T1-weighted images of the cervical spine after gadolinium injection. . a) Preoperative image demonstrating a C4-6 intramedullary ependymoma and b) postoperative image with complete resection. *(Alkhani, A., et al. (2008). Outcome of surgery for intramedullary spinal ependymoma.* Annals of Saudi Medicine*, 28(4), 255-259.)*

Chapter 3: Surgical Techniques

3.1 Preoperative Planning

Preoperative planning is crucial for the successful surgical management of spinal ependymomas. This process includes a comprehensive assessment of the tumor's location, size, and relationship to surrounding structures. Neurophysiological testing such as somatosensory evoked potentials (SSEPs) and motor evoked potentials (MEPs) is often employed preoperatively to assess the functional integrity of neural pathways and to anticipate potential complications.

Patient optimization is essential, with consideration given to factors such as general health, spinal stability, and the extent of neurological deficits. Anesthetic management is crucial, particularly with the need for long surgeries and the potential for intraoperative monitoring. Preoperative multidisciplinary

consultation involving neurosurgeons, neurologists, and radiologists is vital to refine the surgical approach and mitigate risks[^11].

3.2 Surgical Approach

The approach to spinal ependymoma resection depends largely on the tumor's location within the spinal cord:

- **Posterior Midline Approach:** This is the most commonly used technique for tumors located in the cervical, thoracic, and lumbar regions of the spinal cord. A laminectomy is performed to remove a section of the vertebral arch, providing access to the spinal cord and the tumor[^12]. This approach is ideal for tumors with well-defined borders.
- **Anterior or Anterolateral Approach:** Tumors located ventrally or in the **lower** cervical and upper thoracic regions may require an anterior approach. In some cases, corpectomy (removal of a vertebral body) or **discectomy** may be necessary for adequate exposure[^13]. This approach is also favored when the tumor is causing compression of the anterior spinal cord.
- **Combined Anterior-Posterior Approach:** Large, complex tumors that extend over multiple segments or infiltrate both anterior and posterior spinal cord regions may require a combined approach. This allows for optimal access and resection while maintaining spinal stability[^14].

3.3 Tumor Resection

The primary objective during surgery is **gross total resection (GTR)**, which entails the complete removal of the tumor. However, in cases where the tumor is infiltrative or adherent to surrounding neural tissue, the surgeon may need to perform a **subtotal resection (STR)**[^15]. This decision is guided by intraoperative **neurological monitoring** (SSEP and MEP), which helps identify when resection may compromise neural integrity.

The surgical procedure is typically performed under **microscopic guidance**, allowing the surgeon to make precise cuts while avoiding damage to adjacent spinal cord structures. The use of **intraoperative neuromonitoring** enables real-time assessment of sensory and motor function, providing vital feedback and ensuring maximal safe resection without causing irreversible damage[^16].

3.4 Intraoperative Neurophysiological Monitoring

Intraoperative neurophysiological monitoring (IONM) plays a critical role in preventing neurological complications during spinal tumor surgery. It involves continuous monitoring of somatosensory evoked potentials (SSEPs) and motor

evoked potentials (MEPs), as well as free-running electromyography (EMG). These techniques enable the surgical team to detect early signs of spinal cord compromise, including ischemia or mechanical injury, and to modify the surgical approach as needed[^17]. The goal is to preserve spinal cord function while achieving the most complete tumor resection possible.

3.5 Challenges in Tumor Resection

Spinal ependymomas are often characterized by their infiltrative nature, making complete resection challenging. Some tumors infiltrate the pia mater, while others extend into adjacent nerve roots or critical motor pathways, which complicates surgical decisions. For these tumors, a conservative resection strategy is often adopted to preserve neurological function[^18].

In some cases, neurophysiological signals such as MEPs may show diminishing motor activity, which can guide the surgeon to stop resecting further to avoid irreversible damage. Moreover, in cases where the tumor is deeply infiltrative, achieving complete resection might not be feasible, and the goal will shift to obtaining a subtotal resection with close follow-up to monitor for recurrence.

Chapter 4: Postoperative Care

4.1 Postoperative Monitoring

Postoperatively, patients are usually monitored in a **neurointensive care unit (NICU)** for 24-48 hours, particularly after complex spinal tumor resections. Neurological assessments are performed frequently to evaluate any changes in motor, sensory, or sphincter function. **Pain management** strategies include opioids, NSAIDs, and regional nerve blocks to manage discomfort postoperatively[^19]. Early mobilization is encouraged as tolerated, with physiotherapy and occupational therapy starting once the patient's condition stabilizes.

4.2 Complications

Postoperative complications following spinal ependymoma surgery include wound infections, cerebrospinal fluid (CSF) leaks, and spinal instability. Prophylactic antibiotic therapy is administered to reduce the risk of infection, and any CSF leaks are repaired surgically if necessary[^20]. Spinal instability may result from extensive tumor resection, especially if a large portion of the vertebrae

or posterior elements is removed. In such cases, spinal fusion may be required to restore structural integrity and prevent deformity[^21].

4.3 Rehabilitation and Recovery

Rehabilitation is an integral part of the recovery process. Early physical therapy focuses on restoring movement and strength, particularly in patients who had significant preoperative neurological deficits. Occupational therapy is also important for helping patients regain independence in daily activities. Recovery times vary, with patients requiring different durations for rehabilitation depending on the extent of the resection and preoperative function[^22].

4.4 Long-Term Surveillance

Given the potential for recurrence, long-term follow-up is essential. MRI surveillance is typically performed at 3, 6, and 12-month intervals during the first two years after surgery, with extended intervals for patients who remain recurrence-free. Clinical follow-up visits assess neurological status, and additional radiological imaging is employed to detect any signs of tumor regrowth[^23].

Chapter 5: Prognostic Factors and Future Directions

5.1 Prognostic Factors

The prognosis of spinal ependymoma largely depends on factors such as:

- **Tumor Grade**: Higher-grade tumors (grade III) have a poorer prognosis due to their increased risk of recurrence and resistance to treatment.
- **Extent of Resection**: Complete resection (GTR) is associated with significantly better survival rates than subtotal resection (STR)[^24].
- **Patient Age and General Health**: Younger, healthier patients tend to have better outcomes, with higher functional recovery rates post-surgery[^25].
- **Molecular and Genetic Factors**: NF2 mutations and chromosomal abnormalities, such as deletions on chromosomes 1p and 19q, are associated with poorer outcomes[^6].

5.2 Future Directions

Recent advances in molecular biology and genetic profiling offer promising new avenues for the treatment of spinal ependymomas. Targeted therapies that address specific molecular pathways, such as the **PI3K/AKT/mTOR** signaling pathway, could provide more effective treatments for aggressive tumors[^8]. Moreover, ongoing research into **immunotherapy** and **gene therapy** holds potential for improving long-term outcomes, particularly in recurrent or anaplastic ependymomas[^26].

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CHAPTER 2

Cellular and Molecular Mechanisms Contributing to Chemotherapy-Induced Peripheral Neuropathy

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1. Introduction

Cancer is one of the most common diseases and is increasing due to many factors (Zheng et al., 2019). Today there are 27 different main types of cancer. Many cancer treatment methods such as surgical operation, radiotherapy, photodynamic therapy, and chemotherapy are preferred (Kim et al., 2018; Mattiuzzi and Lippi, 2019). Chemotherapy is the most preferred and most important treatment method in recent years and is used for lung, breast, bladder, colorectal, cervical, ovarian, and prostate cancers. Although chemotherapy is effective in cancer treatment, many side effects are observed. Side effects include vomiting, nausea, hair loss, sleep disturbance, weight loss, numbness, tingling, and urinary problems (Eto et al., 2018; van der Valk et al., 2020). Among all these side effects, chemotherapy-induced peripheral neuropathy affects healthy neuronal cells.

2. Chemotherapy-induced Peripheral Neuropathy

Since they have multiple targets and mechanisms of action to kill rapidly dividing cancer cells, drugs used in cancer chemotherapy are a handy tool for stopping cancer progression (Zajaczkowską et al., 2019). Unfortunately, these medications can also have negative and occasionally fatal adverse effects on healthy cells and bodily structures (anemia, diarrhea, nausea, vomiting, infections, neurological abnormalities, exhaustion, hair loss, infertility, pain, and peripheral neuropathy). Depending on the specific substance, chemotherapeutic medicines can damage the structures of the nervous system and result in various neuropathies, including cranial and autonomic, sensory and/or motor, large and small fiber, demyelinating, and axonal (Cioroiu and Weimer, 2017a). Varied drug classes have diverse effects on the nervous system, depending on the drug's unique physical and chemical characteristics and whether it is taken in single or cumulative dosages (Banach et al., 2016). Chemotherapy-induced peripheral neuropathy (CIPN) is characterized by damage to the somatosensory nervous system following chemotherapy treatment. The first symptoms of CIPN are usually numbness, pain, tingling, tingling, and numbness in the distal parts of the lower and upper extremities (Bernhardson et al., 2007; Park et al., 2013; Starobova and Vetter, 2017).

3. Chemotherapy Agents

The most prevalent types of cancer are treated with normal, ordinary drugs called chemotherapeutics, which have neurotoxic effects on the peripheral nervous system. Platinum-based antineoplastics (oxaliplatin, cisplatin), vinca

alkaloids (vincristine and vinblastine), epothilones, taxanes (paclitaxel, docetaxel), proteasome inhibitors (bortezomib), and immunomodulatory medications (thalidomide) are the six primary agent groups that damage peripheral sensory, motor, and autonomic neurons, leading to the development of CIPN (Starobova and Vetter, 2017).

Vinblastine and vincristine are examples of medications that are classified as vinca alkaloids. These medications are mostly used to treat testicular cancer, often referred to as lymphoma, and small lung cancer. Vincristine produces the most severe peripheral neuropathy, whereas vinblastine is less hazardous (Boyette-Davis et al., 2018; Cioroiu and Weimer, 2017b).

Taxanes are generally used in the treatment of non-small cancers such as breast, ovarian, prostate, stomach, head, and neck cancers. These drugs, including paclitaxel and docetaxel, are microtubule-stabilizing agents (Yared and Tkaczuk, 2012).

The authorized proteasome inhibitor regulators ixazomib, carfilzomib, and bortezomib are utilized in therapeutic settings. Bortezomib is the first proteasome inhibitor for clinical use. For the treatment of multiple myeloma, a novel proteasome inhibitor called carfilzomib has been approved. Through the inhibition of proteasome activity, they produce their anti-tumor actions. This causes tumor cells to undergo apoptosis, cell cycle arrest, and the buildup of misfolded proteins (Thawani et al., 2015).

Antineoplastics include the drug groups cisplatin and oxaliplatin. These drugs bind to DNA. Due to their alkyl properties, they show chemotherapeutic effects by forming intra-strand cross-links (Canta et al., 2015). Testicular, ovarian, bladder, and lung cancers are among the many cancers that they are used to treat (Storey et al., 2010).

4. Cellular Targets of Chemotherapy-Induced Neurotoxicity

The process by which chemotherapeutics harms the structures of the nervous system and results in the multifactorial nature of CIPN includes myelin sheath destruction, altered ion channel function, oxidative stress, mitochondrial damage, microtubule disruption, and altered intercellular signaling (Areti et al., 2014).

4.1. Oxidative Stress and Mitochondrial Dysfunction

The membrane-bound organelles called mitochondria are in charge of oxidative phosphorylation, which is how energy is produced (Angelova and

Abramov, 2016). It is also crucial for preserving intracellular calcium levels in healthy physiological settings (Misgeld and Schwarz, 2017). In cancer cells that are growing, platinum-based substances such as cisplatin and oxaliplatin attach directly to the DNA and create platinum adducts (Sałat, 2020; Yamamoto and Egashira, 2021). By directly attaching to the mitochondrial DNA with the same affinity as the nuclear DNA in the DRG neurons, cisplatin interfered with the transcription and replication of the mitochondrial DNA in peripheral neurons, causing off-target consequences (Podratz et al., 2011). Cytochrome c is released from injured mitochondria and DRG neurons undergo apoptosis as a result of the inability of the mitochondria's DNA repair mechanism to repair cisplatin-induced mitochondrial DNA damage (McDonald and Windebank, 2002). Primary afferent myelinated A-fibers and unmyelinated C-fibers show signs of vacuolation and mitochondrial edema in peripheral neuropathy caused by bortezomib. The sciatic nerve's enlarged and vacuolated mitochondria have a significantly decreased ability to produce ATP, indicating a strong cytotoxic effect of bortezomib on the peripheral nerve. In neuroblastoma, paclitaxel causes a fast depolarization of the mitochondria and the release of cytochrome C from the mitochondria by binding with beta-tubulin, the primary constituent of the mitochondrial membrane, and opening the mitochondrial permeability transition pore (mPTP). (Andre et al., 2000; Carré et al., 2002). Mitochondrial dysfunction caused by paclitaxel severely disrupts cellular respiration and ATP production in DRG neurons, ultimately leading to neuronal apoptosis (Duggett et al., 2017). Paclitaxel causes significant demyelination and mitochondrial enlargement in both myelinated and unmyelinated axonal fibers in the distal sciatic nerve (Chine et al., 2019a). The distal axonal terminals of cultured neurons similarly rapidly depolarize their mitochondria when exposed to vincristine, another microtubule-binding drug. Moreover, vincristine causes extensive demyelination and significant mitochondrial enlargement in the distal nerve (Chine et al., 2019c).

4.2. Ion Channels

Neuronal excitability changes as a result of the changing expression of voltage-gated sodium, potassium, and calcium channels (Bennett et al., 2019; Starobova and Vetter, 2017). Neurons require a voltage-gated sodium channel in order to originate and propagate action potentials (Boyette-Davis et al., 2011). Chemotherapeutic medicines can enhance action potential by enhancing the expression of voltage-gated sodium channels in nociceptive neurons (Starobova and Vetter, 2017). Action potential amplification in nociceptive neurons causes hyperexcitability and spontaneous neuronal firing, which heightens pain sensitivity and causes neuropathic pain (Bennett et al., 2019). In the dorsal root

ganglion, paclitaxel therapy enhances the expression of voltage-gated sodium channels, which leads to an increase in action potential firing and hyperexcitability (Calhoun et al., 2003; Li et al., 2014). Following oxaliplatin therapy, patients have shown a marked downregulation of voltage-gated potassium channels, which increases neuronal membrane excitability (Viatchenko-Karpinski et al., 2018; Zhang et al., 2012). Additionally, chemotherapy can increase voltage-gated calcium channels, which alter the action potential of peripheral neurons and result in hyperexcitability (Krukowski et al., 2017; Li et al., 2018).

4.3. Disruption of Cytoskeletal

Taxanes and vinca alkaloids disrupt tubulin polymerization, which causes peripheral neuropathy (Mattar et al., 2024). The microtubule dysfunction caused by taxanes and vinca alkaloids after they bind to tubulin alters the transit of axonal synaptic vesicles and interferes with axon remodeling and regeneration (Bober and Shah, 2015; Gornstein and Schwarz, 2017a; LaPointe et al., 2013; Meng et al., 2019).

To cause cell cycle arrest and ultimately the death of cancerous cells, chemotherapeutic drugs like vincristine and paclitaxel are known to induce or prevent microtubule assembly. These antineoplastic drugs, however, can penetrate the blood-nerve barrier and attach to the β-tubulin of peripheral sensory neurons and sensory nerve fibers, which is the building block of the cytoskeleton in neuronal cells (Kober et al., 2019; Malacrida et al., 2019; Windebank and Grisold, 2008). The strong proteasome inhibitor bortezomib, which has been shown to enhance microtubule polymerization in neuronal cell lines, also increases hyper-stable delta 2 tubulin (D2), which in turn leads to excessive microtubule polymerization in the cell bodies of DRG (dorsal root ganglion) neurons (Pero et al., 2021; Poruchynsky et al., 2008). When cultured DRG neurons are treated with bortezomib, neurite outgrowth is significantly reduced and axonal fragmentation occurs (Staff et al., 2013). Axonal degeneration of intraepidermal nerve fibers (IENFs) is brought on by chemotherapy drugs like paclitaxel, vincristine, and bortezomib (Bennett et al., 2011; Chine et al., 2019b; Geisler et al., 2019). Degeneration of distal sensory nerve fibers, or IENFs, can be caused by loss of plasticity in remodeling axonal terminals (Gornstein and Schwarz, 2017b). This can lead to hypersensitivity and change pain perception (Thomas et al., 2023).

4.4. Intracellular Signaling

Deteriorations in mitochondrial function activate calcium signaling pathways. Neuronal cell pathological disturbances and structural alterations are linked to this triggering. In neuronal and glial cells, variations in the concentration of Ca^{+2} within the cell can impact gene expression, neurotransmitter release, and membrane excitability (Carozzi et al., 2015). A Ca^{+2} chelator called oxaliplatin contributes to the development of peripheral neuropathy (Wang et al., 2012). The threshold potential and membrane resistance fall when the extracellular Ca^{+2} concentration is disturbed because this causes an increase in Na^{+} conductance. In DRG, cisplatin and oxaliplatin cause MAPK-related apoptosis (Scuteri et al., 2009). Paclitaxel may induce the release of Ca2+ from mitochondria, likely through the activation of the mitochondrial permeability transition pore (mPTP), resulting in rapid mitochondrial depolarization (Mironov et al., 2005). Additionally, paclitaxel may promote Ca2+ release from the ER, potentially via the inositol 1,4,5-trisphosphate receptor (IP3R) (Li et al., 2017). This process appears to increase the expression of CaV3.2 channels in rats, and inhibiting these channels can reverse hyperalgesia (Okubo et al., 2011).

5. Conclusion

Despite being the recommended treatment for cancer, chemotherapy can cause harm to healthy neuronal cells, which can lead to chemotherapy-induced peripheral neuropathy in patients. Despite being dependent on the dosage of chemotherapy, this syndrome may continue for months or even years following treatment. It has been proposed that there are elements that contribute to the formation and persistence of CIPN, although the precise process is still unclear. The current approaches to CIPN symptom prevention and treatment (opioids, anticonvulsants, plant-based pharmaceuticals, and laser therapy) are still insufficient. Therefore, investigating treatment alternatives that can provide more long-lasting and effective solutions is essential.

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CHAPTER 3

Changes in Cognitive Functions With Cardiovascular Disease

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Introduction

Cognition refers to the mental process of obtaining knowledge and comprehension through cognition, memory, experience, and sensory perception. Cognitive skills encompass a range of advanced intellectual activities and processes, including attention, memory, knowledge, decision-making, planning, reasoning, judgment, perception, comprehension, language, and visual-spatial abilities (Dhakal and Bobrin, 2023). Cognitive decline refers to the progressive or abrupt deterioration of cognitive abilities, including learning, attention, and memory of past events and details (McNamara et al., 2024).

The World Health Organization's 2021 Global Status Report on Dementia indicates that, in 2019, dementia impacted 55.2 million individuals globally. Researchers anticipate that between 2030 and 2050, the figures will rise to 78 million and 139 million, respectively (WHO, 2019). The aging population is creating a substantial public health concern by escalating the demand for non-pharmacological therapies for moderate cognitive impairment, garnering increased focus from healthcare providers, scholars, and politicians (Wu et al., 2017). Cognitive decline is associated with difficulties in executing daily tasks, negatively impacting patients and their caregivers, and laying a considerable burden on families and society as a whole (Sun et al., 2018).

Neuropathological changes linked to cognitive decline commence prior to clinical symptoms, particularly in older persons. This may provide sufficient time for intervention to prevent systematic cognitive decline and effectively delay dementia (Feldman et al., 2014).

Recent Global Burden of Disease studies reveal that the increase in various illnesses is associated with multiple cognitive disorders, including mild cognitive impairment and dementia (GBD, 2017). Cognitive decline, a major public health concern, is characterized by neuropsychiatric symptoms and persistent difficulties in performing daily tasks. Such disorders adversely affect the lives of patients and their caretakers, imposing considerable strain on families and society. Recognizing and mitigating the impacts of cognitive issues is essential for formulating preventive measures (8,9). Cardiovascular diseases and their associated risk factors, which significantly affect morbidity and mortality, inevitably influence cognitive processes (Pinto and Subramanyam, 2009). This section analyzes cognitive issues linked to cardiovascular risk factors and disorders. Cognition is a physiological capacity that diminishes with age and pertains to memory, thinking, language, and attention (DeCarli, 2003). Cognitive impairments may arise from neurodegenerative disorders, dysthymia/dysphoria,

and vascular issues (Song et al., 2009). Cognitive impairment and diminished cognitive ability can impact social, functional, occupational, and daily activities. Cognitive impairment is prevalent among the elderly and is an age-related issue. The elderly experience roughly 21.5 to 71.3 cognitive issues per 1,000 person-years (Woodford and George, 2007). The anticipated rise in the prevalence of cognitive impairment has significantly differed across several studies (DeCarli, 2003).

Reduced plasma amyloid beta (Aβ) concentrations in cerebrospinal fluid and plasma may signify cognitive deterioration (Song et al., 2009). We employ neuroimaging techniques to diagnose mild cognitive impairment by assessing the structural and functional components of the brain (Pinto, 2009). Imaging examinations can reveal hippocampus volume reduction and temporal lobe atrophy in mild cognitive impairment (Trojanowski et al., 2010). Approximately 70% to 74% of patients exhibit amyloid abnormalities in colony-stimulating factor and imaging assessments (Ward et al., 2012).

The Mini-Mental State Examination (MMSE) is used to screen individuals for dementia, evaluate the extent of cognitive impairment, and track the evolution of the disease (Tombaugh et al., 1992). The MMSE's user-friendliness and brief administration duration (5–10 minutes) have led to its global acceptance (GBD, 2017). The Montreal Cognitive Assessment (MoCA) is a widely utilized screening instrument that requires around 10 minutes to administer. It evaluates visual-spatial abilities, attention, linguistic proficiency, abstract reasoning, delayed recollection, executive functioning, and orientation (Krishnan et al., 2015). The MoCA encompasses more domains than the MMSE, resulting in enhanced sensitivity and specificity (Finney, et al., 2016). The Mini-Cog is among the most rapid cognitive evaluation tests available. It comprises a three-item recall and a clock-drawing assessment. The delayed three-item recall evaluates memory, whereas the clock-drawing test measures cognitive function, language, executive function, and visual-spatial abilities (Gonzalez and Tadi, 2022).

Other cognitive tests that have been written about are the Saint Louis University Mental Status Examination, the Blessed Orientation-Memory-Concentration Test, the Kokmen Brief Mental Status Test, the Memory Impairment Screen, the Ottawa 3DY, the Brief Alzheimer Screen, and the Caregiver-Completed AD8 (Gonzalez and Tadi, 2022).

Numerous risk factors contribute to cognitive decline via diverse processes and varying degrees of impairment (Bellou et al., 2017; Zhang et al., 2022).

Research indicates that hypertension, diabetes, cerebrovascular disease, and coronary artery disease, prevalent risk factors among the elderly, correlate with cognitive impairment (Ou et al., 2020; Kivimäki et al., 2019).

Coronary Artery Disease (CAD), also called ischemic heart disease, is a common cardiovascular disease (CVD). It happens when the coronary blood flow is blocked or interrupted by large atherosclerotic lesions, which can lead to a myocardial infarction or sudden heart failure (Garcia et al., 2016). Recent research has indicated a possible link between the health of people with coronary artery disease and cognitive impairment, which adversely affects their mental performance. Research indicates that the brains of older individuals have significant cerebral shrinkage, hypoperfusion, and white matter involvement. Individuals with CAD exhibit reduced regional brain volume and an elevated risk of cardiovascular disease (Cousineau et al., 2024). Managing age, cardiovascular disease, and cerebrovascular dysfunction is challenging and inadequately comprehended. Coronary artery disease (CAD) and cognitive impairment share several prevalent risk factors, including low-density lipoprotein cholesterol, hypertension, obesity, hyperglycemia, diabetes, and smoking. Individuals often exhibit these risk factors in observational studies. Therefore, further research is necessary to establish the causal relationship between CAD and cognitive impairment (Brown et al., 2024).

The pathways linking CAD to cognitive impairment are intricate. Significant dementias entail vascular dysfunction. Vascular dysregulation may double the likelihood of a neurodegenerative condition progressing to dementia. Atherosclerosis and the breakdown of small blood vessels can cause dementia due to neurovascular disease. This can happen because of microinfarcts and problems with white matter tracts in the cerebral cortex that are not noticeable to the naked eye. This condition may result in cerebral hypoperfusion, microvascular ischemia, increased periventricular spaces, cerebral amyloid angiopathy, and hippocampal sclerosis. Moreover, left ventricular failure and the consequent reduction in cardiac output may result in hypotension and cerebral hypoperfusion (Hachinski et al., 2019). People with CAD are more likely to have increased platelet activation, which we think will cause perivascular cerebral inflammation, cerebral vasoconstriction, and a worsening of carotid artery disease. Platelet activation may help amyloid precursor protein and amyloid-β (Aβ) protein build up in the brain, which can make early-onset Alzheimer's disease worse (Li et al., 2023). Patients with coronary artery disease significantly increase the incidence of cognitive impairment to around 35%. The data is concerning, and the potential factors contributing to cognitive impairment

necessitate additional examination (Balbaid et al., 2020). A prior study indicated that cognitive impairment deteriorated following cardiac catheterization in cardiovascular disease individuals with assessed memory patterns. Ischemic brain damage was a possible etiology. A further potential factor leading to cognitive decline in individuals with cardiovascular disease is the administration of cardiovascular medicines, which may induce cognitive impairment (Whitlock et al., 2019). The National Institute of Neurological Disorders and Stroke-Canadian Stroke Network endorsed a series of standardized measures in a recent study (Hachinski et al., 2006) to assess cognitive performance and investigate dementia and vascular cognitive impairment. Individuals with coronary artery disease and endothelial dysfunction had worse verbal memory. Improvements in global cognition and processing speed significantly correlated with the enhancement of endothelial function during treatment (Hachinski et al., 2006). A separate study indicated that diminished peak oxygen uptake in CAD patients, irrespective of other cardiac risk factors, correlated with worse cognition, especially in executive function. The findings underscore the significance of neurocognitive evaluations in individuals with cardiovascular disease and suggest the potential advantages of lifestyle modifications in this context (Balbaid et al., 2020).

Planning strategies to mitigate cognitive damage and maintain cognitive function in CAD patients is essential. Preliminary memory evaluation in patients may facilitate the commencement of interventions, such as memory training, and may assist in categorizing symptoms that signify illness progression (van Nieuwkerk et al., 2023; Lappalainen et al., 2021). Coronary artery bypass grafting (CABG) exhibits low mortality rates and enhances coronary vascularity and cardiac function. However, CABG significantly increases the incidence of postoperative cognitive dysfunction, including delirium (Spadaccio and Benedetto, 2018). A recent meta-analysis examining cognitive outcomes after coronary artery bypass grafting showed that 43% of patients experienced postoperative cognitive impairment or decline by 4 days, and this persisted at a rate of 39% by 1 month post-surgery. It diminished to roughly 25% 6–12 months post-coronary artery bypass grafting and escalated to about 40% 1–5 years thereafter. Using a standard method along with clinical criteria, it was found that 24% of people who had coronary artery bypass grafting showed signs of delirium within one week. After coronary artery bypass grafting, cognitive deterioration is believed to correlate with a higher incidence of depression, as well as a diminished quality of life, functional capability, and daily activity performance. In elderly individuals, delirium correlates with diminished quality of life and

heightened mortality, prolonged hospital stays, increased readmissions, cognitive deterioration, and dementia (Greaves et al., 2019). Cerebrovascular problems significantly contribute to hypertension-related damage (Pires et al., 2013). Factors such as collagen buildup and elastin degradation link hypertension in humans to structural changes in vascular walls and arterial stiffness. Hypertension can increase pulse pressure and mechanical stress within the cerebrovascular system, prompting adaptive changes to protect downstream microcirculation (Santisteban et al., 2023). Studies demonstrate that remodeling of cerebral small arteries and arterial stiffness are associated with cerebral vascular disease, cognitive decline, and dementia. While intracranial atherosclerosis distinctly links to Alzheimer's disease (AD), extracranial atherosclerosis elevates the risk of cognitive deterioration (van Sloten et al., 2015). A recent proteomic study of autopsy samples from people who had mild cognitive impairment, AD, or intracranial atherosclerosis suggested that cognitive decline may be caused by signaling mechanisms linked to normal myelination and less synaptic regulation and plasticity (van Sloten et al., 2015). Vascular risk factors, particularly hypertension, substantially influence the development of dementia. Notably, up to 50% of patients with AD exhibit cerebrovascular lesions and a diverse range of pathologies upon autopsy. Midlife hypertension is a recognized risk factor for dementia in later life, regardless of genetic predisposition. Thus, clarifying the mechanisms between hypertension and dementia remains a crucial area of research (van Sloten et al., 2015; Santisteban and Iadecola, 2018). The consequences of hypertension are ambiguous and variable; nevertheless, research indicates potential risk mitigation with proper blood pressure (BP) management. There is an urgent need for discoveries, particularly new treatment targets, to maintain cognitive function in hypertension patients. Moreover, the early identification of at-risk patients may serve as an effective preventative strategy for health, perhaps postponing their progression to dementia (Santisteban et al., 2023). Before the results of larger imaging studies are available, it is now simpler to detect small neuroradiological signs of brain damage from high blood pressure (Si-Cheng et al., 2024). Managing arterial hypertension is a prevalent approach to enhancing cognitive health in older adults. The interplay between hypertension and aging, as well as hypertension's contribution to cognitive decline in the elderly, is complex. Primarily, the aging process correlates with hypertension. Furthermore, aging is associated with a widespread decline in various homeostatic mechanisms, such as those regulating cerebral blood flow and microvascular pressure. Furthermore, aging diminishes cellular resilience to stress, exacerbating cellular and molecular damage induced by hemodynamic and oxidative stress associated with elevated

blood pressure. In the end, oxidative stress, endothelial dysfunction, inflammatory responses, and a breach in the blood-brain barrier can all contribute to vascular aging, dysfunction, and organ damage (Ungvari et al., 2021). Consequently, vascular pathologies linked to hypertension may arise from accelerated vascular aging (Santisteban et al., 2023). Chronic high blood pressure may also make it easier for atherosclerotic plaques to form in the brain's larger arteries. This could reduce blood flow to the brain and make it harder for older people to think clearly, which can lead to ischemic strokes (Ungvari et al., 2021).

The Honolulu-Asian Aging Study identified a correlation between vascular cognitive impairment, AD, and midlife blood pressure. Elevated blood pressure was significantly associated with an increased risk of dementia due to vascular cognitive impairment or Alzheimer's disease in individuals without prior hypertension treatment. Dementia was 4.8 times more common in those with hypertension (systolic blood pressure ≥160 mmHg) compared to those with normal blood pressure (Launer et al., 2000). A retrospective cohort study in Northern California, USA, found that midlife hypertension significantly increased the risk of dementia in later life (Whitmer et al., 2005). Likewise, a prospective population-based study in Eastern Finland indicated that midlife hypertension augmented the risk of Alzheimer's disease thereafter (Kivipelto et al., 2001). Furthermore, a prospective cohort study in the United States associated higher systolic blood pressure in younger individuals with a higher risk of dementia (Kennelly et al., 2009). Research from the USA and Japan indicates that hypertension is an independent risk factor for vascular dementia in individuals aged 65 and older (Turana et al., 2019). Additionally, studies have recognized hypertension as a risk factor for mild cognitive impairment in older adults, with a mean age of 75 years (Ungvari et al., 2021). A separate study, after an eight-year follow-up, revealed that men aged 45 to 55 with elevated systolic and diastolic blood pressure exhibited significantly diminished cognitive performance. (Palta et al., 2021). In women, higher SBP was associated with better cognition at a younger age and poorer cognition at an older age. Hypertension negatively impacts memory, mental processing speed, abstract reasoning and/or executive function, and other cognitive areas (Ungvari et al., 2021). Hypertension is higher in low- and middle-income countries than in high-income countries. Similarly, the prevalence of dementia is higher in low- and middle-income countries than in high-income countries. Estimates suggest that 60% of people with dementia lived in low- and middle-income countries in 2001, and this proportion will increase to 71% by 2040. Furthermore, the rates of increase are not uniform; in high-income countries, the number of people with

dementia is projected to increase by 100% between 2001 and 2040, while in India, China, and countries of South Asia and the Western Pacific, an increase of >300% is projected. These results are partly attributable to environmental and lifestyle factors, disease duration, and age-specific incidence (Ungvari et al., 2021;Turana et al., 2019). The prevalence of hypertension and dementia differs among countries. African Americans exhibit elevated hypertension rates and are 1.5 to 4 times more predisposed to dementia compared to non-Hispanic white individuals (Shiekh et al., 2021). Longitudinal data from 34,349 people in U.S. cohort studies indicate that cumulative blood pressure influences racial differences in cognitive decline (Levine et al., 2020). Social determinants of health, such as education, income, healthcare accessibility, and knowledge of hypertension, are fundamental factors influencing these disparities (Havranek et al., 2015). Modifiable lifestyle factors, such as treatment accessibility, medication adherence, physical activity, tobacco consumption, alcohol use, and dietary habits, are crucial in managing hypertension and alleviating cognitive impairments (Santisteban et al., 2023). In developed countries, over fifty percent of the elderly population experiences hypertension alongside other chronic conditions, including type 2 diabetes, chronic kidney disease (CKD), obesity, and cardiovascular diseases, all of which undermine cerebral microvasculature and intensify cognitive decline. These elements collectively exacerbate vascular cognitive impairment and AD in elderly individuals with comorbidities (Colosia et al., 2013). An excessively rapid reduction in blood pressure can lead to cerebral hypoperfusion, especially in individuals with hypertension-related adaptations in cerebral autoregulation. Studies indicate a U-shaped relationship between blood pressure and cognitive function, where abnormally low blood pressure increases the risk of cognitive decline in older adults (Lv et al., 2017; Waldstein et al., 2005). Chronic hypertension may contribute to the development of atherosclerotic plaques in large cerebral arteries due to vascular aging, which can impair cerebral blood flow and increase the risk of ischemic strokes that exacerbate cognitive decline in the elderly. Hypertension adversely affects cognitive domains such as abstract reasoning, executive function, memory, and mental processing speed (Ungvari et al., 2021; Lv et al., 2017; Waldstein et al., 2005).

The adaptive rightward shift of the cerebral autoregulation curve in hypertension makes the brain vulnerable to cerebral hypoperfusion if perfusion pressure is aggressively reduced. Cerebral hypoperfusion and microvascular ischemia may lead to structural brain changes, including enlarged periventricular spaces, cerebral amyloid angiopathy, and hippocampal sclerosis. These changes

can cause left ventricular failure, reduced cardiac output, hypotension, and further cerebral hypoperfusion (Ungvari et al., 2021).

Increased platelet activation in patients with CAD may contribute to perivascular cerebral inflammation, cerebral vasoconstriction, and the progression of carotid artery disease. Additionally, some cardiovascular medications may contribute to cognitive decline. Poorer peak oxygen uptake in CAD patients has been linked to reduced cognition, particularly executive function (Li et al., 2023).

Cardiovascular diseases are significant risk factors for cognitive impairment. Future studies should investigate the mechanisms through which cardiac risk factors affect cognitive functions to inform effective prevention and treatment strategies.

Neuropsychiatric symptoms and ongoing challenges in executing daily tasks mark cognitive decline, a significant public health issue. These conditions adversely affect the lives of patients and caregivers, imposing a considerable burden on families and society. Recognizing cognitive deficits and comprehending their effects is crucial for formulating effective preventive interventions.

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CHAPTER 4

Incidental in Patients Undergong Total Thyroidectomy Effect of Parathyroidectomy on Postoperative Hypocalcaemia

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Introduction

Thyroidectomy is one of the most common operations performed today. One of the most common complications after thyroid surgery is hypocalcaemia due to hypoparathyroidism. Hypocalcaemia may be asymptomatic or life-threatening symptoms may be observed. One of the causes of hypoparathyroidism is incidental parathyroidectomy. matter how meticulously the surgical dissection plan is followed, there is always a risk of incidental parathyroidectomy after thyroidectomy due to the ectopic location of the parathyroid gland. Therefore, ectopic localisation of the parathyroid gland and preservation of its vascular structure must be during surgery. The relationship between incidental parathyroidectomy and postoperative hypocalcaemia is a subject with different opinions in the literature. In our study, a significant relationship was found between incidental parathyroidectomy and postoperative transient hypocalcaemia.

Patients and Methods

For our study, the histopathological results of patients who underwent total thyroidectomy between 1 January 2014 and 1 January 2020 were retrospectively reviewed and 732 patients with total thyroidectomy operated for multinodular goiter and early micropapillary/papillary carcinoma were included in the study. Patients with parathyroid adenoma, Graves' disease, medullary carcinoma, anaplastic carcinoma, follicular carcinoma, advanced papillary carcinoma, neck dissection, reoperation and parathyroid autotransplantation were excluded from the study. Papillary carcinomas larger than 4 cm in size, exceeding the thyroid capsule, having extrathyroidal soft tissue and organ invasion or lymph node metastasis were considered advanced stage. Papillary carcinomas with subcapsular localisation, smaller than 4 cm and without lymph node metastasis were considered early stage. Central lymph node dissection was not performed in any of the patients with multinodular goiter in the benign group and micropapillary/papillary carcinoma in the malignant group and intraoperative nerve monitoring was used in all patient groups. Patients who were found to have parathyroid gland removal on histopathological examination after surgery were included in the incidental parathyroidectomy group.

Gender, age, postoperative histopathological results, presence, number and location of incidental parathyroid gland were recorded. Preoperative calcium, parathormone, albumin and postoperative calcium, corrected calcium, parathormone, albumin parameters obtained between the 4th and 6th hours were analysed. All operations were performed by the same team experienced in this

field. The surgical technique consisted of a mini Kocher necklace incision, preparation of skin flaps,

lateralisation of the scorpion muscles, a dissection plan close to the thyroid capsule, distal ligation of the thyroid vascular structures using energy-based vessel sealing devices. Intraoperatively, all

parathyroid glands were tried to be seen and identified, but no further exploration was performed in cases where no parathyroid gland could be found. In the presence of a traceable parathyroid gland, vascular damage was avoided by preserving the surrounding adipose tissue. At the end of the

operation, positive pressure up to 50 mm Hg was routinely applied by the anaesthesia team to control bleeding. Patients with postoperative corrected calcium values below 8mg/dl were

considered hypocalcaemic and patients with parathormone values below 10pg/ml were considered hypoparathyroid. Patients with postoperative numbness and muscular spasms around the hand and mouth, Trousseau or Chvostek's signs, and cardiac arrhythmia were considered symptomatic, whereas patients without these findings with a corrected calcium value below 8mg/dl were considered as asymptomatic hypocalcaemic cases. IV calcium gluconate replacement and oral vitamin-D preparation were given to patients with symptomatic hypocalcaemia. Asymptomatic hypocalcaemic patients received oral calcium preparation. Vocal cord examinations were performed in all patients on the 1st postoperative day and patients with no complications were discharged. The duration of hospitalisation was 1.2 SD 0.9 days in hypocalcaemic patients and 1.1 SD 0.3 days in normocalcaemic patients.

Findings

A total of 732 people, including 166 males and 566 females aged between 18 and 87 years, were included in the study. The mean age of the patients included in the study was 48.93 SD 12.54.

Incidental parathyroidectomy was detected in 77 patients, while incidental parathyroidectomy was not detected in 655 patients. The incidental parathyroidectomy rate in our study was n=77/732 (10.53%). The location of incidental parathyroidectomies was determined as extracapsular in 55, subcapsular in 11 and intrathyroidal in 11 patients. It was determined that 1 parathyroid gland was removed from 74 patients and 2 parathyroid glands were removed from the remaining 3 patients. In our study, the prevalence of

intrathyroidal parathyroid gland was found to be n=11/732 (1.5%) in all patients. The prevalence of incidental parathyroidectomy in women n=72/566 (12.74%) was higher than that in men n=5/166 (3.01%) (p<0.001).

As shown in Table 1 ın our study, the rate of hypocalcaemia was n=67/732 (9.15%). The rate of hypocalcaemia was significantly higher in patients with incidental parathyroidectomy n=16/77 (20.78%) compared to patients without incidental parathyroidectomy n=51/655 (7.78%) (p<0.001). As shown in Table 2 the rate of hypoparathyroidism in our study was n=141/732 (19.27%). The rate of hypoparathyroidism was significantly higher in patients with incidental parathyroidectomy n=27/77 (35.06%) compared to patients without incidental parathyroidectomy n=114/655 (17.40%) (p<0.001). As shown in Table 3 the rate of hypoparathyroidism was significantly higher in hypocalcaemic patients n=32/67 (48.28%) compared to normocalcaemic patients n=109/665 (16.35%) (p<0.001). As shown in Table 4 calcium and parathormone changes were analysed between the groups. The rate of decrease in parathormone in hypocalcaemic patients (65.18% SD 29.53) was higher than in normocalcaemic patients (35.14% SD 40.42) (p<0.001). As shown in Table 5 the incidental parathyroidectomy rate was n=39/406 (9.61%) in the benign disease group and n=38/326 (11.69%) in the malignant disease group and there was no significant difference between these two groups (p= 0.361).

Table 1 Incidental parathyroidectomy is significantly associated with postoperative hypocalcaemia.

	Incidental parathyroidectomy detected	Incidental parathyroidectomy not detected	Total	P
	n %	n %	n %	
Hypocalcaemia	16 20.78	51 7.78	67 9.15	<0.001
Normocalcaemia	61 79.22	604 92.21	665 90.85	

Table 2 Incidental parathyroidectomy is a risk factor for postoperative hypoparathyroidism.

	Incidental Parathyroidectomy detected	Incidental parathyroidectomy not detected	Total	p
	n %	n %	n %	
Hypoparathyroidism	27 35.06	112 17.09	139 19.27	<0.001
Normoparathyroidism	50 64.94	543 82.91	593 80.73	

Table 3 There is a significant relationship between hypoparathyroidism and postoperative hypocalcaemia.

	Hypocalcaemia	Normocalcaemia	Total	p
	n %	n %	n %	
Hypoparathyroidism	32 48.28	109 16.35	141 19.27	<0.001
Normoparathyroidism	35 51.72	556 83.65	591 80.73	

Table 4 There is a significant relationship between the rate of parathormone decreaşe and hypocalcaemia 4-6 hours after surgery.

	Parathormone decline rate (%)			p
	Average	Standard Deviation	Median	
Hypocalcaemia	65.15	29.53	77.78	<0.001
Normocalcaemia	35.14	40.42	39.63	

Table 5 There is no significant difference between benign and malignant groups in terms of incidental parathyroidectomy.

	Bening	Malign	Total	p
	n %	n %	n %	
Incidental parathyroidectomy detected	39 9.61	38 11.69	77 10.53	<0.361
Incidental parathyroidectomy not detected	367 90.39	287 88.31	655 89.47	

Discussion

Nowadays, total thyroidectomy has become a safe surgical procedure performed in large numbers in surgical clinics. Therefore, it is important to know and recognise the complications of this procedure. The most common complication of total thyroidectomy is hypocalcaemia. In the literature, the rate of transient hypocalcaemia in total thyroidectomies has been found between 6.9-49% [1]. In our study, the rate of transient hypocalcaemia was n=67/732 (9.15%). The fact that our cases were performed by a single team experienced in thyroid surgery helped to minimise the risk related to technique and experience, because the most common cause of postoperative hypocalcaemia is related to surgical treatment [2]. Neck dissection in total thyroidectomy surgery, surgery for Graves' disease, medullary carcinoma, follicular carcinoma, anaplastic carcinoma, advanced papillary carcinoma and reoperation in patients with previous neck surgery are risk factors for hypocalcaemia [3,4,5]. We think that the exclusion of the high-risk group in terms of hypocalcaemia in our study and the fact that central lymph node dissection is not routinely performed in total thyroidectomies performed for early micropapillary/papillary carcinoma are the reasons for the low incidence of transient hypocalcaemia in our study. Transient hypoparathyroidism occurs in cases of manipulation, devascularisation, venous obstruction and incidental removal of parathyroid glands during surgery. The frequency of postoperative transient hypoparathyroidism varies between 19-38% in the literature [6]. In our study, the rate of transient hypoparathyroidism after total thyroidectomy was n=141/732 (19.27%). We attribute the low hypoparathyroidism rate to the fact that a single experienced team performed the operations and the high-risk group was excluded from the study.

A rapid and sensitive method to predict postoperative hypocalcaemia will allow early treatment and shorten the duration of hospitalisation. Many studies have been performed to predict transient hypocalcaemia by taking advantage of the short half-life of parathyroid hormone (4 minutes) and its early postoperative decline [7,8]. In one study, parathyroid hormone levels at 4 and 6 hours postoperatively were found to be more valuable than calcium measurement in predicting the risk of hypocalcaemia [9]. Another study reported that a parathyroid hormone level below 7ng/dl on the first postoperative day was 100% sensitive for permanent hypoparathyroidism [10,11]. In a study, PTH values of the patients were measured at 2, 4, 6, 24 and 48 hours after surgery. <10pg/ml was accepted as the reference value of PTH and PTH levels obtained at 4 and 6 hours after surgery could predict transient hypocalcaemia [12]. In our study, PTH was measured 4-6 hours after surgery and 10ng/dl was accepted as the reference value.

In our study, the rate of hypoparathyroidism was significantly higher in the hypocalcaemic group (n=32/67, 48.28%) compared to the normocalcaemic group (n=109/665, 16.35%) ($p<0.001$).

In a meta-analysis, it was shown that a PTH level more than 65% lower than the preoperative level 6 hours after surgery may be an early marker for postoperative hypocalcaemia, with a sensitivity of 96.4% and a specificity of 91.4% [13]. Similar results were obtained in our study. According to our study, the mean PTH decrease was 65.18% SD 29.53%, median 77.78%, which is a significant indicator for hypocalcaemia ($p<0.001$). In conclusion, early initiation of calcium replacement therapy may decrease the frequency of transient hypocalcaemia and the duration of hospitalisation. The incidental parathyroidectomy rate, which is one of the causes of hypoparathyroidism, varies between 6-28% in experienced surgical teams [14,15,16]. In our study, all operations were performed by a single experienced surgical team, only total thyroidectomy was performed as the surgical technique and incidental parathyroidectomy n=77/732 (10.53%) was found.

In one study, the rates of extracapsular, intracapsular and intrathyroidal parathyroid gland localisation in incidental parathyroidectomies were 58%, 20% and 22%, respectively [17]. In another study, the rate of intrathyroidal parathyroid localisation was found to be 3% in the whole patient group and intrathyroidal localisation was reported in 19.2% of patients with incidental parathyroidectomy [16]. In a cadaveric study, intrathyroidal parathyroid gland was found between 2% and 5% [18]. In our study, parathyroid gland localisation was extracapsular (n=55, 71.43%), intracapsular (n=11, 14.29%) and intrathyroidal (n=11, 14.29%). Intrathyroidal parathyroid rate was found to be 1.53% in the whole patient group. Therefore, it is concluded that the risk of ectopic localisation is always possible in total thyroidectomy operations and surgical technique and experience cannot completely eliminate this risk. Malignant thyroid lesions have anatomical changes compared to benign lesions, which makes the recognition of anatomical structures difficult. Total thyroidectomy, neck dissection and reoperation for malignant lesions are risk factors for incidental parathyroidectomy [19,20]. In a study, it was reported that recognition of parathyroid glands would reduce the risk of incidental parathyroidectomy [21]. In our study, parathyroids were tried to be identified in the extracapsular dissection plan during surgery, but in cases where they could not be identified, further dissection was not performed to find the parathyroid glands. In our study, in 77 patients with incidental parathyroidectomy, the benign group was n=39/406 (9.61%) and the malignant group was n=38/326 (11.69%) and there was no

significant difference between the groups (p=0.361). Among the factors affecting this rate, early stage micropapillary/papillary carcinoma and lack of central lymph node dissection in malignant cases can be counted.

One of the controversial issues in the literature is the effect of incidental parathyroidectomy on hypocalcaemia. In humans, the parathyroid gland is pluriglandular and it has been reported that even a single functional gland can prevent hypoparathyroidism [22]. In a study of 440 patients, 25.2% of the patients total thyroidectomy and 74.8% underwent near-total thyroidectomy, the incidental parathyroidectomy rate was found to be n=48/440 (10.90%) and no significant relationship was found between incidental parathyroidectomy and postoperative transient hypocalcaemia [23]. In a previous study, the rate of hypocalcaemia was significantly higher in patients with incidental parathyroidectomy (n=18/47, 38.3%) than in patients without incidental parathyroidectomy (n=48/240, 20%) [24]. In our study, the rate of hypocalcaemia was significantly higher in patients with incidental parathyroidectomy (n=16/77, 20.78%) than in patients without incidental parathyroidectomy (n=51/655, 7.54%) (p<0.001).

Result

Incidental parathyroidectomy is an important risk factor for transient hypocalcaemia after total thyroidectomy. The correct approach is to perform surgical dissection close to the thyroid capsule to preserve the parathyroid glands. The possibility of ectopic localisation of the parathyroid gland should not be ruled out; therefore, the parathyroid glands that can be detected during total thyroidectomy should not be damaged or manipulated and vascularisation should be preserved. ¸The rate of decrease in parathormone values at 4 and 6 hours after surgery is a significant parameter for the early diagnosis of postoperative transient hypocalcaemia.

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CHAPTER 5

Precision Gene Silencing in Malignant Melanoma: The Therapeutic Potential of CRISPR-Cas9

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Introduction

Malignant melanoma is an aggressive form of skin cancer originating from melanocytes, the pigment-producing cells in the skin (Uong and Zon, 2010). Although it accounts for only about 1% of skin cancer cases, melanoma is responsible for the majority of skin cancer-related deaths due to its high potential for metastasis (Liu and Sheikh, 2014). According to recent statistics, the incidence of melanoma has been rising steadily, with an estimated 100,350 new cases and 6,850 deaths in the United States alone in 2020 (American cancer society, 2024). The primary risk factors for melanoma include excessive ultraviolet (UV) radiation exposure, genetic predisposition, and the presence of numerous or atypical moles (Wunderlich et al. 2024).

Current Treatment Options

Current treatment strategies for malignant melanoma vary depending on the stage and location of the tumor. Early-stage melanomas are often treated with surgical excision, which can be curative. For advanced melanoma, treatment options include targeted therapies, immunotherapies, chemotherapy, and radiation therapy. Targeted therapies are designed to target specific genetic mutations in melanoma cells, such as BRAF inhibitors (e.g., vemurafenib, dabrafenib) and MEK inhibitors (e.g., trametinib) (Subbiah et al. 2020; Teixido et al, 2021; Castellani et al. 2023). Immune checkpoint inhibitors (e.g., anti-CTLA-4, anti-PD-1) have revolutionized the treatment of advanced melanoma by enhancing the body's immune response against tumor cells (Buchbinder and Desai, 2016;Carlino et al. 2021). Although less commonly used due to the advent of targeted and immune therapies, chemotherapeutic agents like dacarbazine can be employed in certain cases (Wilson and Schuchter, 2016). Also radiation therapy is used selectively, often for palliation of symptoms in metastatic melanoma (Fort et al. 2016). Despite these advancements, the prognosis for advanced melanoma remains poor, with many patients eventually developing resistance to therapies. Therefore, there is a critical need for more effective and durable treatment strategies.

The CRISPR-Cas9 System in Cancer Research

CRISPR-Cas9 is a revolutionary genome-editing technology that enables precise modification of DNA sequences within the genome. Derived from a bacterial immune system, CRISPR-Cas9 uses a guide RNA to direct the Cas9 nuclease to specific genomic loci, where it induces double-strand breaks. These breaks can be repaired by the cell's machinery, leading to gene disruption

(knockout) or correction (knock-in). CRISPR-Cas9 has been widely adopted in cancer research for its ability to dissect gene function, identify novel therapeutic targets, and develop potential gene therapies (Ma et al. 2014; Wang et al. 2022).

Potential for Gene Silencing in Melanoma

In malignant melanoma, CRISPR-Cas9 offers a powerful approach for silencing oncogenes or reactivating tumor suppressor genes. By precisely targeting genes such as BRAF, NRAS, PTEN, and others, researchers can investigate their roles in melanoma progression and resistance to treatment (Mirmohammadsadegh et al. 2006; Kelleher and McArthur, 2012). Additionally, CRISPR-Cas9 can be used to explore the interactions between melanoma cells and the immune system, paving the way for new immunotherapeutic strategies (Imani et al. 2024). By synthesizing current research and identifying areas for future investigation, this review aims to underscore the potential of CRISPR-Cas9 as a transformative tool in the fight against malignant melanoma.

Biology of Malignant Melanoma

Molecular and Genetic Basis of Malignant Melanoma

Malignant melanoma arises from the malignant transformation of melanocytes, which are pigment-producing cells located primarily in the skin (Shenenberger, 2012). This transformation is driven by a complex interplay of genetic mutations, environmental factors, and epigenetic changes. Several key genes and signaling pathways are critically involved in the development and progression of melanoma (Valdez-Salazar et al. 2024). The BRAF gene encodes a protein that is part of the MAPK/ERK signaling pathway, which regulates cell division, differentiation, and survival (Hussain et al. 2015). Mutations in BRAF, particularly the BRAFV600E mutation, are present in about 50% of melanomas and lead to constitutive activation of the MAPK pathway, promoting uncontrolled cell proliferation (Castellani et al. 2023). NRAS mutations are found in approximately 15-20% of melanomas (Muñoz-Couselo et al. 2017). NRAS encodes a GTPase that also participates in the MAPK/ERK pathway. Mutant NRAS leads to continuous activation of this pathway, contributing to melanoma growth and survival (Randic et al. 2021). PTEN is a tumor suppressor gene that negatively regulates the PI3K/AKT pathway, which is involved in cell survival and proliferation (Georgescu, 2010). Loss of PTEN function, through mutations or deletions, leads to hyperactivation of the PI3K/AKT pathway, which promotes melanoma progression and resistance to apoptosis (Davies, 2012). The microphthalmia-associated transcription factor (MITF) is a master regulator of

melanocyte development, function, and survival. Amplification or overexpression of MITF is observed in some melanomas and is associated with enhanced cell proliferation and survival (Levy et al. 2006). The CDKN2A gene encodes two important tumor suppressor proteins, p16INK4a and p14ARF, which regulate the cell cycle and apoptosis (Brown et al. 2004). Mutations, deletions, or promoter methylation of CDKN2A are common in melanoma and result in unchecked cell cycle progression and reduced apoptosis (Bennett, 2008; Soltan et al.2023). TP53, often referred to as the "guardian of the genome," is a crucial tumor suppressor gene involved in cell cycle regulation, DNA repair, and apoptosis (Hernández Borrero and El-Deiry, 2021). Although TP53 mutations are less common in melanoma compared to other cancers, they are associated with advanced disease and poor prognosis when present (Hocker et al. 2008; Olivier et al. 2010). Mutations and amplifications in the KIT gene are found in certain subtypes of melanoma, particularly acral and mucosal melanomas. KIT encodes a receptor tyrosine kinase involved in cell signaling, and its aberrant activation contributes to melanoma growth and survival (Pham et al. 2020; Guo et al. 2021). MAPK and PI3K/AKT pathways are frequently dysregulated in melanoma due to mutations in BRAF, NRAS, and PTEN. They play a central role in promoting cell proliferation, survival, and resistance to apoptosis, making them critical targets for therapeutic intervention.

Significance of Gene Silencing in Melanoma

Gene silencing using CRISPR-Cas9 technology offers a promising approach to target specific genetic alterations in melanoma. By selectively knocking out or downregulating oncogenes and restoring the function of tumor suppressor genes, CRISPR-Cas9 can effectively disrupt the molecular pathways driving melanoma progression.

By leveraging CRISPR-Cas9 for targeted gene silencing, researchers can dissect the functional roles of the key genes and pathways in melanoma, identify novel therapeutic targets, and develop more effective treatment strategies. This precision approach holds great potential to improve outcomes for patients with malignant melanoma, particularly those with advanced or treatment-resistant disease.

CRISPR-Cas9 System: Principles and Applications

Overview of the CRISPR-Cas9 System

The CRISPR-Cas9 system, originally discovered as an adaptive immune mechanism in bacteria, has revolutionized genetic research and therapeutic

development. The system allows for precise editing of the genome by introducing double-strand breaks at specific DNA sequences, which can then be repaired in ways that modify the genome (Asmamaw and Zawdie, 2021; Ahumada-Ayala et al. 2023; Alaa et al. 2024).

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats), DNA sequences found in the genomes of bacteria and archaea that provide a form of acquired immunity against viruses (Barrangou and Marraffini, 2014). Cas9 (CRISPR-associated protein 9) is an endonuclease enzyme that introduces double-strand breaks in DNA at specific sites, guided by RNA (Peng et al. 2016). Guide RNA (gRNA) is a synthetic RNA molecule that directs Cas9 to the target DNA sequence. It consists of a scaffold sequence necessary for Cas9 binding and a user-defined spacer sequence of about 20 nucleotides that is complementary to the target DNA (Wang et al. 2016). The mechanism of action of CRISPR-Cas9 system is divided into some sections such as target recognition, DNA cleavage and DNA repair (Xue and Greene, 2021; Li et al. 2023).

For the target recognition, the gRNA binds to the target DNA sequence through complementary base pairing (Konstantakos et al. 2022). After that Cas9, guided by the gRNA, introduces a double-strand break in the DNA at the target site for DNA cleavage (Lemos et al. 2018). At last, the cell's endogenous repair mechanisms fix the break, either by non-homologous end joining (NHEJ), which can introduce small insertions or deletions (indels), or by homology-directed repair (HDR) if a repair template is provided, allowing precise edits (Stinson and Loparo, 2021; Xue and Greene, 2021).

Applications of CRISPR-Cas9 on Malignant Melanoma

The versatility of CRISPR-Cas9 has led to a wide range of applications in biomedical research and therapeutic development, including gene knockout, gene regulation, functional genomics, gene correction and epigenome editing.

To disrupt the function of specific genes by introducing indels that lead to frameshift mutations and loss of gene function (Lalonde et al. 2017). Knockout of the BRAFV600E allele in melanoma cells using CRISPR-Cas9 has been shown to inhibit the MAPK pathway, reduce cell proliferation, and increase apoptosis. This approach helps to study the functional role of BRAF mutations and develop potential therapeutic strategies (Yang et al. 2017; Soria et al. 2021). Modulating the expression of genes without permanently altering the DNA sequence can be achieved using modified versions of Cas9, such as dead Cas9 (dCas9), which can be fused to transcriptional activators or repressors (Moreno

et al. 2018; Bendixen et al. 2023). CRISPR Interference (CRISPRi) uses dCas9 fused to a repressor domain to inhibit gene expression. CRISPRi targeting MITF, a key transcription factor in melanoma, has been used to downregulate its expression, resulting in decreased melanoma cell proliferation and survival (Hartman and Czyz, 2014). CRISPR Activation (CRISPRa) uses dCas9 fused to an activator domain to enhance gene expression. CRISPRa can be employed to upregulate tumor suppressor genes, such as PTEN, to restore their function and inhibit melanoma progression (Moses et al. 2019).

For the functional genomics this system systematically investigates the function of genes and genetic interactions on a genome-wide scale. This is often done through CRISPR-Cas9 screening (Ford et al. 2019). Genome-wide CRISPR-Cas9 knockout screens have been conducted to identify genes essential for melanoma cell survival, proliferation, and resistance to therapy. These screens can uncover novel therapeutic targets and provide insights into melanoma biology (Gautron et al. 2021).

Gene correction is pathogenic mutations by providing a repair template for homology-directed repair (HDR) (Riesenberg et al. 2023). Although less common, CRISPR-Cas9 has potential applications in correcting mutations in genes like CDKN2A or TP53 in melanoma, potentially restoring their tumor suppressor functions (Chehelgerdi et al. 2024).

Using dCas9 fused to epigenetic modifiers modifies the epigenetic state of the genome, such as DNA methylation or histone modifications (O'Geen et al. 2017). Epigenome editing can be used to study the role of epigenetic changes in melanoma development and to identify new epigenetic targets for therapy.

Experimental Approaches and Outcomes of CRISPR-Cas9-Mediated Gene Silencing in Malignant Melanoma

CRISPR-Cas9 technology has been extensively used to investigate the functional roles of various genes in malignant melanoma.

BRAF

The BRAFV600E mutation, present in approximately 50% of melanomas, leads to constitutive activation of the MAPK/ERK signaling pathway, promoting cell proliferation and survival (Castellani et al. 2023). Researchers have designed gRNAs specific to the BRAFV600E allele and used CRISPR-Cas9 to introduce double-strand breaks at the mutation site. Knockout of BRAF was confirmed by sequencing and Western blot analysis to ensure the absence of BRAF protein

expression (Palit et al. 2021). Studies have shown that CRISPR-Cas9-mediated knockout of BRAFV600E significantly reduces melanoma cell proliferation and induces apoptosis (Wu et al. 2020). Tumor growth in xenograft models was also inhibited, demonstrating the potential of BRAF silencing as a therapeutic strategy (Sharma, 2005). BRAF knockout cells exhibited increased sensitivity to MAPK pathway inhibitors, suggesting a combinatorial approach for enhanced efficacy (Shan et al. 2024).

NRAS

NRAS mutations, found in about 15-20% of melanomas, result in continuous activation of the MAPK and PI3K/AKT pathways. gRNAs targeting the NRAS gene were used to disrupt its function via CRISPR-Cas9. The effects of NRAS silencing were assessed using cell viability assays, apoptosis assays, and Western blot analysis. Silencing NRAS led to a marked decrease in cell proliferation and an increase in apoptosis in NRAS-mutant melanoma cells. Tumor growth in animal models was significantly reduced, highlighting the therapeutic potential of NRAS targeting (Muñoz-Couselo et al. 2017; Randic et al. 2023; Phadke and Smalley, 2023).

PTEN

PTEN is a tumor suppressor gene that negatively regulates the PI3K/AKT pathway (Haddadi et al. 2018). Loss of PTEN function is associated with melanoma progression and resistance to therapy (Catalanotti et al. 2017). CRISPR-Cas9 was employed to restore PTEN function in PTEN-null melanoma cells (DuBose et al. 2024). The effects on cell proliferation, apoptosis, and signaling pathway activity were analyzed. Restoration of PTEN expression resulted in decreased PI3K/AKT pathway activity, reduced cell proliferation, and increased apoptosis. In vivo studies showed that PTEN restoration inhibited tumor growth and enhanced sensitivity to PI3K inhibitors (DeGraffenried et al. 2004; Wu et al. 2008).

MITF

MITF is a key transcription factor regulating melanocyte development and survival (Levy et al. 2006). Overexpression or amplification of MITF contributes to melanoma progression (Wiedemann et al. 2019). CRISPR-Cas9 was used to knock down MITF expression in melanoma cells (Sánchez-Del-Campo et al. 2021). Cell proliferation, survival, and differentiation were assessed following MITF silencing (Vlčková et al. 2018). MITF silencing led to a significant reduction in melanoma cell proliferation and survival (Carreira et al. 2006).

Knockdown of MITF also impaired the invasive capabilities of melanoma cells, indicating its role in metastasis.

CDKN2A

CDKN2A encodes the tumor suppressor proteins p16INK4a and p14ARF, which regulate the cell cycle and apoptosis (Laud et al. 2006; Jiao et al. 2018). Loss of CDKN2A function is common in melanoma. CRISPR-Cas9 was used to reintroduce or upregulate CDKN2A in melanoma cells with CDKN2A loss (Young et al. 2014). Effects on cell cycle progression, apoptosis, and tumor growth were studied. Reintroduction of CDKN2A induced cell cycle arrest and increased apoptosis in melanoma cells (Castellano et al. 1997). In vivo studies demonstrated that CDKN2A restoration inhibited tumor growth, supporting its potential as a therapeutic target.

TP53

TP53 is a critical tumor suppressor involved in cell cycle regulation, DNA repair, and apoptosis (Robles and Harris, 2010). TP53 mutations are associated with poor prognosis in melanoma (Khan et al. 2023). CRISPR-Cas9 was used to correct TP53 mutations or enhance TP53 activity in melanoma cells. Cellular responses to DNA damage, apoptosis, and tumor growth were evaluated. Correction of TP53 mutations or enhancement of TP53 activity led to increased apoptosis and sensitivity to DNA-damaging agents (Wang et al. 2023). Tumor growth in xenograft models was significantly inhibited, demonstrating the therapeutic potential of targeting TP53 (Huang, 2021).

DISCUSSION

CRISPR-Cas9-mediated gene silencing has shown significant effectiveness in inhibiting melanoma cell proliferation, inducing cell death, and potentially overcoming drug resistance (Vaghari-Tabari et al. 2022). Targeting key genes such as BRAF, NRAS, PTEN, MITF, CDKN2A, and TP53 provides valuable insights into the molecular mechanisms driving melanoma and identifies promising therapeutic targets. These studies underscore the potential of CRISPR-Cas9 as a powerful tool for advancing melanoma research and developing more effective treatments.

By leveraging CRISPR-Cas9 technology, researchers can continue to dissect the functional roles of critical genes, uncover novel therapeutic strategies, and ultimately improve clinical outcomes for patients with malignant melanoma (Chira et al. 2022).

While CRISPR-Cas9 has shown great promise in advancing melanoma research and therapy, several challenges and limitations need to be addressed to fully realize its potential. By optimizing delivery methods (Du et al. 2023), enhancing specificity (Betof Warner et al. 2023), exploring combination therapies (Luke et al. 2017), and addressing tumor heterogeneity (Margazalli et al. 2019), researchers can develop more effective and safer CRISPR-Cas9-based strategies for treating malignant melanoma. Continued advancements in CRISPR technology and a deeper understanding of melanoma biology will pave the way for innovative treatments and improved patient outcomes.

In conclusion, the potential of CRISPR-Cas9-mediated gene silencing as a therapeutic approach for malignant melanoma is immense. By selectively targeting and modulating the expression of genes crucial to melanoma progression, CRISPR-Cas9 offers a precision medicine approach that can complement existing treatments and provide novel avenues for intervention. The ability to knock out oncogenes, restore tumor suppressor functions, and enhance sensitivity to therapies positions CRISPR-Cas9 as a powerful tool in the fight against melanoma. Ongoing research efforts in CRISPR-Cas9 technology and melanoma biology are essential to translate these promising findings into clinical applications. Continued advancements in delivery methods, specificity enhancement, and combination strategies will further refine CRISPR-Cas9-based therapies. Collaborative efforts among researchers, clinicians, and industry partners are critical to overcoming current limitations and ensuring the safety and efficacy of these innovative treatments. The integration of CRISPR-Cas9-mediated gene silencing into the therapeutic landscape holds the potential to significantly impact future melanoma treatments. By providing a targeted, adaptable, and efficient approach to modulating gene function, CRISPR-Cas9 can contribute to personalized treatment strategies that improve patient outcomes. As research progresses, the adoption of CRISPR-Cas9 in clinical settings could revolutionize melanoma therapy, offering hope for more effective and durable solutions against this aggressive form of cancer.

CRISPR-Cas9-mediated gene silencing represents a promising frontier in melanoma research and therapy. With continued innovation and dedicated research, this technology has the potential to transform the treatment paradigm for malignant melanoma and pave the way for groundbreaking advancements in cancer therapy.

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CHAPTER 6

Antimicrobial Potential of *Lactobacillus* Probiotics: A Natural Alternative to Antibiotics

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Introduction

Probiotics are described by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) as living microorganisms that, when consumed in sufficient quantities via food or dietary supplements, provide beneficial effects on the health of the host (FAO and WHO, 2002). Probiotics, when administered in adequate quantities (a minimum of 10^6 viable CFU/g), contribute to improving the host's gut microbial balance and play a role in metabolic processes (Bezirtzoglou et. al., 2011). Probiotics persist within the gastrointestinal tract and support the stability of the gut microbiota by tolerating acidic conditions, bile salts, and pancreatic secretions, along with their ability to attach to the intestinal epithelium (Vassos et. al., 2009).

Probiotics confer a variety of health benefits by supporting gastrointestinal function, maintaining gut microbiota balance, and reducing the risk of conditions such as diarrhea and inflammatory bowel disease (Gul and Durante-Mangoni, 2024), enhancing immune function and reducing inflammation (Pyo et. al., 2024; Khalesi et. al., 2018), improving metabolic and cardiovascular health by managing conditions like obesity, type 2 diabetes, and hypertension (Gul and Durante-Mangoni, 2024), supporting mental health and cognitive function through the gut-brain axis (Kim et. al., 2020), and potentially benefiting female reproductive health, as well as exhibiting anti-cancer and anti-viral properties (Khalesi et. al., 2018; Pyo et. al., 2024).

Lactic acid bacteria (LAB), classified as Generally Recognized as Safe (GRAS), are widely employed as probiotics in clinical applications (Sieladie et. al., 2011). The genus *Lactobacillus*, comprising several species with notable probiotic properties, plays a crucial role in enhancing human health. *Lactobacillus* strains are commonly consumed as probiotics, where they colonize the gastrointestinal tract, providing a range of health benefits, such as antimicrobial, anti-inflammatory, antioxidant, and immune-regulatory effects (Minj et. al., 2020). *Lactobacillus* strains produce a variety of antimicrobial substances, such as organic acids, hydrogen peroxide, diacetyl, bacteriocins, and antimicrobial peptides (AMPs), which act synergistically to inhibit the growth of various pathogens (Silva et. al., 1987; Arena et al., 2016; Moal et al., 2014).

Antibiotic resistance poses a significant global health risk, compromising the effectiveness of therapies for bacterial infections. The overuse of antibiotics, combined with the slow development of new drugs, results in infections that are increasingly difficult to treat, as well as higher morbidity and mortality rates. This also leads to increased healthcare costs due to prolonged hospitalizations and the

need for more expensive treatments (Nwobodo et. al., 2022). Additionally, resistance genes originating from human activities and livestock farming significantly contribute to the spread of resistance (Kumar et. al., 2021).

The demand for alternative therapeutic strategies is growing due to the rise in antibiotic resistance. *Lactobacillus* strains, known for their potent antimicrobial properties, show significant promise for both medical and industrial applications, particularly in the development of naturally sourced antimicrobial agents. This study aims to investigate the antimicrobial properties of *Lactobacillus* probiotics and the underlying mechanisms responsible for their activity.

Overview of *Lactobacillus*: Probiotic Properties and Health Benefits

The genus *Lactobacillus,* first described in 1901, comprises non-spore-forming, Gram-positive, rod-shaped, anaerobic LAB that have long been essential components of fermented foods and are naturally present in the gastrointestinal and genitourinary systems. These bacteria are capable of enhancing the absorption and bioavailability of minerals, as well as reducing intestinal permeability. A considerable number of the probiotics in use today are derived from the ***Lactobacillus*** genus. This group of microorganisms, commonly referred to as "lactobacilli" is multifunctional, providing a broad spectrum of advantages from enhancing the shelf life of food products (e.g., by producing yogurt or cheese from milk) and supporting health when consumed as probiotic foods or supplements (El-Saadony et. al., 2021; Cichonska ve Ziarno, 2022). With recent taxonomic revisions, the genus Lactobacillus has been reclassified into a total of 25 genera, including 23 new ones, based on genomic and ecological characteristics (Zheng et al., 2020).

The species of Lactobacillus meet the necessary criteria for classification as probiotics and exhibit both nutritional and therapeutic effects. Their ability to survive and proliferate under gastrointestinal conditions enables them to exert beneficial effects, rendering them appropriate for both preventive and therapeutic applications. Consequently, understanding their mechanisms of action is essential for clarifying their roles in both illness prevention and therapeutic intervention (Kang et. al., 2017; Pan et. al., 2017). Lactobacillus species are recognized for their probiotic properties, which include immune modulation, epithelial barrier enhancement, and antipathogenic activities (Zhang et. al.,2018). These bacteria are vital in maintaining gut health by supporting the structural integrity of the intestinal barrier and mucosal defense, in addition to modulating immune responses (Rastogi and Singh, 2022). Additionally, they are capable of surviving

in acidic environments and are generally resistant to certain antibiotics, which enhances their survival and efficacy as probiotics (Goldstein et. al., 2015).

Lactobacillus species have been shown to alleviate symptoms of various inflammatory conditions, including asthma, pulmonary diseases, neuroinflammatory disorders, cardiovascular diseases, and inflammatory bowel disease, while also playing a role in the management of metabolic conditions such as obesity and diabetes by modulating oxidative stress and inflammatory pathways (Rastogi and Singh, 2022). *Lactobacillus plantarum,* an extensively researched species, is commonly utilized in the food industry to improve the safety and prolong the shelf-life of fermented foods, owing to its capacity to generate bioactive compounds that suppress pathogenic microorganisms (Behera et. al., 2018). The *Lactobacillus casei* group, encompassing species like *L.casei*, *L.paracasei*, and *L.rhamnosus*, has been the subject of considerable investigation for its therapeutic potential in addressing or preventing diseases associated with disruptions in the gut microbiome (Hill et. al., 2018).

Antibacterial and antibiofilm activity of *Lactobacillus* probiotics

Lactobacillus probiotics are well known for their health-promoting properties, particularly their broad-spectrum antimicrobial activity against various pathogens. When used alone or in combination, they have the potential to function as potent therapeutic agents for bacterial infections in humans. *L.casei and L.plantarum* have demonstrated notable inhibitory activity on enterotoxigenic *Escherichia coli and Salmonella enterica* (Divyashree et. al., 2021; Wang et al.,, 2020). Furthermore, *L.salivarius* and *L.helveticus* have exhibited strain-dependent inhibitory activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Dobreva et. al., 2024). Additionally, *L.acidophilus*, *L.plantarum*, *L.fermentum*, *L.casei*, and *L.rhamnosus* have demonstrated effectiveness against *P.mirabilis* and *P.vulgaris*, which are associated with urinary tract infections (Goudarzi et. al., 2016). *L.casei* and *L.plantarum* strains exhibit high survival rates and potent antagonistic effects against *S.paratyphi*, suggesting their potential as natural substitutes for chemical preservatives in the food industry (Divyashree et. al., 2021). *L.acidophilus* AD125 demonstrates significant antibacterial properties against *E.coli* O157:H7, providing potential for the prevention and treatment of intestinal disorders associated with this pathogen (Xing et al., 2023). *L.plantarum* has demonstrated potential as a probiotic by suppressing the growth of multidrug-resistant pathogens, including *P.aeruginosa* and MRSA, in diabetic foot infections (Layús et al., 2020).

Probiotic *Lactobacillus* strains derived from the human gut microbiota exhibit promising antimicrobial activity against gastric and enteric bacterial pathogens, including rotavirus, offering a new avenue for the development of novel gastrointestinal anti-infective treatments. Specifically, probiotic *Lactobacillus* strains (*L.rhamnosus*, *L.casei*, *L.johnsonii*, *L.acidophilus*, and *L.reuteri)* have been extensively investigated for their antimicrobial potential (Moal et al., 2014). *Lactobacillus* probiotics have exhibited notable antibacterial effects against a range of pathogenic bacteria. Research indicates that strains such as *L.acidophilus, L.plantarum, L.fermentum,* and *L.rhamnosus* are effective in inhibiting the growth of *E.coli, K.pneumoniae, P.aeruginosa,* and *S.aureus* (Davoodabadi et al., 2015; Radwan, 2022).

L.plantarum 200661, present in fermented foods, exhibits the strongest antibacterial activity and has potential applications as a biofilm disruptor and oral probiotic in functional foods to combat *Streptococcus mutans* (Lim et al., 2020). Furthermore, *Lactobacilli* have demonstrated the ability to prevent dental caries by suppressing the growth of oral streptococci (Jafarzade et al., 2021). Probiotic *L.johnsonii* NBRC 13952 shows considerable ability to suppress the growth and biofilm formation of *Aggregatibacter actinomycetemcomitans,* suggesting its use as an effective agent against periodontal pathogen biofilms (Jaffar and Zamry, 2023). Probiotic *L.rhamnosus* and its biosurfactants effectively prevent *Acinetobacter baumannii* biofilm formation and reduce microbial contamination on medical devices, presenting a promising alternative to synthetic antimicrobial agents (Al-Shamiri et. al., 2023). Cell-free supernatant (CFS) from four (*L.plantarum*, *L.acidophilus*, *L.johnsonii*, and *L.delbrueckii*) LAB with probiotic potential suppresses the growth of *P.aeruginosa* and decreases its biofilm formation, offering a potential strategy to reduce antibiotic usage in hospital-associated infections (Drumond et. al., 2023). Strains of *L.salivarius* and *L.casei* demonstrate strong antibacterial and antibiofilm properties against *E.coli*, indicating their potential as beneficial probiotics for health promotion (Al-Groom, 2023).

The combination of various *Lactobacillus* strains can produce synergistic antibacterial effects, demonstrating growth inhibition indices between 0.56 and 0.74 on *E.coli* and *K.pneumoniae* (Halder & Shyamapada, 2016). The combination of *L.rhamnosus* and *L.acidophilus* has demonstrated efficacy in inhibiting pathogens linked to bacterial vaginosis and aerobic vaginitis (Bertuccini et al., 2017).

L.reuteri synthesizes reuterin, a powerful antimicrobial compound that exhibits effectiveness against both Gram-positive and Gram-negative bacteria, such as enterohemorrhagic *E.coli* and *Vibrio cholerae* (Spinler et al., 2008). *L.casei* NA-2, isolated from northeast sauerkraut, displays bactericidal activity attributed to its exopolysaccharides, which prevent biofilm formation and facilitate the dispersal of pathogenic bacteria (Xu et. al., 2020). Lyophilized CFS from *Lactobacillus* isolates has demonstrated antibiofilm activity against *A.baumannii* and *E.coli*, which could offer potential advantages for functional fermented foods and pharmaceutical applications (Sornsenee et. al., 2021). The lyophilized CFS of *L.reuteri* AN417 exhibits antibacterial properties against *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *S.mutans*, suggesting its potential for preventing and treating periodontal disease (Yang et. al., 2021).

Antibacterial Mechanisms of *Lactobacillus* Probiotics

Lactobacillus strains demonstrate antimicrobial properties via various mechanisms, predominantly through the secretion of antimicrobial substances and competitive interactions with pathogenic microorganisms. *Lactobacillus* strains synthesize a range of antimicrobial agents, such as organic acids, hydrogen peroxide (H_2O_2), bacteriocins, and AMPs, which are capable of suppressing the growth of both Gram-positive and Gram-negative pathogens (Arena et. al., 2016; Moal and Servin, 2014; Silva et al., 1987).

Production of bacteriocin

Bacteriocins are peptide compounds produced by bacteria, with their synthesis by beneficial microorganisms being regarded as a valuable trait of probiotics. For instance, *L.gasseri* LM19, a strain derived from human milk, exhibits inhibitory effects against enteropathogenic organisms and produces a variety of bacteriocins, positioning it as a promising probiotic for supporting gut health (Garcia-Gutierrez et al., 2019). In a similar vein, the bacteriocin Lac-B23, a novel AMP synthesized by *L.plantarum* J23, shows potential for use in food preservation and exhibits antimicrobial properties against a broad spectrum of microorganisms (Zhang et al., 2018). Moreover, several *Lactobacillus* strains produce bacteriocins such as Nisin Z and Sakacin P, which effectively inhibit the growth of pathogens (Nebbia et. al., 2020). Bacteriocins synthesized by *L.fermentum* strains isolated from Mexican cheese demonstrate antimicrobial efficacy against both Gram-positive and Gram-negative microorganisms, emphasizing their potential use as biopreservatives in dairy products (Heredia-Castro et al., 2021). Furthermore, *L.acidophilus* KS400 produces a bacteriocin that demonstrates antimicrobial activity against clinically significant urogenital

pathogens, indicating its potential contribution to maintaining vaginal health (Gaspar et al., 2018). Research on *L.lactis* isolated from marine sediment revealed that its bacteriocin effectively inhibited *E.coli*, *E.faecalis*, *S.aureus*, and *B.subtilis*, while exhibiting no inhibitory effects on other *Lactobacillus* species (Sunaryanto and Tarwadi, 2015).

Production of antimicrobial peptides

AMPs synthesized by *Lactobacillus* species are increasingly being recognized as promising alternatives for conventional antibiotics, particularly amid the growing concern regarding antibiotic resistance. These naturally occurring molecules exhibit inhibitory effects against numerous pathogens, highlighting their potential for both medical treatments and food preservation applications. In this context, *L.fermentum* and other related strains synthesize AMPs that effectively suppress the growth of multidrug-resistant pathogens, including *P.aeruginosa* and *Serratia marcescens* (Pavlova et al., 2020; Kishilova et. al., 2024). Furthermore, some *Lactobacillus* strains synthesize bacteriocin-like inhibitory compounds that exhibit antimicrobial activity against *S.aureus* and *E.coli*. Notably, these protein-based compounds can be partially degraded by enzymatic activity, suggesting their structurally complex nature (Heredia-Castro et. al., 2015). AMPs typically adopt an amphipathic structure, enabling them to interact with and disrupt negatively charged bacterial membranes, resulting in increased membrane permeability and cell lysis. In addition to membrane disruption, AMPs can penetrate cells, inhibiting nucleic acid and protein synthesis, disrupting enzyme function, and interfering with cell division and cell wall biosynthesis (Le et al., 2017; Raheem et al., 2019). Some AMPs form membrane channels, leading to cytoplasmic leakage and cell death. Furthermore, AMPs can prevent biofilm formation and modulate immune responses, enhancing their therapeutic potential (Raheem et al., 2019).

Production of organic acids

Numerous *Lactobacillus* strains, including *L.gasseri*, and *L.crispatus*, exhibit antimicrobial properties by producing lactic acid, which can directly neutralize pathogens through direct interaction and prevent their adhesion to host cells (Atassi et. al., 2019). Moreover, probiotic *Lactobacillus* strains effectively inhibit the growth of *Salmonella enterica* serovar *Typhimurium* SL1344 through the synergistic effects of lactic acid synthesis, the release of non-lactic acid antimicrobial compounds, and environmental pH reduction (Fayo-Messaoudi et. al., 2005). Furthermore, the rapid synthesis of lactic acid is vital for the effective inhibition of *Bacillus cereus* (Røssland et. al., 2005). Besides lactic acid, other

organic acids such as acetic acid, contribute to antimicrobial activity by lowering pH levels, thereby creating an environment that is less conducive to the proliferation of carbapenem-resistant *Enterobacteriaceae*. The acidification of the environment impairs bacterial metabolic processes and compromises membrane integrity (Chen et. al., 2020). Additionally, *Lactobacillus* strains synthesize various organic acids, including acetic, citric, and phenyl-lactic acids, which enhance their antimicrobial properties against the growth of *Listeria monocytogenes* (Šalomskienė et al., 2019).

Production of Hydrogen Peroxide

H_2O_2 is a reactive oxygen species produced by the immune system cells during the inflammatory response, serving as an antimicrobial compound. *L.reuteri*, a probiotic bacterium, known for its anti-inflammatory properties, has the potential to enhance gut health by modulating responses to inflammatory oxidants (Thakur et. al., 2019). H_2O_2 produced by host cells impedes the proliferation of pathogenic bacteria by downregulating the transcription of polysaccharide intercellular adhesins, thereby playing a crucial role in maintaining vaginal health. Some *Lactobacillus* strains generate H_2O_2, which exerts oxidative damage on bacterial membranes, resulting in cell harm. H_2O_2 functions as a preservative in fermented products, disrupting microbial cell membranes and compromising DNA integrity (Šuškovi´c et. al. 2010). H_2O_2-producing *Lactobacillus* strains, together with lactic acid, act synergistically to eliminate intestinal, vaginal, and urinary tract pathogens (Atassi and Servin, 2010).

Competitive Exclusion and Bioactive Metabolites

Lactobacillus strains inhibit pathogen colonization and infection by effectively competing for essential nutrients and binding sites on host tissues (Moal et al., 2014). *Lactobacillus* species employ competitive exclusion by depleting nutrients, adhering to surfaces, and engaging in metabolic interactions to suppress the proliferation of spoilage microorganisms and pathogens across diverse environments (Atassi et. al., 2019; Coconnier et. al., 2000). Furthermore, the antimicrobial properties of *Lactobacillus* strains are also linked to their CFSs, which comprise a combination of organic acids and other bioactive metabolites. These supernatants have demonstrated efficacy in suppressing the growth of bacteria and fungi both in vitro and in food-related applications (Mani-López et. al., 2021).

Conclusion

Lactobacillus strains exhibit potent antimicrobial and antibiofilm activities that can vary even within the same species, highlighting the importance of selecting appropriate strains for specific applications. *Lactobacillus* strains employ a variety of antimicrobial mechanisms, including the synthesis of lactic acid, AMPs, and bacteriocin-like compounds, as well as competitive exclusion and inhibition of pathogen adhesion. These mechanisms collectively contribute to their potential as natural antimicrobial substances in both clinical and food industries. The ability of *Lactobacillus* strains to produce a wide range of antimicrobial compounds offers promising opportunities for the development of natural and effective antimicrobial agents that can serve as an alternative approach to treating antibiotic-resistant pathogens. Nonetheless, additional studies are required to clarify the mechanisms responsible for their antimicrobial properties, especially the involvement of new peptides and other bioactive substances.

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CHAPTER 7

Digital Dentistry's Environmental Impact: Carbon Footprint of Clear Aligners

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Introduction

This study examines the bioethical dilemma surrounding the integration of digital technologies in dentistry, with a specific focus on clear aligners in digital orthodontics and their impact on environmental sustainability. This dilemma arises from the fact that digital technologies in orthodontics, while reducing certain ecological risks, also bring new sustainability challenges.

In healthcare, environmental sustainability involves prioritizing resource renewability, energy efficiency, emission reduction, minimally invasive procedures, waste management, and eco-friendly infrastructure. Healthcare professionals have an ethical duty to consider future patients and the ecosystem (Jameton et al., 2001).

The rapid advancement of digital technologies in healthcare has revolutionized the field, offering more efficient, precise, and personalized care. Dentistry, in particular, has experienced significant transformation with innovations such as three-dimensional (3D) imaging, computer-aided design and manufacturing (CAD/CAM) systems, and digital patient records enhancing treatment quality and accessibility (Gawali et al., 2024). As the healthcare industry grapples with the challenge of reducing its carbon footprint, questions arise regarding the sustainability of digital solutions in dental practice. While these advancements streamline procedures and reduce clinical errors, their environmental impact remains a pressing concern. The production, maintenance, and disposal of digital devices, along with increased energy demand, may offset the benefits these technologies provide (Martin et al., 2022). Conversely, proponents argue that digitization reduces waste, optimizes resource usage, and enhances efficiency, potentially benefiting the environment in the long run (Mittal et al., 2020a).

Oral healthcare, essential for overall health, well-being, and quality of life, must be delivered ethically, safely, and sustainably to minimize environmental impact and protect future healthcare opportunities (Sustainability in Dentistry, 2017).

Dentistry, which provides professional oral health care services, significantly impacts the environment through energy consumption, water usage, plastic waste, radiation exposure, and material disposal. Acknowledging the need for sustainable practices, the dental community is increasingly committed to reducing its environmental footprint and adopting eco-friendly innovations (Martin et al., 2022; Mittal et al., 2020a).

In the 21st century, with the rise of the digital era and the rapid technological improvements in biomaterials, computer-aided design and manufacturing (CAD/CAM), the orthodontic field has integrated modern technology to develop a variety of the contemporary clear aligner systems that offer a more comprehensive approach to orthodontic treatment. The development of clear aligner systems represents a modern approach to orthodontic treatment, integrating digital technologies for enhanced precision and efficiency (Panayi et al., 2024a). The number of adult patients seeking orthodontic treatment is increasing as well as the demand for more esthetic appliances, requiring alternatives to the conventional fixed orthodontic appliance. Therefore, in the last decades, the spotlight has been on the use of clear aligners (Abu-Arqub et al., 2023).

This paper evaluates whether digital technologies in dentistry, particularly clear aligners in digital orthodontics, are beneficial or detrimental to environmental sustainability by considering both potential environmental risks and promising solutions they offer.

Sustainability in Dentistry

The "Sustainability in Dentistry" project by the FDI World Dental Federation promotes sustainable practices globally, emphasizing waste reduction, energy conservation, and the adoption of digital technologies such as electronic patient records, intraoral scanners, and digital radiographs (FDI World Dental Federation, 2024). Such technological transformations not only enhance patient care but also reduce the carbon footprint associated with traditional methods.

The concept of "eco-friendly dentistry" has gained momentum, focusing on reducing waste and pollution, conserving energy, and using biodegradable materials. Sustainability in dentistry represents a broader commitment to social and environmental responsibility (Mittal et al., 2020a; Martin et al., 2021). Oral health professionals play a crucial role in ensuring future generations have access to sufficient natural resources by adopting sustainable practices. By embracing sustainability as a core principle, oral health professionals can contribute to creating a healthier and more sustainable future for all (Duane et al., 2019).

Digital Technologies in Dentistry: Benefits and Challenges

In recent years, digital technology has driven a significant evolution in engineering and consequently transformed various aspects of our daily lives, including medicine and dentistry. Today’s high-tech innovations have made dental practices more reliable, efficient, and cost-effective. Many of these

innovations in dentistry also offer environmental benefits; for example, CAD/CAM systems eliminate the need for impression materials, reducing waste. Additionally, they decrease the number of patient appointments, which in turn lowers carbon emissions by minimizing patient travel (Martin et al., 2021).

Traditional dental practices often rely heavily on physical impressions and radiographs, which require significant amounts of plastic, chemicals, and energy. In contrast, digital records and imaging systems reduce the reliance on physical materials, thereby lessening environmental impact. Moreover, the use of digital radiography over traditional film radiography reduces the consumption of toxic chemicals needed for film development, conserves water and cuts down on lead waste. Three-dimensional (3D) technologies like intraoral scanners and 3D printers have transformed dentistry, offering numerous environmental benefits. They allow for fewer patient visits, as multiple procedures can be combined in a single appointment. This minimizes the need for transportation between dental offices and laboratories, contributing to reduced carbon footprints. The reduction in transportation not only cuts down on greenhouse gas emissions but also decreases the overall energy consumption associated with dental care (Duane et al., 2019a; Duane et al., 2019b; Martin et al., 2021).

However, the sustainability of digital dentistry remains debated. For instance, although digital orthodontics reduce resource consumption and the number of clinic visits, clear aligners become contaminated medical waste after use and are difficult to recycle. Additionally, concerns about microplastics in aligner materials raise questions about their overall environmental impact. (Taneya et al., 2015; Vasamsetty et al., 2020; Mulligan et al., 2021).

Digital Orthodontics and Clear Aligners

Digital orthodontics, which involves the use of digital tools like 3D scanners, simulation software, and 3D printers, has revolutionized orthodontic treatment. These technologies facilitate precise treatment planning and the manufacture of orthodontic appliances, offering greater accuracy compared to traditional methods. For instance, digital simulations can predict the outcome of treatments, allowing for adjustments to be made before actual implementation, thereby reducing errors and waste (Caelli et al., 2023). Traditionally, orthodontic treatment involved metal braces and alloy wires, which could impact patient aesthetics. Advances in digitization and 3D printing have led to the development of clear aligners made from thermoplastic polymers, offering a more aesthetically pleasing alternative (Peter et al., 2022).

Classically, teeth malalignment has been treated with metal braces and alloy wires, which can negatively affect a patient's appearance during treatment. However, advances in digitization, computer simulation, and 3D printing have led to the development of clear aligners as a modern alternative. Made from transparent thermoplastic polymers, clear aligners provide a more aesthetically pleasing option for orthodontic treatment. Unlike metal braces, clear aligners can be easily removed, allowing patients to maintain better oral hygiene (Peter et al., 2022).

Contemporary aligners combine the principles established by early pioneers like Remensnyder, Kesling, and Nahoum with modern CAD/CAM technology. They are custom-made to fit a patient's dental arches and achieve tooth movement through a series of aligners, each incrementally adjusting the teeth by a predetermined amount. Clear aligners are especially beneficial for those seeking an effective orthodontic solution without compromising their appearance (Weir, 2017).

Clear aligners, a significant innovation in digital orthodontics, are increasingly favored over traditional braces due to their aesthetic appeal and comfort. However, their environmental impact is a subject of ongoing debate. On the one hand, digital orthodontics reduces resource and energy consumption by minimizing materials and process steps compared to traditional methods. It also promotes the use of renewable energy sources and reduces the number of clinic visits required, positively affecting patients' carbon footprints. On the other hand, the production and disposal of clear aligners present substantial environmental challenges. Because, after the orthodontist creates and approves a virtual treatment plan, the aligner distributor manufactures the entire set of aligners at once. Simple cases typically require 7 to 20 aligners per arch, while complex cases may need 50 or more (Mittal et al., 2020b; Caelli et al., 2023; Yashodhan et al., 2023; Panayi et al., 2024b).

Clear aligners are increasingly favored due to their comfort and appearance. While digital orthodontics minimize energy and resource consumption, the production and disposal of aligners present environmental challenges. The materials used in aligners contribute to plastic waste, and their disposal remains an unresolved issue (Raj et al., 2024; Freitas, 2022).

Environmental Impact of Clear Aligners

Clear aligners, primarily composed of PET, PETG, or TPU, apart from other petroleum-based polymers that release a wide variety of nanoplastics are non-

biodegradable and contribute to plastic pollution. These materials can release microplastics into the environment, affecting marine life and human health. Given that plastic decomposes very slowly, clear aligners discarded in landfills or oceans present a significant environmental challenge. The majority of aligners are not recycled, and there are few initiatives or guidelines from companies to address this issue (Hartshorne&Wertheimer, 2022; Raj et al, 2024).

Clear aligners are not biodegradable. Once their purpose is served, these aligners become contaminated medical waste, and routine recycling methods often cannot accommodate this substantial volume. The accumulation of plastic waste from clear aligners contributes to the broader issue of plastic pollution, which has far-reaching environmental and health implications. Microplastics, which result from the degradation of larger plastic items, are a global environmental and public health concern. In orthodontics, the use of plastic materials can release microplastics during their use or disposal, further complicating the issue. Research has indicated that exposure to microplastics can lead to various health problems, including inflammation, oxidative stress, and even cell mutation. This raises significant concerns about the long-term safety and sustainability of clear aligner therapy (Freitas, 2022; Mani et al, 2023; Panayi, 2023; Macri et al, 2024).

Current Recycling and Waste Management Strategies of Clear Aligners

The orthodontic industry is beginning to address these challenges with new recycling initiatives and strategies to manage the waste generated by clear aligners. Some clinics have introduced recycling programs that encourage patients to return their used aligners for proper disposal or recycling. These initiatives represent a step in the right direction; however, they are far from a complete solution. The volume of aligners discarded globally is vast, and current recycling efforts are limited to specific locations and require significant patient participation. For a more substantial impact, comprehensive guidelines and policies need to be developed to standardize the disposal and recycling of aligners on a global scale (Gandhi&Veerasekaran, 2023; Ong et al.,2024).

Innovations and Future Directions in Sustainable Orthodontics

The orthodontic industry must explore innovative solutions to reduce the environmental impact of clear aligners and other appliances. Researchers are investigating new materials, such as shape memory polymers and bioactive substances, that could replace conventional plastics and reduce the number of aligners needed, thus lowering plastic consumption. Direct 3D printing of clear

aligners from digital designs is another promising development that could streamline production, reduce waste, and eliminate the need for thermoforming and polishing. This method offers higher precision, better fitness, and improved patient outcomes while minimizing the environmental footprint. However, further research is required to ensure these materials' durability, safety, and cost-effectiveness. Additionally, using recycled materials in 3D printing could enhance sustainability by reducing dependence on virgin plastics and lowering the manufacturing process's environmental impact, making orthodontic practices more eco-friendly (Freitas, 2022; Macri et al, 2024).

Balancing Innovation with Environmental Responsibility

While the development of new materials and technologies in orthodontics presents exciting opportunities, it also brings challenges. Unlike the pharmaceutical industry, where rigorous testing precedes product release, orthodontics sometimes sees the rapid adoption of new machines and materials without comprehensive environmental assessments. This can lead to unforeseen consequences, such as increased plastic waste and environmental pollution. Therefore, the orthodontic community must carefully evaluate the environmental impact of new technologies and prioritize sustainability in their adoption (Katyari et al., 2024).

The Role of Stakeholders in Promoting Sustainability

The responsibility for reducing the environmental impact of orthodontics does not rest solely with manufacturers; it also involves clinicians and patients. Manufacturers need to provide clear guidelines on the disposal and recycling of aligners, while clinicians should educate patients about the environmental impact of their choices and encourage sustainable practices. Patients, on their part, can make informed decisions by choosing treatments that align with their values and the principles of sustainability. Awareness and collaboration among these stakeholders are crucial for driving meaningful change. For example, some clinics have started advising patients on proper aligner disposal methods and recycling options. Expanding these practices globally could help mitigate the environmental impact of orthodontics (Peter et al., 2022; Peter et al, 2025).

Conclusion

Digital technologies in dentistry, particularly clear aligners in orthodontics, offer significant advantages in precision, efficiency, and patient experience. They also present opportunities to reduce waste and environmental impact by minimizing the need for traditional materials and improving workflow efficiency.

However, challenges remain, particularly regarding the disposal of clear aligners and the energy demands of digital systems.

A balanced approach is essential, integrating digital advancements while implementing sustainable waste management and energy-efficient solutions. Future research should focus on developing biodegradable materials for clear aligners and optimizing digital technologies to enhance sustainability. By prioritizing environmental responsibility, the dental industry can leverage digital innovations while minimizing their ecological footprint. Applying a medical waste and recycling protocol to clear aligners returned to orthodontic or dental clinics at the end of treatment is as feasible as it is challenging for aligners remaining with the patient. However, considering that aligners are typically not returned to clinics and remain with patients, they should be included in the sanitation and disposal processes.

The aim of this study is not to criticize the use of clear aligners but to raise awareness within the orthodontic community about the potential dangers of the uncontrolled use of non-recyclable materials and contaminated waste. Considering the risks associated with the improper disposal of clear aligners and the potential for their safe reintegration into the production process to protect the environment, proper disposal methods should be developed. Education on health and environmental issues, commitment to ecological and social values, the development of a more responsible society, the support of the sustainable development of human activities, and the protection of environmental health are all crucial. The importance of raising awareness on this matter among orthodontists is evident.

By investing in research, embracing eco-friendly innovations, and fostering a culture of sustainability, the orthodontic community can help mitigate the environmental challenges associated with clear aligners and other digital orthodontic practices. As awareness grows and sustainable options become more accessible, we can look forward to a future where dental care not only enhances patient health but also supports the health of our planet.

The integration of digital technologies in dentistry, particularly clear aligners, has enhanced patient care by offering efficient, precise, and aesthetically appealing treatment options. However, concerns about their environmental impact remain significant. Clear aligners, made primarily of non-biodegradable plastics like PETG and TPU, contribute to plastic waste and microplastic pollution. While digital tools reduce resource consumption and minimize patient visits, the disposal of aligners as medical waste creates environmental challenges.

Current recycling solutions are insufficient, and sustainable management strategies are needed. Innovations such as shape memory polymers, bioactive materials, and 3D printing are being explored to reduce the environmental footprint of aligners and streamline production. The dental community must prioritize awareness of these issues and adopt more sustainable practices.

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CHAPTER 8

Alternative Treatment and Prevention Methods Against Biofilm Layer in Veterinary Medicine

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1. Introduction

The term "biofilm" is defined as "a community surrounded by a polymer matrix produced by the microorganism itself". A wide variety of structures and compositions may be exhibited. The behaviour of these cells in community is characterised by the formation of an independent ecosystem, with a balanced homeostasis (Donlan & Costerton, 2002; Hall-Stoodley et al., 2012). The formation of biofilms by microorganisms is driven by several factors. Firstly, it provides a protective barrier against adverse environmental conditions. Secondly, it facilitates access to nutrient sources. Thirdly, it enables cooperation with other microorganisms. Finally, it enables synchrony and the revelation of new genetic characteristics and behavioral patterns (Davey & O'toole, 2000; Fetzner, 2015).

The advent of the concept of biofilms in the field of medicine can be traced back to the pioneering work of Gram (1, 2), who, in the course of routine gram staining of crusts from patients suffering from cystic fibrosis and chronically infected with *Pseudomonas aeruginosa*, made the first detection of biofilms. This was followed by the autopsy of these patients, which further contributed to the advancement of the scientific understanding of biofilms (Høiby & Axelsen, 1973).

Costerton and colleagues were able to observe *P. aeruginosa* microcolonies on postmortem cystic fibrosis lungs (Lam et al 1980) and bacterial glycocalyx in nature by electron microscopy. They also showed that this ‘glycocalyx’ structure provides chemical and physical resistance to antibacterial drugs (JW, 1981). The team changed the name of ‘glycocalyx’ to ‘biofilm’ in 1985 (Costerton et al., 1987). Although the biofilm structure formed by different bacteria due to different environmental conditions is not expected to be the same, the first basic biofilm model was established in 1995 as a result of the studies carried out by Costerton et al (Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995). In 1998, confocal laser scanning microscopy (CLSM) technology was combined with the field of molecular genetics, leading to significant advancements in the analysis of biofilm structure and character (Steven L. Percival, Knottenbelt, & Cochrane, 2011).

Biofilms exhibit a high degree of responsiveness to changes in internal and external conditions, with the capacity to rapidly adapt genetically, phenotypically and structurally to these conditions (Steven Lane Percival, Walker, & Hunter, 2000). Nevertheless, these adaptation capabilities remain unattainable *in vitro*. Consequently, researchers have been unable to replicate industrial or medical biofilms with fidelity under laboratory conditions. Consequently, endeavors to

establish a 'gold standard' biofilm model have met with failure (Steven L. Percival et al., 2011).

One of the fundamental components of the biofilm structure is the 'matrix'. In a biofilm formed under *in vitro* conditions, the matrix can constitute approximately 90% of the biomass. The remaining components consist of water and cells (H. Flemming & Wingender, 2010). The matrix structure also contains a mixture of various biopolymers, including polysaccharides, lipids, extracellular DNA (eDNA) and proteins (H.-C. Flemming, Neu, & Wozniak, 2007; H. Flemming & Wingender, 2010; Mayer et al., 1999). In addition to these components, cell-dependent secreted biopolymer structures, such as fimbriae, flagella and pili, also contribute to the composition of the matrix (Zogaj, Nimtz, Rohde, Bokranz, & Römling, 2001).

Biofilms do not only occur on body surfaces. They can also envelop various surfaces including tubes and implants, such as intravenous catheters, teeth and gums, lungs, ears, urogenital system and wounds (Potera, 1999).

2. Biofilm Formation Mechanism and Stages

The formation of a mature biofilm is a multi-step process that depends on several variables. These include the type of microorganism, the surface on which the film is formed, environmental factors and the genes required for the biofilm (Carpentier & Cerf, 1995; Dunne Jr, 2002).

The biofilm formation process can be subdivided into five fundamental stages (Donlan & Costerton, 2002).

1. The initial (or preparatory) film development on the surface.

2.The movement of microorganisms towards each other.

3.The adhesion (reversible or irreversible).

4.Growth and division of organisms with microcolony and biofilm formation as a result of colonization on the surface; phenotype and genotype changes.

5. Disaggregation of biofilm structure

In their natural environment, biofilm-forming bacteria do not directly adhere to the surface. Instead, they undergo a preparatory phase, termed the "pre-film" or "preparation film," which typically enhances the conditions of the original surface on which they subsequently form a biofilm (Mittelman, 1996).

The initial film formation phase is characterised by a thorough examination of the surface to ensure that it is sufficiently nutrient- and trace element-rich. During this phase, the physicochemical properties of the surface to which the bacteria will attach are optimised (Sauer et al., 2022).

Once the surface becomes conducive to attachment through the formation of a pre-film, the transport of microbial cells and nutrients to the surface is achieved through a number of well-established fluid dynamics. These include mass transfer, thermal effects (i.e. Brownian motion and molecular diffusion) and gravitational effects (i.e. differential settling and sedimentation) (Characklis, 1984).

Following the transportation of cells and nutrients to the surface, adhesion then occurs. Adhesion is a two-stage process, which can be categorised as reversible or irreversible (Zobell, 1943). The occurrence of reversible adhesion is characterised by the formation of weak bonds (Rittman, 1982), and the presence of nutrients essential for survival on the surface is subsequently investigated (İlhan & Ekinci, 2009). At this stage, the microorganism is not in full contact with the surface and weakly adheres with hydrophobic Van der Walls bonds (Duran, 2011). It is important to note that reversible binding is generally followed by non-reversible binding (Rittman, 1982). In the context of irreversible binding, a multitude of interactions, including dipole-dipole, hydrophobic, ion-dipole, ionic, covalent bonds, and hydrogen bonds, occur between the microorganism and the surface (Poulsen, 1999). The specific nature of these bonds, i.e. their reversibility or irreversibility, is determined by the distance between the bacteria and the surface (H. J. Busscher & Weerkamp, 1987).

Following irreversible attachment, cells undergo a process of growth and reproduction. This process of cell development gives rise to the formation of cell communities known as 'microcolony'. These microcolonies then attract other bacteria in a process known as 'chemotaxis' (Blenkinsopp & Costerton, 1991).

3. Biofilm Durability Mechanisms

The intricate architecture of the biofilm, in conjunction with the physiological traits of the microorganisms within it, engenders a high level of tolerance to antimicrobial agents such as antibiotics, disinfectants, germicides, and antifungals (Salmon et al., 1991; Mah & O'Toole, 2001). The resistance of biofilm-forming microorganisms to minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of antimicrobial agents is

generally 100-1000 times higher than their free forms. This finding suggests that their resistance to disinfectants is likely to be 150-3000 times greater (Wood, Leech, & Ohman, 2006).

The microorganisms present within the biofilm are embedded within a matrix, which is produced by the cells and is an extracellular polymeric substance. The matrix provides both nutrient storage for the organisms and mechanical protection against adverse conditions in the external environment. In addition, the matrix structure functions as a structure that allows microorganisms to be in close proximity to each other and plays a role in facilitating chemical communication and metabolite exchange between cells. The coexistence of diverse microorganisms in the biofilm suggests that these organisms transition from a prokaryotic state to a community organization, a transition that has been likened to the cell differentiation observed in eukaryotic organisms (Nikolaev & Plakunov, 2007; Penesyan, Paulsen, Kjelleberg, & Gillings, 2021; Shapiro, 1998; Watnick & Kolter, 2000).

The resistance of biofilm structure against antibiotics can be analyzed under two different concepts: resistance and tolerance. Antibiotic resistance is defined as the ability of a microorganism to proliferate and persist in the presence of elevated drug concentrations over an extended duration (Brauner, Fridman, Gefen, & Balaban, 2016). Tolerance is defined as the capacity of bacteria to survive when temporarily exposed to increasing concentrations of antibiotics, even those above the MIC value. However, it should be noted that biofilm resistance to antibiotics does not depend on only one of these two concepts. Rather, these mechanisms work in combination to resist antibiotics. This defence mechanism varies according to the type of bacteria, the type of antimicrobial agent and the structure of the biofilm (Hall & Mah, 2017).

The mechanisms underlying antibiotic resistance include QS, mutation, the presence of a thick exopolysaccharide layer, and horizontal gene transfer (HGT). The development of antibiotic tolerance is attributed to the presence of physiological and metabolic diversity within the biofilm (Uruén, Chopo-Escuin, Tommassen, Mainar-Jaime, & Arenas, 2020).

QS is hypothesised to contribute to the resistance of biofilms to antimicrobials. In natural conditions treated with QS inhibitors, biofilms formed by QS mutants or bacteria are more sensitive to antibiotics. A study demonstrated that *P. aeruginosa* biofilms formed by a QS-deficient mutant strain lacking *lasR* and *rhlR* genes were significantly more sensitive to tobramycin than biofilms formed under natural conditions (Bjarnsholt et al., 2005).

Mutations in the bacterial genome may play a role in increasing resistance to antibiotics (Woodford & Ellington, 2007). Mutation can also occur spontaneously without any environmental pressure. Bacteria resulting from these spontaneous mutations form a minority of the population (Schroeder, Yeesin, Simmons, & Wang, 2018). The mutation rate may increase rapidly as a result of exposure to agents that cause oxidative stress. Oxidative stress, in turn, gives rise to the formation of reactive oxygen species (ROS). These radicals have been demonstrated to directly cause mutations and damage to DNA. However, when exposed to non-lethal doses of bactericidal antibiotics, ROS accumulation is low and the microorganism can increase its resistance to the antimicrobial agent through induction of synthesis of multidrug efflux pumps and mutagenesis (Van Acker & Coenye, 2017). Depending on the biofilm structure, resistance develops by a similar mechanism as a result of exposure of microorganisms in the biofilm to non-lethal doses of antibiotics (Leong, Hsia, & Miller, 1986; Schaaper & Dunn, 1987).

The matrix constitutes the initial component of the biofilm with which antimicrobial agents come into contact. The matrix structure, which constitutes approximately 75-90% of the biofilm, acts as a diffusion barrier (R. Huang, Li, & Gregory, 2011). In order for the antimicrobial agent to penetrate the biofilm, it must overcome this thick exopolysaccharide layer (Fletcher, 1992). The antibiotic, unable to penetrate the matrix rapidly, enters the biofilm slowly and in limited quantities. This phenomenon is a contributing factor to the emergence of antimicrobial resistance (Szomolay, Klapper, Dockery, & Stewart, 2005).

Horizontal gene transfer (HGT) is a feature that enables the exchange of antimicrobial resistance genes between bacteria through five different mechanisms. The three most widely recognised of these five mechanisms are conjugation (direct gene transfer between cells), transformation (uptake of DNA from the environment) and transduction (gene exchange between bacterial cells via bacteriophages). Two further mechanisms involve the release of membrane vesicles that act as DNA reservoirs or long membranous structures called nanotubes used for direct cell-to-cell contact. Horizontal gene transfer has been observed to occur at a higher rate in microorganisms in biofilms than in their free form (Hausner & Wuertz, 1999).

The structure and organization of the biofilm also produces distribution gradients of nutrients, oxygen, pH, signaling molecules and waste products. In the deeper and inner layers of the biofilm, oxygen and nutrients become more difficult to access and these resources are depleted over time. As a result, various

physiological conditions occur within the biofilm, including different metabolic (aerobic, microaerobic and fermentative) and growth rates (H.-C. Flemming et al., 2016; PS, 2008). Sometime after the discovery of antibiotics, it was discovered that resting cells were less sensitive to penicillin. This phenomenon was named drug indifference (Lee, Foley, & Epstein, 1944; Mc Dermott, 1958). Again, non-dividing and resting cells were found to be completely resistant to ampicillin and tetracycline. Although ciprofloxacin and streptomycin are active against resting cells, their activity levels are lower than the active growth period of the microorganism (Levin & Rozen, 2006). It has also been suggested that the presence of drug-insensitive, slow-growing (or resting) cells may be the cause of relapse after antibiotic treatment in some bacterial infections (Clement et al., 2005; Fitoussi et al., 1997).

The bacterial community, which causes infection in the living organism and forms a biofilm, can bypass or escape the host's immune system in various ways. Although the relationship between leukocytes and biofilms is not fully known (Leid, Shirtliff, Costerton, Stoodley, & Paul, 2002), various enzymes produced by leukocytes cannot enter the biofilm due to the matrix structure. At the same time, a decrease in phagocytic capacity is observed. This phenomenon is called inhibited phagocytosis, macrophages and neutrophils cannot engulf biofilm forming bacteria (Leid, 2009).

4. Clinical Importance of Biofilm in Veterinary Medicine

It is evident that the risk of infection and biofilm formation is more prevalent in animal species than in humans, given the disparity in breeding and living conditions. Biofilms have been associated with numerous infectious diseases in animals, including mastitis, endometritis, chronic wounds and periodontal diseases. In addition to these direct effects, biofilm infections also exert significant indirect effects on the livestock sector, given their persistent and chronic nature, resulting in economic losses (AbduLLahi, Igwenagu, Mu'azu, Aliyu, & Umar, 2016; Princy Choudhary, 2020; Raheel, Hassan, Salem, & Salam, 2020).

Recent studies have demonstrated the prevalence of microbial biofilms in canine, feline and equine wounds. Nevertheless, the significance of these biofilms and the factors that regulate and promote their formation remain to be fully elucidated (König, Klopfleisch, Höper, & Gruber, 2014). While a significant number of studies on biofilms have been conducted on rodents, rabbits, pigs, dogs, horses and other animals, there is a paucity of research determining the clinical value of bacterial biofilms in veterinary medicine. Nevertheless, it is

frequently observed in veterinary clinics that animals, like humans, suffer from chronic wounds with biofilm formation (Jørgensen, Bjarnsholt, & Jacobsen, 2021).

Although oral infections are rare, chronic periodontal infections (especially dental caries, dental calculus) caused by biofilms of different bacterial species are one of the most common diseases in adult cats and dogs. It is known to affect approximately 80% of animals (Kačírová, Maďar, Štrkolcová, Maďari, & Nemcová, 2019; Zambori et al., 2012).

Biofilm formation is observed not only on living surfaces but also on inanimate surfaces such as medical devices. Biofilm formation on medical devices/tools that remain stationary for a long time can lead to serious chronic infections (Roberts, 2013). In the medical field, biofilm-associated infections are frequently reported on urinary catheters (Stickler, 2008), orthopaedic implants (Esteban et al., 2010), artificial heart valves (Venditti, 2009), dental implants (H. Busscher, Rinastiti, Siswomihardjo, & Van der Mei, 2010) and eye lenses (Behlau & Gilmore, 2008). Bacteria capable of forming biofilms on these devices/instruments can be transmitted from the patient's own skin, healthcare workers, tap water or other environmental sources (Roberts, 2013).

As a result, polymeric substances, exoenzymes and toxins in the biofilm structure delay wound healing and body repair, prolong the duration of inflammation in the living organism and cause chronic, persistent infections in the host (Costerton, Stewart, & Greenberg, 1999; Donlan & Costerton, 2002; Parsek & Singh, 2003).

5. Current Antibiofilm Approaches

In the present day, antimicrobial resistance represents a constantly growing threat. Of particular concern is the group of bacteria consisting of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp. and *Escherichia coli* (ESKAPEE), which show remarkable resistance to antibiotics. These bacterial genera have been identified by the World Health Organisation (WHO) among the top 10 antimicrobial resistance threats to global health. Moreover, the formation of biofilms by bacteria plays a pivotal role in the development of resistance to antimicrobial agents (Reza, Sutton, & Rahman, 2019). It has been reported that the amount of antibiotic required to destroy the biofilmed form of a bacterium is greater than the amount required to destroy its free form (Chen, Thomsen, Winkler, & Xu, 2020; Sedlacek & Walker, 2007). Consequently, the

development of novel, alternative strategies to combat antimicrobial resistance in pathogenic microorganisms has emerged as a pressing concern within the scientific community (Lu et al., 2019). As a result, many alternative treatments to antibiotics are being investigated. These include probiotics, bacteriophages, hyperbaric oxygen therapy, cold plasma therapy, phytochemicals and honey.

5.1. Probiotics

WHO defines probiotics as 'live microorganisms that provide health benefits when given to the host in sufficient quantities' (Reid, Gadir, & Dhir, 2019). The most well-known bacterial species as probiotic microorganisms are *Lactobacillus* spp., *Bifidobacterium* spp., *Escherichia* spp., *Enterococcus* spp., *Bacillus* spp. and *Streptococcus* spp. Some *Saccharomyces* spp. yeasts have also been used as probiotics (Alvarez-Olmos & Oberhelman, 2001; Gibson & Roberfroid, 1995; Jin, Marquardt, & Zhao, 2000). These microorganisms are found in high amounts in fermented foods and processed milk (Shi, Balakrishnan, Thiagarajah, Ismail, & Yin, 2016). Probiotics have many beneficial effects such as regulating and improving the gastrointestinal microflora of the host, strengthening the immune system and reducing serum cholesterol levels at an optimal level. In addition, probiotics are also used in cancer prevention, treatment of diarrhea associated with irregular bowel syndrome, treatment of depression and improvement of lactose metabolism (Nagpal et al., 2012; Saarela, Mogensen, Fonden, Mättö, & Mattila-Sandholm, 2000).

In addition to these effects, probiotics are known to have inhibitory effects on Gram positive and Gram negative bacteria through different mechanisms (F.-C. Huang, Lu, & Liao, 2020). Although these mechanisms are thought to produce antimicrobial compounds, to negatively affect their adhesion and nutrition by entering into a competitive relationship with pathogenic microorganisms in the environment, to increase the barrier strength by improving the function of the intestinal wall, and to regulate immunity, the principles of action of probiotics are still not fully understood today (Mohammad Yousef Memar, 2022).

In a study conducted on Holstein cows in Japan, probiotic consumption was proven to have a prophylactic effect against mastitis with biofilm formation. *Bacillus subtilis* C-3102 strain was added to the ratio of the experimental group starting one month before birth until the end of lactation and somatic cell count (SHC) was compared in the milk of the experimental group and the control group in the third month of the postpartum period. As a result of the measurement, it was observed that the amount of somatic cells in the milk of the experimental group was significantly lower than the control group (Urakawa et al., 2022).

In a study conducted in China, it was proved that *B. subtilis*, which is present in the natural flora of the breast, reduces the colonisation and biofilm formation of *S. aureus*. *B. subtilis* and *S. aureus* were isolated from mastitic and healthy animals, and these bacteria were inoculated into mice. As a result of the study on 60 mice, it was determined that *B. subtilis* reduced biofilm formation by regulating QS (Qiu et al., 2022).

5.2. Bacteriophages

It is recorded in historical documents that some river sources are healing for leprosy caused by *Mycobacterium leprae* in humans (Keen, 2012). British scientist Ernest Hankin observed in 1896 in the Ganges and Yamuna Rivers (India) that these waters had an 'unknown' antimicrobial effect against *Vibrio chlorea* (Adhya & Merril, 2006). The French-Canadian scientist Felix d'Herelle discovered bacteriogens, or 'phages' for short, during a study to control locusts in Mexico in 1910 and described them as viruses capable of lysing bacterial cells (Duckworth, 1976).

With the discovery of penicillin, phage therapy has been an option ignored by the scientific world (Eaton & Bayne-Jones, 1934). However, the development of resistance to antibiotics today has led phages to come to the forefront as an important alternative option in treatment. Phages have serious advantages over antibiotics in the treatment of bacterial infections. Since antibiotics are not agent-specific, they can cause secondary infections by damaging the normal flora, whereas phages do not have such effects since they are agent-specific. In addition, phages do not need to reach a certain concentration in the living body to show their effect, side effects are not observed and they do not carry the risk of resistance development (Ho, 2001; Matsuzaki et al., 2005; Sulakvelidze, Alavidze, & Morris Jr, 2001; Weber-Dąbrowska, Mulczyk, & Górski, 2001).

Various studies have shown that phages prevent or penetrate biofilms formed by mastitis agents *in vitro* and *in vivo*. These studies show that bacteriophages may be a stand-alone treatment option or may increase therapeutic efficacy as a supportive treatment in antibiotic use (Song et al., 2021; Teng et al., 2022).

Due to this potential, several research studies have been conducted on the isolation, characterisation and safety and efficacy testing of therapeutic phages in appropriate model systems for bovine mastitis with biofilm formation and MDR (Nale and McEwan 2023). In an *in vitro* study in the milk of cows with mastitis, it was noted that agent-specific bacteriophage significantly reduced the activity

of biofilm-forming MRSA (Mohammadian, Rahmani, Bidarian, & Khoramian, 2022).

5.3. Hyperbaric Oxygen Therapy

The chemical microenvironment around the biofilm is determined by many factors including bacterial metabolic activity, solute exchange with the environment, diffusion properties and limited bacterial mobility in the exopolymeric matrix of biofilms. One of these factors is the oxygen (O_2) level in the biofilm. It is known that there is a correlation between the O_2 level deep in the matrix structure and the O_2 level on the surface. The presence of different O_2 levels in different layers in the biofilm structure also allows the observation of various species that differ in terms of oxygen consumption (Jensen et al 2017). Under *in vitro* conditions, oxygen can reach up to 50 μm depth in a biofilm population formed with atmospheric O_2, while below this level has been shown to be suitable for microaerophilic and anaerobic microorganisms (Walters III, Roe, Bugnicourt, Franklin, & Stewart, 2003).

The sensitivity of most bacteria to antibiotics is determined by their metabolic activity (Cozens et al., 1986). The relationship between bacterial metabolism and antimicrobial susceptibility stems from the ability of low levels of metabolic activity to reduce the uptake of antimicrobial agents and downregulate the activity of antibiotic targets (Van Acker & Coenye, 2017). Aerobic respiration is also one of the factors determining metabolic activity (Lobritz et al., 2015). For this reason, it is thought that the O_2 concentration in the microenvironment of the biofilm may affect the level of antibiotic susceptibility (Stewart, 2003). In biofilm, aerobic respiration has been proven to increase susceptibility to antibiotics by removing O_2 from the biofilm (Borriello et al., 2004).

In addition, the presence of O_2 can cause lethal effect resulting from the formation of reactive oxygen species (ROS) at cytotoxic levels induced by antibiotics (Brochmann et al., 2014; Dwyer et al., 2014).

In infections with biofilm formation, bacterial aerobic respiration can be activated by alleviating O_2 limitation to combat hypoxia (oxygen deficiency) induced antibiotic tolerance. Thus, the sensitivity of microorganisms forming the biofilm to various antibiotics targeting metabolically active bacteria can be increased (Kolpen et al., 2016). It has been observed that the addition of O_2 to the biofilm structure by hyperbaric O_2 therapy (HBOT) significantly increases the bactericidal activity of various antibiotics *in vitro* (Lerche et al., 2017).

5.4. Cold Plasma Therapy

The substance formed by charging a neutral gas with energy until it ionizes and becomes electrically conductive is called plasma. Plasma is also defined as the fourth state of matter. In modern medicine, high-temperature plasmas are used for the sterilization of medical devices and implants. However, cold atmospheric pressure plasmas (CAP) can also be used for the treatment of living tissues and have therefore become the focus of medical research in recent years. Besides therapeutic applications, CAP is also used for surface modification and biological decontamination (Bernhardt et al., 2019). In healthcare, cold plasma can also refer to 'hot' plasma applied in pulsed bursts to prevent tissue heating. As long as the temperature of the tissue of the area does not exceed 40 C^o at any time during the application of the plasma, it can be classified as cold plasma therapy (von Woedtke, Emmert, Metelmann, Rupf, & Weltmann, 2020).

Plasma and plasma-activated fluids (PAL) are highly effective in sterilising surfaces contaminated with free bacteria, but microorganisms in biofilms are naturally more tolerant to sterilisation with plasma and PAL (Flynn et al., 2015). The tolerance of microorganisms in the biofilm to plasma varies depending on the maturity, thickness and microorganism of the biofilm structure (Ermolaeva et al., 2011). Plasma has been reported to be effective against ESKAPEE bacteria (Abdo, Schmitt-John, & Richter, 2021).

Cold plasma creates an environment containing atomic oxygen (O), superoxide (O_2^-), hydroxyl radicals (OH), singlet oxygen ($_1O_2$), ozone (O_3), hydrogen peroxide (H_2O_2), nitrous oxide (NO), nitrite/nitrate (NO_2^- / NO_3^-), nitrogen pentaoxide (N_2O_5), peroxynitrite ($ONOO^-$). This environment is called reactive oxygen and nitrogen species (RONS) (Nicol et al., 2020; Salgado, Fabbri, Dickenson, Hasan, & Walsh, 2021). High purity inert gases produce higher levels of reactive nitrogen species (RNS), but when other molecular gases (O_2, air, H_2O) are added to the inert gas, there is a large change in the production of reactive oxygen species (ROS) and a lower rate of RNS is produced. This allows the tailoring of specific reactive species in the plasma to ensure effective killing of different microbial species (Lietz & Kushner, 2018).

These molecules in the plasma react with complex biological components of the biofilm, such as water, ions, polysaccharides, eDNA, lipids and proteins, the composition of which may differ between bacterial species. Unlike antibiotics, which have a specific mechanism of action against bacteria, which microorganisms can then adapt to and resist, cold plasma produces a large number of reactive species and particles that cause damage in a variety of ways.

This difference gives cold plasma an advantage over conventional antimicrobials because bacteria cannot mount even a single response to resist or reverse the damage caused by cold plasma. It has also been proven that the presence of antioxidants and free radical scavengers such as ascorbic acid and α-tocopherol during plasma exposure provides antimicrobial effect and lipid peroxidation in *E. coli* and completely prevents DNA oxidation (Joshi et al., 2011). The ability of plasma to erode biofilm is another important process to physically remove the source of infection, which can be categorised as the antibiofilm activity of plasma (Xu et al., 2015).

In a study, the efficacy of cold plasma against *E. faecalis* and *S. aureus* suspensions grown on agar media, as well as against *E. faecalis* biofilm on nitrate membrane filters, was evaluated. Following cold plasma treatment, zones of inhibition were observed in agar cultures, and it was reported that the size of these zones increased as the treatment period prolonged (Cao et al., 2011).

5.5. Phytochemicals

Plants produce primary metabolites such as amino acids, fatty acids, carbohydrates and organic acids to survive and fulfil their necessary functions (Tian, Xu, Liu, Fan, & Pan, 2018). In addition to these metabolites, substances called secondary metabolites are also produced at lower concentrations in order for the plant to defend itself. These substances protect plants against most herbivores and microorganisms. At the same time, it also provides an advantage in the competition of the plant with other plants (Lopes et al., 2020). Phytochemicals are also among these secondary metabolites. These are metabolites that have biological activity and do not have nutritional properties (Jimenez-Garcia et al., 2018).

Phytochemicals have been the subject of investigation in order to identify new antimicrobial agents that differ from antibiotics and are effective against a range of organisms, including fungi, yeasts, bacteria and viruses (Wintola & Afolayan, 2015). A wide range of phytochemicals, including various classes of compounds, have been identified in leaves, roots, fruits, seeds, bark, stem bark and flowers of plants. These phytochemicals have the potential to serve as effective, economical and safe antimicrobial agents (Aung, Kristanti, Aminah, Takaya, & Ramadhan, 2020). The scientific community has expressed particular interest in plant extracts and their phytochemicals due to their cost-effectiveness, environmental friendliness, wide structural diversity and the possibility of reducing resistance to antimicrobial drugs (Giaouris & Simões, 2018).

Phytochemicals have been shown to inhibit DNA and RNA synthesis, change the hydrophobicity and permeability of bacterial membranes, destroy the structure of microbial membranes, inhibit peptidoglycan synthesis and interfere with QS by various mechanisms (Monte, Abreu, Borges, Simões, & Simões, 2014; Mori, Nishino, Enoki, & Tawata, 1987; Nazzaro, Fratianni, De Martino, Coppola, & De Feo, 2013).

Antibacterial and antibiofilm properties of essential oils in animals with mastitis were determined in a study conducted in cows with subclinical mastitis in Argentina. In this study, *E. coli*, *Bacillus pumilus* and *E. faecium* isolates were obtained from 15 cows and the oil obtained from the extract of *Minthostachys verticillata*, an endemic plant, was used as essential oil. As a result of the study, it was revealed that the essential oil obtained from *Minthostachys verticillata* inhibited the growth of biofilm-forming bacteria and disrupted the mature biofilm structure by 36.51-89.60% (Cerioli, Moliva, Cariddi, & Reinoso, 2018).

In a study conducted in China, the antimicrobial and antibiofilm effects of tea saponin on *Streptococcus agalactiae* isolated from the milk of cows with mastitis were investigated. As a result of the research, the inhibitory effect of the addition of tea saponin at a rate of 2 mg / ml to the biofilm structure of *S. agalactiae* formed in Tryptic Soy Broth on biofilm formation was proven (Shang, Wang, & Xue, 2020).

Quercetin (a plant flavonol from the flavonoid group found in capers, red onions and cabbage), which is among the phytochemicals showing antibiofilm character, shows an effect by reducing the adhesion strength in biofilm formation by inhibiting alginate production (Górniak, Bartoszewski, & Króliczewski, 2019).

In a study with usnic acid obtained from lichen species, it was shown that this substance, another phytochemical, has antimicrobial properties by resisting biofilm formed by *S. aureus* and other Gram-positive organisms. It was demonstrated that the formation of *S. aureus* biofilm on polymer surfaces modified with usnic acid was successfully inhibited for up to 6 days under harsh conditions with high bacterial concentrations (Francolini, Norris, Piozzi, Donelli, & Stoodley, 2004).

It has also been reported that ‘emodin’, often derived from rhubarb, restricts the biofilm development abilities of *P. aeruginosa*, *E. coli* and *S. aureus* by reducing the expression of key genes involved in biofilm formation (Borges et al., 2016).

Antimicrobial and antibiofilm effects of various phytochemicals on *Staphylococcus* species were investigated. Thirty different *Staphylococcus* strains were obtained from 100 cows with subclinical mastitis. Cinnamaldehyde (cinnamon), eugenol (clove), carvacrol (thyme), thymol (thyme) and citral (lemon) were used as phytochemicals in the *in vitro* study. As a result of the study, antimicrobial and antibiofilm effects of these substances were revealed (Unlu et al., 2018).

5.6. Honey

Honey, a sweet and flavourful natural product produced by bees from plant nectar, plant secretions and secretions of plant-sucking insects (Alvarez-Suarez, Gasparrini, Forbes-Hernández, Mazzoni, & Giampieri, 2014), was used for medicinal purposes in ancient India, Egypt, Greece and the Islamic world (Eteraf-Oskouei & Najafi, 2013). Nowadays, scientists consider honey to be a useful natural substance as a complement to modern therapy (Mărgăoan et al., 2021), thanks to its suppression of uncontrolled cell proliferation (Fernandez-Cabezudo et al., 2013), antimicrobial activity (Almasaudi, 2021) and antioxidant effect (Erejuwa, Sulaiman, & Ab Wahab, 2012).

The significance of numerous honey species in clinical applications has prompted the interest of scientists. A notable species is Manuka honey, which is produced from the nectar of Manuka trees (*Leptospermum scoparium*) by honeybees of the genus Apis mellifera. It possesses a distinctive monofloral character. The antibiofilm property of Manuka honey is attributable to elevated phenolic acid levels, hyperosmolarity, hydrogen peroxide levels and methylglyoxal (MGO), the active component of Manuka honey (Johnston, McBride, Dahiya, Owusu-Apenten, & Nigam, 2018; Ooi et al., 2019). This honey has been shown to exhibit an antibiofilm property without the risk of triggering antibacterial resistance (Johnston et al., 2018) and has been shown to promote the synthesis of several cytokines including TNF-α, IL-1β and IL-6 via TLR4 (Tonks et al., 2003).

In a study, multifloral honey obtained from local honey producers in the region was injected into the udder of three cows showing subclinical mastitis and isolated *S. aureus* and *P. aeruginosa*, which form biofilms, in order to observe the antibiofilm effect *in vivo*. Furthermore, 5ml of honey was added to Mueller Hinton Agars inoculated with *S. aureus* and *P. aeruginosa* to measure *in vitro* MIC. The study concluded with the observation that locally obtained honey exhibited robust antibacterial properties against both *S. aureus* and *P. aeruginosa*. In addition, the *in vivo* infusion of honey into the mammary gland was found to

result in a substantial reduction in the total bacterial count (Benhanifia, Ayad, & Mohamed, 2020).

Conclusion

The formation of biofilms by pathogenic bacteria is a significant challenge in the treatment of infections in humans and animals. This problem persists due to the inadequacy of various methods employed in the fight against bacterial infections involving biofilm formation. Despite extensive research on biofilm structure, the underlying mechanisms of this complex structure remain to be fully elucidated.

The era of antibiotic discovery, which commenced in 1928 with the treatment of infectious diseases, is drawing to a close. This is primarily due to the development of resistance by bacteria to these agents. Moreover, the identification of novel antibiotics appears to be a challenging process, both in terms of time and financial investment. The endeavor to combat biofilm formations, wherein bacteria exhibit heightened resistance to antibiotics in comparison to their free state, is becoming increasingly arduous due to the diminishing availability of effective antibiotics. Furthermore, given the intricate nature of the biofilm structure, which facilitates the development of resistance to antimicrobial agents, it becomes imperative to explore alternative control methodologies.

The formation of biofilms in bacterial infections in animals has been demonstrated to increase the severity of the infection by rendering treatment difficult and creating a significant economic burden on animal breeders due to yield losses and treatment costs. The persistence of this structure and its ability to recur render complete recovery a considerable challenge. Furthermore, the development of resistance to available antibiotics, which is already a concern in the veterinary field, is further exacerbated by this process. Consequently, there is a global focus on researching alternative methods and substances capable of disrupting the biofilm structure and inactivating the microorganisms it contains.

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CHAPTER 9

Acute Hypertensive Crisis in Cerebrovascular Diseases: Emergency Management Approaches

Erkan Boğa

Introduction

Cerebrovascular diseases are among the leading causes of death and disability worldwide, imposing a significant economic and social burden on individuals and societies (1). These diseases typically manifest as ischemic or hemorrhagic strokes caused by pathological changes in the cerebral vasculature. Hypertension is the most significant and modifiable risk factor for cerebrovascular diseases (2). Uncontrolled chronic hypertension weakens arterial walls, promotes the progression of atherosclerosis, and ultimately impairs cerebral blood flow. In acute cases, hypertensive crises can occur, adversely affecting the patient's overall clinical course (3).

A hypertensive crisis is characterized by a sudden and dangerous increase in blood pressure. Systolic blood pressure of ≥180 mmHg or diastolic blood pressure of ≥120 mmHg often triggers target organ damage (4). Hypertensive crises are categorized into hypertensive emergencies, requiring immediate intervention due to associated organ damage, and hypertensive urgencies, where blood pressure control is needed without evidence of organ damage (5). In acute cerebrovascular diseases, hypertensive crises present a complex clinical picture. Blood pressure control requires a delicate balance between preventing hematoma expansion in hemorrhagic strokes and maintaining cerebral perfusion in ischemic strokes (6).

The primary challenge in managing hypertensive crises is to lower blood pressure while preserving cerebral perfusion. The brain is an organ that requires stable blood flow. Excessive and rapid reductions in blood pressure can lead to cerebral hypoperfusion, causing irreversible damage to ischemic tissues (7). Conversely, uncontrolled hypertension increases intracranial pressure, worsens neurological complications, and adversely affects the prognosis. Therefore, managing hypertensive crises requires an individualized approach, considering patient characteristics, stroke type, and comorbidities.

Pathophysiologically, hypertensive crises result from a combination of factors, including disruption of cerebral autoregulation, sympathetic nervous system activation, oxidative stress, and inflammatory processes. Cerebral autoregulation normally ensures stable blood flow to the brain, but this mechanism becomes insufficient during hypertensive crises. This leads to both hyperperfusion and hypoperfusion, with adverse consequences. Hyperperfusion causes disruption of the blood-brain barrier and cerebral edema, while hypoperfusion results in irreversible damage to ischemic tissues. Additionally, cardiac complications, reduced renal perfusion, and inflammation triggered by

sympathetic system activation further complicate the systemic effects of hypertensive crises (8).

Advanced technologies and biomarkers are becoming increasingly important in the diagnosis and management of hypertensive crises. For instance, near-infrared spectroscopy (NIRS) is an innovative method for real-time monitoring of cerebral oxygenation (9). Moreover, new biomarkers have the potential to improve early diagnosis and guide treatment strategies for hypertensive crises. However, further research is needed to expand and optimize the use of these technologies effectively.

The management of hypertensive crises requires a multidisciplinary approach. Collaboration among cardiologists, neurologists, nephrologists, and intensive care specialists is crucial to preventing complications and improving treatment outcomes. However (10), implementing current guidelines presents various challenges. Aligning guidelines with individualized clinical decisions, optimizing treatment targets, and enhancing patient adherence are essential in this process. Additionally, preventive strategies such as lifestyle modifications and regular medication use play a critical role in the long-term management of hypertensive crises.

This chapter aims to provide a comprehensive examination of hypertensive crises in acute cerebrovascular diseases. It will address the pathophysiology, diagnostic criteria, management strategies, and challenges encountered during the process. Furthermore, individualized treatment approaches and recommendations for future research will be discussed. By contributing to the development of more effective strategies in hypertensive crisis management, this chapter seeks to guide healthcare professionals and researchers.

Pathophysiology of Hypertensive Crises

Hypertensive crises are characterized by a sudden and excessive increase in systemic blood pressure, leading to potential organ damage through a complex pathophysiological process. This process typically arises from a combination of vascular, neurological, and systemic mechanisms. Understanding the pathophysiology of hypertensive crises is crucial for their effective management.

1. Disruption of Cerebral Autoregulation

Cerebral autoregulation is a fundamental mechanism that maintains stable cerebral blood flow despite changes in systemic blood pressure. Under normal conditions, cerebral autoregulation sustains blood flow when systemic blood

pressure is within the range of 60-160 mmHg (11). However, during hypertensive crises, blood pressure exceeding this range disrupts autoregulation, resulting in:

- **Hyperperfusion:** Elevated systemic blood pressure leads to excessive blood flow to the brain, causing disruption of the blood-brain barrier, extravasation of plasma proteins, and the development of cerebral edema. Cerebral edema increases intracranial pressure and triggers neurological impairments (12).
- **Hypoperfusion:** Disruption of autoregulation reduces blood flow to ischemic regions, exacerbating ischemic tissue damage, particularly in individuals with pre-existing vascular disease.

2. Endothelial Dysfunction

Endothelial dysfunction in hypertensive crises arises from an imbalance between vasoconstrictive and vasodilatory factors. Normally, endothelial cells produce nitric oxide (NO), a potent vasodilator. However, during hypertensive crises:

- **Reduced Nitric Oxide Production:** Increased oxidative stress reduces NO synthesis, leading to the production of reactive oxygen species (ROS) instead.
- **Increased Vasoconstriction:** Endothelial cells produce higher levels of vasoconstrictive substances, such as endothelin-1, which elevate peripheral vascular resistance and exacerbate hypertension.

Endothelial dysfunction contributes to impaired organ perfusion and aggravates organ damage.

3. Activation of the Sympathetic Nervous System

Sympathetic nervous system (SNS) activation is a key trigger for the excessive rise in blood pressure during hypertensive crises. Excessive SNS activation results in:

- **Increased Cardiac Output:** Elevated heart rate and myocardial contractility increase cardiac workload.
- **Increased Vascular Resistance:** Arterial vasoconstriction raises systemic vascular resistance, increasing the risk of organ damage.

- **Activation of the Renin-Angiotensin-Aldosterone System (RAAS):** SNS activation overstimulates the RAAS, leading to enhanced vasoconstriction and fluid retention, which further elevate blood pressure.

These mechanisms amplify the systemic effects of hypertensive crises and worsen organ damage.

4. Inflammatory and Oxidative Stress Mechanisms

Inflammation and oxidative stress play critical roles in the pathophysiology of hypertensive crises. Elevated blood pressure and endothelial dysfunction trigger the release of pro-inflammatory cytokines and reactive oxygen species (ROS):

- **Pro-inflammatory Cytokines:** TNF-α, IL-6, and other inflammatory mediators promote vascular inflammation, increasing endothelial damage and vascular permeability.
- **Reactive Oxygen Species (ROS):** Increased ROS production causes cellular damage, including lipid peroxidation and protein oxidation. ROS also reduces NO bioavailability, impairing vasodilation.

These processes exacerbate damage at both microvascular and macrovascular levels, intensifying the systemic impact of hypertensive crises.

5. Target Organ Damage Mechanisms

Target organ damage during hypertensive crises commonly affects high-perfusion organs such as the brain, heart, kidneys, and retina:

- **Brain:** Hypertensive encephalopathy arises from blood-brain barrier disruption and cerebral edema, leading to neurological symptoms such as headaches, altered consciousness, and seizures.
- **Heart:** Cardiac complications, including myocardial ischemia, acute left ventricular failure, and pulmonary edema, are frequently observed.
- **Kidneys:** Acute kidney injury results from reduced renal perfusion and a decrease in glomerular filtration rate.
- **Retina:** Hypertensive retinopathy, characterized by damage to retinal vessels, can lead to vision loss.

6. Disruption of the Blood-Brain Barrier

The blood-brain barrier normally acts as a selective filter protecting brain tissue from systemic circulation (13). During hypertensive crises, increased permeability of the blood-brain barrier leads to:

- Leakage of plasma proteins and fluids into cerebral tissue,
- Development of cerebral edema,
- Worsening of neurological impairments.

The pathophysiology of hypertensive crises involves a series of complex mechanisms, including cerebral autoregulatory failure, endothelial dysfunction, sympathetic nervous system activation, inflammatory responses, and oxidative stress. These processes culminate in target organ damage and potentially fatal outcomes. Understanding these mechanisms is essential for developing individualized treatment approaches and improving patient outcomes in the management of hypertensive crises.

Emergency Management of Hypertensive Crisis

Hypertensive crises are medical emergencies characterized by a sudden and dangerous rise in blood pressure, posing a risk of target organ damage. In acute cerebrovascular diseases, hypertensive crises can exacerbate both neurological and systemic complications, potentially leading to fatal outcomes if not managed appropriately. Emergency management in these cases aims to carefully control blood pressure, preserve cerebral perfusion, and prevent target organ damage (14).

1. General Principles of Emergency Management in Hypertensive Crisis

Emergency management is tailored based on patient characteristics, the type of hypertensive crisis, and any comorbid conditions. The general principles are as follows:

1. **Target Blood Pressure Levels:**
    - Blood pressure should be cautiously reduced to prevent target organ damage and improve neurological prognosis.
    - **Hypertensive Emergencies:** Mean arterial pressure should be reduced by 20-25% within the first hour and then gradually normalized over 24-48 hours.

- **Hypertensive Urgencies:** A less aggressive approach is adopted, with blood pressure gradually reduced over 24-48 hours.

2. **Rate of Blood Pressure Reduction:**
    - Sudden reductions can cause cerebral hypoperfusion, leading to irreversible damage in ischemic tissues.
    - Blood pressure should be lowered slowly and in a controlled manner.
3. **Assessment of Target Organ Damage:**
    - Neurological evaluation, cardiac function, renal function, and ophthalmic examination should be conducted to assess the presence of target organ damage.

2. Management of Hypertensive Crisis in Acute Cerebrovascular Diseases

The management of hypertensive crises in acute cerebrovascular diseases varies depending on the specific type of disease. Strategies differ for ischemic and hemorrhagic strokes.

A. Acute Ischemic Stroke

Careful management of blood pressure in ischemic stroke is critical for preserving perfusion in the penumbra region and preventing the risk of hemorrhagic transformation. Key strategies include:

1. **Blood Pressure Control:**
    - **Candidates for Reperfusion Therapy (Thrombolysis or Mechanical Thrombectomy):**
        - Blood pressure should be reduced to below 185/110 mmHg.
        - Intravenous antihypertensive agents are used to achieve this target.
    - **Patients Not Receiving Reperfusion Therapy:**
        - Intervention is recommended if blood pressure exceeds 220/120 mmHg.
        - Lower levels typically do not warrant intervention.

2. **Pharmacological Treatment:**
    - **Nicardipine:** Preferred for its ease of titration and rapid effect.
    - **Labetalol:** Frequently used as an effective and well-tolerated agent.
    - **Esmolol:** Provides rapid control in acute situations.
3. **Prevention of Complications:**
    - Avoid hypotension and closely monitor cerebral perfusion.
    - Minimize the risk of cerebral edema and neurological deterioration.

B. Acute Hemorrhagic Stroke

Hypertension in hemorrhagic stroke can increase hematoma expansion and worsen mortality. Aggressive blood pressure control is necessary.

1. **Blood Pressure Control:**
    - Systolic blood pressure should be reduced to below 140 mmHg within the first hour.
    - Blood pressure should be lowered in a controlled manner to avoid sudden drops.
2. **Pharmacological Treatment:**
    - **Nicardipine:** A reliable and fast-acting agent.
    - **Labetalol:** Commonly used intravenous antihypertensive drug for hemorrhagic strokes.
    - **Hydralazine:** Can be used as an alternative in selected patients.
3. **Management of Complications:**
    - Monitoring should be performed to prevent increased intracranial pressure.
    - Maintain target blood pressure levels to limit hematoma expansion.

3. Management of Hypertensive Encephalopathy

Hypertensive encephalopathy is a neurological complication of hypertensive crises, characterized by headaches, nausea, vomiting, visual disturbances, and altered consciousness. Treatment strategies include:

1. **Blood Pressure Reduction:**
    - Blood pressure should be reduced by 20-25% within the first hour.
    - Cerebral perfusion must be maintained during this process.
2. **Pharmacological Treatment:**
    - **Nicardipine and Labetalol:** Preferred first-line agents.
    - **Fenoldopam:** An alternative intravenous agent.
3. **Management of Complications:**
    - Dexamethasone or mannitol may be used in the presence of cerebral edema.
    - Neurological functions should be regularly monitored.

4. Pharmacological Management

The primary intravenous antihypertensive agents used in hypertensive crises include:

1. **Nicardipine:** A calcium channel blocker with rapid action and reliability.
2. **Labetalol:** Blocks both alpha and beta-adrenergic receptors, controlling both heart rate and blood pressure.
3. **Esmolol:** Particularly useful in cases with accompanying cardiac complications.
4. **Hydralazine:** A direct vasodilator that effectively lowers blood pressure.
5. **Nitroprusside:** A potent vasodilator, though its use should be cautious due to its potential to increase cerebral blood flow.

5. Patient Monitoring and Supportive Care

Patient monitoring and supportive care are essential in managing hypertensive crises:

- **Hemodynamic Monitoring:** Continuous monitoring of blood pressure, heart rate, and oxygen saturation.
- **Neurological Evaluation:** Frequent reassessment of neurological status.
- **Laboratory Testing:** Monitoring renal function, electrolyte levels, and blood biochemistry.

Emergency management of hypertensive crises in acute cerebrovascular diseases requires careful planning and an individualized approach. Controlled reduction of blood pressure is critical to improving neurological prognosis and preventing organ damage. A multidisciplinary approach based on current guidelines ensures successful management of these conditions. Additionally, patient education and long-term measures, such as lifestyle modifications, play a complementary role in preventing the recurrence of hypertensive crises.

Conclusion

The management of hypertensive crises in acute cerebrovascular diseases requires patient-specific, individualized approaches. During the emergency treatment of hypertensive crises, careful blood pressure reduction aims to prevent target organ damage while preserving cerebral perfusion. Adopting different management strategies for ischemic and hemorrhagic strokes is crucial for preventing complications and improving neurological outcomes.

In recent years, advancements in imaging techniques and the increasing use of biomarkers in hypertensive crisis management have enabled the development of more precise treatment approaches. However, challenges in applying existing guidelines have emphasized the importance of multidisciplinary collaboration and personalized treatment strategies based on patient-specific characteristics.

During the management of hypertensive crises, hemodynamic and neurological monitoring are as important as the effectiveness of pharmacological treatments. The antihypertensive drugs used must be effective, fast-acting, and safe, playing a decisive role in the treatment process. Furthermore, in the long term, patient education, lifestyle modifications, and regular follow-up are essential to reduce the risk of recurrent hypertensive crises.

In conclusion, the effective management of hypertensive crises requires the combined use of evidence-based guidelines, technological innovations, and multidisciplinary approaches. Future research in this field is expected to further optimize treatment protocols and improve patient outcomes. Managing hypertensive crises is not only a clinical challenge but also a multidisciplinary effort requiring continuous development and collaboration.

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CHAPTER 10

Community-Acquired Pneumonia and Covid-19 Pneumonia:

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1.Introduction

The novel coronavirus (COVID-19) first emerged in Wuhan, China, in December 2019 and was declared a pandemic by the World Health Organization (WHO) on March 11, 2020. Throughout the COVID-19 pandemic, various sources have reported different figures regarding the global death toll. As of January 1, 2023, more than 656 million confirmed cases and over 6.6 million deaths have been documented worldwide. However, some experts suggest that the actual number of deaths may be higher than the officially reported figures, with estimates indicating that COVID-19-related fatalities could have exceeded 20 million globally (Jangnin et al.2024; Jeffrey et al.2024).

The management of symptoms in patients diagnosed with COVID-19 is one of the primary objectives of healthcare services. During the treatment process, antiviral drugs are primarily administered, and patients are isolated to prevent the spread of the virus (Kader et al.2024; Teopiz et al.2024).

As a newly identified disease, COVID-19 has also introduced a new dimension to nursing care. Nurses providing care to COVID-19 patients primarily manage symptoms based on their clinical experience (Majrashi et al.2021; Tolksdorf et al.2022). It has been emphasized that nurses, who are at the forefront of the fight against the pandemic, require evidence-based practices to ensure effective care. Among the complications caused by COVID-19, pneumonia is particularly noteworthy. Studies indicate that approximately 7% of COVID-19 cases develop pneumonia, the majority of whom require intensive care. Moreover, a significant proportion of COVID-19-related deaths are attributed to pneumonia (Hamadi et al.2021; Majrashi et al.2021; Tolksdorf et al.2022).

Nursing care for patients with COVID-19 pneumonia encompasses both COVID-19 protocols and established pneumonia management strategies (Attaway et al.2021;Majrashi et al.2021).The symptoms of viral pneumonia caused by COVID-19 are similar to those of influenza and other respiratory tract infections, which are particularly prevalent during the winter months. However, the laboratory and radiological findings of COVID-19 pneumonia differ from those of conventional pneumonia (Hamadi et al.2021;Lv et al.2021). Accurate diagnosis of COVID-19 and the appropriate administration of treatment and nursing care rely heavily on clinical manifestations. As the pandemic progresses, nursing care practices for COVID-19 pneumonia continue to evolve. Therefore, nurses require updated guidelines to effectively manage the disease and its emerging complications.

This study aims to contribute to the nursing care of COVID-19 pneumonia by identifying the similarities and differences between the nursing care practices for COVID-19 pneumonia and non-COVID-19 pneumonia.

2. Methods

2.1 Study Design and Participants

This study was designed as a case-control study within the framework of the thesis titled *"Comparison of Clinical Course and Nursing Care in Pneumonia Developing After COVID-19 and Community-Acquired Pneumonia."* The study aims to evaluate the differences in nursing care provided to patients diagnosed with community-acquired pneumonia (n=5) and COVID-19-induced pneumonia (n=5) who were hospitalized in the Chest Diseases Service of Istanbul University-Cerrahpaşa Faculty of Medicine in May 2020.

Ethical approval for the study was obtained from the Non-Invasive Clinical Research Ethics Committee of Istanbul University-Cerrahpaşa (Date: 07.11.2024, Ethical Approval Number: 2024/191).

2.2 Research Inclusion and Exclusion Criteria

The inclusion criteria for the study were as follows:

Being over 18 years of age.

Having a confirmed COVID-19 diagnosis based on a positive RNA test.

Presence of ground-glass opacity on computed tomography (CT) scans, considered compatible with COVID-19 pneumonia.

Diagnosis of non-COVID-19 pneumonia (community-acquired pneumonia).

Pregnant women were excluded due to the complexity of physiological and immunological factors. Additionally, patients with incomplete file records were not included in the analysis.

2.3 Data Collection

In this study, clinical charts, nursing records, laboratory findings, chest X-ray reports, and nursing care plans of patients hospitalized with COVID-19-induced pneumonia and community-acquired pneumonia in May 2020 were retrospectively reviewed.

To minimize potential effects on laboratory results, only patients with a similar disease duration were evaluated. Clinical, radiological, and laboratory data were retrieved from hospital records, and the researcher did not interact directly with the patients or their families.

A total of ten patients with complete medical records and nursing care plans were included in the study.

2.4 Statistical Analysis

Categorical data were described as continuous data as median with interquartile range (IQR) and percentages (%). Nonparametric comparative test for continuous data and $\chi 2$ test for categorical data were used to compare variables between groups. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using SPSS Statistics version 22.0 software.

3. Results

3.1 Demographic Characteristics of Cases

Demographic characteristics of cases are shown in Table 1, 5 COVID-19 pneumonia patients and 5 non-COVID-19 pneumonia patients had included in this study. The mean age was 52.80± 13.28 (35-88) years and 55.85± 14.79 (25-79) years in COVID-19 and non-COVID-19 patients. There were 2(40%) female COVID-19 pneumonia patients, and 1(20%) female non-COVID-19 pneumonia patients. COVID-19 pneumonia patients have the highest hypertension 3 (60%), non-COVID-19 patients have COPD 3 (60%) comorbidity.

Table 1: Characteristics of COVID-19 Pneumonia Patients and Non-COVID-19 Pneumonia Patients

Variable	**COVID-19 Patients (n=5)**	**Non-COVID-19 (n=5)**
Age median years	52.80± 13.28 (35-88)	55.85± 14.79 (25-79)
Male n (%)	3 (60)	4 (80)
Comorbidities n(%)		
Hypertension	3 (80)	4 (80)
Chronic obstructive pulmonary disease	1 (20)	1 (20)
Rheumatoid arthritis	1 (20)	-

COVID-19, coronavirus 2019

3.2 Illness Features of Patients

In patients diagnosed with COVID-19, dyspnea (100%) and myalgia or arthralgia (100%) were the most common symptoms, while fever (60%), cough (60%), fatigue/weakness (60%), sore throat (60%), and headache (60%) were also frequently observed. In non-COVID-19 pneumonia patients, the most common symptoms were fever (60%) and cough (60%), while less common symptoms included headache (20%) and sore throat (20%). Upon admission, among COVID-19 patients, 3 (60%) had bilateral involvement, and 4 (80%) had multiple lobular ground-glass opacities (Table 2). In comparison, among non-COVID-19 pneumonia patients, 2 (40%) had bilateral involvement, and except for 1 (20%) patient who had multiple patchy and mottling shadows with partial ground-glass opacity, no similar findings were observed (Table 2).

Table 2: Clinical Characteristics of COVID-19 Pneumonia Patients and Non-COVID-19 Pneumonia Patients

Variable n(%)*	**COVID-19 Patients (n=5)**	**Non-COVID-19 (n=5)**
Symptoms and signs		
Fever	3 (60)	3 (60)
Cough	3 (60)	3 (60)
Fatigue-weakness	3 (60)	1 (20)
Sore throat	3 (60)	1 (20)
Headache	3 (60)	1 (20)
Dyspnea	5 (100)	2 (40)
Myalgia or artralgia	5 (100)	1 (20)
Chest CT findings		
Unilateral pneumonia	2 (40)	4 (80)
Bilateral pneumonia	3 (60)	1 (20)
Multiple mottling and ground-glass opacity	4 (80)	1 (20)

COVID-19, coronavirus 2019; CT, computed tomography,

* More than one answer was given and percentages were taken from n.

3.3 Laboratory Test Results of the Patients

Laboratory test results of the patients are shown in Table 3. In this study, white blood cell (WBC) counts of investigated COVID-19 pneumonia patients were in the normal range (8.99±4.20) but non-COVID-19 pneumonia patients were not in the normal (12.36±0.72). There was a statistical difference between patients. Compared with non- COVID-19 pneumonia, COVID-19 pneumonia patients had higher levels of ratio of neutrophils (>0.05), aspartate aminotransferase (AST) (<0.001), alanine aminotransferase (ALT) (<0.001), lactate dehydrogenase (LDH) (<0.001), C-reactive protein (CRP) (<0.001), D-dimer (<0.001), procalcitonin (<0.001). In addition, a proportion of COVID-19 pneumonia patients but no non-COVID-19 pneumonia patients had abnormally increased AST (10.0-274.0), ALT (7.0-854.0), LDH (164-3547), CRP (5.10- 310.90), D-dimer (151-6212), procalcitonin (195-433). SpO2 of COVID-19 pneumonia patients had 78-97%, non-COVID-19 pneumonia patients had 80-94% (p>0.05) (Table 3).

Table 3: Laboratory findings of patients with COVID-19 pneumonia and patients with Non- COVID-19 pneumonia.

Laboratory test results	**COVID-19 Patientes (n=5)**	**Non-COVID-19 Patientes (n=5)**	**P***
White blood cell (WBC 4-10x109/L) mean (range)	8.49±5.24 (3.20-22.78)	11.28±1.47 (11.45-13.74)	<0.001
Ratio of neutrophils (45-75%) mean (range)	68.24±15.74 (2.5-85.0)	54.12±17.58 (27.4-84.3)	<0.05
Aspartate aminotransferase (AST) (15-40 U/L) mean (range)	54.05±45.68 (11.0-247.0)	22.45±8.78 (12.4-34.0)	<0.001
Alanine aminotransferase (ALT) (9-50 U/L) mean (range)	78.04±14.54 (8.0-5.5)	21.45±6.87 (12.0-38.0)	<0.001
Lactate deydrogenase (LDH) (120-250 U/L) mean (range)	479.25±47.45 (167.0-3577.0)	158.57±25.87 (145-225)	<0.001
C-reactive protein (CRP) (0-4 mg/L) mean (range)	78.12±48.12 (4.10-210.80)	48.87±48.12 (22.00-154.00)	<0.001
Procalcitonin (0-0.05 µg/L) mean (range)	3.78±4.78 (0.03-18.54)	0.20±0.12 (0.24-0.78)	<0.001
D-dimer (0-500 mg/L) mean (range)	1120±1548.02 (147-6845)	214.28±141.47 (195-453)	<0.001

COVID-19, coronavirus 2019, P*: Mann- Whitney U test

3.4 Nursing Care and Treatments

Nursing Care and treatments of the patients are shown in Table 4. Similar nursing practices applied to COVID-19 and non-COVID-19 patients; isolated room, maintain rest-sound sleep, maintain personal hygiene, oral care, monitor vital signs, management fever, maintain SpO2>90%, maintain acid-base balance, 30-45 degrees head-end elevation, assessing the risk of pressure sores, provide fluids and nutritious high protein diet with vitamins and minerals. However different nursing practices applied to COVID-19; pron position, follow-up of patients who need dialysis treatment due to pneumonia, follow-up coagulation profile (Procalcitonin, LDH, D-dimer), liver-renal function (ALT, AST, Creatine, Urea, Albumin), assessing signs of DVT and psychological support. 4 patients (80%) received corticosteroid, 3 patients (60%) received expectorant, 3 patients (60%) received vitamin C or B complex, 5 patients (100%) received anticoagulant. All of the COVID- 19 pneumonia patients received the antiviral drug, while non-COVID-19 pneumonia patients received antibiotics.

4. Discussion

In this study, the nursing care of patients with COVID-19 pneumonia and non-COVID-19 pneumonia was retrospectively examined. It was determined that the majority of the patients included in the study had hypertension and diabetes (Table 1). This finding is consistent with previous studies in the literature suggesting that COVID-19 pneumonia is more prevalent among elderly individuals with chronic conditions such as hypertension and diabetes (Attaway et al. 2021; Lv et al.2021).

Upon examining the clinical findings, dyspnea, fever, cough, and fatigue were identified as the most frequently observed symptoms in both patient groups. However, while dyspnea was found to be the most common symptom in patients with COVID-19 pneumonia, fever was the predominant symptom in those with non-COVID-19 pneumonia (Table 2). These symptoms have also been frequently reported in cases of influenza and other respiratory infections (Macias et al.2021). Furthermore, the patient data obtained in this study revealed significant differences in computed tomography (CT) imaging findings between COVID-19 pneumonia and non-COVID-19 pneumonia patients. Specifically, a large proportion of COVID-19 pneumonia patients exhibited bilateral pneumonia characterized by multiple nodular infiltrations and ground-glass opacities on CT scans (Table 2). This finding aligns with previous studies in the literature (Jeffrey, et al.2024; Kader et al.2024). The evaluation of these radiological and clinical findings related to COVID-19 pneumonia may contribute significantly to nursing

care processes. It is well established that placing critically ill COVID-19 pneumonia patients in the prone position is recommended based on the assessment of their symptoms and clinical findings (Attaway et al.2021;Majrashi et al.2021). However, more comprehensive studies are needed to integrate such clinical indicators into nursing care practices effectively.

Regarding laboratory findings, alterations in white blood cell (WBC) and neutrophil counts were observed in most patients with COVID-19 pneumonia and non-COVID-19 pneumonia (Table 3). This result suggests that COVID-19 pneumonia, like other respiratory viral infections, triggers an inflammatory immune response (Zhang et al.2022).

Additionally, significantly elevated levels of lactate dehydrogenase (LDH), C-reactive protein (CRP), and procalcitonin (PCT) were detected in patients with COVID-19 pneumonia (Table 3). LDH and CRP are recognized as inflammatory markers in various pulmonary diseases [17,18]. In particular, in cases with increased disease severity and bacterial coinfection, PCT levels exceeding 0.5 μg/L have been reported (Ozbay et al.2023). Recent studies have also demonstrated that patients with COVID-19 requiring intensive care exhibit significantly higher levels of LDH, CRP, and PCT (Pore et al.2021; Macias et al.2021). These findings are crucial for assessing the clinical progression of COVID-19 pneumonia and guiding nursing care practices. Further research is warranted to strengthen these observations and enhance nursing interventions for patients with COVID-19 pneumonia. In the COVID-19 pandemic, the early identification of patients who may require intensive care can provide significant benefits in terms of saving time in nursing care and effectively managing the intensive care process. The findings of this study indicate that the levels of liver function markers (ALT and AST) are higher in COVID-19 pneumonia patients compared to non-COVID-19 pneumonia patients (Table 3). Recent studies have also demonstrated that ALT and AST levels increase with disease severity (Hamadi et al.2021; Ozbay et al.2023). The elevation of liver enzymes may be an important parameter to monitor in COVID-19 patients during the recovery process. Therefore, nurses should emphasize the importance of liver function monitoring during discharge education for patients diagnosed with COVID-19 pneumonia.

The study data also revealed that D-dimer levels were higher in COVID-19 pneumonia patients than in non-COVID-19 pneumonia patients (Table 3). Dynamic changes in D-dimer levels during the disease course have been reported as an indicator of poor prognosis in patients in China (He et al.2021; Yang et

al.2021). Furthermore, a strong correlation has been observed between D-dimer levels and the severity of COVID-19 (He et al.2021). Nurses can monitor changes in D-dimer levels to assess the clinical progression of COVID-19 patients and adjust care accordingly.

When the nursing care provided to COVID-19 pneumonia and non-COVID-19 pneumonia patients was evaluated, it was determined that, in addition to similar nursing interventions, different approaches were applied based on laboratory and radiological findings. In this study, all COVID-19 pneumonia patients underwent regular monitoring of liver function tests (AST, ALT, LDH) and coagulation markers (PCT, D-dimer), whereas this monitoring was less frequent in non-COVID-19 pneumonia patients. Large-scale studies conducted in China have similarly reported elevated levels of AST, ALT, and LDH in patients with severe COVID-19 (Macias et al.2021;Ozbay et al.2023;Pore et al.2021; Zhang et al.2022).

Liver damage observed in COVID-19 patients may be associated with direct viral infection of hepatocytes. Pathological studies have confirmed the presence of SARS-CoV-2 in liver tissues of COVID-19 patients, demonstrating that angiotensin-converting enzyme 2 (ACE2), the viral entry receptor, is expressed in liver cells [24-28]. Therefore, it is recommended that COVID-19 pneumonia patients undergo regular monitoring of AST, ALT, and LDH levels to prevent potential liver damage and enable early treatment initiation.

Additionally, the analysis of patient records showed that all COVID-19 pneumonia patients received anticoagulant therapy. The elevated coagulation markers in COVID-19 patients highlight the necessity of anticoagulant treatment (Hamadi et al.2021;Tolksdorf et al.2022).

Moreover, the study determined that deep vein thrombosis (DVT) risk assessment was conducted as part of the nursing care provided to COVID-19 pneumonia patients. Research has reported the presence of both large and small venous and arterial thrombi in COVID-19 patients (Zhang et al.2022; Ozbay et al.2023). Nurses should remain vigilant regarding deep vein thrombosis and carefully assess COVID-19 pneumonia patients for signs of clotting, such as unilateral swelling or redness (Majrashi et al.2021;Teopiz et al. 2024).

According to the findings of this study, certain pharmacological treatments, including antiviral drugs, corticosteroids, and anticoagulant therapy, are administered to COVID-19 pneumonia patients, distinguishing them from non-COVID-19 pneumonia patients. The antiviral drugs used have been reported to

exhibit anti-inflammatory and immunomodulatory effects and have been found effective in the management of COVID-19. However, some antiviral drugs are known to cause atrioventricular blocks, cardiomyopathies, and renal damage. Nurses should closely monitor patients for these adverse effects through careful cardiac monitoring and regular QT interval assessments (Labrague et al.2022).

For adult patients with COVID-19, low-dose corticosteroid therapy is recommended. However, given the potential side effects of corticosteroid therapy, it is crucial to regularly monitor vital signs and assess for adverse reactions.This issue warrants further investigation. Additionally, incorporating family members into the patient education process aligns with international patient safety standards and reflects the significance that nurses place on patient care both during hospitalization and after discharge (Labrague et al.2022; Majrashi et al.2021).

In the nursing care plans analyzed in this study, it was found that 80% of COVID-19 pneumonia patients required psychological support, distinguishing them from non-COVID-19 pneumonia patients. The uncertainty, fear, and anxiety associated with COVID-19 as a novel disease have been shown to have negative psychological effects on patients. Studies have indicated that COVID-19 has detrimental effects on mental health, exacerbating the severity of symptoms (Majrashi et al.2021;Tsamakis et al.2021).

Nurses should prioritize not only the improvement of physiological symptoms but also the psychological well-being of individuals infected with COVID-19.

5. Conclusion

Therefore, enhancing nursing care plans with strategies for coping with stress may serve as a valuable guide.Based on the findings of this study, future research should adopt a larger sample size and a longitudinal follow-up approach to better evaluate the impact of COVID-19 pneumonia on the treatment process. The implications of laboratory findings for nursing care should also be investigated more comprehensively at specific intervals throughout the treatment process.

Acknowledgments

This study is derived from a section of the thesis titled *"Comparison of Clinical Course and Nursing Care in Pneumonia Developing After COVID-19 and Community-Acquired Pneumonia."*

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CHAPTER 11

Dyspnea: A Cornerstone in Heart Failure Nursing Care

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Introduction

Heart Failure (HF) is a condition in which the heart is unable to pump blood in an amount sufficient to meet the metabolic demands of the tissues or does so at the cost of elevated cardiac filling pressures. HF is a complex clinical syndrome characterized by structural and functional impairment of the heart, left ventricular dysfunction, fluid retention, activity intolerance, and reduced quality of life, accompanied by neurohormonal activation, necessitating high-quality nursing care (Campbell et al. 2021).

HF is a prevalent disease affecting approximately 15 million people worldwide. Its incidence increases with age, rising from 1–2% in individuals aged 50–60 to approximately 10% in those over 75. Nearly 80% of all HF cases occur in individuals aged 65 and older. According to the Framingham study, among individuals aged 50–59, 3 out of every 1,000 men and 2 out of every 1,000 women are diagnosed with HF, while in the 80–89 age group, these numbers rise to 27 and 22 per 1,000, respectively. In general, due to increased survival rates both in the general population and among individuals with cardiovascular diseases, the number of HF cases continues to rise (Emmons-Bell et al. 2022;Joseph et al. 2020; Norhammar et al. 2023).

HF presents with systemic symptoms such as tachycardia, cardiomegaly, and peripheral and pulmonary edema; however, dyspnea is the most common symptom leading to emergency department visits and recurrent hospitalizations (Henriques et al.2024; Sá, M et al. 2022). As HF progresses, dyspnea worsens, significantly affecting individuals' physical, psychological, and social well-being. Managing dyspnea in HF patients is a crucial aspect of patient care and requires a multidisciplinary approach (Butts et al.2025; Hu et al.2023). Nurses play a critical role in alleviating dyspnea symptoms and enhancing patients' coping abilities. In this process, nurses aim to improve patients' quality of life by implementing various care interventions, including assessing dyspnea severity, teaching breathing techniques, positioning, medication management, fluid and salt restriction, patient education, and psychosocial support (Henriques et al. 2024).

Pathophysiology of Dyspnea

Dyspnea often arises due to pulmonary congestion associated with HF, where the activation of "J" receptors located in the lung parenchyma triggers signals to the central nervous system's respiratory center, leading to increased respiratory effort. However, the presence of dyspnea in cardiac conditions without

pulmonary congestion, as well as in other systemic diseases, indicates that mechanoreceptors are not the sole mechanism. Additionally, hypoxia, stimuli from respiratory muscles and the diaphragm, and direct central activation play significant roles in the pathogenesis of dyspnea (Fukushi et al. 2021).

Types of Dyspnea

Dyspnea is the most significant symptom of HF. Its severity progresses as follows: exertional dyspnea, paroxysmal nocturnal dyspnea (PND), orthopnea, dyspnea at rest, and acute pulmonary edema (Fukushi et al. 2021;Santus et al. 2023).

Exertional dyspnea: As HF progresses, the amount and duration of physical activity required to induce dyspnea gradually decrease. Although exertional dyspnea is characteristic of HF, it may also be observed in individuals with other conditions affecting mobility, such as severe angina pectoris or orthopedic disorders (Neder et al. 2022).

Paroxysmal nocturnal dyspnea (PND): PND refers to sudden episodes of dyspnea occurring at night. The patient wakes up abruptly, experiencing severe respiratory distress, air hunger, and a sensation of suffocation (King 2023). The patient typically sits upright on the edge of the bed with their legs dangling and attempts to breathe deeply. Congestion of the bronchial mucosa and compression of small airways due to interstitial pulmonary edema can lead to bronchospasm, further exacerbating respiratory difficulty. The redistribution of interstitial fluid accumulated in gravity-dependent areas, an increase in thoracic blood volume upon lying down, and a reduction in adrenergic support for left ventricular function during sleep are key mechanisms in the pathogenesis of PND (Fukushi et al. 2021; King 2023).

Cheyne-Stokes Respiration: A type of periodic breathing, Cheyne-Stokes respiration is frequently observed in HF patients and is often associated with PND (King 2023).

Orthopnea: Orthopnea refers to difficulty breathing when lying flat, relieved by sitting upright. Patients may alleviate their symptoms by using multiple pillows to elevate their head. In advanced stages of HF, orthopnea can become severely distressing, forcing patients to remain seated throughout the day and night. This condition arises due to the redistribution of extracellular fluid from the lower extremities and abdomen to the thoracic region when lying down. The failing ventricle is unable to accommodate the increased venous return, leading

to elevated pulmonary venous (Fukushi et al. 2021; King 2023; Neder et al. 2022).

Stages of Dyspnea

Dyspnea is classified into stages based on the intensity of exertion required to induce symptoms or the individual's exercise tolerance:

Stage 1: Dyspnea occurs during rapid movement on a flat surface or when climbing a slight incline.

Stage 2: Dyspnea occurs when walking on a flat surface alongside peers.

Stage 3: Dyspnea occurs even at a self-regulated pace, necessitating frequent pauses.

Stage 4: Dyspnea is present even at rest (Fukushi et al. 2021; King 2023; Neder et al. 2022).

Heart Failure and Dyspnea

HF is the leading cardiac condition resulting in dyspnea and represents the terminal clinical stage of many cardiovascular diseases. Dyspnea is the most distressing symptom for most HF patients. When it occurs in response to routine physical exertion, many patients instinctively reduce their physical activity to avoid this discomfort. During patient history-taking, evaluating daily activity levels, assessing whether they have decreased over time, and determining when the patient first noticed their symptoms can help gauge the severity of dyspnea. The gradual progression of dyspnea can also be associated with pulmonary diseases (such as asthma, pleural disease, or effusions), anxiety, poor physical fitness, or obesity (Ertuğrul and Ünsar 2023).

Many HF patients instinctively sleep with multiple pillows to avoid the unpleasant sensation of dyspnea. However, dyspnea often occurs a few hours after falling asleep and typically recurs throughout the night. Historically, all forms of exercise, whether mild or intense, were considered harmful to HF patients (Gharzeddine et al. 2021). However, since the late 1980s, carefully structured exercise programs for patients with moderate HF have been observed to be beneficial. The first randomized controlled trial on exercise in HF patients demonstrated that warm-up exercises increased exercise tolerance and improved symptoms of dyspnea and fatigue (Ng et al.2021;.

Dyspnea significantly impacts all aspects of life for HF patients, typically developing gradually over months or years, leading to a decline in quality of life (Niklasson et al. 2022). In recent years, studies on HF management by nurses have increased in Türkiye (Sarıgül et al.2024; Sayın et al.2021;Seckin et al.2024). However, in the United States, where HF prevalence is high, specialized nursing clinics have contributed to a 4% reduction in hospital admissions and a two-day decrease in hospital stays for HF patients. Another study conducted in North America, involving nurses and other healthcare professionals, reported a significant 56% reduction in hospital admissions and length of stay. In this follow-up study, patients also experienced an improvement in their quality of life (Driscoll et al.2022).

Nurses play a crucial role in providing care for HF patients and alleviating the increasing burden of the disease. To deliver effective nursing care, it is essential for nurses to understand the pathophysiology of HF as well as the physical and emotional responses associated with the disease. Providing patients and their families with education and support is also a fundamental aspect of nursing care. There is no single definitive treatment for HF, and medication regimens often require dose adjustments and modifications throughout the disease course, further complicating patient care (Henriques et al.2024;Srimookda et al.2021).

The Role of Nurses in Dyspnea Management

Active involvement of nurses in dyspnea management helps patients better control their symptoms and contributes to reducing hospital admissions. Moreover, expanding patient education and home care practices enables HF patients to develop self-management skills for their health. This section will discuss nursing approaches to dyspnea management in HF patients and examine the impact of evidence-based nursing interventions on patient care (Bierle et al.2021;Srimookda et al.2021;Katayıfçı et al. 2022;Ng et al.2021; Gaertner et al.2023).

Dyspnea as a Sensory Perception

Dyspnea is a sensory experience similar to pain, characterized by an individual's awareness of breathing and difficulty in taking breaths. Another definition describes it as a greater-than-expected sensation of breathing difficulty during a given level of activity. The sensation of dyspnea, defined as difficult or uncomfortable breathing, is a normal response experienced at a moderate level in healthy individuals following intense physical exertion. However, experiencing discomfort during rest or with activities that were previously manageable is

considered abnormal. Dyspnea occurring at rest requires medical intervention (Schwartzstein 2022).

In exertion-related dyspnea, individuals often restrict their activity to avoid discomfort. Assessing individuals' daily activity levels and having them describe their routine activities can provide valuable insights into the extent to which dyspnea affects their lives. It is important to determine whether there has been a subtle decrease in activity levels due to dyspnea. The timing of symptom onset is also significant. Dyspnea is a common symptom in both pulmonary and cardiac disorders, particularly when lung clearance function declines or airway obstruction increases. It can manifest at varying degrees, from mild to severe, and may occur following physical exertion or even at rest (Chang et al.2023).

Collaboration of Nurses and Cardiologists in Home Care Plans

The complexities associated with HF management can be reduced through collaborative home care plans involving nurses and cardiologists. A study evaluating the effects of nurse-led follow-up after patient discharge found that cardiac events occurred less frequently among patients after 12 months. In the same study, intensive home monitoring of middle-aged patients with severe HF was associated with improved quality of life and a decrease in readmissions (Wu et al.2024). Nurse follow-ups conducted via telephone have also led to a reduction in hospital visits. The multidisciplinary discharge planning approach taken by nurses provides significant economic benefits in reducing hospital readmissions. Implementing care plans, especially for advanced HF patients, has enhanced positive feedback. Overall, such care plans have been shown to reduce recurrent hospitalizations by 30% (İlaslan et al.2022; Wu et al.2024).

The primary aim of nursing care is to positively impact the individual's quality of life across physical, social, and psychological dimensions. In this approach, the individual is at the center and actively participates in decisions regarding their care. They assume responsibility for their own care, which necessitates comprehensive nursing interventions that involve the individual directly. To achieve this, self-care must be promoted, and necessary educational programs must be implemented for patients. Education tailored to symptom management can facilitate adherence (Katayıfçı et al. 2024; Ng et al.2021).

Post-discharge follow-up programs have been shown to improve symptom management and increase treatment compliance among patients. Structured education programs for HF patients enable them to control symptoms such as dyspnea, fatigue, and edema while also contributing to the prevention of

complications associated with the disease. Research indicates that providing individualized education enhances medication adherence and facilitates lifestyle changes such as salt and fluid restriction (Bierle et al..2021; Henquries et al.2024).

Notably, patient follow-up programs conducted with a multidisciplinary approach have been reported to reduce hospital readmission rates and support patients' physical and psychosocial well-being. The active role of nurses in the care of HF patients ensures the continuity of patient education and assists individuals in developing self-care skills. In this process, educating patients about symptom management, medication use, diet, and physical activity is of paramount importance (Emmons-Bell et al.2023;Santus et al.2023).

Moreover, with the increasing prevalence of remote patient monitoring, regular follow-up through phone calls and digital health applications enhances the chances for early interventions, significantly reducing hospitalizations and emergency department visits. In this regard, it is critical for nurses to stay updated on evidence-based practices in caring for HF patients, adopt patient-centered approaches, and encourage individuals' active participation in disease management to enhance the effectiveness of care (Campbell et al.2021;Joseph et al.2020). Below, the nursing interventions for managing dyspnea in HF patients will be discussed.

Assessment of Dyspnea

Before planning dyspnea management for HF patients, nurses should conduct a comprehensive assessment, including the following elements:

1. **Severity and Duration of Dyspnea**: The patient's level of breathlessness related to physical activity should be determined using scales like the New York Heart Association Functional Classification.
2. **Factors Causing Dyspnea**: It should be assessed whether dyspnea occurs at rest or during exertion, and potential triggers should be identified.
3. **OrthoPnea and Paroxysmal Nocturnal Dyspnea (PND)**: Evaluation should include whether the patient experiences breathlessness while lying down or during sleep.
4. **Vital Signs and Physical Examination**: Assessment of respiratory rate, oxygen saturation, blood pressure, pulse, lung auscultation (e.g., crackles, wheezing), and presence of edema.
5. **Patient Diaries**: Patients should be encouraged to keep a diary to track symptoms such as dyspnea, weight changes, and edema (Fukushi et al.2021; King et al.2023).

Nursing Interventions

1. **Positioning**:
   Patients should be positioned in a Semi-Fowler or high Fowler position to alleviate dyspnea symptoms. For patients experiencing orthopnea, elevating the head and chest with multiple pillows is recommended. Patients should be encouraged to lean slightly forward while sitting to facilitate breathing(Campbell et al.2021; Henriques et al.2024).

2. **Breathing Techniques and Exercises**:

   - **Pursed-Lip Breathing**: This technique slows down breathing and reduces air trapping in the alveoli, helping to relieve dyspnea.
   - **Diaphragmatic Breathing**: Encourages patients to use their abdominal muscles for more effective breathing.
   - **Slow and Controlled Breathing Techniques**: Recommended for patients to manage stress and anxiety (Srimookda et al.2021; Ertuğrul et al.2023).

3. **Oxygen Support and Respiratory Devices**:

Oxygen therapy should be administered to patients identified with hypoxemia. For patients with severe dyspnea, oxygen support via nasal cannula or mask should be provided. In cases of pulmonary edema, respiratory support through Positive Airway Pressure (CPAP, BiPAP) devices may be recommended (Santus et al.2021; Driscoll et al.2022).

Fluid and Sodium Restriction

Patients should be advised to limit their daily fluid intake to 1.5–2 liters. It should be explained that excessive fluid intake can increase pulmonary edema. Sodium consumption should be reduced to below 2 grams per day, as sodium intake can lead to fluid retention and consequently dyspnea (Bierle et al.2021;Fukushi et al. 2021).

Medication Management and Monitoring

- **Diuretic Therapy**: Patients should be informed about the importance of regular diuretic use.
- Regular use of beta-blockers, ACE inhibitors, and other cardiovascular medications should be ensured, and their side effects should be

monitored. Nurses should assess the effectiveness of medications and any potential side effects, reporting them to the healthcare team as necessary (Campbell et al. 2021;do Sá Sá et al.2022).

Patient Education and Lifestyle Modifications

- **Physical Activity**: Light to moderate exercise (such as walking and stretching) should be encouraged, while avoiding excessive exertion.
- **Smoking and Alcohol Consumption**: Patients should be encouraged to quit smoking and reduce alcohol intake, as these can worsen respiratory function.
- **Weight Monitoring**: Daily weight measurements should be conducted, with a recommendation to consult a doctor if weight gain occurs.
- **Psychosocial Support**: Patients are at high risk for anxiety and depression due to dyspnea; thus, psychosocial support should be provided (Fukushi et al. 2021; Butts et al.2025).

Management of Emergencies

If patients experience sudden or worsening dyspnea, tachycardia, cyanosis, altered consciousness, or signs of pulmonary edema, they should immediately contact the healthcare team. Patients should be made aware of emergency symptoms and advised to seek hospital care if necessary.

Conclusion and Recommendations

Dyspnea is a common symptom in heart failure (HF) patients that negatively impacts quality of life. Nurses should perform a comprehensive assessment for dyspnea management in HF patients, implementing various nursing interventions such as positioning, breathing techniques, medication management, patient education, and psychosocial support. Additionally, multidisciplinary teamwork can improve care processes for HF patients, reduce readmission rates, and enhance patients' quality of life.

Management of dyspnea in HF patients should be supported by individualized care plans that encourage patients to take responsibility for their own care. The active role of nurses in this process can positively influence the prognosis of HF patients, contributing to reduced mortality and morbidity rates (Henriques et al.2024).

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CHAPTER 12

The Effectiveness of Macronutrient Supplements in Athletes

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1. Macronutrient Requirements in Athletes

Athletes have increased energy and nutrient requirements compared to sedentary individuals due to their higher levels of physical activity. It is also known that the environmental conditions under which athletes perform can alter their energy and nutrient needs. Meeting these increased energy and nutrient requirements with an adequate diet is crucial for maintaining the athlete's overall health, improving training and competition performance, reducing injury risks, accelerating post-performance recovery, and enhancing metabolic adaptation (Potgieter, 2013).

1.1. Carbohydrate Requirements in Athletes

Carbohydrates are the primary source of energy. Specifically, glucose is the main and ideal energy source for the brain and central nervous system. Carbohydrates also play an important role in regulating blood glucose levels and controlling appetite. The most important function of carbohydrates for athletes is their ability to regulate fatigue perception, motor skills, and concentration, all of which directly affect performance. Therefore, adequate carbohydrate intake through the diet supports both general health and performance. Additionally, the sufficient amount of carbohydrates in the diet prevents the use of proteins as an energy source and ensures that fats are utilized efficiently for energy production (Burke, Hawley, Wong, & Jeukendrup, 2011; Koenig et al., 2019).

Muscle glycogen stores and blood glucose levels are the primary sources of energy for muscle contraction. However, if glycogen stores in the body are not supplemented during performance, they are depleted within 1.5 to 3 hours. The limited nature of glycogen stores makes carbohydrates a limiting factor for performance (Burke et al., 2011). Therefore, starting performance with adequate glycogen stores and ensuring appropriate carbohydrate intake at regular intervals during prolonged performances, lasting more than one hour, are important for the athlete's performance. Starting performance with low muscle glycogen levels can lead to problems such as hitting the wall, hypoglycemia, leg heaviness, lack of concentration, dizziness, fainting, and a decrease in recovery speed, which can negatively affect both performance and overall health (Potgieter, 2013).

For recreational athletes with no performance goals, it is sufficient to obtain 45-55% of total energy from carbohydrates in the diet or to add 3-5 g/kg/day to the diet. However, for professional athletes, carbohydrate requirements vary depending on the type, duration, and intensity of the sport (Kerksick et al., 2018). As the duration and intensity of the sport increase, carbohydrate needs also increase (Table 1).

Table 1. Carbohydrate Requirements Based on the Type, Duration, and Intensity of the Sport

Physical Activity Level	Carbohydrate Requirements
General physical activity: 3-4 days per week, 30-60 minutes per day	3-5 g/kg/day
Moderate-to-high intensity exercise: 5-6 days per week, 2-3 hours per day	5-8 g/kg/day
High-intensity, strenuous exercises: 5-6 days per week, 3-6 hours per day, more than one training session per day	8-10 g/kg/day

References: Potgieter, 2013

1.2. Protein Requirements

Proteins support muscle protein synthesis, reduce breakdown, and repair damage. Considering all these effects, adequate intake becomes essential for athletes' overall health and performance (Potgieter, 2013; Egan, 2016).

Protein requirements in athletes vary depending on the type, intensity, and duration of the sport, but they are generally higher compared to sedentary individuals. The daily amount of protein is typically calculated based on grams per kilogram of body weight. For sedentary individuals, 0.8 g/kg/day, for recreational athletes, 1 g/kg/day, and for professional athletes, 1.2-2 g/kg/day are considered appropriate protein intake levels. Although meeting these increased values through the daily diet may seem challenging, an appropriately prepared nutrition plan allows athletes to meet their protein requirements sufficiently through food, often without the need for supplements (Potgieter, 2013; Egan, 2016).

1.3. Fat Requirements

Fat requirements in athletes are similar to those of sedentary individuals. They are important for maintaining overall health, ensuring adequate energy intake, sufficient intake of essential fatty acids and fat-soluble vitamins, and replenishing intramuscular triglyceride stores. Approximately 20-35% of total energy intake should come from fats, and it should not fall below 20% (Potgieter, 2013).

2. Macronutrient Supplements

Athletes may use various ergogenic aids to meet their increased nutrient needs and enhance performance. Among these, dietary supplements, which are nutrition-based ergogenic aids, are frequently preferred. The use of dietary supplements among athletes has rapidly increased in recent years. Studies have shown that depending on the type of sport, the athlete's performance level, and the type of supplement, between 40% and 100% of athletes use various supplements (Garthe & Maughan, 2018). Furthermore, the production and variety of dietary supplements have significantly increased in recent years. The growing number and diversity of these products have raised concerns about their reliability and effectiveness. Research has evaluated these products in terms of the performance or health benefits they promise, their reliability, and whether they are legal. Some studies have found that certain products have no impact on performance, may even have negative health effects, and may contain illegal substances (doping). Based on these findings, guidelines have been created in which dietary supplements are classified according to evidence levels to ensure the safety of both athletes and the sport. Macronutrient supplements are among those that provide evidence-based benefits (Australian Institute of Sport, 2021; Bird, 2024).

Although athletes' increased energy and macronutrient needs should primarily be met through food, in certain special situations, this may not be possible. In such cases, the use of evidance based nutritional supplements is recommended. These are primarily grouped as carbohydrate-containing sports drinks, gels, candies, and bars, isolated protein supplements, and mixed macronutrient supplements that can serve as meal replacements (Australian Institute of Sport, 2021).

2.1. Carbohydrate Supplements

Carbohydrate supplements are typically marketed as sports drinks, gels, candies, and bars. They are used according to the athlete's needs before, during, and after performance (Australian Institute of Sport, 2021). Adequate and properly planned carbohydrate intake positively impacts the athlete’s overall health and reduces the risk of injury, making the appropriate and sufficient use of these products beneficial for the athlete (Jeukendrup, 2013; Burke & Maughan, 2015; Abreu, 2023).

When evaluating the effects of these products on performance, their positive impacts have been explained by two mechanisms. The first is the provision of carbohydrates, the primary energy source for muscles, which supports the continuous replenishment of glycogen stores, thereby contributing to the energy intake necessary for exercise and metabolism (Phillips et al., 2011; Stellingwerff & Cox, 2014). The second mechanism explains that carbohydrates delay fatigue perception by stimulating the reward center in the brain and the central nervous system (Jeukendrup, 2013; Burke & Maughan, 2015). A study conducted by Silva et al. found that rinsing the mouth with a carbohydrate solution stimulated the central nervous system, and rinsing with carbohydrate at specific intervals delayed perceived exertion (Silva et al., 2014). With these two mechanisms, appropriate carbohydrate supplementation leads to positive outcomes in athlete performance.

In addition to the performance effects of carbohydrate supplements, there are also positive health benefits. A 2017 review found that among various nutritional strategies and physical therapies used by athletes to regulate immune responses after exercise, the most effective method was carbohydrate supplementation. It was stated that consuming carbohydrates before, during, and after prolonged intense exercise reduced the increased cytokine profile typically caused by exercise stress and helped regulate immune cells such as monocytes, lymphocytes, and neutrophils (Peake et al., 2017). In another study, long-distance runners who consumed carbohydrates before and during exercise were found to have significantly lower post-exercise bone resorption levels compared to the placebo group. It was noted that the appropriate use of carbohydrate supplements helped preserve bone mineral density in athletes (Sale et al., 2015).

To observe all these positive effects, carbohydrate supplements must be consumed at the appropriate time, in the right amount, and with the correct type. As the duration of the exercise increases, the need for these products, particularly during exercise, becomes greater. However, as the required amount of the product increases, the absorption capacity of the product must also be considered. Studies have shown that consuming products containing only one type of monosaccharide, such as glucose, at more than 60 grams per hour results in the saturation of glucose transporters (SGLT1), causing a slowdown in absorption. Therefore, consuming products at more than 60 grams per hour can lead to gastrointestinal issues and negatively impact performance. Some products on the market contain various types of monosaccharides, such as glucose-fructose, which use different transport systems (Australian Institute of Sport, 2021; Jeukendrup, 2010). In a related study, when carbohydrate needs exceeded 60

grams per hour, replacing glucose-based products with glucose-fructose-based products resulted in more positive effects on both performance and gastrointestinal system outcomes (Jeukendrup, 2010).

The type of carbohydrate product to be given to the athlete before, during, and after performance also varies depending on the athlete's needs. If the athlete's fluid needs are higher than their carbohydrate needs, more liquid forms should be preferred; if the carbohydrate needs are higher than the fluid needs, denser gel forms should be preferred (Table 2).

Table 2. Carbohydrate Supplements That Can Be Used According to the Duration and Intensity of Exercise

Type of Exercise	**Duration of Exercise**	**Targeted Carbohydrate Supplementation**	
Short-Duration Daily Exercise	<45 minutes	Carbohydrate supplementation is not required.	
High-intensity exercise	45-75 minutes	A small amount or mouth rinsing is sufficient.	The requirement can be met with sports drinks. Mouth rinsing should be done every 10-20 minutes by holding for 10 seconds.
Endurance exercises	1-2.5 hours	30-60 gram/hour	The need can be met with liquid or solid carbohydrate supplements. The selected product should support the athlete's hydration and not cause gastrointestinal system issues.

Ultra- Endurance exercises	>2.5 hour	90 gram/hour	It is appropriate to use products containing different types of monosaccharides that require multiple transport systems.

References: (Australian Institute of Sport, 2021)

Any carbohydrate supplement used excessively and without proper guidance can disrupt the energy balance of the diet and may lead to dental and gastrointestinal system problems (Australian Institute of Sport, 2021).

2.2. Isolated Protein Supplements

Proteins play a key role in muscle mass gain and protein synthesis. Isolated protein supplements positively affect both anaerobic and aerobic performance by increasing muscle mass and strength (Pasiakos et al., 2015).

There are five different types of isolated protein supplements available on the market. Some of these are derived from animal sources, while others are plant-based. The type of product provided to the athlete depends on the athlete's specific needs (Table 3).

Table 3. Types of Isolated Protein Supplements

Whey	Milk protein is a high biological value protein, constituting 20% of the protein content in milk. It is rich in branched-chain amino acids, particularly leucine, and helps support muscle protein synthesis.
Casein	It is a high biological value protein that makes up 80% of milk protein content. It coagulates in the acidic environment of the stomach, slowing down the digestion and absorption of amino acids into the body. Due to its slow digestion property, it is recommended as a nighttime meal.
Egg albumin (egg white)	It is a high biological value protein that contains no fat or carbohydrates.
Soy protein	It is a rapidly digested high biological value, plant-based protein. It is cheaper than whey protein and is often used in sports bars. It is low in leucine, although products enriched with leucine are available in the market. It helps support muscle protein synthesis. It can be especially preferred by vegan-vegetarian athletes.
Plant-based proteins (rice etc.)	They are low biological value, plant-based proteins. The biological value can be increased by combining plant sources, enriching with leucine and other amino acids, or by increasing the serving size. They can be especially preferred by vegan-vegetarian athletes.

Rererances: (Australian Institute of Sport, 2021)

Studies evaluating the effects of animal- and plant-based isolated protein supplements on athlete health and performance have shown that different types of products provide similar benefits. In a study by Joy et al., rice and whey protein supplements were compared. When evaluating the effects of the two different

protein types individually, no significant differences were found (Joy et al., 2013). Similarly, in another study, the effectiveness of high-quality and low-quality protein supplements was assessed. At the end of the study, no difference was found between the effects of the protein types (Rindom et al., 2016). A 2019 review found that there was no significant difference in the effects of protein types on endurance performance; however, all products with high leucine content had significantly more positive effects on muscle protein synthesis. The study particularly noted that protein supplements enriched with leucine were more effective than consuming leucine as a separate product (Huecker et al., 2019).

When planning protein intake in athletes' diets, priority should be given to obtaining the required amount from food sources, with isolated protein supplements being used only in necessary situations. These supplements can be useful when quick-digesting protein sources are needed, such as when enriching meals with low-protein options (e.g., breakfast or snacks), when the athlete has a poor appetite, when high-quality proteins are not accessible, or during high-protein diets aimed at weight loss. However, excessive and unintentional use of isolated protein products, without following guidelines, can disrupt the energy balance in the diet, lead to inadequate intake of nutrients such as calcium, iron, and zinc found in natural protein sources, and, due to their small volume and high energy content, can cause a lack of satiety, potentially resulting in unwanted weight gain (Australian Institute of Sport, 2021).

2.3. Mixed Macronutrient Supplements

Mixed macronutrient supplements are compact and practical products that contain varying amounts of macronutrients and micronutrients. They are available in powder, bar, and liquid forms on the market. These supplements can provide 25-50% of the daily reference nutrient intake. The type of product provided to the athlete depends on their specific needs (Table 4).

Table 4. Types of Mixed Macronutrient Supplements

High-carbohydrate powder/bar	Suitable for pre-, during-, and post-workout use.
High-carbohydrate and high-energy powder/bar	It contains more fat compared to other products. It is suitable for athletes with high energy requirements due to their dense energy content.
High-protein and high-energy powder/bar/liquid	It is suitable for athletes aiming for muscle gain. It is ideal for athletes with high energy needs due to its high energy content.
High-protein and low-energy powder/bar/liquid	It is suitable for athletes with high protein needs alongside low energy requirements. It is appropriate for athletes aiming for fat loss and muscle mass gain.
Meal and snack replacement products	These products contain nuts, fruits, and grains. They are suitable for use when a regular meal is not accessible.

References: (Australian Institute of Sport, 2021)

Mixed macronutrient supplements are ideal alternatives in situations such as ultra-marathon events or other long-duration activities, athletes experiencing appetite suppression due to stress, when the entire daily energy requirement cannot be consumed, when food access is difficult or not possible, or when athletes cannot carry or safely transport their meals. However, the excessive and unconscious use of mixed macronutrient supplements without following guidelines can disrupt the energy and nutrient balance in the diet (Australian Institute of Sport, 2021).

In conclusion, the goal of athlete nutrition is to maintain health and enhance performance. Nutritional supplements can be used by athletes to

meet increased energy and nutrient needs or to boost performance. When using these supplements, it is recommended to ensure that they deliver their promised positive effects and are safe. Evidence-based supplements should be prioritized. Among the evidence-based nutritional supplements, those containing macronutrients, when used appropriately and as needed, help protect the athlete's health while also providing positive contributions to their performance.

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CHAPTER 13

The Significance of Cryobiology in Fish

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Introduction

The behavior of spermatozoa at low temperatures has been researched for millennia. Cryobiology is related to theriogenology, molecular biology, engineering, mathematics, human and veterinary medicine, and has studied land and sea species. Until cryoprotective agents' discovery, effective freezing methods relied on inhibiting the growth of extracellular ice, and this goal was achieved by combining the injection of chemical agents with rapid cooling, a process known as vitrification (Luyet and Hodapp, 1938; Luyet and Gehenio, 1940; Benson et al., 2012). Viable cells and tissues can be preserved for a long period at extremely low temperatures via cryopreservation. Stem cell therapies, assisted reproductive technology, germplasm preservation, animal husbandry, and the preservation of endangered species and plant biotechnology are all significantly impacted by cryopreservation (Pegg, 2002; Chatterjee et al., 2017). Cryobiology's scientific foundations date back to the 1950s, when glycerol's ability to protect fowl sperm from freezing was first discovered (Polge et al., 1949). Soon after, the initial analyses of fish sperm cryopreservation were published (Blaxter, 1953). Biological materials can be preserved generally at -196°C in liquid nitrogen. These materials continue to function after thawing. This procedure for sperm cryopreservation includes the collection of gametes, sperm dilution, sperm evaluation, supplementation of cryoprotectants, equilibration, freezing and thawing, and fertility (Tiersch, 2000).

Fish Species

The term "fish" refers to more than 25,000 species. Invertebrates like oysters, abalone, and corals must be further included to extend this classification to aquatic organisms. Freshwater species' sperm typically become more active and mobile under hypoosmotic pressure. Sperm from maritime species respond differently, increasing their motility as increased osmotic pressure increases. Anatomy of the female has evolved into the working environment for the gametes in this group of live-bearing fishes of the genus Xiphophorus, and as a result, the osmotic pressure of sperm activation has changed (Huang et al., 2004; Yang et al., 2006). The very tiny size of the fish genome makes it the ideal model for investigating human genetic problems (Barbazuk et al., 2000). More than 200 fish species, including carp, salmonids, catfish, cichlids, medakas, whitefish, pike, milkfish, grouper, cod, and zebrafish, had their sperm successfully managed and cryopreserved (Babiak et al., 1995; Suquet et al., 2000; Van et al., 2006; Kopeika et al., 2007; Tsai et al., 2010). Spermatozoa from frozen-thawed organisms are

more fertile and resilient than those from freshwater species (Afreen and Ucak, 2020).

Importance of Cryopreservation in Fish

The application of cryopreservation to fisheries and aquaculture offers increased gamete transport between different points, lower numbers of fish-bearing males, gives progeny availability more time, and selective propagation should be used to preserve a high number of families and keep genetic resources safe. Water pollution and overfishing have led to an alarming state of the fish population (Afreen and Ucak, 2020). Fish farming and aquatic germplasm cryopreservation are two methods for preserving endangered species. By using these techniques, genetically significant traits can be preserved and protected from loss due to illnesses and natural calamities. Cryopreserving semen has allowed the total preservation of numerous domestic and wild fish species. For the past three decades, scientists have made numerous attempts to freeze fish embryos, but they have been ineffective (Streit et al., 2014). By preserving the genomes of threatened species, the genetic diversity of aquatic resources can be preserved (Afreen and Ucak, 2020). Many different cells, including gametes, embryos, somatic cells, spermatogonia, and primordial germ cells, are used in the cryobanking of fish germplasm. It is possible to save endangered fish species' natural reservoirs using germline cryopreservation. Sperm is typically used in fish aquaculture to propagate and manage species such as salmonids, silurids, and cyprinids (Tsvetkova et al., 1996; Afreen ve Ucak, 2020). Only eastern oyster eggs and sea urchin and eastern oyster larvae are successfully cryopreserved in different fishes (Tervit et al., 2005; Adams et **al., 2006).** Procedures for cryopreservation must be customized for each species and type of cell and be founded on a complete knowledge of cryobiological characteristics (Tiersch et al., 2007). For the majority of aquatic species, cryopreservation has not progressed far beyond the stage of exploratory study, and the techniques for effective cryopreservation are still not well defined. To represent and preserve a wide range of genetic variation for each species, biological resources other than sperm or somatic cells are not generally banked for the majority of aquatic species. Therefore, to assure reproducible recovery of cryopreserved materials, new methods and standardization are required for repository-level applications. Furthermore, the maintenance of novel biomaterials, such as aquatic species' eggs and embryos, calls for the advancement of new techniques. Biomedical researchers value zebrafish (Tavares and Santos Lopes, 2013), medaka (Kirchmaier et al., 2015; Walter and Obara, 2015), Xiphophorus (Malitschek et al., 1995; Schartl et al., 2013; Schartl and Walter, 2016; Shen et al., 2016), and

Xenopus (Grainger, 2012) to a greater extent because these species offer important insights into the mechanisms underlying human health and disease. Aquatic species can be produced in transgenic, knockout, and mutant lines, giving researchers a wide range of tools for study. Even if it were expensive to produce these lines, there are still no regular, trustworthy, or cost-effective methods for keeping these scientific treasures safe for the long term. Although cryopreservation of sperm is the only and most effective method for the long-term upkeep of many aquatic models, aquatic researchers are currently without access to other strategies, such as additional germplasm preservation (oocyte, embryo, ovarian tissue, testicular tissue, larvae, or embryonic stem cells) or reproductive engineering technologies (Hagedorn et al., 2019). Cryobanks of endangered and significant species are one of the most efficient strategies to safeguard genetic resources. The overfishing of fish stocks has been limited to the population. Dimethylsulfoxide (DMSO) is employed most frequently in the process of cryopreserving sperm. According to scientists, ascorbic acid and lysine can act as free radical scavengers and lessen freeze-related damage to sperm, protect motility, DNA integrity, and capacity to fertilize oocytes (Katkov, 2006). By offering sperm on demand and making the timing of induced spawning easier, cryopreservation can be used to enhance current hatchery operations. By removing the requirement to keep live males, frozen sperm can improve the hatchery's efficient use of resources and open up new prospects. This could free up resources to be used with females and larvae. The preservation of frozen sperm can safeguard priceless genetic lineages like those of threatened animals, research models, or enhanced farmed strains. Cryopreservation makes way for quick genetic advancement (Tiersch et al., 2007).

Cryobanking and Germplasm Cryopreservation

Cryobanking has significant implications for the reproduction of aquacultured marine and freshwater species. For each species and each type of cell, specific cryopreservation procedures must be developed. Diverse cell types, such as sperm, somatic cells, spermatogonia, and primordial germ cells, as well as oocytes and embryos, have been studied in research on fish germplasm cryobanking. Sperm cryopreservation is the most often used method because fish spermatozoa have a small size and good resistance to freezing (Martínez-Paramo et al., 2017). Despite proven techniques being available, there is a limited application of cryopreserved sperm in aquaculture (Tiersch et al., 2007). There are some challenges to expanding a cryopreservation industry that has restricted commercialization in aquatic species, such as aquatic species' sperm being subjected to drastically different physical and chemical circumstances than

mammalian sperm (McKaye et al., 1994). For aquatic species and livestock, the first successful sperm cryopreservation attempts happened around the same period. However, the market for cryopreserved livestock sperm has grown to be worth billions of dollars worldwide. Despite research on more than 200 species, the cryopreservation of aquatic species sperm largely remains a research activity with limited commercial applications. The majority of studies have been on large-bodied culture and recreational fishes like salmonids, carp, and catfish, as well as mollusks such as commercially significant abalone and oyster species. However, sperm cryopreservation in tiny fishes like zebrafish and endangered species has only been the subject of a small number of research studies (Tiersch et al., 2007). The use of fish sperm cryopreservation procedures in restocking and conservation initiatives is a key objective. There have been different taxa for which preservation approaches have been created (Horvath et al., 2005; Ciereszko et al., 2006; Sarvi et al., 2006; Martínez-Páramo et al., 2009; Nynca et al., 2015). Additionally, research has been done to assess how cryopreservation affects some species' genetic diversity (Van Der Walt et al., 1993; Martinez-Páramo et al., 2009). There are various reasons for the deficiency of knowledge on the use of cryopreservation of sperm in fish. Aquatic species raised in captivity and those that are considered "wild" can both be cryopreserved with conservation in mind. According to the IUCN Red List, 5,161 aquatic species are vulnerable. These species could benefit from cryopreservation when ex-situ programs for restocking or even for the conservation of wild populations were implemented (Martínez-Paramo et al., 2017). The creation of sperm banks has proven successful in several commercially significant aquatic species, mainly teleost fishes, but the technology has not yet developed to the point where it can be used for shellfish farming. One of the key ex-situ techniques for not only the preservation of germplasm but also for enhancing the quality of gene resources and enhancing fish production in captivity is the cryopreservation of gametes (Martinez et al., 2013; Maria et al., 2015; Martinez et al., 2016). In particular, groupers, salmonids, and some commercial and endangered fish species currently have their sperm banks (Diwan et al., 2020). The basic goal of gamete cryobanking is to extend gamete life without significantly reducing their ability to reproduce. Even though cryopreservation of fish eggs and embryos is highly important given the function that mitochondrial DNA plays, attempts to do so have had little to no success (Ahammad et al., 2003; Ahammad et al., 2004). The majority of research done in recent years has been on freshwater species. The fact that most marine species inherently reproduce in tanks reduces the requirement for assisted reproductive techniques and is one of the reasons why research on and deployment of cryopreservation techniques at the production level is lacking.

Of course, there are certain exceptions, including halibut and turbot, where significant sperm cryopreservation research has been done (Chereguini et al., 2003; Babiak et al., 2008).

Cryopreservation of gametes and embryos has grown into a very lucrative industry in mammals; however, this technology has not yet reached this level in aquatic species. The concept of creating selective breeding programs for fish to increase production in aquaculture has received considerable attention over the past few years (Lind et al., 2012). The preservation of the genetic material of certain strains with biotechnological interest by cryopreserving sperm from individuals with poor efficiency or in fish model organisms like zebrafish for the recovery of endangered species. The improvement of extenders to enhance gamete quality, to ensure adequate sperm motility, or for short-term sperm storage when cryopreservation is not required all techniques are standardized for efficient fish reproduction. For several fish species, cryopreservation services are currently offered. Cryopreservation of fish embryos hasn't been accomplished for decades because fish embryos have low surface-to-volume ratios, large yolk sizes, low membrane permeabilities, and high chilling sensitivity. If it were possible to successfully freeze fish embryos, both the father's and mother's genomes would be preserved. It would also be much easier to set up and run genetic selection programs in fish farms.

Effective cryopreservation and cryobanking of aquatic species' reproductive cells have numerous advantages for aquaculture, biodiversity preservation, and biomedicine. The world's over 5000 aquatic animal species, including fish, mollusks, crabs, and corals, are all in danger. Additionally, this has raised interest in developing cryobanks for environmental preservation. Numerous types of cells have been examined in terms of cryopreservation. To achieve the best possible survival, cryopreservation procedures must be carefully created for each species and each cell type (Diwan et al., 2020). There hasn't been much research done on the cryopreservation of fish and shellfish eggs and embryos, with mixed results. Oocytes and embryos are also vulnerable to chilling damage. While fish sperm cryobanking has been largely successful, egg and embryo cryobanking has not been as successful (Diwan et al., 2020). Studies on aquatic species' sperm cryopreservation have a history comparable to that of studies on domestic livestock animals (Blaxter, 1953) however, fish and shellfish cryopreservation production scale and public acceptance trail far behind. The last 60 years have focused on study methodology for aquatic species (Tiersch, 2011).

Cryopreservation of fish embryos is undoubtedly challenging due to the numerous obstacles that must be overcome. Fish oocyte cryopreservation has so recently received increased attention (Isayeva et al., 2004; Zhang et al., 2005). Studies on fish oocyte cryopreservation have been conducted by several researchers, mainly on zebrafish (Guan et al., 2010; Anil et al., 2011), as well as various marine and freshwater species. Because of their smaller size, which aids in the effective permeability of water and cryoprotectants, low yolk content, and holoblastic cleavage of the developing embryos, shellfish embryos are easier to cryopreserve than fish embryos (Robles et al., 2008; Diwan et al., 2020).

The chromosomes in the egg are reportedly more susceptible to harm than the sperm. Additionally, sperm and eggs' loss of membrane integrity is a significant risk factor during the freeze/thaw process. According to recent research, freezing causes certain important enzymes to change or break down. There is a need for research, in particular, on the membrane permeability of the egg/embryos for various extenders, the effect of freezing on the retention of structural composition, the use of microinjections of different cryoprotectants and antifreeze proteins, as well as the application of osmotic and hydrostatic pressures to enhance cryosolution permeability. Therefore, more research is necessary to advance cryobiology so that it can be used to cryopreserve not just spermatozoa but also eggs, embryos, and larvae of fish and shellfish species that are economically significant. One of the main obstacles to establishing the aquaculture sector is the lack of enough spawners and seeds to generate seeds at the necessary period. Even if spawners are available, maintaining and managing them becomes challenging and expensive. Therefore, it is urgently necessary to develop appropriate technology for the cryopreservation and cryobanking of fertile gametes to produce aquatic creatures in captivity as needed (Diwan et al., 2020).

The potential genetic significance of aquatic organisms has frequently been disregarded. Even though there are more than 32,000 different species of fish (Barton, 2007), just 95 of these fish, crustaceans, and mollusks are used as the main food source for people. Most aquatic organisms have not had access to genetic resources in any way. Aquatic species may gain from genetic material management techniques like cryopreservation but have not been raised for genetic improvement. Germplasm serves as a medium of exchange, enabling the production, upkeep, and dissemination of genetic resources' many values (Torres and Tiersch, 2018).

Fish germplasm cryopreservation is a field that would significantly benefit from methods that make research more easily repeatable. Since its inception, about 65 years ago, countless protocols have been created for countless species (Cabrita et al., 2009; Tiersch, 2011). The majority of protocols were created without standardized methodologies, terminology, or reporting criteria due to the lack of community consensus and the wide variance in training and goals, which resulted in inconsistent experiments and meaningless or dubious comparisons (Martinez-Paramo et al., 2016; Torres et al., 2016).

Numerous hundreds of cryopreservation procedures have been created in response to the enormous diversity of aquatic species (Cabrita et al., 2009; Tiersch and Green, 2011). The differences between protocols typically range from the lack of, or at best, different methods to estimate sperm concentration to the non-standard addition of different types and doses of buffers and cryoprotectants, the use of different rates and methods of cooling, and the use of numerous methods to evaluate various aspects of sperm quality (Torres and Tiersch, 2018).

By making broodstock management simpler, germplasm cryobanking has significant applications in the reproductive procedures of cultured marine and freshwater aquatic species. Maintaining a significant laboratory model of fish species has also shown its utility. With the aid of reproductive biotechnologies like germ cell xenotransplantation, cryobanking has also proven to be a tool for preserving the genetic resources of a variety of species. It also plays a significant part in genetic selection programs, biodiversity preservation, and assisted reproduction. The genetic material of these species may be safely preserved using cryopreservation, giving scientists the chance to maintain representative samples and further recreate the original strain, population, or diversity. Technical expertise in genetics, reproductive physiology, cryobiology, and data administration is necessary for managing these banks (Martinez-Paramo et al., 2017).

Researchers have been interested in the possible use of this technique for freshwater and marine species since the first cryopreservation of fish sperm. Around the world, freezing procedures have been established for a wide range of species; many studies have concentrated on species from temperate areas with seasonal reproduction. Spermatozoa have some benefits over other cell types because of their size and relatively good resistance to chilling; cryopreservation of sperm is the most popular method in water animals. Although salmonids, cyprinids, and sturgeons continue to dominate most recent findings, establishing

cryopreservation techniques for species from different regions is becoming more and more popular (Viveiros and Godinho, 2009; Maria et al., 2015).

Many cryoprotectants were used in the cryopreservation techniques; 10% DMSO was the most efficient agent for the majority of the species in terms of motility and fertility rates. Additionally, 10% glycerol and 15% trehalose had a positive effect. High reproductive rates were produced by adding substances like BSA and cholesterol that interact with the plasma membrane (Cabrita et al., 2009; He et al., 2011; Martinez-Paramo et al., 2017). While 15% propylene glycol achieved comparable fertility of summer flounder, Paralichthys dentatus, 15% propylene glycol has been shown to offer the greatest results for flatfish sperm in terms of post-thaw quality (Martinez-Paramo et al., 2017). Due to their great size, limited membrane permeability, and cooling sensitivity, fish embryo cryopreservation is challenging (Martinez-Paramo et al., 2017). Since oocytes and follicles in fish are preferable parts for cryopreservation than embryos because they are smaller, have higher membrane permeability, are less sensitive to chilling, and have a simpler membrane system, more recent studies have concentrated on this topic (Isayeva et al., 2004; Zhang et al., 2005). Generally studies on fish oocyte cryopreservation have primarily focused on some species like zebrafish (Zhang et al., 2007; Guan et al., 2010; Anil et al., 2011; Godoy et al., 2013). There have been several studies to preserve various cell types that could ensure complete individual genome cryobanking, numerous studies on fish oocytes and embryos; still, preservation of the maternal genome is inadequate. Primordial germ cells, spermatogonia, and oogonia have been investigated and successfully cryopreserved in several fish species (Yoshizaki et al., 2011; Martinez-Paramo et al., 2017). With the use of reproductive biotechnological technologies, these cells can provide a good opportunity to preserve individual genomes (Yoshizaki et al., 2011).

Result

Since somatic cells are diploid, they have the advantage of passing both the maternal and paternal genomes on to future generations. Additionally, somatic cells can be obtained without regard to the fish's gender or age and still have the same value for genome preservation (Akimenko et al., 2003; McDonald et al., 2013). This is crucial when dealing with endangered species of fish or rare specimens because any serious harm should not degrade the priceless creature. Additionally, fin cells are among the finest donors for nuclear transfer in fish regeneration (Siripattarapravat et al., 2011).

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CHAPTER 14

Physiological Effects of Panax Ginseng, the Miraculous Herb of the Far East

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1.Introduction

The use of the ginseng plant, a member of the Araliaceae family, in traditional Chinese medicine dates back nearly 5,000 years. Derived from the Chinese word "zhensheng" meaning "plant person" because of its anthropomorphic form, ginseng root is believed to contain the three human essences: of body, mind and spirit. This is why ginseng roots are considered the "master of plants" (Yun, 2001).

A total of 8 to 13 species, including Panax ginseng, which means "cure-all", are called "Asian-Chinese ginseng", while Panax quinquefolius, which grows in the north of the American continent, is called "American ginseng". Siberian (Eleutherococcus senticosus) and Brazilian ginsengs (Pfaffia paniculata) are not actually considered as ginsengs, even though they are a type of Panax species. This is because they contain eleutherococcus, but not ginsenosides, which are the main active ingredients (Baeg and So, 2013).

The ginseng plant reaches full maturity after 6 years of growth and the ginseng obtained from the growing roots of the plant during this process is actually obtained from the root extract of the plant (REF PICTURE). P. ginseng contains many active components, the main ones being polysaccharides, ginsenosides, peptides and ligands. The most active component among these components is ginsenosides, which give the plant its name (Kiefer and Pantuso, 2003)

This plant, which is growing in popularity worldwide, is now on the shelves in shopping malls in almost all countries. It is widely used as a supportive treatment for many diseases such as mental or physical fatigue, weakness, exhaustion syndrome, distraction, liver, kidney and heart diseases and cancer (Choi BY, 2018).

In many experimental studies conducted with P. ginseng, it has been observed that it increases learning ability, shows antidepressant effect, reduces lipid peroxidation, protein and DNA damage in cell membrane, regulates the circulatory system and keeps blood pressure in balance (Park et al, 2022; Wang Z et al., 2023). In addition, many studies have reported that ginseng intake has a protective effect on experimental liver and kidney damage caused by various drugs, accelerates wound healing, supports the immune system in chemotherapy patients and increases sexual power in men (Lee et al., 2017; Hyun et al., 2022; Yang K, et al., 2023). In this review, the physiological effects of P. ginseng on organs and systems and current studies in this field are included.

2.Pharmacological Properties

Ginseng is orally administered and metabolized by intestinal flora through deglycosylation, oxygenation and hydration. These reactions occurring in the intestine are carried out by Bacteroides, Bifidobacterium, Eubacterium, Clostridium, Lactobacillus, Peptostreptococcus, Fusobacterium and Prevotella (Yun TK,2000). In terms of chemical structure, depending on their aglycone moieties, these glycosides are divided into 20(S)-protopanaxadiol (PPD; ginsenosides Rb1, Rb2, Rg3, Rc and Rd), and 20(S)-protopanaxatriol (PPT; ginsenosides Re, Rg1, Rg2 and Rh1), which contain many subgroups (Kim et al., 2013; Zhang et al. 2023).

In a pharmacokinetic study in humans, the mean compound K conversion activity for ginsenoside Rb after ingestion of Panax ginseng powder form (12 g) was reported to be 1381.1 ± 427.8 mmol/(h-g). The passage of compound K into the blood begins 4 hours after ginseng administration and reaches its maximum level 9 to 14 hours after administration (Lee et al., 2009).

Ginsenosides are metabolized in the liver by the oxidation reaction of cytochrome P-450 isoform 3A4 (CYP3A4) and enter the enterohepatic circulation (Yang et al., 2018). There are many in vitro studies on the effects of P. ginseng components or metabolites on human or animal cell systems and liver or intestinal microsomes. Unfortunately, all these studies report different independent results. According to these studies, ginsenoside or substrates were reported to inhibit, induce or have no effect on CYP3A4 synthesis. Similar results have been reported for other cytochrome isoforms CYP1A2, CYP2C9, CYP2C19 and CYP2D6 (Hu et al., 2023; Yang et al., 2018; Goey etal., 2013).

The reasons for the difference between the reported results are suggested to be the use of ginsenosides and compounds used in in vitro studies at higher concentrations than the doses recommended for humans or differences in the exposure time of cells or tissues to active substances. The main route of excretion of ginsenosides is through feces and only 0.2-1.2% is excreted in the urine without being metabolized. (Hu et al., 2023; Goey et al., 2013; Yun TK,2000).

3.Physiological Effects

3.1-Antioxidant effect

P. ginseng contains many phytochemicals with potent antioxidative effects that protect the organism against damage caused by oxidative stress. Many in vitro tests have been performed to examine the antioxidant activity of these

phytochemicals and as a result, it has been found that they usually show their free radical scavenging activity through hydrogen atom transfer and/or electron transfer mechanisms (Yang et al., 2011, Park et al., 2010). Yuan et al. (2012) reported that administration of red ginseng oil to HepG2 cells decreased the radical formation and oxidative stress induced by 2,2-azobis (2-amidino-propane) dihydrochloride and Cu2þ ion. Again Park et al. (2010) showed that ginsenosoid administration inhibited the formation of intracellular reactive oxygen species induced by amyloid beta peptide in PC12 cells.

In addition to the strong antioxidant capacity of P. ginseng, it is thought to contribute positively by increasing the levels of primary antioxidant enzymes in the organism. In this regard, it was found that the addition of ginseng to the medium containing HepG2 cell lines treated with H2O2, a compound that damages cells, caused the CAT, SOD and GPx enzyme activity levels of the cells to remain constant (Cui et al., 2018). Similarly, it was reported that ginseng applications increased the decreased CAT, SOD2 and GPx enzyme levels in the liver tissue in liver damage induced by CCl4 in mice and thus reduced the damage to the liver (Ning et al., 2018).

3.2-Nervous system and behavior

P. ginseng has beneficial effects on nerve cells due to its antioxidant, anti-inflammatory, lipid peroxidation inhibiting and neuroplasticity enhancing effects. Due to these effects, the use of ginseng may contribute positively to neurodegenerative or other nervous system diseases (Jin et al, 2019). On the other hand, there are also reports that ginseng intake may affect the nervous system by regulating the levels of neurotransmitters commonly found in the nervous system such as serotonin, dopamine and norepinephrine. There are many in vivo and in vitro studies to examine all these effects of P. ginseng on the nervous system and nerve cells (Lu et al., 2022; Nah SY 2019; Rokot et al., 2016).

In a study investigating the protective effect of Rd applications on ischemic brain injury induced by experimental middle cerebral artery occlusion in aged mice, it was reported that it significantly reduced both cortical and striatal infarct volume; improved neurological functions, prevented mitochondrial damage and suppressed lipid peroxidation. This study showed that Rd applications may act as a neuroprotective agent against focal ischemia that may be caused by redox-induced damage in aged brain tissue (Ye et al., 2013).

Patel and Rauf (2017) have been reported that the adaptogenic properties of people who use P. ginseng increase, and that these people cope with stress and

recover and regain balance faster. As it is known, long-term exposure to stress adversely affects many systems in the body, but causes the greatest damage to the nervous system. There are many studies reporting that the use of P. ginseng reduces stress levels and even increases resistance to stress. In a study investigating adaptation by providing cold tolerance, both young and old rats were administered Rb1 intraperitoneally (i.p.) at concentrations of 2.5 and 5.0 mg/kg. As a result of this study, it was found that Rb1 administration increased thermogenesis and cold tolerance in both young and old rats, while Rg1 administration was reported to be ineffective in the same study (Wang and Lee, 2000). In another study, it was found that 20 mg/kg Rb1 administration to mice improved adaptation by recognizing objects in mice and reduced immobility time in the forced swimming test. The researchers interpreted the reason for this situation as that Rb1 administration increased neuronal 5-HT concentration and tryptophan hydroxylase enzyme activity in the brain tissue of mice, while decreasing MAO activity (Hao et al., 2011).

On the other hand, the positive effects of P. ginseng on cognitive functions have also been reported. It is thought to increase cognitive functions due to the protective effect of ginsenosides in its content on neurons. Many studies in this field have shown that ginseng has a healing effect on memory and attention deficits. In a study conducted by Kennedy et al. (2003), 15 healthy volunteers were given a single dose of 200 mg P. ginseng (G115) extract and electrical conduction in the brain was measured. It was found that cerebral electrical conduction in the brain of volunteers receiving G115 was more regular than in the control groups. In another study investigating memory quality in 20 healthy adults, 200, 400 and 600 mg of G115 were given for 5 days and it was determined that the best memory quality was obtained at a dose of 400 mg (Scholey and Kennedy, 2002). Planned a study in which rats (Liu et al., 2011) were given Rb1 at a concentration of 2 mg/kg for 30 days in a Morris water maze setup to examine the effects of P. ginseng on spatial and cognitive performance. As a result of the study, it was found that Rb1 significantly increased the survival of cells in the dentate gyrus and CA3 region of the hippocampus, but had no significant effect on cell proliferation in hippocampal sub-regions.. According to these results, it has been suggested that ginseng may facilitate spatial learning and memory and may be used as a therapeutic agent for dementia patients with memory loss.

P. ginseng also supports the energy metabolism of cells. As a result of this situation, the use of ginseng causes a decrease in the feeling of fatigue in people. As a result, this indirectly has a positive effect on the nervous system and causes vitality and alertness in people who use ginseng. Taking advantage of this effect

of P. ginseng, some researchers recommend taking P. ginseng in the treatment of severe psychogenic illnesses such as depression. In a study (Xu et al., 2010) comparing the antidepressant effect of 20(S)-protopanaxadiol, the intestinal metabolite of ginseng, with fluoxetine, it was found that 20(S) PPD given orally showed antidepressant properties as strong as fluoxetine, decreased oxidative stress values in brain tissue and serum corticosterone levels, and decreased monoamine neurotransmitter reuptake activity. These results showed that this metabolite of ginseng has a very strong antidepressant effect.

In a study investigating its effects on feeding behavior, rats were administered Rb1 intracerebroventrically at a dose of 0.05-0.2 μmol. It was reported that Rb1 administration dose-dependently decreased food intake; suppressed feeding at the highest dose of 0.2 μmol; increased plasma glucose levels of rats, but did not change insulin levels (Etou et al., 1988).

3.3-Heart and vascular system

P. ginseng has some important regulatory effects on the cardiovascular system due to the various mechanisms of action of its active components. Ginsenosides increase blood circulation by inhibiting ROS formation, increasing nitric oxide (NO) production, improving vasomotor tone and regulating blood lipid profiles to prevent atherosclerosis (Zheng et al., 2012). The active components of ginseng responsible for all these effects are G-Ro, G-Rb1, G-Re, G-Rgl, G-Rg3, PT (Liu et al., 2020).

There are many studies on the possible effects of ginseng on the cardiovascular system. In one of these studies, it was reported that the administration of ginseng extract to patients with acute heart attack resulted in an improvement in the coronary blood flow of these patients and an increase in the number of angiogenic cells that provide this improvement in flow (Zheng et al., 2012).. In a study in which diabetes-induced rats were given intravenous Rb1, it was observed that apoptosis and caspase-3 activities in myocytes decreased and infarct size decreased (Wu et al., 2011). It has been shown that Rb1 given intravenously at a dose of 40 mg/kg before the experiment decreased the severity of the infarction, significantly decreased creatine kinase, lactate dehydrogenase and troponin T levels, and increased protein kinase (Akt) phosphorylation in the damage caused by ischemia-reperfusion in rat heart (Wang et al., 2008). When all these results are evaluated together, it is thought that ginseng improves blood circulation of tissues by inhibiting platelet aggregation or coagulation.

When the effects of ginseng on vascular endothelial cells are examined, it has been observed that ginsenosides may play a role in the formation of angiogenesis with their angiomodulatory and neurotropic effects by acting on Pl3 K/Akt-dependent kinase 1/2 and eNOS pathways that regulate extracellular stimulation in these cells (Lee et al., 2016). Re, also a type of ginsenosoid, activates K+ channels in vascular smooth muscle cells through Pl3 K/Akt and NO pathways (Lü et al, 2019). In a study conducted in 2011, 80 volunteers using antihypertensive drugs were additionally given 3 g P. ginseng daily in powder form for 3 months. As a result of this study, in which its possible effect on atherosclerosis was investigated, it was reported that there was no significant change in the systolic blood pressure of the patients, and a significant decrease in the diastolic blood pressure of all patients in the study groups and in the systolic blood pressure of the placebo group (Rhee et al., 2011). When all the results obtained are evaluated, it is suggested that ginsenosides may have a protective effect on vascular endothelial cells through cellular stimulation pathways.

In addition, some compounds such as Rd from ginsenosides have been reported to prevent atherosclerosis by regulating lipid profile in mice (Li et al., 2011). Intraperitoneal administration of Rd at a dose of 20 mg/kg for 12 weeks to apolipoprotein E deficient (apoE(-/-)) mice prevented the development of atherosclerosis ((Xue et al., 2021). In an in vitro study, Rg1 and Rh2 were also found to have a protective effect against oxidation-induced damage to the erythrocyte membrane (Li and Liu, 2008).

On the other hand, Engels et al. (2003) reported that long-term dietary intake of G115 had no positive effect on heart rate regulation in a study conducted with a group of 38 healthy volunteers.

3.4-Digestive system and metabolism

Ginseng can affect gastrointestinal secretion and peristaltic movements through various mechanisms caused by the active substances in its content. It can regulate digestive activity by changing the sensitivity of receptors in the digestive tract or the release rate of neurotransmitters released in stimulation (Yang et al. 2020). For example, it has been reported that ginseng may increase the release of acetylcholine, which causes contraction in the smooth muscles of the digestive canal, or inhibit the effect of serotonin, which blocks contractions (Zhao et al., 2024). Xu and Huang (2012) reported that ginsenosides have a direct effect on peristalsis by increasing the contractility of intestinal smooth muscles. These effects reduce the risk of constipation by allowing the excretion of digestive wastes along the canal.

It is known that there is a bidirectional highly dynamic communication network between the CNS and the digestive tract. Based on this reality, ginseng is thought to affect motility and secretions by regulating the release activity of certain neurotransmitters, hormones and signaling molecules that mediate the effects of the CNS on the digestive tract (Iqbal et al., 2024).

Studies with ginseng have shown that ginseng intake has positive effects on the diversity of various intestinal flora such as bacteria and yeast. These flora effects may contribute to the treatment of certain digestive disorders such as constipation, diarrhea and abdominal pain (Yu et al., 2021; Iqbal et al., 2024). Although clinical evidence supporting these reports is limited for the time being, it has been observed that ginseng added to the diets of some patients reduces digestive complaints. There are also some reports that the use of ginseng in the treatment of irritable bowel syndrome and peptic ulcer may have positive effects. However, there is a need for some supportive studies on their clinical use and safety (Yu et al., 2021;Wang et al., 2023)

There are many studies reporting that P. ginseng administration to humans and animals regulates liver enzyme activities. In particular, in studies in which ginseng was administered to subjects before damage as a protector; it was observed that it regulated fluctuations in liver enzymes, thus protecting liver function and reducing damage (Kim TW 2016; Yang et al., 2023). In fact, in a study we conducted, we found that P. ginseng applications improved the tissue damage caused by CCl4-induced liver damage and that these improvements were reflected both biochemically in enzyme levels and histologically in tissues (Karakus et al., 2011). It is suggested that ginseng reduces fat accumulation in the liver by regulating enzyme systems involved in lipid metabolism. Many studies have reported that P. ginseng acts as an anti-hyperlipidemic agent and accordingly decreases serum total cholesterol, LDL and trglyceride levels and increases HDL levels (Yu et al., 2021). In one study, it was reported that Rb2, a type of ginseng saponin, increased bile acid synthesis and cholesterol excretion in rabbits fed with cholesterol for a long period of time, thus decreasing blood cholesterol levels (Lee et al., 2013). In another study, it was observed that administration of ginseng to healthy rats increased hepatic cholesterogenesis but not cholesterol breakdown and excretion in feces (Joo CN, 1992). Park et al. (1987) reported that ginsenoside administration increased the number and synthesis of LDL receptors in rats. In contrast, Ismail et al. (1999) reported that ginseng G-115 given to rabbits did not show a significant positive hypolipidemic or antioxidant effect.

3.5-Diabetes

It has been reported in many studies that saponins and ginsenosides contained in P. ginseng have the effect of reducing increased blood glucose levels and stimulating insulin release. However, the data obtained from the studies on the effects of ginseng on diabetes to date show that ginseng can adjust blood glucose in four ways. In the first of these, ginseng is thought to regulate blood glucose regulation by improving cellular functions and increasing sensitivity to insülin (Luo and Luo, 2009; Mancuso and Santangelo 2017; Chen et al., 2019). For example Kim et al., (2016) reported that administration of ginseng to mice with STZ-induced diabetes induced proliferation of beta cells in the pancreas and increased insulin secretion, thereby controlling blood glucose. In a similar study, Wang et al. (2022) reported that administration of ginseng extract to diabetic mice may increase sensitivity to insulin, possibly by increasing IRS-1 and AKT activation pathways in cells.

Secondly, ginseng may increase the glucose uptake of cells by increasing the expression of glucose transporter (GLUT) receptor proteins in cells. In a study conducted on rats with diabetes, oral administration of Re ginsenoside at concentrations of 5, 10 or 20 mg/kg caused a significant decrease in blood glucose, cholesterol and triglyceride levels of rats. It was also found that glutathione and malondialdehyde levels in the eye and kidney tissue of the animals belonging to the same group decreased to normal (Cho et al., 2006). In another study in which 21% fermented red ginseng was administered to ob/ob mice for 16 weeks, body weight decreased significantly in the first 4-week results compared to the control group, and at the end of the application, it was found that blood glucose decreased significantly (Cheon et al., 2015). In this study, it was found that ginseng administration significantly increased GLUT1 and GLUT4 expressions in liver and muscle tissue. This result supports the thesis that ginseng can regulate elevated blood glucose by increasing glucose uptake in skeletal muscle and liver tissue. In a study conducted to investigate the effects of ginseng on glucose tolerance in type-2 diabetic patients, 20 type-2 diabetic patients were given two capsules containing 369 mg ginseng powder three times a day. At the end of the four-week study, it was observed that insulin resistance and fasting blood glucose of the patients in the group decreased compared to the placebo group, but there was no significant change in glucose and insulin responses, oxidative stress and antioxidant values in the Oral Glucose Tolerance Test (OGTT) (Ma et al., 2008).

Thirdly, it has been reported that administration of ginseng polysaccharides such as ginsenoside Rg1 to STZ-induced diabetic rats (Yokozawa et al., 1985) suppressed oxidative stress by increasing SOD activities and decreasing MDA formation in the tissues of the patients and contributed positively to the treatment. Jung et al., (2021) found that administration of pectin lyase modified red ginseng extract to diabetic rats for 6 weeks significantly decreased the levels of urinary albumin, 8-hydroxy-20-deoxyguanosine and advanced glycolization end products of diabetic rats.

As the last fourth mechanism, ginsenosides administered in diabetic mouse or rat models were found to regulate the expression of mediators involved in inflammation such as TNF-α and eNOS. This suggests that ginseng may prevent the development of insulin resistance that may occur due to inflammation (Park et al., 2014).

3.6-Erectile dysfunction

Although drugs such as phosphodiesterase inhibitors are the first-line agents in erectile dysfunction (ED), the contraindications they cause in some patients and the low success rate in patients with diabetes or prostate surgery force patients to investigate some alternative treatment methods such as ginseng (Argiolas et al., 2023). Although the mechanism of action of ginseng on erectile dysfunction is not known exactly, studies have reported that ginseng applications may cause vasodilatation and consequently erection by increasing nitric oxide release in the smooth muscles of penile cavernous tissue (Lee et al., 2021). Related to this, in studies on the effects of active components of ginseng on smooth muscles of rat and rabbit corpus cavernosum in isolated organ systems, it was observed that they caused relaxation in smooth muscles depending on the dose (Leisegang and Finelli 2021; Ratan et al., 2021). In another study, the effect of ginseng on cavernous tissue was studied in rats with metabolic syndrome. At the end of the twelve-week treatment, the intracavernous pressure (ICP) of the ginseng group was found to be higher than the saline group (Lee et al., 2021)).

There are studies reporting that the use of ginseng reduces complaints related to erectile dysfunction. For example, in the meta-analyses of 7 randomized trials investigating the use of 600-1000 mg ginseng 3 times a day for 4-12 weeks in patients with psychogenic, organic or mixed ED, it was observed that there was a significant improvement in the complaints of the patients due to the use of ginseng compared to the placebo groups, with the most benefit in the psychogenic ED group (Jang et al., 2008). In another study, P. ginseng was given three times a day to examine the effectiveness of P. ginseng on a total of 60 patients with

mild or moderate ED symptoms. It was shown that the five-item scoring determined by the International Index of Erectile Function (IIEF) of patients receiving P. ginseng was statistically higher compared to the pretreatment status (Rosen et al., 1998).

3.7-Kidney

It is thought that Panax ginseng may provide protection by preventing oxidative stress, inflammation, epithelial-mesenchymal transitions and fibrosis in kidney tissue (Liu et al., 2020; Shi et al., 2020; Xie et al., 2020; Zhu et al., 2020; Li et al., 2021). The actin-rich foot-like structures found in the structure of podocytes contribute to the renal filtration barrier. Decreased protein expression in the structures that make up the podocyte feet causes proteuria, kidney damage and ultimately failure (Leeuwis et al., 2010). It is reported that ginsenosoid applications increase the formation of these receptor proteins, heal the damage and thus have a protective effect on the kidneys. Fan et al., (2021) reported in their study that the use of ginseng has a protective effect by increasing the synthesis of actin, which participates in the structure of podocytes that form the glomerular filtration barrier. Sun et al., (2011) studied both in vivo and in vitro in rats with diabetic nephropathy that high glucose may cause oxidative stress-related apoptosis in podocytes. In these studies, they found that 10 weeks of ginseng administration increased the number of podocytes and related mRNA expressions and had a protective effect against the complications caused by diabetes.

The structure and thickness of the glomerular basement membrane may vary in renal diseases. Ginsenoside Rg1 applications reduce IL-1β-induced podocyte inflammation and apoptosis by regulating the nuclear factor E2-related factor 2 (Nrf2) pathway (Xie et al., 2020; Zhu et al., 2020; Li et al., 2021). On the other hand, some in vitro studies have shown that Rg 1 ginsenoid administration plays a positive role in improving epithelial-mesenchymal transition (EMT) of tubule cells. Epithelial-mesenchymal transition is an important biological process in the differential diagnosis of tubulointerstitial renal fibrosis in which changes along epithelial and mesenchymal cell lines are examined (Guo et al., 2019). As a matter of fact, Ni et al. (2017) reported that ginsenoside Rg l prevented EMT damage by activating PI3K/AKT pathway and inhibiting NF-κB pathway in tubule epithelial cell line HK-2s against inflammation and apoptosis induced by lipopolysaccharide treatment. Similar results were found in the study conducted by Yang et al., (2019).

3.8-Menopause

Although hormone replacement therapy is usually applied in menopause, patients may resort to many supportive therapies including acupuncture, aromatherapy and herbal cures to prevent adverse effects. Many herbs, especially sage, centaury, fennel and ginseng, are widely used in the elimination of side effects that occur in menopause and their clinical effects are being investigated (Ru et al., 2015).

It is reported that the use of ginseng in menopause may correct hormonal imbalance, control vasomotor symptoms and prevent hot flashes or night sweats. Another positive effect of Panax ginseng on menopause is that it increases its adaptogenic properties in individuals using it. As a reflection of this situation, emotional fluctuations that occur during menopause are reported to be less in people who use ginseng (Kim et al., 2013; Kim et al., 2015). Many studies have also reported that ginseng can help people cope with stress and fight depression. In a study conducted on 384 postmenopausal women, a significant difference was found in the depression and peacefulness levels of the group taking ginseng, but no difference was observed in vasomotor symptoms such as hot flashes (Wiklund et al., 1999).

It has been suggested that the use of P. ginseng may increase calcium absorption from the intestine and kidneys, increase bone density and consequently reduce the risk of postmenopausal osteoporosis (Amato et al., 2002). Polan et al., (2004) reported that the possible mechanism of action of ginseng on menopausal symptoms is similar to the hormone estrogen.

3.9-Cancer

Although many studies have been conducted on the use of P. ginseng against cancer, the evidence obtained has not yet been conclusive. In vitro and in vivo studies in this field indicate that the protective properties of ginseng against cancer may be due to the antioxidant and anti-inflammatory effects of ginsenosides in its content (Cao et al., 2019). In vitro studies with many active substances contained in ginseng have reported that it inhibits the growth of cancer cells or causes apoptosis (programmed cell death) in cancer cells (Chen et al., 2016; Lv et al., 2023). Ginseng is also thought to regulate the severity of the immune system's reaction against cancer cells due to its immunomodulatory effect (Lee at al., 2018).

Some different results have been obtained in clinical studies on the use of ginseng in the prevention or treatment of cancer. While some studies in this field

report potential benefits of ginseng use, some studies report no significant effect and even some negative results. Due to its possible interaction with drugs used during cancer treatment and its side effects, it is recommended that people should consult their doctor before adding ginseng or any herbal supplements to their treatment protocols (Wang et al., 2016; Mancuso and Santangelo 2017)

It is also known that the incidence of various cancers such as stomach and lung is lower in ginseng users compared to non-users. Chang et al., (2003) reported in a study that the use of ginseng inhibits the formation of vessels that indicate the development and spread of cancerous tissues and increases the effects of chemotearpetic drugs such as mitomycin and tamoxifen.

3.10-Immune system

Plant-derived antioxidant compounds containing flavonoids, terpenoids, lignans, polyphenolics, sulfites and saponins that support the immune system can be used as immune system supporting agents in the treatment of chronic diseases. There are many in vitro and in vivo studies showing that ginseng, one of the plants with these compounds, supports the immune system (Cao et al., 2019; Lee at al., 2018).

In vitro and in vivo studies on spleen cells of mice showed that oral administration of P. ginseng at a concentration of 2 g/kg stimulated B-lymphocyte proliferation, interleukin (IL)-2, IL-10 and interferon-gamma production (Liou et al., 2006). In another study, Miller et al., (2012) reported that ginseng administration increased the number of natural killer cells in mouse spleen and bone marrow. Rhule et al., (2006) found that the immunomodulatory effect of ginseng inhibited LPS-induced TNF-α and IL-6 production in a concentration-dependent manner in a study on RAW264.7 macrophages cells.

The positive effect of ginsenosides on phagocytic activity was also studied on RAW264.7 macrophage cell lines and the results obtained show that it supports the immune system. In another study, it was reported that ginseng aqueous extract stimulated the synthesis of inducible nitric oxide (i-NOS), a reactive nitrogen produced by macrophages and used against pathogenic microorganisms (Kim et al., 2013). In another study with the same cells, it was reported that ginseng extract increased the production of many cytokines including IL-6, IL-1α, IL-1β, TNF-α (Um et al., 2020).

In studies examining the effects of ginseng on the cytotoxicity of natural killer cells, it was reported to increase the activation of cells taken from both normal and AIDS patients. Similarly, in a study in which 20 healthy individuals were

given 1 g of ginseng extract daily for 14 days, it was found that the cytotoxicity of the cells of the subjects increased (Scaglione et al., 1990).

There are many in vitro, in vivo and experimental clinical studies investigating the effects of ginseng on humoral immunity. Liou et al., (2006) found that serum IgG levels decreased, IgA levels increased and IgM levels remained the same in a study in which male mice were given oral ginseng extract for five days. In addition, there are many in vitro and in vivo studies on the use of ginseng and active ingredients in virus and fungal infections. For example, Sung et al., (2008) reported that the antifungal activity of ginseng is caused by its disruption of the membrane integrity of the fungal cell. Lee et al., (2008) reported that administration of ginseng extract on the viability of alveolar epithelial A549 cells in H1N1 virus infection in humans reduced virus-induced cytokine secretion and reactive oxygen species formation, which may increase the success in the treatment of H1N1 virus infection.

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CHAPTER 15

Genes and Signaling Pathways Involved in Proliferation and Differentiation of Stem Cells

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1. Introduction

Embryonic stem cells or pluripotent stem cells have indefinite division and differentiation properties for all somatic cells derived from the three embroynic germ layers: ectoderm, mesoderm, and endoderm (1). These cells originate from the inner cell mass (ICM) at the blastocyst stage during early embryonic stage, serving as fundamental components in the development of all multicellular organisms (2). The primary characteristics distinguishing embryonic stem cells from somatic (differentiated) cells are their self-renewal ability and their potential to differentiate into diverse cell lineages. Upon losing their totipotent properties, embryonic stem cells acquire a pluripotent identity, enabling them to differentiate into various tissue and organ cells when cultured in vitro. This differentiation process facilitates their transformation into specific cell types, including neurons, cardiomyocytes (heart muscle cells), hepatocytes (liver cells), and pancreatic hormonal cells. Consequently, embryonic stem cells hold significant promise in fields such as regenerative medicine, disease modeling, and drug development. Embryonic stem cells sustain their pluripotency through asymmetric cell division, producing both identical stem cells and precursor cells capable of differentiating into distinct cell lineages. This equilibrium between renewal and differentiation is meticulously regulated by a series of signaling pathways that govern the cell proliferation and tissue development. These pathways mediate the responses of stem cells to external environmental factors and intracellular regulatory mechanisms. It is recognized that the mechanisms controlling cell differentiation across different organisms during evolution are analogous, with certain signaling networks collaborating to ensure cellular regulation. Notably, developmental signaling pathways such as Wnt, Notch, Hedgehog, and BMP have been demonstrated to play pivotal roles in maintaining pluripotency and directing the differentiation of embryonic stem cells. A comprehensive understanding of these biological processes will enhance the development of cell-based therapeutic approaches, the modeling of genetic diseases, and the formulation of more effective treatment strategies in regenerative medicine.

Signaling pathways that regulate the pluripotency and self-renewal capacity of embryonic stem cells are pivotal in controlling cellular differentiation processes (3). These signaling networks comprise intricate mechanisms that interact with both intracellular and extracellular factors to direct the cell cycle, proliferation, and differentiation processes. The Transforming Growth Factor Beta (TGF-β)/Activin/Nodal signaling pathway operates through Smad 2/3/4

proteins, which are crucial for maintaining pluripotency, and also modulates cell growth and survival by activating the Mitogen-Activated Protein Kinase (MAPK) and Akt signaling pathways. The Wnt/β-catenin signaling pathway contributes to determining cellular fate by interacting with the β-catenin molecule, which sustains pluripotency in embryonic stem cells. This process is regulated by the transcription activator TCF1 and the repressor molecule TCF3, thereby controlling whether stem cells remain pluripotent or proceed to differentiation. Additionally, the Notch signaling pathway, which promotes the differentiation of embryonic stem cells but negatively impacts proliferation, plays a critical role in tissue organization by regulating intercellular communication (4). The Bone Morphogenetic Protein (BMP) signaling pathway functions as a mechanism that directs differentiation processes by suppressing stem cell proliferation. The Hippo and Leukemia Inhibitory Factor (LIF)/Signal Transducer and Activator of Transcription (STAT) signaling pathways are the primary signaling mechanisms responsible for maintaining the self-renewal capacity of embryonic stem cells and preserving the undifferentiated cell state. Genes that sustain the pluripotent state include Oct-4, Sox-2, and Nanog, which encode key transcription factors essential for the self-renewal and differentiation capabilities of embryonic stem cells (5). Furthermore, surface markers such as SSEA-3/4, TRA-1-60, and TRA-1-81 are significant glycoproteins that define the phenotypic characteristics of embryonic stem cells. Proteins synthesized by these genes serve as biomarkers for the identification and characterization of embryonic stem cells. A more comprehensive understanding of these mechanisms will contribute to the development of novel strategies for regenerative medicine, genetic engineering, and cellular therapies.

Mesenchymal stem cells (MSCs), akin to other stem cells, possess the ability for self-renewal, albeit with a more restricted differentiation potential compared to embryonic stem cells. These cells exhibit multipotent proliferation and have the capacity to differentiate into specific cell lineages. A notable characteristic of mesenchymal stem cells is their ability to differentiate into cells derived from connective tissue, including cartilage (chondrocytes), bone (osteoblasts) (6), adipose tissue (adipocytes), and neural cells. Bone marrow, adipose tissue, umbilical cord, synovial fluid, dental pulp, and amniotic fluid are among the primary sources of mesenchymal stem cells in adult organisms. Notably, bone marrow-derived mesenchymal stem cells are prominently utilized in regeneration and tissue engineering applications. The differentiation processes of mesenchymal stem cells are governed by various signaling pathways. The Bone Morphogenetic Protein (BMP) signaling pathway directs MSCs towards

chondrogenic (cartilage cell) differentiation, while the Transforming Growth Factor Beta (TGF-β), BMP, and Fibroblast Growth Factor (FGF) signaling pathways guide these cells towards osteogenic (bone cell) and adipogenic (fat cell) lineages. Additionally, the WNT/β-catenin, Notch, and Mitogen-Activated Protein Kinase (MAPK) signaling pathways are recognized as critical regulators of both differentiation and self-renewal mechanisms in mesenchymal stem cells. Beyond these signaling mechanisms, the RunX2/Cbfa1 and Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ2) genes control the osteoblastic or adipocytic differentiation and proliferation processes of mesenchymal stem cells. Proteins synthesized from specific genes play a pivotal role for determining cellular fate, acting as specific transcription factors for bone and adipose tissue development. An enhanced understanding of mesenchymal stem cell biology will contribute to the advancement of innovative therapeutic approaches in clinical areas such as skeletal tissue engineering, cartilage regeneration, treatment of neurodegenerative diseases, and immunotherapies.

The processes of differentiation and proliferation in embryonic stem cells, mesenchymal stem cells, and other stem cell types are meticulously regulated by specific signaling pathways and genetic mechanisms. Signaling pathways such as TGF-β/Activin/Nodal, Wnt/β-catenin, Notch, BMP, Hippo, and LIF/STAT, along with related transcription factors including Oct-4, Sox-2, Nanog, RunX2/Cbfa1, and PPAR-γ2, play pivotal roles in determining cellular fate. These pathways interact to ensure that cells maintain a balance between self-renewal and differentiation. While some signaling pathways are activated, others are suppressed, allowing stem cells to elicit appropriate cellular responses. Through these dynamic regulatory mechanisms, stem cells either sustain the cell pool via asymmetric and/or symmetric division or select a specific differentiation pathway, demonstrating the capacity to differentiate into over 200 cell types present in the organism. The precise regulation of these proliferation and differentiation processes by genetic and epigenetic mechanisms is crucial for maintaining the functional integrity of stem cells. However, disruption of signaling pathways can trgiger to the loss of normal biological roles in stem cells, resulting in abnormal proliferation and uncontrolled differentiation. Notably, dysregulated activation of developmental signaling pathways can make stem cells to differentiate into cancer stem cells or initiate oncogenic processes in the affected tissues. Therefore, a deeper understanding of the interplay between stem cell biology and signaling pathways will contribute to the development of novel strategies in regenerative medicine, cancer therapy, and cellular therapy approaches.

2. Signalling Pathways Related to Proliferation and Differentiantion of Stem Cells

2.1. WNT/β-Catenin Signalling Pathway

The Wnt gene family comprises 19 distinct genes that regulate biological mechanisms, including cell growth, differentiation, tissue homeostasis, and embryonic development (7). These genes exert their influence on cellular mechanisms through two primary pathways: the β-catenin-dependent canonical Wnt signaling pathway and the β-catenin-independent non-canonical Wnt signaling pathway. Each Wnt gene fulfills specific roles in developmental processes; for instance, Wnt7b promotes osteoblastogenesis, while Wnt10b is expressed in the bone marrow microenvironment to perform particular functions. The Wnt signaling pathway governs critical mechanisms such as cell proliferation, differentiation, apoptosis, and cellular and tissue regeneration in embryonic, mesenchymal, or other stem cell types through the involvement of proteins synthesized from these genes in signaling processes. Notably, in osteoblastic and chondrogenic differentiation processes, the Wnt signaling pathway plays a significant role and is instrumental in skeletal development and tissue repair. In the activation process of the Wnt signaling pathway, co-receptor proteins on the membrane surface of stem cells also contribute to the regulation of signaling. One such protein is the LRP membrane protein, synthesized from the gene of the same name. Specifically, the LRP5 protein contributes to the canonical Wnt signaling pathway and regulates chondroblastic and osteoblastic proliferation and differentiation. Additionally, various Wnt proteins participate in this regulatory process; for example, Wnt7b activates the canonical Wnt signaling pathway by stimulating the LRP protein and mediates the initiation of chondroblastogenesis and osteoblastogenesis. Through this mechanism, cell types of cartilage and bone tissues can be derived from stem cells. The canonical Wnt signaling pathway is known to be involved in the regulation of Transforming Growth Factor Beta (TGF-β) and Bone Morphogenetic Protein (BMP) molecules. However, any disruption in this control mechanism may lead to hypertrophy and abnormal growth processes in osteocyte and chondrocyte cells. Furthermore, the interaction of Fibroblast Growth Factor Receptor 1 (FGFR1) with the Wnt signaling pathway plays a critical role in skeletal development and tissue homeostasis by contributing to the orderly progression of chondrogenic differentiation and proliferation processes.

In contrast to the canonical Wnt pathway, the non-canonical Wnt signaling pathway modulates cellular processes through alternative signaling mechanisms

rather than a β-catenin-dependent route. This pathway engages with molecules such as c-Jun N-terminal kinase (JNK), calcium (Ca^{2+}), the Rho GTPase family, diacylglycerol (DAG), and protein kinase C (PKC) to orchestrate cellular signaling processes. The inhibition of canonical Wnt signaling and the activation of the non-canonical pathway are pivotal in determining cellular fate. Specifically, this pathway plays significant roles in maintaining cell polarity, morphogenesis, cell movement, tissue homeostasis, and immune response. Within this framework, the Wnt signaling pathway is particularly influential in osteogenic (bone formation) and adipogenic (adipose tissue formation) differentiation and proliferation processes, with specific Wnt ligands and receptors involved in regulating these biological processes. Interactions between ligands such as Wnt1, Wnt4, Wnt5a, Wnt11, and Wnt16 and receptors such as FZD1, FZD3, FZD4, FZD5, and FZD6, which belong to the Frizzled (FZD) receptor family, facilitate the activation of this signaling pathway. Furthermore, specific microRNAs, such as miR-27 and miR-29a, are recognized for their crucial roles in the intracellular regulation of the Wnt signaling pathway. These microRNAs influence osteoblastic and adipoblastic differentiation processes by modulating gene expression within the Wnt pathway. However, it is acknowledged that the regulatory and activator genes, proteins, and signaling molecules involved in this pathway may exhibit varying effects under in vivo and in vitro conditions. Consequently, it is evident that the Wnt signaling pathway is contingent upon the cellular microenvironment, cell type, and organismal-level factors, necessitating further research to comprehensively elucidate its mechanism..

2.2. TGF-β Signalling Pathway

The Transforming Growth Factor Beta (TGF-β) signaling pathway serves as a pivotal regulatory mechanism governing cell differentiation, growth, and the continuous of cellular homeostasis in both embryonic and adult stem cells (8). The TGF-β superfamily encompasses a broad array of proteins, including numerous growth factors and morphogens, which are integral to processes such as the development of the skeletal system and the maintenance of postnatal skeletal homeostasis. TGF-β-activated signaling pathways are instrumental in regulating cell proliferation and differentiation and are implicated in various biological processes, including tissue remodeling, immune response modulation, and tumor suppression. Stem cells synthesize molecules such as angiopoietin-2, granulocyte colony-stimulating factor (G-CSF), hepatocyte growth factor (HGF), interleukin-6 (IL-6), interleukin-8 (IL-8), and platelet-derived growth factor (PDGF-BB) following the activation of the TGF-β signaling pathway, with

contributions from the MEK/MAPK and PI3K/Akt signaling pathways. This mechanism orchestrates the interaction of cells with their microenvironment, facilitating tissue regeneration and the functional maintenance of stem cells. Proteins within the TGF-β superfamily, such as Bone Morphogenetic Proteins (BMP) and Growth Differentiation Factors (GDF), are crucial in directing stem cell differentiation. Specifically, SMAD proteins regulate the expression of genes responsible for osteogenic and chondrogenic differentiation through MAPK, p38, ERK-1, and JNK proteins. These signaling networks mediate the differentiation of stem cells into bone (osteoblasts), cartilage (chondrocytes), and other mesenchymal cell lines. TGF-β2, a member of the TGF-β family, plays a vital role in endothelial cell formation and initiates the epithelial-mesenchymal transition (EMT) mechanism, endowing epithelial cells with mesenchymal characteristics (9). This process is intricately linked to various biological events during embryonic development, such as tissue remodeling, wound healing, and cancer metastasis. Additionally, Nodal and Activin A molecules of the TGF-β family are crucial during the gastrulation stage, regulating the formation of endodermal and mesodermal layers during embryonic development. A deeper understanding of these molecules' functions will contribute to the advancement of new strategies in regenerative medicine, organ engineering, and stem cell-based therapies.

Bone Morphogenetic Proteins (BMPs), members of the TGF-β superfamily, constitute a principal group of growth and differentiation factors that regulate developmental processes and homeostatic mechanisms in bone, cartilage, and other connective tissues. During embryonic development, the BMP signaling pathway is instrumental in the formation of the skeletal system and the coordination of chondrogenesis and osteogenesis, playing a pivotal role in cell proliferation, mesenchymal cell guidance, and the shaping of the cellular microenvironment. Distinct BMP molecules exhibit unique expression profiles during cell differentiation processes, contingent upon developmental stages. For instance, BMP-2 is predominantly expressed in periosteal and osteogenic regions, BMP-4 in the perichondrium, and BMP-6 in prehypertrophic chondrocytes. The specific roles of these BMP members in particular tissues and cell types are crucial for the proper development of the skeletal system and the formation of functional bone structures. The TGF-β/BMP signaling pathway regulates differentiation processes by interacting with various transcription factors and other signaling pathways to determine cell fate. The TGF-β signaling pathway influences the pluripotent nature of stem cells by regulating the expression of the NANOG gene through Activin and Fibroblast Growth Factor (FGF) molecules.

Concurrently, it enhances the expression of chondrogenic genes such as Type II Collagen (Col2a1) and Sox9 in collaboration with the MAPK signaling pathway, and activates osteogenic genes like Noggin and Frzb through synergistic interactions with the Wnt/β-catenin signaling pathway. Consequently, the expression of these genes directs the osteogenic and chondrogenic differentiation of mesenchymal cells derived from stem cells, leading to the formation of bone and cartilage tissues (10). The TGF-β/BMP signaling pathway plays a regulatory role in mesenchymal cell condensation, intercellular matrix synthesis, and terminal differentiation processes through synergistic or antagonistic interactions with other signaling pathways. A comprehensive understanding of these processes will provide a significant foundation for the treatment of skeletal diseases, bone regeneration, and tissue engineering applications.

2.3. Notch Signalling Pathway

The Notch signaling pathway is an evolutionarily conserved signal transduction mechanism in multicellular organisms, playing a pivotal role in determining cellular fate and maintaining tissue homeostasis through cell-cell interactions. Active since embryonic development, this pathway regulates the division and differentiation of stem cells into various cells and is involved in both organogenesis and tissue regeneration and repair in adults. The Notch signaling pathway is particularly significant as a determinant in the control of cardiomyocyte differentiation (11). The Notch receptor, a primary component of the Notch signaling pathway, is a transmembrane protein activated in a ligand-dependent manner on the cell surface. The interaction of this receptor with Delta-like ligand (DLL) and Jagged ligands is a critical step that initiates the signal transduction mechanism. Upon binding to the ligand, the Notch receptor undergoes proteolytic cleavage by the enzyme γ-secretase, resulting in the release of the Notch intracellular domain (NICD; Notch Intracellular Domain) and its transport from the cytoplasm to the nucleus. Functioning as a transcription factor in the nucleus, NICD regulates the expression of hes (Hairy/Enhancer of Split) and hey (Hes-related) family genes and activates target genes. Activation of the Notch signaling pathway specifically promotes the development of cardiac muscle cells by increasing the expression of cardiomyogenic genes while inhibiting the activity of the RunX2 transcription factor to suppress osteoblastic differentiation. This mechanism reveals the negative regulatory effect of Notch signaling on bone formation processes.

The Notch signaling pathway also plays a crucial role in the development and homeostasis of the nervous system (12). Notch activation on stem cells in the

spinal cord promotes proliferation, maintaining the neural progenitor cell pool. Furthermore, although it enhances neural differentiation, it lacks the ability to regulate the migration of cells from the spinal cord to other regions. This suggests that Notch signaling plays a site-specific regulatory role for neural progenitor cells. However, the function of the Notch signaling pathway may vary depending on the cellular microenvironment (niche). Under hypoxic conditions, the Notch signaling pathway contributes to bone development and regeneration processes by regulating osteogenic differentiation. Activation of hypoxia-associated genes leads to differential effects of the Notch signaling pathway in specific cells. In summary, the Notch signaling pathway is a versatile signaling network that plays specific roles in different tissues and cell types and determines cell fate. It is known to act through various regulatory mechanisms in many cell types, from cardiomyocytes to neural progenitor cells, osteoblasts to chondrocytes, from embryonic development to adulthood.

2.4. Fibroblast Growth Factor (FGF) Signaling Pathway

The fibroblast growth factor (FGF)-dependent signaling pathway constitutes a fundamental cellular mechanism that mediates the loss of pluripotency in embryonic stem cells, facilitating their differentiation into specific cell lineages and the establishment of stable cell lines. FGF signaling is pivotal in regulating cell proliferation, directing differentiation processes, and maintaining tissue homeostasis. Activation of the FGF signaling pathway in embryonic stem cells results in the loss of pluripotent properties and the initiation of differentiation (13). The suppression of this process by various inhibitory molecules in in vitro culture media, or the predominance of alternative signaling pathways (e.g., Wnt or TGF-β) in vivo, aids in maintaining the pluripotency of embryonic stem cells. FGF ligands initiate signaling by binding to FGF receptors (FGFRs), which are transmembrane proteins with tyrosine kinase activity. Their activation triggers intracellular signaling pathways and initiates gene expression changes that determine cell fate. Activation of FGF receptors stimulates the Mitogen-Activated Protein Kinase (MAPK)/Extracellular Signal-Regulated Kinase 1/2 (ERK1/2), Phosphoinositide 3-Kinase (PI3K)/Akt, and Signal Transducer and Activator of Transcription (STAT) signaling pathways to regulate the cell cycle, maintain cell survival, and promote differentiation processes. Notably, the MAPK-ERK1/2 signaling pathway mediates the acquisition of specific cellular identities by promoting neural cell differentiation. During embryonic development, the influence of the FGF signaling pathway on ectodermal cells is

crucial for the proliferation and differentiation of neural progenitor cells into specific nerve cells.

The influence of the FGF signaling pathway extends beyond ectodermal derivatives, playing a pivotal role in the fate determination of mesodermal cells through autocrine and/or paracrine mechanisms. FGF serves as a crucial regulator in the differentiation of muscle, skeletal, and connective tissues. Specifically, certain FGF ligands, such as FGF8 and FGF10, guide the differentiation of mesodermal cells into muscle cells, connective tissue fibroblasts, and osteoblasts. This mechanism is essential for skeletal development, muscle tissue remodeling, and the maintenance of connective tissue homeostasis. For optimal functionality, the FGF signaling pathway must interact with the Transforming Growth Factor Beta (TGF-β) and Wnt signaling pathways. These pathways contribute to signal integration in cell proliferation, differentiation, and tissue morphogenesis, thereby influencing cell fate. The FGF signaling pathway collaborates with Wnt signaling to direct cells regionally to specific tissues during embryonic development. Notably, the synergistic interaction with the Wnt/β-catenin signaling pathway enhances skeletal development and osteoblastic differentiation. FGF also cooperates with the TGF-β signaling pathway to guide endodermal and mesodermal cells towards specific tissue types. This process facilitates the smooth progression of developmental processes by aligning the genetic programming of cells with environmental cues.

2.5. Platelet-Derived Growth Factor (PDGF) Signaling Pathway

The platelet-derived growth factor (PDGF)-dependent signaling pathway constitutes a principal mitogenic signaling mechanism that regulates cellular actions. Notably, it plays a pivotal role in angiogenesis and the stimulation of new vessel formation. This pathway is mediated by four distinct PDGF ligands (PDGF-A, PDGF-B, PDGF-C, and PDGF-D) and PDGF receptors, which belong to two receptor tyrosine kinase families (PDGFR-α and PDGFR-β). Upon ligand binding, receptor autophosphorylation occurs through homodimerization or heterodimerization, thereby activating intracellular signaling cascades. This activation modulates cellular responses by initiating various intracellular signaling pathways, particularly the Mitogen-Activated Protein Kinase (MAPK) and Extracellular Signal-Regulated Kinase 1/2 (ERK1/2) pathways. Specifically, the interaction between PDGF-A and PDGFR-α is crucial in embryogenesis and organogenesis, as it stimulates the proliferation of mesenchymal precursor cells. Conversely, the interaction between PDGF-B and PDGFR-β enhances the

mitogenic activity of stem cells and augments their migratory capacity. The PDGF signaling pathway initiates the production of reactive oxygen species (ROS) through the activation of the cPLA2 enzyme, following the activation of intracellular downstream signaling mechanisms. ROS function as essential second messengers that facilitate cell proliferation and advance cell cycle progression to the G1-S phase; however, their excessive production can result in heightened oxidative stress. A significant regulator of the PDGF signaling pathway is the Vascular Endothelial Growth Factor (VEGF) signaling pathway. VEGF facilitates angiogenesis in endothelial cells, whereas the PDGF signaling pathway contributes to vascular stabilization by aiding the recruitment of pericytes and smooth muscle cells. This interaction enables the coordinated advancement of new vessel formation processes. Furthermore, the effects of the PDGF signaling pathway on cell differentiation are tissue-specific. For instance, it promotes preadipocyte proliferation during adipogenesis, while it inhibits terminal differentiation and encourages cartilage tissue development during chondrogenesis. In the context of osteogenesis, unlike the Epidermal Growth Factor (EGF), it inhibits osteogenic differentiation.In conclusion, the PDGF signaling pathway serves as a crucial regulator of cellular proliferation, differentiation, and migration processes, and is recognized as a significant biological target, particularly in tissue repair, angiogenesis, and regenerative medicine (14, 15). A comprehensive understanding of the mechanisms underlying this signaling pathway may facilitate the development of novel therapeutic strategies in stem cell biology, cancer research, and regenerative medicine applications. The effects of PDGF across various tissues and its interactions with other growth factors are vital for tissue engineering and clinical applications. In this context, targeting the regulatory mechanisms of the PDGF signaling pathway may enable the development of innovative approaches for the treatment of diseases such as pathological angiogenesis, fibrosis, and cancer.

2.6. Epidermal Growth Factor (EGF) Signaling Pathway

Epidermal Growth Factor (EGF) is a potent mitogen that plays a crucial role in cellular proliferation, differentiation, migration, and survival, exerting its effects specifically through a tyrosine kinase receptor known as ErbB-1 (EGFR). EGFR, which is highly expressed in mesenchymal stem cells, becomes activated through autophosphorylation upon interaction with the EGF ligand, initiating a series of intracellular signaling cascades. This signaling specifically involves the Mitogen-Activated Protein Kinase (MAPK)/Extracellular Signal-Regulated Kinase (ERK) and Phosphoinositide 3-Kinase (PI3K)/Akt pathways. Activation of the MAPK-ERK pathway enhances the proliferation of mesenchymal stem

cells by promoting cell cycle progression, whereas the PI3K-Akt signaling pathway engages anti-apoptotic mechanisms that promote cellular survival. Through these mechanisms, the EGF signaling pathway facilitates the growth of epithelial cells and mesenchymal stem cells while suppressing differentiation processes to a certain extent. This pathway is of significant importance for the proliferation and protection of mesenchymal stem cells, particularly in regenerative medicine applications. However, over-activation of this mechanism has been associated with cancer cell proliferation and tumor development, necessitating careful regulation of the signaling pathway. Although the effects of the EGF signaling pathway on bone development have not been fully elucidated, available evidence suggests that this pathway has a suppressive effect on osteoblast and chondrocyte differentiation. The inhibition of bone formation by EGFR activation is primarily attributed to the inhibition of the expression of osteogenic transcription factors such as Runx2 and Osterix. Suppression of these factors, which are critical for the differentiation of osteoblasts, can lead to a slowing or arrest of the osteogenesis process. Concurrently, EGF signaling interacts with Transforming Growth Factor-beta (TGF-β) to inhibit the chondrogenic direction of mesenchymal stem cells. By reducing the positive regulatory effect of TGF-β on chondrogenesis, EGF is thought to suppress the formation of cartilage tissue. Considering these mechanisms, it appears that the EGF signaling pathway functions as a negative regulator of bone and cartilage tissue development, and this effect should be considered in tissue engineering applications. EGF receptor-mediated signal transduction promotes the proliferation of mesenchymal stem cells but suppresses osteoblast and chondrocyte differentiation. This is an important research topic, particularly in biomedical fields such as bone tissue engineering, tissue regeneration, and cartilage repair, and targeting the EGF signaling pathway may contribute to the development of therapeutic approaches. Furthermore, since over-activation of the EGFR signaling pathway has been directly associated with cancer development, abnormal tissue growth, and metastasis processes, it is necessary to modulate this signaling pathway in a controlled manner. Future studies may enable the development of new therapeutic strategies to optimize bone regeneration and repair processes through specific targeting of the EGF signaling pathway.

2.7.Hedgehog Signaling Pathway

The Hedgehog (Hh) signaling pathway is a crucial mechanism for intercellular communication, playing a vital role in embryonic development and tissue repair. By facilitating signal transmission from embryonic stem cells, it regulates

processes such as organogenesis and cellular differentiation. Hedgehog proteins, categorized as Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh), are integral components of this pathway, orchestrating developmental processes through cellular receptor-mediated signaling. Activation of the Hedgehog signaling pathway is initiated via the transmembrane receptor Patched (Ptch). Under normal circumstances, Ptch inhibits the receptor Smoothened (Smo). However, upon binding of Hedgehog ligands to Ptch, this inhibition is relieved, leading to Smo activation. Activated Smo initiates intracellular signal transduction pathways and modulates transcription factors of the Gli family. Gli transcription factors are pivotal in regulating cell proliferation, differentiation, and organ morphogenesis by controlling the expression of target genes. Dysregulation of the Hedgehog signaling pathway is directly associated with tumorigenesis and the regulation of cancer stem cells. During embryogenesis, Hedgehog signaling proteins are expressed at varying concentrations across different embryonic regions, establishing a morphogenetic gradient. This gradient ensures proper tissue and organ development in the embryo. An imbalance in the Hedgehog signaling pathway can result in aberrant cellular proliferation, particularly in basal cell carcinoma (BCC), medulloblastoma, and other solid tumors. Overactivation of Gli transcription factors can lead to excessive proliferation of cancer stem cells and tumor formation. Therefore, maintaining Hedgehog signaling pathway activity within specific limits is crucial for preserving normal cellular homeostasis. In cancer biology, targeting the Hedgehog signaling pathway can inhibit excessive stem cell proliferation and prevent cancer cell dissemination (16). Beyond its embryonic effects, the Hedgehog signaling pathway also plays critical roles in adult stem cell populations, such as hematopoietic and neural stem cells. This pathway contributes to tissue regeneration and regenerative processes by regulating the proliferation, maintenance of potency, and differentiation mechanisms of adult stem cells. Notably, in nervous system development, Hedgehog signaling governs the proliferation and maturation of neural progenitor cells. It also aids in maintaining hematopoietic stem cells within their bone marrow niche and regulates immune system cell regeneration. Further research is necessary to elucidate the therapeutic potential of the Hedgehog signaling pathway in maintaining tissue homeostasis in adults and in regenerative medicine. Future studies may facilitate the development of novel therapeutic strategies in regenerative medicine, cancer therapy, and tissue engineering by devising therapeutic approaches targeting the Hedgehog signaling pathway.

3. Transcription Factors Affecting Stem Cell Differentiation

The proliferation and differentiation of stem cells are biological events that play a critical role in organismal development and tissue regeneration. These processes are tightly controlled by specific transcription factors and regulated by signaling pathways that direct differentiation processes. In bone and cartilage tissue formation, CBFA-1/RunX2, Osterix (Osx) and Sox9 are known to be the most important transcription factors that direct cells to a specific lineage (17). RunX2 and Osterix, which regulate osteogenic differentiation, are essential for the development and mineralization of bone tissue, while Sox9 transcription factor is one of the most important regulators of cartilage tissue formation. These factors interact with relevant signaling pathways to determine cellular fate and play important roles in bone-cartilage transformation processes. CBFA-1/RunX2, which directs osteoblast differentiation, is a master regulator of the bone formation process and promotes the maturation of osteoblasts by increasing the expression of osteogenic genes. Activation of RunX2 is mediated through the Mitogen-Activated Protein Kinase (MAPK)/Extracellular Signal-Regulated Kinase (ERK) signaling pathway via Laminin-5 and initiates the osteogenic differentiation mechanism (18). This process contributes to the maturation of osteoblast precursor cells, while also increasing the expression of genes that promote the production of bone matrix proteins. Deficiency or suppression of RunX2 disrupts osteogenesis and leads to severe defects in skeletal development. Osterix (Osx), which is activated following RunX2 activation, is a critical transcription factor that functions in a RunX2-dependent manner and directs the terminal differentiation of osteoblasts. In the absence of Osterix, osteoblast cells fail to reach the terminal differentiation stage and bone formation is inhibited. Thus, RunX2 and Osterix function as two important interdependent regulators of osteogenic differentiation (19).

Sox9, the most important transcription factor regulating chondrogenic differentiation, promotes maturation of chondrocytes by increasing the expression of cartilage matrix proteins. Activation of Sox9 interacts with the Wnt/β-catenin signaling pathway and the mutual regulatory effect of these two signaling pathways is considered to be a key component of the mechanism of chondrogenesis (20). High levels of Sox9 promote chondrocyte proliferation, whereas overactivation of the Wnt/β-catenin signaling pathway suppresses chondrocyte maturation. Disruption of this balance leads to reduced chondrocyte proliferation, delayed hypertrophic chondrocyte differentiation and impaired endochondral bone formation. This process is one of the fundamental

mechanisms driving bone-cartilage transformation during embryonic development and sheds light on the development of new therapeutic strategies in the field of tissue engineering. The Wnt signaling pathway plays a critical role in the chondrogenic differentiation process and promotes hypertrophy of chondrocytes, particularly through the Wnt8c and Wnt9a molecules. These molecules promote hypertrophic maturation of chondrocytes by increasing the expression of type X collagen and RunX2. Chondrocyte hypertrophy is a necessary step in endochondral bone formation and the regular progression of this process is of great importance for skeletal development (21). However, overactivation of the Wnt signaling pathway can lead to premature differentiation of chondrocytes and dysregulation of bone formation. Therefore, maintaining the Wnt signaling pathway at certain levels is vital for maintaining bone-cartilage balance.

In the process of chondrogenic differentiation, Transforming Growth Factor-beta (TGF-β) signaling pathway also contributes to the regulation of Sox9 expression (22). TGF-β activation promotes chondrocyte maturation by enhancing Sox9 binding to target genes. However, suppression of Sox9 expression by stimulation of Wnt8c and Wnt9a accelerates the conversion of cartilage cells into hypertrophic chondrocytes. This suggests that the repression of Sox9 in bone development must be timed in a specific manner. Through this mechanism, the endochondral ossification process is completed by decreasing the expression of Sox9 at later stages of the bone formation process. Maintaining the balance between TGF-β and Wnt signaling pathways is crucial for the healthy progression of chondrogenic cell differentiation (23). Effective regulation of transcription factors such as RunX2, Osterix and Sox9 in osteogenic and chondrogenic differentiation processes is essential for the normal development of bone and cartilage tissue. Signaling pathways in which these factors interact with each other ensure that cells are directed to a specific lineage and that tissue homeostasis is maintained. In particular, balanced regulation of intracellular signaling pathways such as Wnt/β-catenin, MAPK/ERK and TGF-β is of great importance for healthy skeletal development and regenerative processes. A better understanding of the processes of osteogenesis and chondrogenesis may contribute to the development of novel therapeutic approaches in the fields of bone diseases, cartilage degeneration and tissue engineering. Future studies may allow the development of novel biomedical strategies that enhance bone and cartilage tissue regeneration by targeting these signaling pathways

4. Conclusion

Genetic and signal transduction mechanisms that direct the proliferation and differentiation of stem cells play a critical role in organismal development and maintenance of tissue homeostasis. By directing cells to specific lineages, these mechanisms form the cornerstones of biological processes that regulate tissue and organ formation. Embryonic stem cells (ESCs) and mesenchymal stem cells (MSCs) have great potential in regenerative medicine, tissue engineering and disease modeling thanks to their pluripotent and multipotent properties. Signaling pathways such as Wnt/β-catenin, TGF-β/Activin/Nodal, Notch, Hedgehog, BMP and FGF function as master regulatory mechanisms that control the self-renewal capacity and differentiation of stem cells. These pathways play a key role in determining cell fate, working together with transcription factors and epigenetic regulators. For example, transcription factors such as RunX2 and Osterix promote osteogenic differentiation, while Sox9 is the key regulator driving chondrogenic differentiation. Wnt/β-catenin signaling promotes osteoblastic differentiation, while its over-activation can lead to premature differentiation of chondrocytes and unregulated progression of bone formation. Furthermore, dysregulation of the Hedgehog signaling pathway can trigger abnormal cellular proliferation that can lead to tumor formation. Thus, the balanced functioning of these molecular networks regulating stem cell biology is essential for healthy tissue development and regeneration. Given the vast possibilities that cell signaling pathways offer for tissue engineering and biomedical research, a more detailed understanding of these mechanisms is crucial for the development of therapeutic approaches for cancer, degenerative diseases and traumatic tissue loss.

Recent research emphasizes the importance of targeting signaling pathways in the integration of stem cells into clinical applications. Modulation of specific signaling mechanisms may contribute to the development of more effective therapeutic approaches in tissue engineering and regenerative medicine by directing stem cells to the desired cellular lineages. For example, activation of TGF-β and BMP signaling pathways in specific combinations appears to be an important strategy for directing osteogenic and chondrogenic differentiation of mesenchymal stem cells. However, the effects of EGF and PDGF signaling pathways on different cell types are important for enhancing the proliferation and migration capacity of mesenchymal stem cells. However, since unbalanced activation of these signaling mechanisms can trigger the proliferation of cancer stem cells and lead to tumor formation, these processes need to be carefully controlled in clinical applications. A deeper understanding of stem cell biology

could lead to the development of new therapies in areas such as cancer therapies, treatment of neurodegenerative diseases, cartilage and bone tissue engineering. Future studies may increase the clinical success rate of stem cell-based therapies through more specific and targeted manipulation of cell signaling networks. In this context, biotechnological advances and genetic engineering techniques offer great potential to improve the safety and efficacy of stem cell therapies.

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CHAPTER 16

In Health Care Institutions Marketing Product and Product Development

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1) Introduction

Before the 1970s, both the legal regulations that prevented competition in the health sector in economic terms and the attitudes of health personnel delayed marketing practices in the health sector. However, from the mid-1980s onwards, increasing competition, consumer awareness and empowerment, and cost increases forced health professionals to consider marketing, and as a result, they tried to adapt marketing techniques and methods to health institutions (Baktır and Yıldırım, 2024: 185). In the 1990s, health services became market-oriented and the importance of the marketing concept within health institutions increased. In the 2000s and today, with the increase in competition and patient demands, marketing of health services has become a necessity for health institutions to survive financially. Therefore, the concept of marketing has a very important role in the presentation of health services, patient satisfaction, and service quality (Cazacu and Oprescu, 2015: 61). However, when health services are compared to other physical products, although they have some similarities, they contain many differences. Due to these features, product-oriented marketing approaches and traditional marketing mix elements have been inadequate in health services marketing and have necessitated the preparation of a different marketing plan and the use of new marketing mix elements. In this study, products and product development strategies in the marketing of health institutions are discussed (Tengilimoğlu, 2000: 190).

2. The Concept of Product in Healthcare

The product is the goods, services and ideas offered by the organizations. Products are offered to the market to meet a need. Based on this, it can be stated that the product is everything that can be offered to the market (idea, service, object etc.). When we look at the concept of product in health services, the product covers not only the products we mentioned, which are used outside of the traditional product, but also all areas of medical services, including insurance services offered by various institutions. In health services, the product is customized according to the person and is mostly given meaning by the personnel who do the job. This situation points to the human element in the marketing mix (Cengiz, 2014: 11).

Since products in health services are more of a service, their intangibility makes them stand out from other services when compared to other services. This difference from other services can create some marketing problems in health services marketing. For example; a person may not be able to evaluate a product

that they have not experienced before purchasing, after consuming it. A patient who has an examination or surgery may not be able to evaluate whether the procedures performed on them are of high quality. In another example; since the health service product can be purchased instantly, it can be a one-time experience compared to other services. The person receiving the service may have to manage the situation without prior experience due to the sudden situation that occurs to him (Tazegül and Çil, 2021: 152).

In addition to the intangible nature of health care products, features such as simultaneous production and consumption, no service is similar to another, demand cannot be predicted, patient expectations are prioritized, and there are many service providers can cause a significant difference between patient expectations and the care received. Such a difference causes customer dissatisfaction and related problems (Purcarea, 2019: 93).

n physical product marketing, the customer has the right to control the product and its consumption. However, in health services, the patient leaves himself in the hands of the person who will provide the service and has to trust him. Most of the time, it is not he who decides how and what service he will receive, but his doctor. In addition, since there is no guarantee in advance about the quality of the service to be received, he can only seek his rights against the doctor's malpractice.

In addition, patients are not at a level where they can evaluate medical quality. Since the quality of the service output cannot be touched, it is not possible for a patient to evaluate the quality of an orthopedic surgery. Only an expert in the field can evaluate the quality of such interventions. Patients also evaluate according to the quality they perceive. The perceived quality level also varies from patient to patient (Tengilimoğlu, 2016: 342). Accordingly, what we call product in health services can be expressed as the benefit provided as a result of changes in the health of patients.

Health services can be seen as a combination of goods or services that can be called a bundle of many elements. The number of elements that comprise the service may vary depending on the situation. The number of elements together may also vary depending on the nature of the service.

2.1. Core Product, Tangible Product and Extended Product Concepts in Healthcare

While providing health services, the product classification is made as core product, tangible product and extended product.

2.1.1. Core Product

The benefit that a product or service provides to the customer is called the core product. The answer to the question, "What needs did the patients meet with the service they received?" determines what the core product is. For example; maternity services are provided in hospitals. However, patients do not make purchases here about the birth itself, but about the pain and discomfort of the birth. A physical therapy center may offer rehabilitation services, and the patient may buy there in hopes that their dysfunctional leg will return to its former state. The question of which needs the service actually meets allows the determination of the core product. This can explain not only the features of the service but also the benefits of the product.

2.1.2. Tangible Product

The core product presented to the patient is the presented state. For example; the patient who comes to the ENT clinic with earache wants his earache to be relieved. Here, the concrete product is shaped as the room where the treatment is performed and the unit used by the doctor. The person came to the ENT clinic with earache, was taken to the examination room, and his examination was performed. As a result of the examination, the treatment to be performed was explained to the patient, and the necessary procedural treatment was applied. The patient's earache was cured and the need was met.

The concrete product has 5 basic features that can be explained with this example.

1- It has a certain style. A hygienic, comfortable treatment room and a clinic with high-tech equipment ensure that the person trusts the service they receive.

2- It has a number of features. A comfortable waiting room that will minimize the patient's distress while waiting for the service, internet access, informative television, magazines and refreshments, etc.

3- It keeps its existence alive with its quality.

4- It has a presentation that makes the patient feel that the service they receive is the best when they compare the place they go to with other places.

5- It may be under the roof of a brand. For example; Like XXX Hospital ENT Diseases Center (Bülbül, 2022: 351).

2.1.3. Extended Product

These are additional features that come with the product that prepare the environment for the better fulfillment of the basic purposes of the product. Hospital managers offer added value and opportunities to the target market with the concrete products and extended products that include additional features. For example; postpartum services provided to mothers who have given birth, training on the health of the mother and the baby, and free health services provided to the baby within the first year are additional services. This concept ensures that the product or service offered is perceived as attractive and different.

3. Service Components in Healthcare

Hospitals are institutions that provide many services, none of which are the same. However, there are some limitations in determining service units due to the different characteristics of health services. Despite this, the following points should be taken into consideration when creating the service components to be offered in hospitals.

1- Service components should indicate the market,

2- Priority should be given to services that can compete with competitors,

3- Service components should be created in a way that can keep up with the changes in the market,

4- Internal and external environmental conditions of the business should be taken into consideration.

Service components can be realized in health services based on the definitions of width, length and depth of service put forward by Kotler.

In the concept we can call product line, the need is the interconnected service group that purchases and distributes the need. For example; X Hospital has a product line that provides service in the fields of ENT, Physical Therapy and Rehabilitation, Cardiology and Neurology. Depending on how the hospitals will be structured, each hospital may have different product lines. The length of the service provided is the number of 4 different specialties specified in that line. The fact that there are four different specialties in the X hospital mentioned above also shows the length of the service. Other sub-branches under each specialty also tell about the breadth of the service. For example, if different fields such as ear, nose, throat surgery and microsurgery are offered under the ENT specialty branch, the breadth of ENT is 4 (Gümüş, 2018: 224).

The concept of depth of service expresses the size of the service in itself. For example; Having 15 beds in the field of cardiology or having 2 operating rooms and 3 specialists for microsurgery gives us information about the depth of the service. When considered from the perspective of hospitals, the concept of depth is a meter used for comparison.

The wider the product lines of a health institution, the different perspectives of patients towards that institution will be. Providing the service in a broad framework attracts attention in terms of influencing patients and this makes the institution that much more successful. Therefore, patients apply to the same institution for all their illnesses. The depth of the service itself provides benefits to the institution in both market segmentation and product diversification, while ensuring that the needs of the patients are met more comfortably.

Service components are reviewed regularly as they determine the place of the hospitals in the market compared to their competitors. Service units with decreasing demand and requiring high costs are eliminated in continuous controls. However, when making this decision, the management takes into consideration how other services will be affected by the decision, in addition to the cost element (Tatarlı, 2007: 16).

4. Product/Service Development in Healthcare

Product development, which is a subject that all kinds of organizations that produce goods or services work on for a period of time, is an important subject. When we look at the concept of marketing, it can be said that marketing exists from the design of the product to its development. Accordingly, it is possible to say that the product development process starts with the design process of the service in health services. Since the person receiving the service is also at the center of marketing, products are developed according to the requests and needs of the people who need the service while determining the product roadmap. Especially the health sector has a rapidly renewing environment, which brings new service needs to the agenda. The service should be developed to meet new needs, and a planned process should be carried out in order to obtain reliable and successful results during development.

There are many stakeholders who can help a health institution that will provide new services arising from a need. The organization should analyze these well and support itself by applying appropriate information collection methods from those receiving service from the organization. The working personnel group is another group whose opinions would be useful to seek. Since this group can be in constant

interaction with the service recipients, it has the opportunity to monitor and listen to the requests and expectations of the service recipients. This will provide the employees with the opportunity to make different analyses for the new products and services to be produced by the organization and will provide foresight on issues such as target audience, price, profit and sales revenue prediction for the products to be produced by the organization.

4.1. Core Product Strategies

Basic product strategies that can be used in marketing can be classified as single product - single message, same product - different message, different product - same message, different product - different message and creating a new product.

4.1.1. One Product - One Message

The product is marketed in different markets with the same message to provide the same benefit. For example; Ultrasound gel is marketed with the same message for the same purpose no matter where it is used.

4.1.2. Same Product - Different Message

The same products may be used differently in different markets. In these cases, the message given to the consumer may also differ. For example; Cotton is used in both hospitals and hairdressers. However, although the product is the same, the purpose may not be the same.

4.1.3. Different Product - Same Message

The product is produced differently in foreign markets, but since it is used for the same purpose, the same message is used in all markets. For example; a fast food chain serves the same message in different countries and with different types of products (red meat or pork).

4.1.4. Different Product - Different Message

As the environment in which the product is used changes, the message to be given about the product also changes. This makes production, communication and control difficult and causes costs to increase.

4.1.5. Creating a New Product

It can be said that in order for the strategies to be implemented, the product that the company produces domestically will be sold abroad when customers buy

and use it. In cases where the customer portfolio that the company has determined as a target does not have the opportunity to purchase the existing product, the company may consider creating and producing the product that the purchasing power of the portfolio it has determined is sufficient to meet the determined need.

It can be said that Turkish companies have an advantage in terms of their geographical location in order for the strategy to be implemented successfully. However, it can be said that the strategy requires high-budget R&D studies and high costs for new production as a disadvantage.

If the company produces short-lived products, it should work to create new and accepted products that are compatible with the market and conduct research on which markets its other products are suitable for (Özdemir, 1990: 87).

5. New Product/Service Concept in Healthcare

The concept of a new product is perceived in two different ways, by management and by consumers.

5.1. New Product/Service Concept from Management Perspective

Let's consider the concept of new product with various meanings from a management perspective.

5.1.1. All New Products: It is a product that is completely new and has a new function and requires a new market search. Completely new products are realized with a new discovery. Since they are new to the business and the market to be entered, their risks are also great. R&D studies require high costs. Examples of new product development studies are diseases that are new and whose treatments are being investigated such as Covid, AIDS and cancer, etc.

5.1.2. Functionally Enhanced Products: These studies are preferred over creating a completely new product. They are new changes that improve a function of an existing product. R&D studies are applied to renew functions. There are different ways to renew the function of the product. Without the need for a new technology for the product, the technology used for the product can be developed or improved by using higher quality input (Bülbül, 2022: 355).

5.1.3. Products with New Functions Added: Improving the functions of an existing product is achieved by developing its application. Taking dental MRI at the same time in a dental unit, adding a new function to the dental unit device are examples of these products.

5.1.4. Products Obtained by Developing Product Types: It is entering the market with products that are not completely new to the market, by producing new products in a certain product class. The company may not have any experience in technology and marketing in terms of producing and launching these products.

5.1.5. Low Cost Products: It is the design of products that have the same function as another product but are produced at a lower cost and a low price strategy is determined. By applying more affordable prices, customers who could not buy the product before can be encouraged to buy it. For example, reducing the costs of certain extra treatments (hyperbaric oxygen) for diabetic patients and offering them to the patient allows reaching more patients suffering from this disease.

5.1.6. Re-Edited Products: It is the redesigning of an existing old product and offering it to the market as a new product. These products are called new products by replacing an old product. For example, old model devices used in hospitals are taken and redesigned with advanced technology and offered.

5.2. New Product/Service Concept for Consumers

In terms of consumers' perceptions and behavioral changes regarding the new product and how they should use the new product, continuous innovation products are evaluated in three separate categories: continuous dynamic innovation products and discontinuous innovation products.

5.2.1. Continuous Innovation Products: It is the name given to products that are created with minor changes or through copying. Customers continue to use the product without the need for additional learning.

The fact that the usage remains the same despite the change in the model of a blood pressure monitor prevents blood pressure patients from changing their behavior while using the device.

5.2.2. Continuously Dynamic Innovation Products: When purchasing and applying dynamic innovation products, there is a small change in behavior. Although not extensive, a need for learning develops. It will be easy to continue to learn and apply these new features of a dental unit to which new features have been added (Timur, 2002: 42).

5.2.3. Discontinuous Innovation Products: Completely new products also have no equivalent. New behaviors are exhibited when purchasing and applying products. Special training is required for the use of products. Chips placed in the brain for ear diseases can be exemplified as a product type (Tengilimoğlu, 2016: 167).

5.3. Stages of New Product Development Process in Healthcare

Healthcare facilities and other businesses determine the process they will follow from beginning to end by making the necessary organization and planning the elements for the new product or service to be produced. Creating a new product or service is a long process and requires high costs. The stages in this process can be examined under six headings:

5.3.1. Gathering New Product/Service Ideas: It includes ongoing R&D studies in line with the expectations and demands of the target market and the goals determined by the organization. Proposals for the production of new goods and services are collected at this stage. Ideas about the new product to be received from all internal and external stakeholders covering the business are combined here (Tengilimoğlu, 2016: 182).

5.3.2. Sorting Out New Product/Service Ideas: In this phase, the decisions taken regarding the new product and service and the paths to be followed are important. Because the decisions to be taken after this phase will progress with the right choices to be made in this phase. In this phase, the new ideas collected are sorted and those that are compatible with the interests and goals of the business are separated from the others and placed in a certain order. If the wrong choices are made, this situation puts the business in difficulty and causes the business assets to be wasted by creating negativities in the target market (Kuru, 2008: 12)

5.3.3. Creating and Testing the Product/Service Concept: Before making decisions about the product or product to be developed, the business reaches the concept of the product or service by objectifying the product or service. After this concept is reached, the ideas about the product or product are filtered and the most suitable ideas are separated. For these separated ideas, the product to be created one by one, the product order, rationality and sales opportunities, etc. are evaluated to see whether they are suitable for the business.

5.3.4. Development of Product/Service: In this phase, the idea of a product or service ceases to be an idea and is objectified. The objectified product or service is developed in different physical forms. The marketing unit determines

the distinguishing features related to the product or service. These features include various issues such as the physical structure of the product or service and what it will be produced from. These are determined by making decisions about the internal and external stakeholders and resources of the business. Assistance can be received from both the R&D unit within the company and independent R&D organizations regarding product development (Tengili-moğlu, 2016: 185). A design is made for the product and a prototype is produced, and a trial is conducted in the production area and, if necessary, outside the production area to test whether the product is effective and safe.

5.3.5. Marketing Challenge: The product whose prototype was made in the previous phase is produced, even if it is small. The small production should be evaluated under the conditions of the target market and converted into real production numbers. First of all, the aim is to minimize all the risks that may be negative when production is definitely started by working in a limited market that has the elements of the real target market. With this marketing trial, businesses try out the entire marketing plan together with the product they will produce. In other words, how the business will advertise the product, how it will carry out distribution activities, pricing policy, brand strategies and product positioning studies are also tried out in this phase (Tengilimoğlu, 2016: 186).

5.3.6. Market Launch: By creating a sales force, continuing promotional activities, organizing marketing services, creating distribution channels and with all this formation, target market trials, products or services that are understood to be successful enough to find a place in the market are produced at a level that can appeal to a wide audience and presented to the market through distribution channels (Catana and Toma, 2021: 487).

6. Conclusion and Evaluation

When we look at the marketing concept, which is based on the principle of offering products or services to meet the demands, expectations and needs of consumers and the desire for reciprocity in most cases, in the health services sector, we can say that it does not happen in this direction. In the health sector, the diagnosis made by the relevant physician and the treatment applied are at the forefront rather than mutual demands and needs. Although this situation is a situation that occurs depending on the person, of course, in the health sector, health businesses that know their market well and can meet the demands and needs of the consumer will be more successful in this field and increase their customer portfolio (Akkılıç, 2002: 208).

Marketing management is often seen as demand management. The task of marketing managers in health services is to develop strategies that match supply and demand by taking into account different demand structures and fluctuations for products and services.

While developing these marketing programs, health managers should take into account the characteristics of the services and bring together the marketing mix elements in a way that will meet and satisfy the demands and needs of the target market and at the same time realize the organizational goals of the health business. In this process, they should cooperate with other department managers and even benefit from potential customers. (Tengilimoğlu, 2016: 245)

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CHAPTER 17

Section XIV Microbiota and Respiratory System

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MICROBIOTA AND RESPIRATORY SYSTEM

GUT-LUNG AXIS

The intestinal system, which constitutes an important part of the human body, creates an environment rich in microbial diversity and numbers (1). Different microorganisms and their metabolites find a living space in the human body. It is known that the human colon contains a concentration of 1 trillion bacterial microbiota per gram. In general, proteobacteria, bacteroidetes and firmicutes anaerobic microorganisms are abundant in the microbiota (2). Metabolite products of the intestinal microbiota are indispensable for human homeostasis. First of all, the microbiota has vital effects on the host it lives in, such as immunity and respiration. In addition, host microorganisms are also affected by the reactions of the body in which they live. Drugs used, diet, probiotic consumption and environmental factors affect the host's metabolites. The effect of metabolites in allergic conditions such as asthma continues to be a topic of curiosity day by day (1).

When the people who make up a society are examined, it is seen that the microbial diversity of each individual is different depending on age (3). The microbiota of postnatal babies is less diverse than that of adults. Microbiota, which is a dynamic ecosystem, increases the diversity of the individual throughout the process leading to adulthood. Short-chain fatty acids (SCFA) are a microbial metabolite and can regulate the host's immune behavior through epigenetics. Therefore, the mother's microbiota may affect the baby, especially the development of allergic diseases (4). If we focus on what affects the baby microbiota; Birth type, diet and mother's microbiota come first. It is known that both the nutrition of the individual and the environmental factors he is exposed to as he progresses towards adulthood contribute to the microbiota diversity (5). All these physiological mechanisms demonstrate the strong connection between host and host.

The gut-lung axis is precisely regulated during respiratory diseases (6). Intestinal microbiota plays an active role in initiating the immune response in the lungs, which contain the mucosal region. The lung-gut axis appears as two important authorities that affect each other during pulmonary diseases (Figure 1) (2).

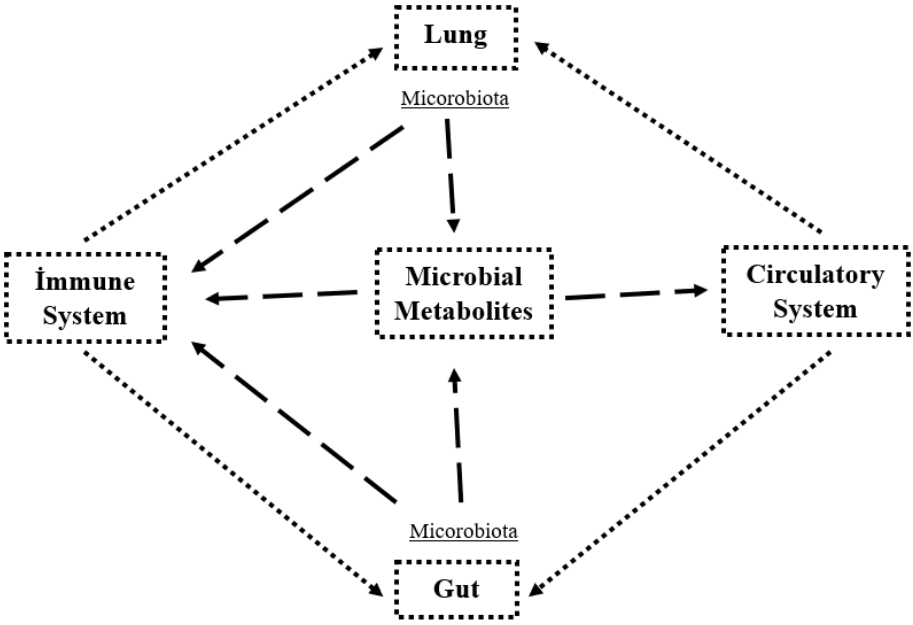

FIGURE 1. Gut-Lung Axis (2)

If the intestine and lung tissue are examined, we can see that they have a common embryonic origin. We see that the gut-lung axis is a valid and important pathway in the body. Around this axis, mucosal co-immunity connects the two systems. The human body is based on a dynamic homeostatic balance thanks to the blood and lymph circulation system. It is obvious that the immune response seen in the mucosal area, thanks to the circulatory system, also creates a strong bridge between the intestine and the lung.

Anatomically, the tight junction regions are provided by proteins such as zonula occludens, occludin and claudin-18 (2). Any disorder in the structure and functions of these proteins increases paracellular permeability. Mucus secreted by the goblet cells protects the inner surface of the intestine by creating a chemical barrier. This barrier formed in the intestines does not allow pathogens to pass through, thanks to the mucosal lymphoid tissue. While the barrier maintains a utilitarian relationship with beneficial bacteria, it neutralizes harmful pathogens and metabolites (7).

The human body comes into contact with millions of microorganisms through daily breathing and nutrition. Both the respiratory tract and the intestines create a living space for microorganisms. This high amount of exposure profoundly affects human homeostasis. To survive, microorganisms consume certain

nutrients and release certain metabolites as products (8). These released metabolites affect the immune structure of both the lungs and intestines, enabling the development of certain diseases. During the disease, the intestine-lung axis is actively working. Thanks to this axis, the microbiota establishes an uninterrupted communication between organs. Studies show that homeostasis is negatively affected as a result of disruption of the intestinal microbiota balance (dysbiosis) (9). A decrease in body immunity may lead to an increase in respiratory lung disease symptoms. It is known that intestinal diseases accompany respiratory pathologies in which the alveoli and lung epithelial tissue are damaged. In addition, pulmonary diseases can be seen more frequently in individuals with irritable bowel syndrome, which is characterized by intestinal motility problems (4).

The inner surface of our intestines is perfectly protected by a barrier. The living spaces of host microorganisms are limited to a certain extent. If the intestinal barrier is disrupted, both microorganisms and the metabolites they produce may spread and exacerbate pulmonary diseases. When the integrity of the intestinal mucosa is disrupted, bacteria can enter both the systemic blood and lymphatic circulation and cause infection. While the intestinal microbiota flora is very beneficial for human homeostasis, it can cause respiratory diseases. The resulting systemic infection disrupts the microbiota balance, causing diarrhea and the immune system collapses. Inflammation occurring in the vulnerable body causes serious damage to human homeostasis (10).

Thanks to advanced health technologies in the twenty-first century, we can analyze in detail the homeostatic process (cause-interaction-effect) trio that takes place in the human body. Nutrition, which has had a very important place throughout human history, has become more important in researching respiratory diseases today. The effect of the intestinal microbiota on the respiratory system, which is essential for life, is becoming a subject of interest and curiosity day by day.

GUT MICROBIOTA AND ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS)

Acute respiratory distress syndrome (ARDS) can be defined as the inflammatory reaction of the body after damage to the lungs. Damage to the alveolar composition ultimately results in gas diffusion disorder. This change in the pulmonary system includes hypoxemia as well as pulmonary roles and pneumonia (11). The COVID-19 pandemic has increased the prevalence of ARDS globally. ARDS has direct effects on the intestinal microbiota. It causes

damaging effects on the intestinal mucosal barrier by affecting the diversity of microorganisms that make up the microbiota. ARDS negatively affects the body's inflammation reaction and immunity by affecting the metabolites produced by the microbiota. Changes in the intestinal microbiota may affect the initial stages and progression of ARDS (2). Intestinal microbiota refers to a flora in which both beneficial and harmful bacteria and viruses are in balance. Intestinal dysbiosis that may occur causes disruption of physiological homeostasis. This imbalance not only collapses immune function, but also prevents the synthesis of essential substances that the body needs.

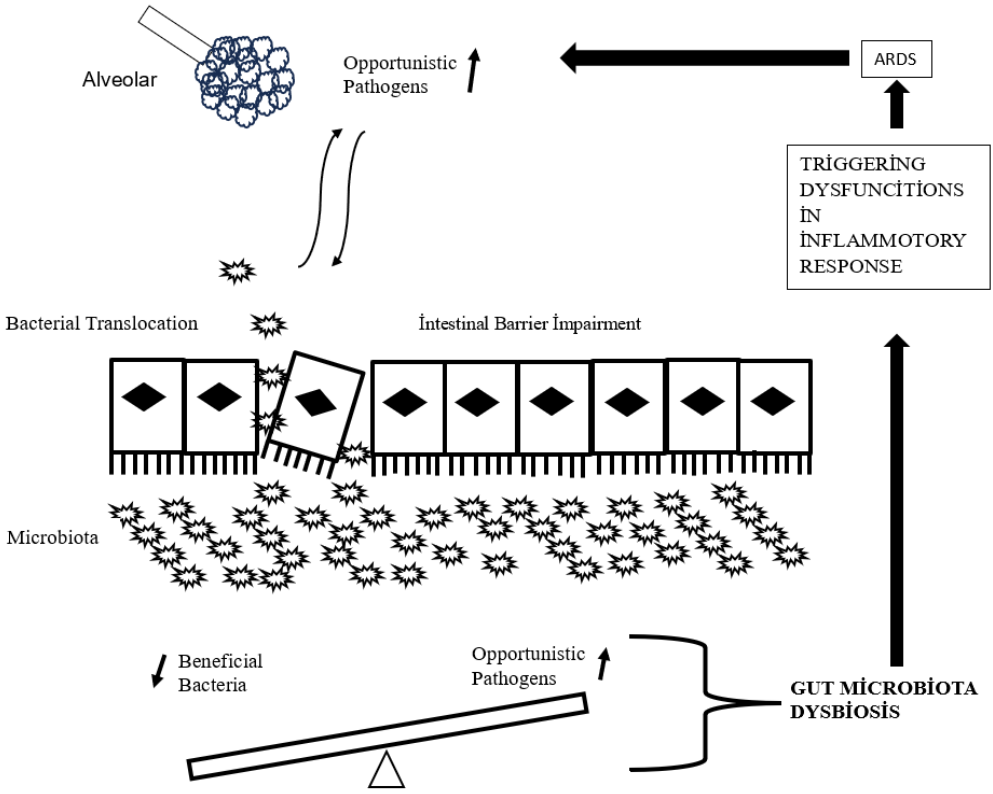

FIGURE 2. Relationship between gut microbiota and ARDS (2)

The gut-lung axis constitutes a complex interaction of two organs, both physiologically and pathologically. Essentially, the imbalance seen in the intestines affects immune and inflammation reactions, causing exacerbation of pulmonary diseases. As a result of the erosion of the intestinal barrier, permeability increases and the production of beneficial metabolites is prevented. In particular, increased intestinal permeability allows pathogens to enter the

blood and lymph circulation, causing systemic inflammation. Therefore, this increases the rate of progression of respiratory diseases (Figure 2), (2).

Studies conducted during the COVID-19 epidemic showed that ACE_2 receptors are abundant in both organs forming the axis. It was determined that intestinal dysbiosis reduces the effect of inflammatory reactions, leaving the living body vulnerable to the effects of SARS-COV-2. A study conducted on mice showed that lung damage increased by disrupting the homeostatic balance between Th1 and Th2 cells as a result of disruption of the intestinal microbiota. In a similar study, it was stated that alveolar macrophages suffered permanent damage (12).

In order to express that the gut-lung axis is healthy, it is necessary for both lung and intestinal microbiota to be in physiological balance. Gut dysbiosis may disrupt the microbiota of the lungs by affecting the systemic inflammatory response (13). Conversely, lung infections can also disrupt the intestinal microbiota flora. This vicious cycle can cause serious damage to our respiratory system, which is essential for life. In light of all this information, the health of the intestinal microbiota appears to be an important factor in the course of ARDS cases.

GUT MICROBIOTA AND PULMONARY FIBROSIS

Pulmonary fibrosis can be defined as structural and functional damage to lung tissue among respiratory system diseases. It is characterized by excessive fluid accumulation in the interstitial space as a result of damage to alveolar epithelial cells. A widespread inflammation follows the disease process. The resulting alveolitis causes fibrosis of the lungs along with extensive scar tissue (14). Structural damage to the lungs prevents gas exchange between the alveoli and the pulmonary blood vessel. Oxygen and carbon dioxide diffusion efficiency, which are vital for humans, decreases. Pulmonary fibrosis is very common in the advanced stages of interstitial lung diseases (15).

The gut-lung axis is based on the theory that both organs are interconnected by the mucosal immune system and the blood-lymphatic circulatory system. Change in intestinal microbiota content and disruption of the mucosal barrier may have negative effects on the lungs. Disruption of intestinal microorganism flora can weaken the inflammatory reaction of the lungs through circulation. Metabolites produced by microorganisms can collapse systemic immunity (16). Conversely, the microbiota change occurring in the lungs may tip the balance in the intestinal flora towards harmful bacteria. As a result of the popularity of the

gut-lung axis, current studies show that the intestinal microbiota has a high impact on pulmonary fibrosis (2). As microbiota and metabolites are examined in more detail, it brings a new perspective to the treatment of lung diseases such as pulmonary fibrosis. In particular, the mucosal immune system creates an excellent barrier that protects both the intestines and the lungs. The barrier not only protects the organ against harmful pathogens, but also provides a safe living space for beneficial microorganisms and their metabolites (2).

Microbiota and metabolites found in the lungs; It causes pulmonary fibrosis by activating lung fibroblasts by affecting macrophages, neutrophils and fibrogenic factors (17). They do this by triggering the apoptosis of lung epithelial cells through the immune system. The contribution of intestinal microorganisms and metabolites to gastrointestinal immunity is well known. If intestinal microbiota homeostasis is disrupted, the intestinal barrier may be seriously damaged. In addition, the intestinal microbiota may cause the secretion of cytokines that can cause fibrosis in the lungs through blood and lymph circulation. The immune and circulatory systems serve as a bridge in this process.

Studies on mice have shown that there is a close link between the intestinal microbiota and pulmonary fibrosis (2). It has been shown that antibiotic use at an early age may disrupt the intestinal microbiota and contribute to the development of pulmonary fibrosis at later ages. Systemic sclerosis is an autoimmune connective tissue disease. It is linked with impairment of the immune system and progressive fibrosis. When examined, a high degree of intestinal dysbiosis is observed in these patients. This correlation between intestinal dysbiosis and pulmonary fibrosis reveals the close relationship between lung fibrosis and intestinal microbiota.

Cross-sectional studies on cystic fibrosis patients have revealed that intestinal microbiota richness and balance are very important in the course of the disease. In light of all this, we see that it is obvious that intestinal microorganisms and their metabolites have serious effects on the lungs. It is well known that the healthy intestinal microbiota contributes greatly to both inflammatory reactions and immune system homeostasis.

GUT MICROBIOTA AND ASTHMA

Increasing tobacco use and smoke exposure exacerbates asthma, which is chronic inflammation of the respiratory tract. Many reasons such as increased air pollution in the world, familial and individual sensitivity increase the incidence of allergic asthma (18). In the modern age, the use of antibiotics by pregnant

mothers and the increasing consumption of baby foods with different contents directly have negative effects on the microbiota. All these external factors disrupt the homeostasis of the mother and her baby. It is known that the change in body balance directly affects the gastrointestinal system through diet (19). Studies show that the decrease in microbiota diversity in the intestinal flora increases the baby's incidence of allergic diseases. In addition, antibiotic treatment is applied to children diagnosed with asthma. As a result of the antibiotics used, the intestinal microbiota is seriously disrupted. In this way, both the number and diversity of microorganisms that make up the microbiota decrease. As a result, the body shows an extreme reaction to allergenic stimuli due to the disrupted microbiota. In vivo studies conducted in different rat species have shown that beneficial bacterial supplementation can enrich the microbiota flora and reduce asthma reactions (19). The health of the gastrointestinal tract is crucial to the homeostatic process of pulmonary diseases. Current research can open a new window to the world of science on preventing asthma and managing the process more easily by regulating the intestinal microbiota.

GUT MICROBIOTA AND CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

Chronic obstructive pulmonary disease (COPD) is associated with a persistent air flow limitation of the air ways as well as widespread inflammation. It emerges as a fundamental public health problem due to its increased prevalence worldwide and its decrease in quality of life (20).

There are very few studies on COPD and intestinal microbiota. However, the fact that people diagnosed with CAOD are chronic smokers suggests its effects on the intestinal microbiota. Chronic cigarette consumption has negative effects on the intestinal microbiota. Studies show that smoking reduces the richness and number of intestinal microorganism flora.

The decrease in the number of beneficial bacteria causes intestinal dysbiosis. Damage to the intestinal barrier as a result of dysbiosis can weaken immunity and reduce the efficiency of lung inflammatory reactions. Decreased immunity may exacerbate respiratory distress in lung diseases such as CAOD (21). Considering the gut-lung axis, the health of the gut microbiota has a very important place in the fight against inflammation of the lungs and the body. More studies are needed to elucidate the interaction of COPD and gut microbiota.

GUT MICROBIOTA AND CYSTIC FIBROSIS

Cystic fibrosis is defined as an autosomal recessive disease. It is known that mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) lies at the basis of the disease (22). The two areas first affected in cystic fibrosis patients are the lungs and intestines. This shows that both systems have a strong mutual interaction. When patients diagnosed with cystic fibrosis are examined, it is seen that the diversity and number of intestinal microbiota decreases. The number of beneficial bacteria decreased and the number of microorganisms producing harmful metabolites increased.

In mouse studies, the increased number of Mycobacteria and Bacteroides fragilis bacteria is correlated with the loss of transmembrane conductance regulators in cystic fibrosis (23). In addition, changes in the intestinal microbiota may cause exacerbation of lung symptoms seen in cystic fibrosis patients. Recent studies show that cystic fibrosis patients taking probiotic supplements manage the process better by increasing the richness and diversity of their intestinal microbiota. More studies are needed to determine the type and dose of probiotics to be administered, especially in cystic fibrosis patients. Current studies on the gut-lung axis hold new hopes in the cystic fibrosis treatment process.

GUT MICROBIOTA AND LUNG CANCER

Lung cancer is a malignant tumor that primarily affects the respiratory tree. As a result of the uncontrolled proliferation of the cells that make up the lung tissue, the tumor begins to take up space anatomically. It can then metastasize to surrounding tissues and organs through the circulation (24). Although it is quite common, it accounts for approximately fifteen percent of all cancer types. It constitutes approximately twenty-five percent of deaths due to cancer. Chest pain is common, as well as hemoptysis originating from the bronchial mucosa and lung glands. With the disruption of the intestinal microbiota, systemic inflammation occurs in the body. The resulting metabolites weaken the immune system by disrupting homeostasis. Studies highlight the effect of microbiota dysbiosis on the formation and progression of lung cancer. The change in microbiota flora is noticeable in different stages of lung cancer (25).

Maintaining the microbiota balance in lung cancer treatment may have positive results on the course of the disease. Thanks to the strong communication of the gut-lung axis, the immune system can be strengthened by enriching the microbiota composition. As a result of improvements in the microbiota, the number of beneficial intestinal bacteria, which are essential for body homeostasis,

can be increased and the course of the disease can be turned to a positive side. All current research shows hope that a healthy microbiota can increase the effect of drugs and radiotherapy used in the lung cancer process. Although the limited number of studies on intestinal microbiota and lung cancer are satisfactory, more studies are needed to detail the subject.

CONCLUSION

When the literature is examined, it is seen that there is a tight and intricate connection between the intestinal microbiota and the respiratory system's healthy and disorders. Increasing studies over the last quarter century have enabled us to better understand the gut-lung axis. Considering current studies, it is believed that intestinal microbiota dysbiosis plays an important role in the onset and progression of lung diseases. The richness and metabolite balance of the intestinal microbiota flora may affect the exacerbation of respiratory diseases. A healthy microbiota can positively affect the course of lung diseases by strengthening the immune system. Although the link between the two systems has been demonstrated, more information is needed. From the perspective of human homeostasis, the relationship between the Gut-Lung axis should be investigated in detail. The literature should be enriched by revealing more physiological mechanisms. Moreover, it should be encouraged to stay away from all kinds of factors that will harm the health of gut-lung axis, such as bad habits, uncontrolled drug use, environmental pollution and unconscious nutrition.

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CHAPTER 18

Evaluation of the General Knowledge of the Healthcare Workers on İnfection Control Precautions in Mogadishu-Somalia

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1. Introduction

Standard Precautions (SP) are minimal infection prevention practices that include all patient care. These applications have been developed to protect the patient and prevent transmission. SP includes the following main topics: hand hygiene, personal protective equipment, prevention of respiratory tract contamination, prevention of stab wounds, safe injection practices, sterilization of instruments and devices, and disinfection of environmental surfaces (1-3). Our hands are the primary factor in the spread of healthcare-associated infections

(HCAI). Hand hygiene is the most effective method to reduce antibiotic resistance and the rate of HCAI. The World Health Organization (WHO) recommends hand hygiene in five indications. 1) before patient contact, 2) after patient contact, 3) before clean or aseptic procedure, 4) after patient zone contact, and 5) after contact with body fluids and glove removal. Alcohol-based disinfectant is sufficient as long as there is no visible contamination on the hand. After taking 3 - 5 mL into the palm, all surfaces of both hands should be disinfected by rubbing vigorously. It is also necessary to wait 20 seconds for the alcohol to dry. However, hand hygiene compliance of healthcare workers (HCWs) is often below the desired level (4-9). In this study, we aim to evaluate the compliance and awareness of Mogadishu healthcare workers to standard infection control prevention (IPC).

2. Materials and Methods

2.1.Study Design and Data Collection

This cross-sectional survey study was carried out between 19 - 30 December 2022 voluntarily after the ethics committee's approval. There are 950 HCWs in our center. There is no previous similar study in Somalia. The minimum number of participants was determined to be 274 using Raosoft Calculator. A 95% confidence interval and 5% margin of error were accepted. A face-to-face survey method was used. The questionnaire was prepared in Somali. Translated into English after the application was finished. Then, the data was added to the Excel and IBM SPSS Statistics 26.0 (IBM corporation) programs.

2.2. Questionnaire Design and Evaluation

A check box for voluntary participation was required in the introduction section of the questionnaire. Patients without consent were not included in the study. Staff who were not health workers were excluded. Somalia employees were also excluded. Incompletely answered questionnaires were also excluded.

All participants were 20 years of age or older. The questionnaire consisted of 30 questions and was planned into three sections. After the questionnaire was prepared, it was developed using some studies (10-13). In the first part, the participants were asked 8 questions about sociodemographic structure (age, gender, marital status, educational status, occupation, department, working period, and frequency of education about infection control measures). In the second part, 14 questions about infection control measures (hand hygiene, standard precautions, medical waste management) were prepared. Yes or no answers were requested. In the third part, 8 questions were asked about the frequency of compliance with infection control measures (always, sometimes, rarely, never). For the general knowledge questions in the second part, the level of knowledge was evaluated by giving 1 point for correct answers and 0 points for incorrect answers. The knowledge score of the volunteers over a total of 14 points was compared with sociodemographic variables. In addition, those with ≥70% of the total score were divided into two groups: good knowledge and those 24 with <70% as poor knowledge (14). These two groups were also compared with sociodemographic variables. In the third part, the frequency of compliance was scored as always: 3 points, sometimes: 2 points, rarely: 1 point, and never: 0 points. Total behavior score was calculated. The effect of sociodemographic parameters and level of knowledge on behavior was evaluated.

2.3.Ethical Approval

An application was made to the Ethics Committee of Mogadishu, Somalia Training and Research Hospital. (Approval no: MSTH/12756). Patient names or identifying information were not requested in the survey. Thus, the data that belonged to the patient remained confidential.

2.4.Statistical Analysis

All data was loaded into IBM SPSS Statistics 26.0 (IBM corporation). Frequency and percentage were calculated for categorical variables, and mean (standard deviation) and median (interquartile range; IQR) were calculated for continuous variables. While the normal distribution evaluation of the data was made with Kolmogorov-Smirnov and Histogram test, homogeneity evaluation was done with Levene's test. The subgroups' significance level over the total knowledge score and attitude score was determined by the Pearson Chi-Square test, Fisher's Exact Test, Kruskal-Wallis H test, Mann Whitney U test, and One Way Anova Bonferroni test. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. Demographic Data

A total of 274 volunteer participants were included in the study. Half were male, and half were female. 70.7% were in the 20-30 age group. Fifty-four percent were single. 52.9% of the volunteers were nurses. 97.2% had a doctorate level of education. 41.6% of the participants were ward staff. 32.8% stated that they received monthly training on infection control measures (Table 1).

Table 1. Sociodemographic Parameters, Number, Percentage

Parameters		**N**	**%**
Overall	**Categories**	274	100
Age	20-30	193	70.4
	31-50	79	28.9
	>50	2	0.7
Gender	Male	137	50
	Female	137	50
Martial status	Married	110	40.1
	Single	148	54.0
	Divorced	16	5.8
Profession	Doctor	83	30.3
	Nurse	145	52.9
	Assistant heatlh personel	46	16.8
Educational status	Bachelor	4	1.5
	Master	12	4.4
	Doctorate	258	94.2
Department	Intensive Care Unit	75	27.4
	Service	114	41.6
	Others	85	31
Experience	1-5 years	186	67.9
	6-10 years	82	29.9
	>10 years	6	2.2

The Frequency of Training

Volunteer participants were questioned about the frequency of training they received on IPC. One-third reported receiving training once a month, while one-tenth reported receiving no training at all (Figure 1).

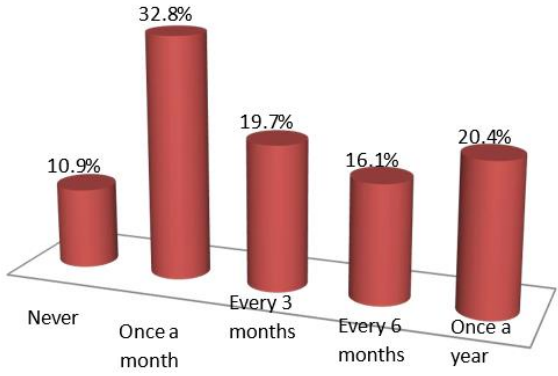

Figure 1. Frequency of Training

3.3. İnfection Control Precautions

In the second part of the questionnaire, the correct answer rates of the multiple-choice questions about infection control measures were summarized in Table 2. It was understood that 75.9% of the participants provided alcohol-based hygiene in a situation where hand washing was indicated. It was determined that 56.2% of the participants used non-sterile gloves before contact with all patients without discrimination. It was also found that most participants (81.8%) used the wrong protective equipment when contact isolation was required. 61.3% of the participants stated that they closed the lids of sharps after use and threw them in the waste bin. This is a very high rate in terms of needle injuries.

Table 2. İnfection Control Precautions

Questions	Responses	N	(%)
1. Considering that every patient is infected, standard precautions must be followed.	Correct Incorrect	257 17	93.8 6.2
2. It is sufficient to use alcohol-based disinfectant when there is visible contamination.	Correct Incorrect	66 208	24.1 75.9
3. Alcohol-based disinfectant must be used before and after wearing gloves.	Correct Incorrect	241 33	88 12
4. All patients must wear non-sterile gloves before contact.	Correct Incorrect	120 154	43.8 56.2
5. Gloves must be changed between two patients and between two procedures.	Correct Incorrect	247 27	90.1 9.9
6. Sterile gloves must be used when interventional procedures are required for the patient.	Correct Incorrect	222 52	81 19
7. Surgical masks are for droplet isolation and are disposable.	Correct Incorrect	207 67	75.5 24.5
8. N95 masks are for respiratory isolation and are disposable.	Correct Incorrect	213 61	77.7 22.3
9. Gloves, masks, aprons, glasses, and bonnets must be used in cases where respiratory isolation is required.	Correct Incorrect	248 26	90.5 9.5
10. Gloves, masks, aprons, glasses, and bonnets must be used in all cases where contact isolation is required.	Correct Incorrect	50 224	18.2 81.8
11. After using the sharp and piercing tools related to the patient, the covers must be closed again and thrown into the waste bin.	Correct Incorrect	106 168	38.7 61.3
12. Any kind of paper, cardboard, plastic, or glass packaging that is not contaminated with the patient's body fluids and secretions is waste and must be thrown into the blue bag.	Correct Incorrect	190 84	69.3 30.7
13. The first thing to do after a sharp injury is to wash the injured area with soap and water.	Correct Incorrect	152 122	55.5 44.5

14. After ¾ of the sharps waste boxes are filled, they must be closed and thrown into the medical waste bag.	Correct	229	83.6
	Incorrect	45	16.4

3.4. Knowledge Level and Behavior of Participants

Sociodemographic variables were compared with health workers' total knowledge score (0-14 points). No significant difference was observed between gender (p=0.276), age (p=0.209), profession (p=0.068), marital status (p=0.183), education (p=0.493), frequency of education (p=0.181), and knowledge level. However, there was a significant difference between department (p=0.045) and experience parameters (p=0.000), as well as knowledge level (Table 3).

Table 3. Comparison of Sociodemographic Parameters and İnfection Control Precautions Knowledge Level

Variables		**Good knowledge**		**Poor knowledge**		***p-values***
		N	**%**	**N**	**%**	
Overall	**Categories**	146	53.3	128	46.7	-
Gender	Male	78	28.5	59	21.5	0.276
	Female	68	24.8	69	25.2	
Age	20-30	107	39	86	32	0.209
	31-40	39	14.3	40	14	
	>50	0	0	2	0.7	
Profession	Doctor	53	14.9	30	11	0.068
	Nurse	70	25.5	75	27.3	
	AHP	23	8.4	23	8.4	
Marital status	Married	59	21.5	51	18.6	0.183
	Single	82	30.1	66	24	
	Divorced	5	1.8	11	4.1	
Education	Bachelor	3	1.1	1	0.4	0.493
	Master	5	1.8	7	2.3	
	Doctorate	138	50.3	120	44	
Department	ICUs	34	12.4	41	15	0.045
	Services	60	22	54	19.7	

	Others	52	18.9	33	12	
Experience	1-5 years	117	42.7	69	25.2	0.000
	6-10 years	27	9.9	55	20.1	
	>10 years	2	0.7	4	1.4	
Training	Never	12	4.2	18	6.6	0.181
	Once a month	51	18.7	39	14.3	
	Once 3 month	29	10.7	25	9	
	Once 6 month	19	6.9	25	9	
	Once a year	35	12.8	21	7.8	

ICU: Intensive care unit, AHP: Assistant health personnel.

The level of knowledge differs according to department and experience. Knowledge score is higher in others than in ICU (p=0.004). There is no difference between Service and ICU or others. The total knowledge score of those with 1-5 years of working experience was higher than those with 6-10 years of experience (p=0.002). There was no correlation between education level and knowledge score. When behavior scores were analyzed, it was observed that Bachelors scored significantly higher than Masters (p=0.033). There was a significant correlation between the frequency of training on infection control measures and behavior (p=0.007). A significant difference was observed in the behavioral status of those who received training once a month compared to those with less training frequency. There was also a significant relationship between knowledge level and behavior (p=0.029) (Table 4).

Table 4. Comparison of Total Knowledge Score and Total Attitude Score with Sociodemographic Variables

Parameters	**Knowledge Score, *p*-values**	**Attitude Score, *p*-values**
Age	0.398	0.183
Gender	0.272	0.434
Martial status	0.060	0.066
Profession	0.112	0.433
Educational status (master vs bachelor)	0.193	**0.033**
Department (ıntensive care unit vs others)	**0.004**	0.749
Experience (6-10 vs 1-5)	**0.002**	0.121
Frequency of training (others vs once a month)	0.290	**0.007**
Knowledge level (poor vs good)	-	**0.029**

4. Discussion

This is the first study of infection control measures among XXX HCWs. A recent survey conducted in six different WHO regions examined the training frequency on IPC. In Africa, the rate of always or sometimes accessing training was found to be below 40%, which was reported to be the lowest rate in the world (15). There are various studies on IPC in different regions of Africa. In a study conducted in Ghana, 58% of healthcare workers, and in another study conducted in Nigeria, 70-80% were found to have sufficient knowledge about IPC (16-18). Data in Ethiopia show that this rate is between 54-60%. When healthcare workers' compliance with IPC was evaluated, 32-55% compliance was found (19-21). In our study, the frequency of education about IPC was quite low. The participants were mostly aware of the indications for alcohol-based hygiene, but they also used alcohol-based hygiene when hand washing was necessary. More than half of the volunteers found non-sterile gloves unnecessary. It was also found that the use of personal protective equipment was largely incorrect, and more than half of the volunteers were misinformed about the use of sharps. The frequency of training on infection control measures is one of the main reasons for these deficiencies. After increasing the frequency of the necessary training, the error rate will decrease significantly with the informed and unannounced observations of the infection control committee unit in the field.

The level of knowledge about infection control measures directly affects compliance with IPC. In a study conducted on HCWs in a region of Ethiopia, the probability of good practice of HCWs who received training on infection prevention was 2.2 times higher (AOR: 2.19, 95% CI; 1.01-4.75) than those who did not receive training (22). There are different studies on the effect of gender on compliance with IPC. In a study conducted in Ghana, compliance was higher in the male gender, but in a study conducted in nursing students in Australia, no difference was found in gender (13, 23). In the Ghanaian study, age, professional rank, duration of practice, having received IPC training before, presence of an IPC committee, knowledge about IPC, and compliance with IPC protocols were not statistically significantly related (13). However, in different studies conducted on this subject, a direct relationship between compliance with IPC and the mentioned criteria has been reported (21, 23-26). Like the Ghanaian study, age, gender, marital status, department, work experience, and professional rank were not associated with IPC adherence. A positive correlation was found between the training frequency, educational status, and level of knowledge about IPC and compliance with IPC. Notably, compliance with IPC was not significantly different, although ICU staff had higher knowledge scores. Considering the presence of multidrug-resistant infections in the ICU, the study's data suggests a high risk of patient-to-patient transmission from HCWs. Another important point is that although the knowledge score was higher in those with less work experience, the effect on compliance remained the same. It is understood that it will not be possible to reach a sufficient level of compliance only by increasing the frequency of IPC training. Increasing the frequency of field observation by the infection control committee unit will positively affect compliance with IPC.

5.1. Study limitations

Of course, our study has some limitations. First, since the face-to-face survey method is used, we cannot guarantee that the participants will answer the questions biasedly. Participants were not asked to provide their names and identities to reduce the risk of biased answers. In addition, the study was conducted only among HCWs working in a single center in the xxx region of xxx. It is not possible to reflect the whole country. Another limitation is that similar publications were used while preparing the survey questions instead of on an internationally recognized scale. However, the study must provide the first data for regional data. Another important point is that it will guide the multi-center, large-participation studies to be conducted in the region. The study also brought to light our deficiencies in IPC in our center. The education unit, infection control

committee unit, and infectious disease specialists re-planned and implemented the study.

6. Conclusion

As a result of our study, serious deficiencies in IPC were identified in xxx HCWs. In particular, significant relationships were found between training and compliance. Increasing the frequency of IPC training and field observations will seriously contribute to eliminating the deficiencies. The results we obtained are very important as they are the first data in the region. However, since it is a study conducted in a single center, it is impossible to reflect the region in general. Multicenter, large-scale studies that include HCWs from various regions of Somalia will more clearly reflect the geography of IPC.

Declarations:

Ethics approval and consent to participate

The study was approved by the Mogadishu-Somalia-Tukey Recep Tayyip Erdogan Noninterventional Clinical Research Ethics Committee Unit. At the meeting dated December 19, 2022, and numbered 2022/742, ethical permission was received (Approval no: MSTH /12756) and following the ethics of research, the participants were informed, and their consent was received.

Consent for publication

All participants' consent was received for the use of the data for the study.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare no competing interests.

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CHAPTER 19

Advances in Nanoencapsulation of Essential Oils for Therapeutic Appliations

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1. INTRODUCTION

The demand for natural therapeutic agents has grown in recent years, driven by concerns over the side effects and resistance associated with conventional pharmaceuticals. Essential oils (EOs) extracted from plants have demonstrated a wide range of biological activities, making them promising alternatives or complementary treatments to synthetic drugs. These volatile plant-derived compounds are composed of numerous chemical constituents, including terpenes, phenolics, and aldehydes, which contribute to their antimicrobial, anti-inflammatory, and antioxidant properties.

Essential oils (EOs) are volatile, highly concentrated oils that are extracted from the various parts of plants such as leaves, flowers, stems, roots, bark, and seeds. They are a mixture of complex bioactive molecules, primarily terpenes, phenolics, and aldehydes, which provide them with their typical aroma and therapeutic properties. Essential oils are extracted by various techniques such as steam distillation, cold pressing, and solvent extraction, the most common being steam distillation (Albuquerque et al., 2022; Baser & Buchbauer, 2009; de Sousa et al., 2023).

Essential oils have been widely utilized in traditional medicine for their antimicrobial, anti-inflammatory, antioxidant, and analgesic effects. Due to their natural origins and therapeutic benefits, they are frequently employed in aromatherapy, cosmetics, and the pharmaceutical industry. They exhibit significant bioactivities that make them effective in managing a variety of conditions, including microbial infections, respiratory disorders, stress-related ailments, and skin diseases. Their ability to modulate physiological and biochemical pathways has led to growing interest in their application in modern medicine (de Sousa et al., 2023; Lammari, Louaer, Meniai, & Elaissari, 2020).

However, despite their potential, essential oils face challenges related to volatility, limited solubility, and chemical degradation upon exposure to environmental factors such as heat, light, and oxygen. These limitations significantly reduce their therapeutic efficacy and restrict their broader application in medicine. Nanotechnology offers innovative strategies for enhancing the delivery, stability, and bioactivity of EOs, making them viable candidates for pharmaceutical development. By incorporating essential oils into nanocarriers such as liposomes and nanoemulsions, researchers aim to overcome these challenges and optimize their therapeutic potential (Aprotosoaie, Gille, Trifan, Luca, & Miron, 2017; Baser & Buchbauer, 2009).

1.1. Importance of Essential Oils in Medicine

Essential oils have been widely used in traditional medicine and modern pharmacology for their antimicrobial, anti-inflammatory, and antioxidant properties. Many studies have confirmed their effectiveness in managing various diseases, including bacterial and fungal infections, inflammatory conditions, and even cancer. These volatile compounds contain complex mixtures of terpenes, phenolics, and other bioactive molecules that contribute to their therapeutic properties. EOs have shown broad-spectrum antibacterial and antifungal activities, making them promising candidates for alternative antimicrobial treatments, particularly in response to rising antibiotic resistance. The increasing prevalence of multidrug-resistant pathogens has prompted scientists to explore natural compounds as alternative solutions. Furthermore, their antioxidant properties make them valuable in protecting cells against oxidative stress, which plays a key role in chronic diseases such as cancer and neurodegeneration. Their ability to neutralize free radicals and reduce oxidative damage suggests potential applications in preventing age-related diseases and supporting overall health (Baser & Buchbauer, 2009; Bilia et al., 2014; Guidotti-Takeuchi et al., 2022; Oprea et al., 2022).

Despite these benefits, EOs are prone to degradation from heat, light, and oxygen exposure, limiting their practical applications. This necessitates the development of advanced drug delivery systems to ensure their efficacy, stability, and controlled release within biological systems (de Sousa et al., 2023).

1.2. Challenges in Essential Oil Applications

Despite their potent bioactivities, essential oils face several challenges that hinder their therapeutic applications. These include:

- **Volatility and Degradation**: Essential oils contain highly volatile compounds that can rapidly evaporate, leading to reduced efficacy and instability during storage. This makes their direct use in pharmaceuticals difficult without suitable stabilization techniques (Oprea et al., 2022).
- **Low Water Solubility**: Most essential oil components are hydrophobic, meaning they do not readily dissolve in biological fluids. This significantly limits their absorption and bioavailability when administered orally or topically (Liao et al., 2021).

- **Short Shelf-Life**: Exposure to oxygen, heat, and light can degrade the chemical composition of essential oils, reducing their potency and effectiveness over time. Proper storage and encapsulation are necessary to maintain their activity (Yammine, Chihib, Gharsallaoui, Ismail, & Karam, 2024).

- **Variability in Composition**: The phytochemical composition of essential oils varies based on factors such as plant species, geographic origin, climate, and extraction methods. These variations lead to inconsistencies in their therapeutic effects, making standardization difficult (Oprea et al., 2022).

- **Potential Toxicity and Irritation**: Some essential oils contain components that may cause irritation or adverse effects when applied in high concentrations. For example, compounds such as phenols and aldehydes can be cytotoxic or cause allergic reactions in sensitive individuals. This necessitates precise formulation and dosage control to ensure safety and efficacy (Liao et al., 2021).

Addressing these challenges requires advanced formulation strategies such as nanoencapsulation, which can enhance the stability, solubility, and controlled release of essential oils, improving their therapeutic potential while minimizing unwanted side effects (de Sousa et al., 2023).

1.3. Role of Nanotechnology in Enhancing Bioavailability

Nanotechnology provides effective methods to improve the pharmacokinetics of essential oils by enhancing their solubility, stability, and targeted delivery. Liposomal formulations and nanoemulsions act as carriers that protect essential oils from environmental degradation while facilitating controlled and sustained release. By encapsulating EOs in nanosized delivery systems, their bioavailability can be significantly increased, allowing for improved therapeutic outcomes and reduced toxicity.

Nanocarriers can also be engineered to provide site-specific delivery, ensuring that essential oils exert their therapeutic effects at the intended location while minimizing systemic exposure. This is particularly beneficial in cancer therapy, where targeted drug delivery can improve treatment outcomes and reduce side effects associated with chemotherapy. Additionally, nanoformulations enable prolonged retention of essential oils in biological systems, allowing for sustained

release and enhanced therapeutic efficacy (de Sousa et al., 2023; Guidotti-Takeuchi et al., 2022; Liao et al., 2021).

1.4. Overview of Nanoencapsulation Techniques

Nanoencapsulation is a process designed to incorporate bioactive compounds within nanocarriers, offering protection against degradation while optimizing their controlled release. Various nanocarriers such as liposomes, nanoemulsions, and polymeric nanoparticles are used for this purpose. Liposomal encapsulation enhances the cellular uptake of essential oils by mimicking biological membranes, while nanoemulsions improve dispersion in aqueous environments. These techniques offer significant advantages in prolonging the shelf life and efficacy of essential oils in therapeutic applications.

Nanoencapsulation also facilitates the combination of essential oils with other bioactive compounds to enhance their therapeutic potential. For instance, co-encapsulation of essential oils with other plant-derived polyphenols or synthetic drugs can result in synergistic effects, improving treatment efficacy and expanding their application in various medical fields (Albuquerque et al., 2022; Yammine et al., 2024).

2. NANOENCAPSULATION TECHNIQUES

Nanoencapsulation refers to the process of enclosing bioactive compounds in nanocarriers such as liposomes, nanoemulsions, solid lipid nanoparticles, and polymeric nanoparticles. These nanocarriers improve the solubility, bioavailability, and controlled release of EOs, optimizing their therapeutic benefits. The field of nanotechnology has revolutionized drug delivery by offering controlled release and targeted administration, significantly enhancing therapeutic efficacy while minimizing side effects (Albuquerque et al., 2022; Liao et al., 2021; Yammine et al., 2024).

2.1. Liposomal Formulations

Liposomes are spherical vesicles composed of phospholipid bilayers that encapsulate hydrophilic or lipophilic compounds. These structures are biocompatible, biodegradable, and non-toxic, making them ideal carriers for drug delivery. Liposomes can be classified based on their composition and size, with small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), and multilamellar vesicles (MLVs) being the most common types. Various studies have demonstrated the ability of liposomal encapsulation to enhance the

bioactivity of essential oils. degradation (Çağdaş, Sezer, & Bucak, 2014; Chavda et al., 2022; Liao et al., 2021).

Entrapment of essential oils in liposomal systems has been found to exhibit enhanced antimicrobial activity against a broad spectrum of pathogens, such as bacteria, fungi, and viruses. Direct contact of liposomal phospholipid bilayer with microbial membranes results in microbial membrane disruption and cell lysis. Liposomal encapsulation also ensures enhanced sustained release of essential oils, which exhibit antimicrobial activity for extended periods.

Studies have shown that liposomal preparations of essential oils hold enormous promise in the battle against antibiotic-resistant bacteria and can be a future alternative to conventional antibiotics. Their ability to penetrate biofilms also renders them useful in the treatment of chronic infections, including wound infections, respiratory infections, and oral infections.

Liposomal encapsulation also enhances the antioxidant activity of essential oils by preventing their oxidation and degradation. Essential oils are rich in phenolic compounds, which are strong antioxidants with the ability to scavenge free radicals and reduce oxidative stress-induced damage. Liposomal formulations provide a protective cover against environmental factors that can degrade such bioactive molecules, resulting in extended antioxidant activity.

The increased bioavailability of liposomal essential oils provides increased cellular uptake, thereby increasing efficacy against oxidative stress. This feature is particularly beneficial in skincare products, neuroprotective therapies, and the prevention of age-related degenerative diseases.

Liposomal encapsulation therefore offers a multi-dimensional solution to the optimization of the therapeutic efficacy of essential oils. Through increased stability, targeted delivery, and prolonged bioactivity, liposomes are prospective carriers of essential oils for antimicrobial and antioxidant therapy.

Moreover, Liposomal encapsulation improves the physicochemical properties of essential oils, such as solubility, stability, and permeability, making them more effective in therapeutic applications. Furthermore, liposomes protect essential oils from oxidation and degradation, extending their shelf life and ensuring sustained therapeutic effects.

2.2. Nanoemulsions

Nanoemulsions are stable dispersions of oil and water stabilized by surfactants. They provide improved solubility and bioavailability of EOs, extending their therapeutic efficacy. These nanosystems have gained significant attention due to their small droplet size, which enhances the absorption and bioactivity of encapsulated compounds (Alam, Ansari, Alqarni, Salkini, & Raish, 2023; Barradas & De Holanda E Silva, 2021; Oprea et al., 2022).

Nanoemulsions have demonstrated superior antimicrobial efficacy by enhancing the ability of essential oils to disrupt microbial membranes, inhibit bacterial growth, and interfere with biofilm formation. The nanoscale size of nanoemulsion droplets enables better penetration into bacterial cell walls, leading to increased permeability and disruption of microbial structures. Furthermore, nanoemulsions improve the sustained release of essential oils, ensuring prolonged antimicrobial activity.

Studies have established that nanoemulsions of encapsulated essential oils exhibit outstanding efficacy against Gram-negative and Gram-positive bacteria and against fungal disease agents and a few viruses. The nanoemulsions' antimicrobial activities position them at the vanguard of a superior asset in the formulation of future disease states, treatments resistant to antibiotics and wound-healing and food preservation processes (Barradas & De Holanda E Silva, 2021; da Silva, do Rosário, Neto, Lelis, & Conte-Junior, 2023; Gupta, Eral, Hatton, & Doyle, 2016).

The antioxidant effect of the essential oils is optimized with nanoencapsulation. The essential oils include phenol compounds that active free radicals antioxidants of high potency scavenge free radicals and minimize oxidative injury and cellular injury. The susceptibility and stability of the oils toward degradation constrain the application of the oils in therapeutic use.

Nanoemulsions protect such antioxidants against degradation caused by external factors such as oxygen, temperature, and light. Encapsulating the nanoemulsions of the oils improves the stability and bioavailability of the oils and extends the shelf life of the antioxidant activities. The such nanoemulsions' properties specifically find application in skincare creams and lotions and neuroprotectant therapeutics and functional foods, where oxidative stress plays an integral role in disease development (Haro-González, Martínez-Velázquez, Castillo-Herrera, & Espinosa-Andrews, 2024; Perumalsamy et al., 2022; Singh et al., 2017).

Nanoemulsion encapsulation, therefore, becomes a flexible strategy for the enhancement of the antimicrobial and antioxidant properties of the essential oils and making them functional in the area of pharmaceuticals, cosmetics, and nutraceuticals.

Nanoemulsions enhance the delivery of essential oils by providing better dispersion in biological fluids, leading to increased cellular penetration and improved bioavailability. Additionally, their ability to control the release of bioactive compounds allows for prolonged therapeutic effects while reducing the risk of toxicity (Guidotti-Takeuchi et al., 2022; Liao et al., 2021).

3. ANTIMICROBIAL POTENTIAL OF NANOENCAPSULATED ESSENTIAL OILS

Essential oils have long been recognized for their broad-spectrum antimicrobial activity against bacteria, fungi, and viruses. However, their efficacy is often limited due to poor water solubility, rapid volatilization, and instability under environmental conditions. Nanoencapsulation techniques significantly enhance the antimicrobial potential of EOs by improving their bioavailability, controlled release, and targeted delivery. This section explores the antimicrobial properties of nanoencapsulated essential oils and their applications in treating infectious diseases (de Sousa et al., 2023; Guidotti-Takeuchi et al., 2022; Liao et al., 2021; Sitarek et al., 2017).

3.1. Mechanisms of Antimicrobial Action

Nanoencapsulated essential oils exert their antimicrobial effects through multiple mechanisms:

- **Cell Membrane Disruption**: Essential oils contain bioactive compounds such as phenolics and terpenes, which integrate into bacterial and fungal cell membranes, disrupting their integrity and leading to cell lysis (Aprotosoaie et al., 2017).
- **Inhibition of Enzyme Activity**: Many essential oils interfere with key metabolic enzymes within microbial cells, disrupting cellular processes such as respiration and nutrient uptake (Bilia et al., 2014).
- **Generation of Reactive Oxygen Species (ROS)**: The oxidative stress induced by certain essential oils damages microbial DNA and proteins, leading to cell death (Pérez-González et al., 2019).

- **Biofilm Inhibition**: Encapsulated essential oils have demonstrated the ability to disrupt biofilm formation, preventing the adherence of bacteria and fungi to surfaces and making infections more susceptible to treatment (Čutović et al., 2023; da Silva et al., 2023).

3.2. Antibacterial Activity

The rise of antibiotic-resistant bacterial strains has increased the demand for alternative antimicrobial agents. Nanoencapsulated essential oils exhibit potent antibacterial activity against a wide range of Gram-positive and Gram-negative bacteria (Guidotti-Takeuchi et al., 2022; Sitarek et al., 2017).

- **Gram-Positive Bacteria**: Nanoemulsions of Thymus capitatus and Origanum vulgare EO have shown enhanced antibacterial effects against Staphylococcus aureus and Bacillus subtilis. Liposomal formulations of Lavandula angustifolia have also demonstrated efficacy against Enterococcus faecalis, a common pathogen responsible for hospital-acquired infections.

- **Gram-Negative Bacteria**: Nanoencapsulated Salvia officinalis EO has exhibited significant activity against Escherichia coli and Pseudomonas aeruginosa, both of which are known for their antibiotic resistance and involvement in urinary tract and respiratory infections.

3.3. Antifungal Activity

Fungal infections, including candidiasis and dermatophytoses, are increasingly challenging to treat due to drug resistance. Encapsulated essential oils have shown promising antifungal properties, overcoming many limitations of traditional antifungal treatments (Caprari et al., 2023).

- **Candida spp.**: Nanoencapsulated Origanum vulgare EO has been highly effective against Candida albicans, a common fungal pathogen responsible for oral and vaginal infections. The encapsulation enhances its penetration into fungal biofilms, improving treatment efficacy.

- **Dermatophytes**: Essential oils encapsulated in polymeric nanoparticles, such as Thymus capitatus EO, have demonstrated inhibitory effects against dermatophytes, the fungi responsible for skin infections like athlete’s foot and ringworm.

3.4. Antiviral Activity

Several studies have suggested that nanoencapsulated essential oils can exert antiviral effects by interfering with viral replication, blocking viral entry into host cells, and modulating immune responses (Kazlauskaite et al., 2023).

- **Herpes Simplex Virus (HSV)**: Liposomal formulations of Lavandula angustifolia EO have shown antiviral activity against HSV by reducing viral replication and lesion formation.
- **Influenza Virus**: Nanoemulsions of Salvia rosmarinus EO have demonstrated potential in inhibiting influenza virus activity by preventing viral attachment and entry into host cells.

3.5. Applications in Medicine and Pharmaceuticals

Nanoencapsulation has expanded the potential applications of essential oils in antimicrobial therapy. Some of the most promising applications include (Pontes-Quero, Esteban-Rubio, Pérez Cano, Aguilar, & Vázquez-Lasa, 2021; Prakash et al., 2018):

- **Topical Treatments**: Encapsulated essential oils in creams and gels are used for treating skin infections, wound healing, and fungal diseases.
- **Oral Formulations**: Nanoemulsions have been developed for oral administration to combat gastrointestinal infections while ensuring controlled release and improved absorption.
- **Inhalable Nanoformulations**: Encapsulated essential oils are being explored as potential inhalable therapies for respiratory infections, offering targeted antimicrobial effects in lung tissues.

3.6. Future Perspectives in Antimicrobial Research

Although significant progress has been made in the application of nanoencapsulated essential oils for antimicrobial purposes, further research is necessary to:

- Conduct clinical trials to validate their efficacy and safety in human subjects.

- Investigate synergistic effects between nanoencapsulated essential oils and conventional antibiotics to combat multidrug-resistant infections.
- Develop advanced delivery systems to enhance targeted antimicrobial therapy while minimizing side effects.

With the increasing global challenge of antibiotic resistance, nanoencapsulated essential oils provide a promising alternative to conventional antimicrobial treatments. Future advancements in nanotechnology and bioengineering are expected to further optimize these formulations, broadening their clinical applications in infectious disease management (Guidotti-Takeuchi et al., 2022; Sitarek et al., 2017).

4. ANTIOXIDANT ACTIVITIES OF NANOENCAPSULATED ESSENTIAL OILS

4.1. Role of Antioxidants in Health

Antioxidants are essential in neutralizing oxidative stress, which contributes to various chronic diseases such as cardiovascular diseases, neurodegenerative disorders, and cancer. Oxidative stress results from an imbalance between reactive oxygen species (ROS) and the body's antioxidant defenses. Essential oils, rich in bioactive compounds such as flavonoids, terpenes, and polyphenols, have demonstrated potent antioxidant activities by scavenging free radicals and reducing oxidative damage (Asensio et al., 2017; Bhavikatti et al., 2024; Sitarek et al., 2017; Trinh et al., 2024).

4.2. Mechanisms of Antioxidant Action

The antioxidant properties of essential oils function through multiple mechanisms:

- **Scavenging Free Radicals**: Bioactive compounds in essential oils donate electrons to neutralize harmful ROS, preventing cellular damage.
- **Metal Chelation**: Essential oils bind to transition metals, reducing their ability to catalyze oxidative reactions.
- **Enhancement of Cellular Antioxidant Enzymes**: Nanoencapsulated essential oils have been found to upregulate antioxidant enzymes such as superoxide dismutase (SOD), catalase

(CAT), and glutathione peroxidase (GPx), which contribute to cellular defense mechanisms.

4.3. Nanoencapsulation Enhancing Antioxidant Properties

Nanoencapsulation enhances the antioxidant efficacy of essential oils by improving their stability, bioavailability, and controlled release. Various nanoencapsulation techniques, including liposomes, nanoemulsions, and solid lipid nanoparticles (SLNs), help protect antioxidant compounds from degradation and ensure prolonged activity (Caprari et al., 2023; Trinh et al., 2024).

- **Liposomes** provide a phospholipid bilayer that preserves the structural integrity of antioxidant compounds, preventing their oxidation before reaching target tissues.
- **Nanoemulsions** enhance the solubility and dispersion of hydrophobic antioxidants, increasing their bioavailability and efficacy in biological systems.
- **SLNs** protect antioxidant molecules by embedding them in solid lipid matrices, allowing for controlled and sustained release over time.

4.4. Applications of Antioxidant Nanoencapsulated Essential Oils

The enhanced antioxidant potential of nanoencapsulated essential oils has applications in multiple fields:

- **Pharmaceuticals**: Used in formulations for neuroprotective, cardioprotective, and anti-aging therapies.
- **Cosmetics**: Incorporated in skincare products to prevent oxidative damage and promote skin health.
- **Food Industry**: Used as natural preservatives to extend shelf life and enhance the nutritional value of food products.

5. STABILITY OF NANOENCAPSULATED ESSENTIAL OILS

5.1. Stability Enhancement through Liposomal Encapsulation

Liposomal formulations have been widely employed to enhance the stability of essential oils. Liposomes are spherical vesicles composed of phospholipid bilayers that can encapsulate hydrophobic and hydrophilic substances, providing a protective barrier against external factors. Studies have demonstrated that

encapsulating essential oils such as Origanum vulgare and Thymus capitatus in liposomes significantly improves their chemical stability by reducing volatility and preventing oxidation.

One key factor influencing stability in liposomal systems is the composition of the phospholipid bilayer. Variations in phospholipid types, such as Phospholipon 90H, Lipoid S100, and Phospholipon 85G, have been explored, with findings indicating that Phospholipon 90H liposomes offer superior stability due to their rigid bilayer structure. Additionally, encapsulation efficiency plays a crucial role, as higher efficiency ensures a greater proportion of the essential oil remains protected over time. Liposomal formulations have been shown to maintain essential oil integrity for extended periods, with minimal degradation observed over six months of storage at controlled temperature.

5.2. Stability in Nanoemulsion Systems

Nanoemulsions have also emerged as effective carriers for enhancing the stability of essential oils. These systems consist of oil-in-water or water-in-oil dispersions stabilized by surfactants, offering improved solubility and bioavailability. Nanoemulsions prepared via high-pressure homogenization have demonstrated increased resistance to oxidative degradation, particularly for Salvia officinalis and Salvia rosmarinus essential oils.

Stability assessments of nanoemulsions encapsulating Origanum vulgare and Thymus capitatus essential oils revealed that these formulations maintained physicochemical integrity over prolonged storage, with no significant changes in droplet size, polydispersity index (PDI), or zeta potential. Furthermore, encapsulated essential oils exhibited enhanced cytotoxic and antimicrobial properties compared to their free counterparts, reinforcing the stability advantage of nanoemulsification (Guidotti-Takeuchi et al., 2022).

5.3. Comparative Analysis and Future Perspectives

When comparing liposomal and nanoemulsion systems, both offer substantial stability benefits, yet each has distinct advantages. Liposomal formulations provide a controlled release mechanism and structural integrity, making them suitable for targeted drug delivery. Conversely, nanoemulsions are advantageous for improving solubility and bioavailability, especially in aqueous environments. Both systems have shown promise in preserving the bioactivity of essential oils while extending their shelf life.

Further studies are needed to optimize formulation parameters, such as lipid composition, surfactant selection, and storage conditions, to maximize the stability of encapsulated essential oils. Additionally, in vivo studies are crucial to validate these findings and support the clinical translation of these advanced delivery systems. The integration of these nanosystems into pharmaceutical and cosmetic industries holds great potential for enhancing the therapeutic application of essential oils while ensuring long-term stability and efficacy.

6. CONCLUSION AND FUTURE PERSPECTIVES

6.1. Conclusion

The application of nanoencapsulation techniques has revolutionized the utilization of essential oils in medicine, cosmetics, and the food industry. By addressing key challenges such as instability, volatility, and poor bioavailability, nanoformulations enable essential oils to be more effective in therapeutic applications. The studies reviewed in this chapter confirm that liposomal, nanoemulsion, and other nanoparticle-based delivery systems enhance the bioactivity of essential oils, allowing for controlled release, increased stability, and improved solubility.

Furthermore, nanoencapsulation provides essential oils with a protective barrier against environmental factors such as oxidation, heat, and light, ensuring prolonged shelf life and sustained therapeutic effects. These advancements pave the way for the broader adoption of essential oils as natural alternatives to conventional pharmaceuticals, particularly in antimicrobial, anticancer, and antioxidant therapies.

However, while in vitro and in vivo studies have demonstrated promising results, large-scale clinical trials are needed to validate the efficacy and safety of these nanoencapsulated formulations. Understanding the pharmacokinetics and pharmacodynamics of nanoencapsulated essential oils remains critical for optimizing their therapeutic potential.

6.2. Future Perspectives

The future of nanoencapsulated essential oils is bright, with ongoing research focusing on improving formulation techniques, enhancing targeted delivery, and optimizing biocompatibility. Some of the key future directions include:

Personalized Medicine: Advances in nanotechnology may lead to personalized essential oil formulations tailored to individual patient's needs, ensuring maximum therapeutic benefits with minimal side effects.

Combination Therapies: Integrating nanoencapsulated essential oils with conventional pharmaceuticals and other natural compounds could result in synergistic effects, enhancing treatment outcomes for chronic diseases such as cancer, neurodegenerative disorders, and infectious diseases.

Smart Drug Delivery Systems: The development of stimuli-responsive nanocarriers that release essential oils in response to specific physiological conditions (e.g., pH, temperature) could further enhance their efficacy and safety profiles.

Regulatory Approvals and Market Expansion: As scientific validation progresses, efforts must be made to obtain regulatory approvals for nanoencapsulated essential oils, facilitating their integration into mainstream medical treatments, nutraceuticals, and personal care products.

Environmental Sustainability: Future research should focus on eco-friendly and biodegradable nanocarrier materials to ensure that nanoencapsulation technologies align with sustainable development goals.

Overall, nanoencapsulation presents a significant opportunity to harness the full potential of essential oils in various applications. Continued interdisciplinary research and collaboration between scientists, pharmaceutical industries, and regulatory bodies will be essential in advancing this promising field and bringing innovative nanoformulations to market for widespread use.

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CHAPTER 20

Comparative Morphological and Cytochemical Analysis of Fish Blood Cells From Diverse Habitats

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1. INTRODUCTION

Vertebrates in the animal kingdom are divided into five classes: fish, amphibians, reptiles, birds, and mammals. Among these, fish, amphibians, and reptiles are cold-blooded (poikilothermic or ectothermic) organisms with body temperatures that vary according to the ambient temperature. In contrast, birds and mammals are warm-blooded (homeothermic or endothermic) organisms with stable body temperatures (1).

Fish are aquatic vertebrates that breathe through their gills, possess fins and scales, and exhibit a remarkable diversity. Today, fish species are broadly classified into three main groups: jawless fish, jawed cartilaginous fish, and jawed bony fish. The term "fish" is commonly used to refer to a group within the phylum Chordata, serving as an appropriate descriptor for poikilothermic aquatic vertebrates (2). Scientifically, the term "fish" primarily applies to Agnatha (jawless fish), Chondrichthyes (cartilaginous fish, including sharks and rays), and Sarcopterygii (bony lobe-finned fish), and Actinopterygii (bony ray-finned fish) (3).

Jawed fish are divided into Chondrichthyes (cartilaginous fish) and Osteichthyes (bony fish) (4). Over 95% of fish species, which comprise roughly half of vertebrates, belong to bony fish, with more than 23,500 species, and ray-finned fish are the dominant group within them. Teleosts constitute 99.8% of ray-finned fish species (5, 6). Bony fish exhibit a wide variety of species in terms of shape, size, lifespan, and adaptations. About 60% live in the sea, while the remainder live in freshwater. Some (approximately 1%) move between saltwater and freshwater during their life cycle (7). In terms of feeding habits, fish are classified into four main groups: carnivores, herbivores, omnivores, and limnivores (8).

1.1. Differences in Peripheral Blood Cells of Fish Compared to Mammals

Based on classical Romanowsky stains (Leishman, Wright, and May Grunwald-Giemsa), veterinary hematology has identified erythrocytes, thrombocytes, and leukocytes in fish. However, classical staining methods have not always been considered reliable for the cellular classification of leukocytes. In some fish, neutrophils have been found, while in others, heterophils are present. A few fish species have been reported to contain both cell types (9, 10). To date, studies on fish blood cells have raised numerous issues, both in terms of classification and the techniques used (11).

The cellular components of fish blood are distinctly different from those of higher vertebrates due to the presence of nucleated erythrocytes and nucleated thrombocytes (12). Nearly all fish possess nucleated erythrocytes, with notable exceptions, such as Maurolicus müelleri (lanternfish), a teleost with small, nucleated erythrocytes, and the Antarctic icefish (family Channichthyidae), which lacks erythrocytes or hemoglobin in its blood (13). In fish, mature erythrocytes are typically oval and disk-shaped with a compact nucleus. The average erythrocyte cell size varies across different systematic fish groups. Teleost erythrocytes generally measure around 8-15 microns (14).

Leukocytes, in the evolutionary process, are represented as key cells for innate immunity. These cells protect against commonly encountered pathogens through phagocytic functions as well as antimicrobial enzymes and peptides. Fish contain a wide variety of leukocytes, and the structural heterogeneity of these cells is observed even among closely related species. Fish leukocytes generally show very little differentiation compared to mammalian leukocytes, making it difficult to distinguish one cell type from another (15).

Due to the high species diversity, the functional and phylogenetic significance of fish leukocytes has not yet been fully resolved, and comparative studies are still needed. The present study aims to examine the morphology and cytochemical properties of peripheral blood cells in marine fish species, such as sea bass and gilthead seabream, as well as in the freshwater species, rainbow trout.

2. MATERIALS AND METHODS

The study material consisted of the blood of sea bass (Dicentrarchus labrax L., 1758), gilthead seabream (Sparus aurata L., 1758), and rainbow trout (Oncorhynchus mykiss Walbaum, 1792). The taxonomic classification of the material in the vertebrate systematics is as follows (16).

Phylum: Chordata (Chordates)

Group 1: Acrania (Acraniates)

Group 2: Craniata (Vertebrates)

Subphylum 1: Agnatha (Jawless fish)

Class: Cyclostomata (Round-mouthed fish)

Subphylum 2: Gnathostomata (Jawed fish)

Superclass: Pisces (Fish)

Class 1: Chondrichthyes (Cartilaginous fish)

Subclass: Elasmobranchii (Sharks, rays, and skates)

Subclass: Holocephali (Chimaeras, ghost sharks)

Class 2: Osteichthyes (Bony fish)

Subclass: Actinopterygii (Ray-finned fish)

Superorder: Teleostei (Typical bony fish)

Order: Perciformes (Perch-like fish)

Family: Moronidae

Dicentrarchus labrax (sea bass)

Family: Sparidae

Sparus aurata (gilthead seabream)

Order: Salmoniformes (Salmonids)

Family: Salmonidae

Subfamily: Salmoninae

Oncorhynchus mykiss (rainbow trout)

Eight fish, with a body weight of approximately 300-400 g, were collected during November-December. The characteristics of the facilities from which the fish samples were obtained are provided in Table 1. Healthy fish, anesthetized with MS222 (tricaine methanesulfonate) (1g/10L Sandoz), were sampled by drawing 1 cc of blood from the tail (caudal) vein using a 23G needle. The blood was collected into tubes containing heparin (0,75 mg per 1 ml of blood) and EDTA (Ethylenediaminetetraacetic acid) (1 mg per 1 ml of blood) (11, 17).

Heparinized and EDTA-treated blood samples were used to prepare blood smears for each fish, which were air-dried at room temperature. The peripheral blood samples were then stained using the methods listed below, each serving specific purposes. These include:

- May-Grünwald Giemsa (MGG) (18) was used for general appearance and morphological identification,

- Congo Red (CR) (19) was applied to identify eosinophils by staining the basic components of their proteins,
- Periodic Acid Schiff's (PAS) (Mc Manus, 1948) was used to investigate the presence of glycogen and neutral mucosubstances,
- Sudan Black (SB) (18, 20) was employed to determine the lipid content of cells,
- Toluidine Blue pH4 (TB pH4) (21) was used to detect the content of acidic mucosubstances in basophils.
- Methyl-Green Pyronin (MGP) (18, 22) was used to differentiate plasma cells by staining their DNA and RNA content,
- Alpha naphthyl acetate esterase Ph: 5.8 (ANAE) (23-26) was applied to reveal the non-specific esterase content of cells,
- The sABC method (27) was used to identify T lymphocytes in sea bass with the DLT 15 antibody. Blood smears were incubated for 18 h at room temperature with mAb DLT15 (1:10, from Giuseppe Scapigliati, Università degli Studi della Tuscia, Italy).
- Stained smears were analyzed under a light microscope, and results were captured with an image analysis system (CellSense/Olympus).

Table 1. Details of the facilities from which the fish samples were collected

	Sea bass	Gilthead seabream	Rainbow trout
Company Name	1. Okyanus Seafood Transp. Feed Tourism Ind.&Trade Ltd. Milas/MUĞLA 2. EGEMAR Seafood Food Ind. & Trade Inc. (LATMOS) Didim/AYDIN	1. Okyanus Seafood Transp. Feed Tourism Ind.&Trade Ltd. Milas/MUĞLA 2. EGEMAR Seafood Food Ind. & Trade Inc. (LATMOS) Didim/AYDIN	ERPE Food Transp. Industry &Trade Ltd. Bozdoğan/ AYDIN
Fish Feed	Normfeed Company/ Aquanorm	Normfeed Company/ Aquanorm	Gençsoy/ Gümüşdoğa/ Özpekler Company
Fish Origin	Kılıç Fish Hatchery	Kılıç Fish Hatchery	Acar Fish Production Facility
Water Temperature	19,6 °C	19,6 °C	16,0 °C
Salinity	%38	%38	Freshwater

3. RESULTS

The morphological and cytological examination of the fish blood cells was based on mammalian blood knowledge. MGG-stained smears clearly identified erythrocytes, rubricytes, lymphocytes, monocytes, and thrombocytes, but granulocyte differentiation was challenging. Therefore, cytochemical staining was examined. The effect of heparin and EDTA on staining was analyzed, and the best results for each species and staining method were recorded (Table 2). Granulocytes were classified as neutrophils, heterophils, eosinophils, and basophils, like mammals and birds.

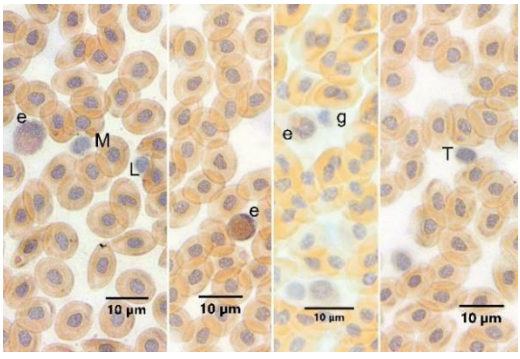

Figure 1B. Eosinophils in EDTA-treated sea bass blood fixed with formalin vapor.e: Eosinophil, L: Lymphocyte, T: Thrombocyte, M: Monocyte, g: Granulocyte. CR staining method.

3.1.2. Periodic Acid-Schiff (*PAS) Staining Method*

In heparinized blood samples, PAS (+) cells with different morphologies were detected in smears fixed with acetone. Granulated cells with large cytoplasm were identified as granulocyte 1, with their nuclei mostly being oval or round (Fig. 2A). Cells with smaller cytoplasm and oval or lobed nuclei were recorded as granulocyte 2 (Fig. 2B).

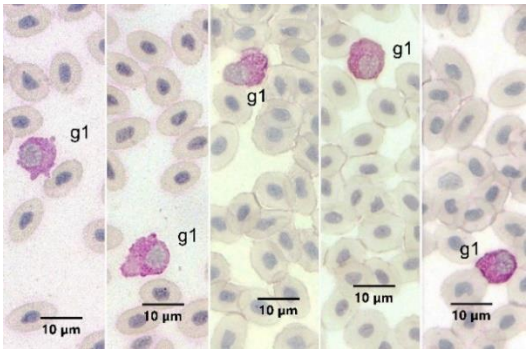

Figure 2A. PAS-positive cells in heparinized sea bass blood fixed with acetone. g1: Granulocyte type 1. PAS staining method.

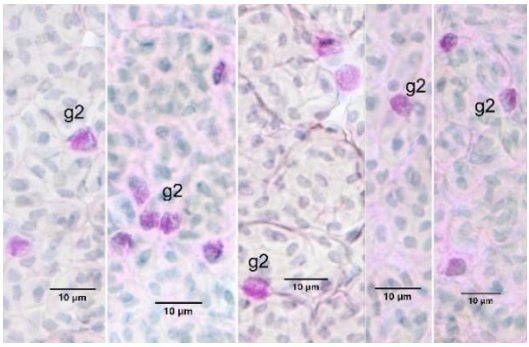

Figure 2B. PAS-positive cells in the blood of sea bass fixed with acetone and anticoagulated with heparin. g2: Granulocyte type 2. PAS staining method

In heparinized blood, methanol-fixed smears showed cells with varying morphologies, similar to acetone-fixed ones. Large-cytoplasm cells were classified as granulocyte 1, with irregular-shaped nuclei (Fig. 3A), while smaller-cytoplasm, lobed-nucleus cells were classified as granulocyte 2 (Fig. 3B). Acetone fixation in heparinized sea bass blood gave better results than methanol fixation.

EDTA anticoagulated smears fixed with acetone (Fig. 4A, B) showed PAS-positive granulocyte types 1 and 2, as did those fixed with methanol (Fig. 5A, B). Methanol fixation slightly impaired PAS staining. No significant differences were found between heparin and EDTA anticoagulants in sea bass blood smears, but acetone fixation yielded slightly better results than methanol.

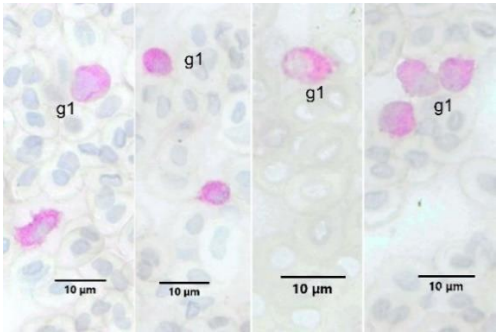

Figure 3A. PAS-positive cells in sea bass blood anticoagulated with heparin and fixed with methanol. g1: Granulocyte type 1. PAS staining method.

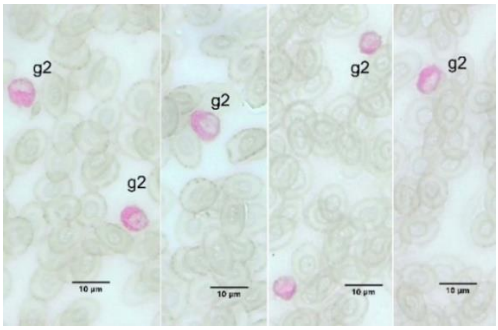

Figure 3B. PAS-positive cells in sea bass blood anticoagulated with heparin and fixed with methanol. g2: Granulocyte type 2. PAS staining method.

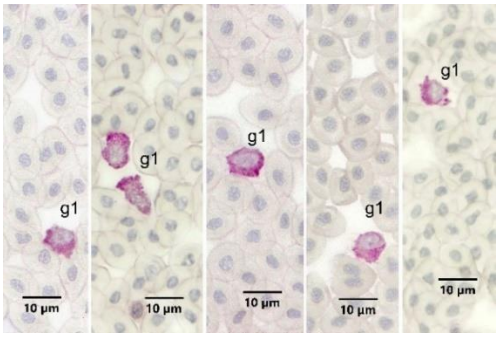

Figure 4A. PAS-positive cells in sea bass blood fixed with acetone and anticoagulated with EDTA. g1: Granulocyte type 1. PAS staining method.

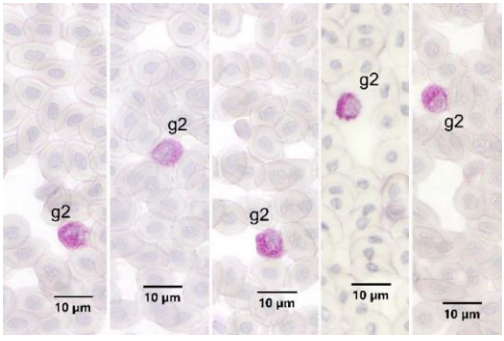

Figure 4B. PAS-positive cells in the blood of sea bass fixed with acetone and anticoagulated with EDTA. g2: Granulocyte type 2. PAS staining method.

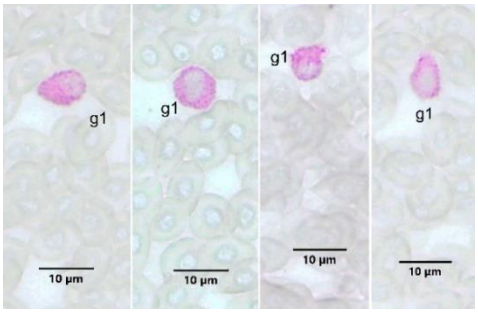

Figure 5A. PAS-positive cells in the blood of sea bass fixed with methanol and anticoagulated with EDTA. g1: Granulocyte type 1. PAS staining method

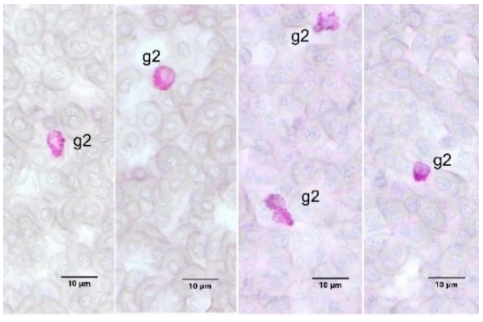

Figure 5B. PAS-positive cells in the blood of sea bass fixed with methanol and anticoagulated with EDTA. g2: Granulocyte type 2. PAS staining method

3.1.3. Sudan Black (SB) Staining Method

SB (+) granulocytes were detected in blood smears fixed in formalin vapor and prepared with both heparin (Fig. 6A) and EDTA (Fig 6B) anticoagulants. It was observed that cell sizes and the number of granules varied, granules were rod-shaped, and the nuclei were either lobed or round-oval in shape. It was recorded that there was no difference between heparin and EDTA anticoagulants for SB staining in the blood smears of sea bass.

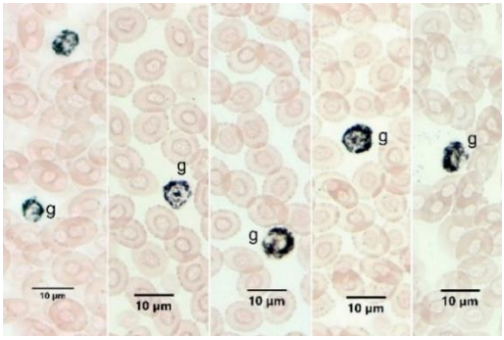

Figure 6A. Heparin anticoagulated sea bass blood showing SB-positive cells. g: Granulocyte. SB staining method.

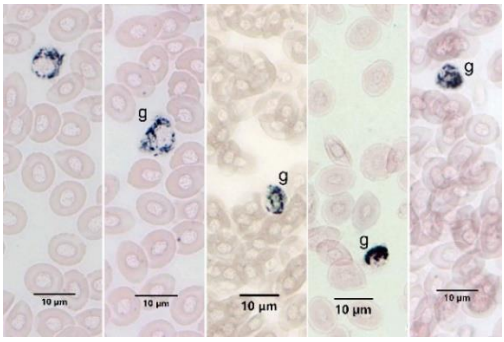

Figure 6B. SB-positive cells in sea bass blood with EDTA anticoagulant. g: Granulocyte. SB staining method.

3.1.4. Toluidine Blue (TB) (pH 4) Staining Method

Heparin (Fig. 7A) and EDTA (Fig. 7B) anticoagulated blood smears were methanol-fixed and stained with TB pH 4. The nuclei of all cells and cytoplasm of some were basophilic. Blue-dark purple granules characteristic of basophils were sought, but instead, a few cells with light blue cytoplasm and fine blue granules were observed, considered basophils. No significant difference was found between heparin and EDTA in TB staining of sea bass blood smears.

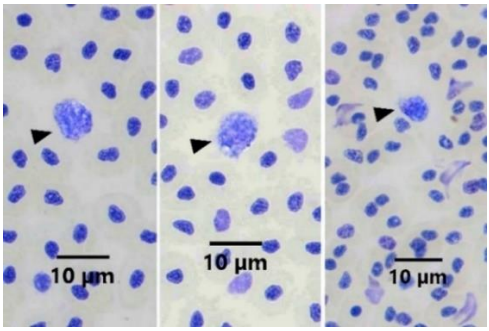

Figure 7A. TB-positive reaction in sea bass blood with heparin anticoagulant. Arrowheads: TB-positive granular cells. Toluidine blue pH 4 staining method.

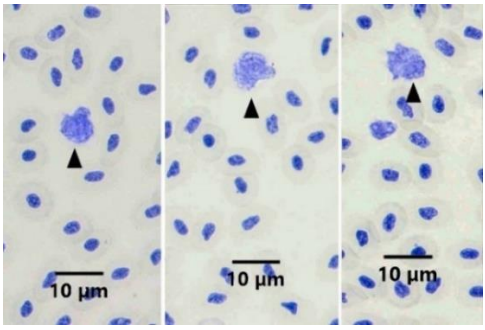

Figure 7B. TB-positive reaction in sea bass blood with EDTA anticoagulant. Arrowheads: TB-positive granular cells. Toluidine blue pH 4 staining method.

3.1.5. *May-Grünwald Giemsa (MGG) Staining Method*

After methanol fixation, the MGG staining method was applied to blood smears with heparin and EDTA. EDTA smears showed clearer images. Erythrocytes appeared oval, with pink cytoplasm and centrally located oval nuclei (Fig. 8). Some nuclei were horseshoe-shaped, lobed, or twisted. Immature erythrocytes, with lighter cytoplasm and round shapes, were also observed. Thrombocytes in sea bass were observed to be spindle-shaped with varying widths (Fig. 9A, B). Although their cytoplasmic amounts and staining characteristics were largely similar, variability was noted, with some thrombocytes showing a small amount of blue cytoplasm and others exhibiting slightly eosinophilic cytoplasm.

In sea bass, lymphocytes (Fig. 9A, B), were observed to be round with a small amount of basophilic cytoplasm. It was noted that both small and large lymphocytes were present, with the cytoplasm sometimes exhibiting pseudopod-like structures.

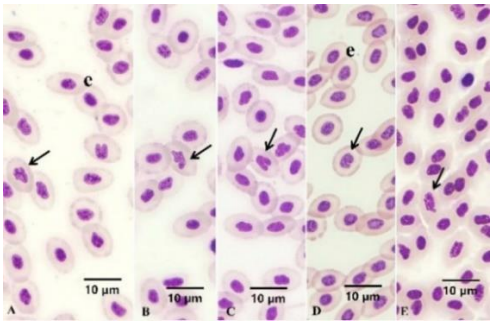

Figure 8. Erythrocytes with differently shaped nuclei in sea bass blood. A-C: Heparin, D, E: EDTA. e: Normal nuclei, arrows: Abnormal nuclei. MGG staining.

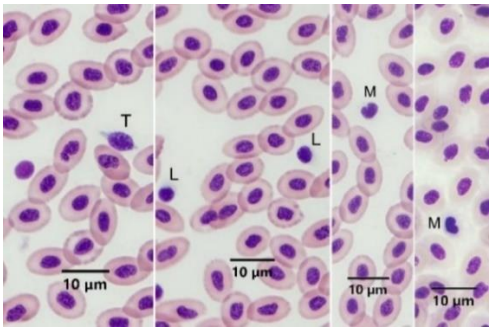

Figure 9A. Appearance of blood cells in sea bass with EDTA. T: Thrombocyte, L: Lymphocyte, M: Monocyte. MGG staining method.

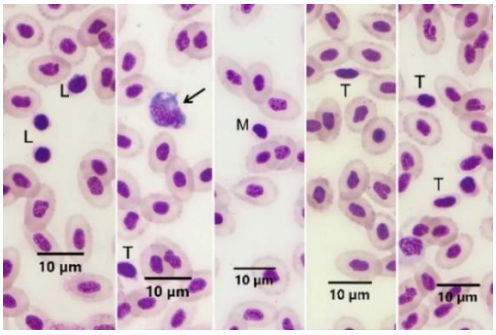

Figure 9B. Blood cells in sea bass with heparin. T: Thrombocyte, L: Lymphocyte, M: Monocyte, Arrow: Monocyte precursor. MGG staining.

Monocytes, another type of agranulocyte, were generally observed to have a kidney-shaped nucleus and a small amount of basophilic cytoplasm (Fig. 9A, B; Fig. 10A). Some of them exhibited vacuole-like structures in their cytoplasm. Additionally, cells with dark blue cytoplasm, often having deeply indented, fragmented, large, and dark purple-stained nuclei and pseudopod-like extensions, were identified as macrophages.

In sea bass, both EDTA (Fig. 10A) and heparin anticoagulated blood smears (Fig. 10B) were examined for granulocytes. After lymphocytes, monocytes, and thrombocytes were identified, it was observed that the granules in the remaining cells were not stained by the MGG method. Consequently, the differentiation of

neutrophils, eosinophils, or basophils could not be performed with MGG staining. Based on the results of previous CR, PAS, and SB cytochemical staining methods, these cells were generally classified as granulocytes. The cytoplasm of granulocytes was lightly basophilic (Fig. 10A, B), and their nuclei were found to be oval, horseshoe-shaped, or lobed, with lobes sometimes folding over each other (Fig. 10A, B). No basophilic granulocytes were observed in the MGG-stained sea bass smears.

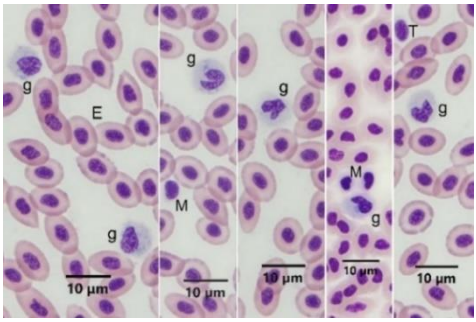

Figure 10A. Appearance of blood cells in sea bass with EDTA. E: Erythrocytes, g: Granulocytes, M: Monocytes, T: Thrombocytes. MGG staining metodu.

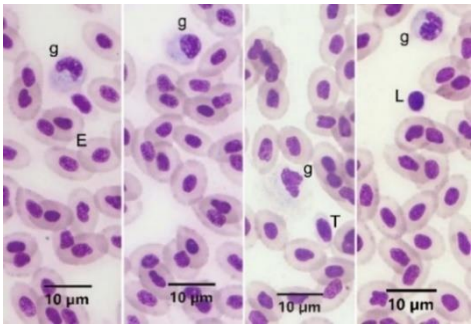

Figure 10B. Appearance of blood cells in sea bass with heparin. E: Erythrocytes, g: Granulocytes, T: Thrombocytes, L: Lymphocytes. MGG staining method.

3.1.6. *Alpha naphthyl acetate esterase (ANAE) pH 5.8 Staining Method*

In the blood smears of sea bass with heparin anticoagulant, following glutaraldehyde-acetone fixation and application of the ANAE enzyme staining, brownish positive reactions were observed in some lymphocytes (Fig. 11). These cells were identified as T lymphocytes. It was also noted that some lymphocytes did not show a positive reaction (Fig. 11, arrowhead).

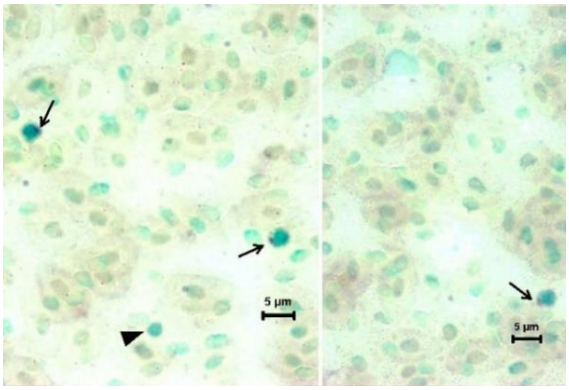

Figure 11. ANAE-positive lymphocytes (arrows) in heparinized sea bass blood. Arrowhead: ANAE-negative lymphocyte. ANAE pH 5.8 staining.

3.1.7. Streptavidin-biotin-peroxidase complex (sABC) Staining Method

T-cell monoclone antibody (DLT15) was used in heparinized smears for sABC staining, which resulted in the identification of reaction-positive T lymphocytes (Fig. 12).

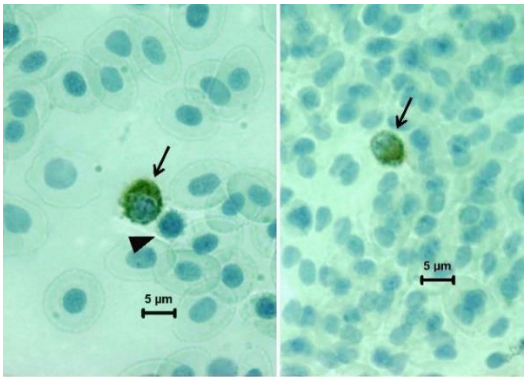

Figure 12. Appearance of DLT 15-positive T lymphocytes (arrows) in heparinized sea bass blood smears. Arrowhead: DLT15-negative lymphocyte. Strept-ABC staining method.

3.1.8. *Methyl Green- Pyronin (MGP) Staining Method*

After Carnoy fixation, a few MGP-positive cells were observed on the smears stained with the MGP method (Fig. 13). These cells were noted to vary in size. No significant difference was observed between smears prepared with heparin and EDTA. MGP-positive cells with eccentric nuclei were considered plasma cells, while those with lobulated nuclei were thought to be immature granulocytes.

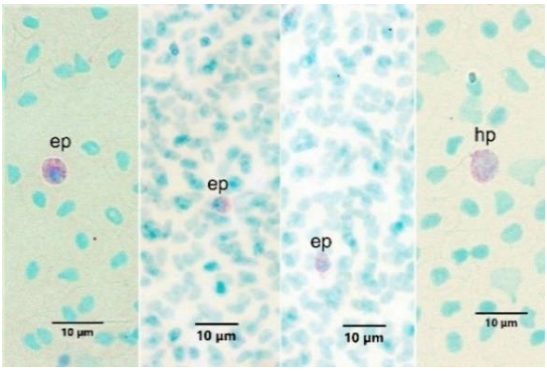

Figure 13. MGP-positive cells in sea bass blood. Ep: MGP-positive cells in blood with EDTA. Hp: MGP-positive cells in blood with heparin. MGP staining.

3.2. Gilthead Seabream Blood

3.2.1. *CR Staining Method*

To investigate eosinophils, CR staining was applied to smears with EDTA (Fig. 14) and heparin (Fig. 15A, B), fixed with formaldehyde vapor. Eosinophils in gilthead seabream blood were CR-positive, with orange-stained granules and slightly basophilic cytoplasm. Eosinophil sizes varied, with some being small, and their nuclei were round, oval, or flattened, with some bilobed. Staining differences were noted based on anticoagulant, with some eosinophils showing granule lysis in EDTA-treated blood (Fig. 14).

In gilthead seabream, when heparin anticoagulant was used, eosinophils exhibited a more distinct and homogeneous appearance (Fig. 15A). Additionally, CR-negative cells were identified (Fig. 15B).

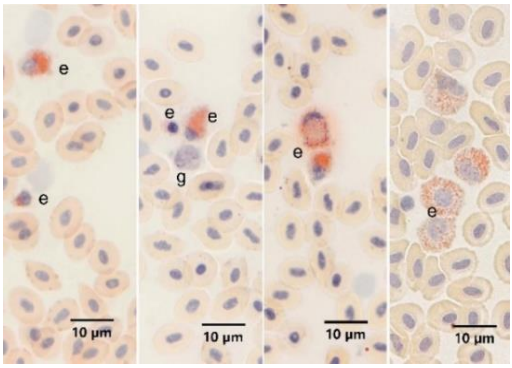

Figure 14. Appearance of eosinophils in gilthead seabream blood with EDTA.

e: Eosinophil, g: Granulocyte. CR staining method.

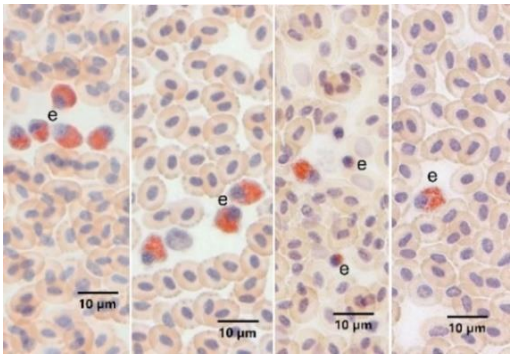

Figure 15A. Appearance of eosinophils in gilthead seabream blood with heparin. e: Eosinophil. CR staining method.

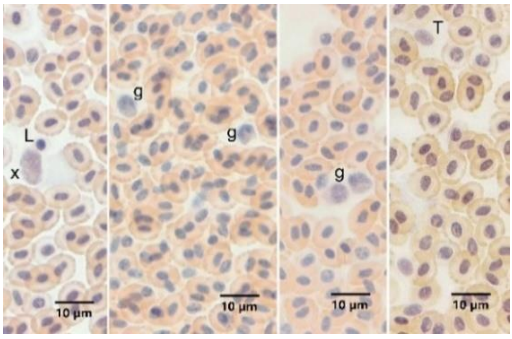

Figure 15B. Cells showing negative reaction in gilthead seabream blood with heparin. L: Lymphocyte, x: Possible precursor cell, g: Granulocyte, T: Thrombocyte. CR staining method.

3.2.2. *PAS Staining Method*

In gilthead seabream, PAS staining was applied to blood smears fixed with acetone or methanol and anticoagulated with heparin or EDTA to detect neutral mucosubstances like glycogen. Positivity was observed in both acetone and methanol fixations with EDTA. Methanol fixation (Fig. 16A) showed slightly better staining than acetone fixation (Fig. 16B), but clarity was not fully achieved. Large, round or oval-shaped cells with round or oval nuclei were classified as granulocyte type 1, while smaller cells with oval or lobed nuclei were classified as granulocyte type 2.

PAS staining of granulocyte type 1 cells in EDTA-anticoagulated blood was difficult, whereas heparin-anticoagulated blood yielded much better results. In heparin blood, positivity was observed in both acetone and methanol fixations, with methanol fixation (Fig.17A, B) giving slightly better results than acetone (Fig. 18).

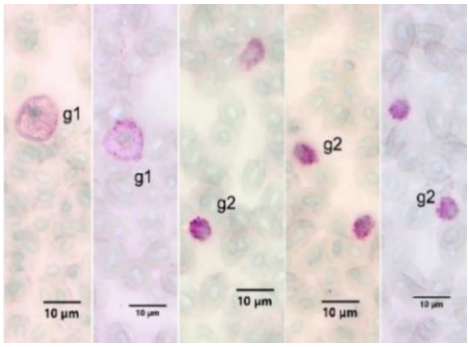

Figure 16A. PAS-positive cells in gilthead seabream fixed with methanol and anticoagulated with EDTA. g1: Granulocyte type 1; g2: Granulocyte type 2. PAS staining method.

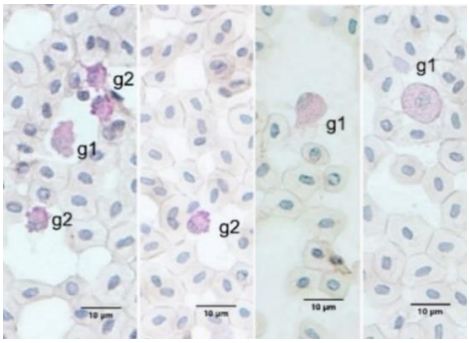

Figure 16B. PAS-positive cells in gilthead seabream blood fixed with acetone and anticoagulated with EDTA. g1: Granulocyte type 1, g2: Granulocyte type 2. PAS staining method

In heparin-anticoagulated blood, as in EDTA-anticoagulated blood, large, round or oval-shaped cells with similarly round or oval nuclei were identified as granulocyte type 1, while smaller cells with oval or lobed nuclei were classified as granulocyte type 2. The large granulocyte type 1 cells were considered eosinophils. Granulocyte type 2 cells were mostly neutrophils, with the possibility of small eosinophils or basophils. PAS staining in gilthead seabream blood showed optimal results with heparin anticoagulant and methanol fixation.

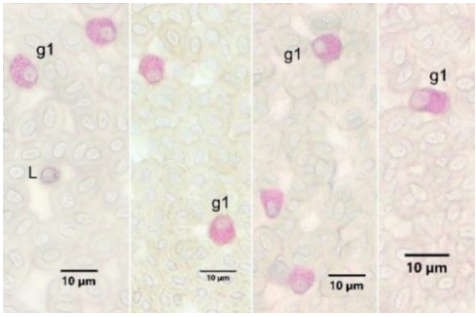

Figure 17A. PAS-positive cells in gilthead seabream blood fixed with methanol and anticoagulated with heparin. g1: Granulocyte type 1, L: Lymphocyte. PAS staining method.

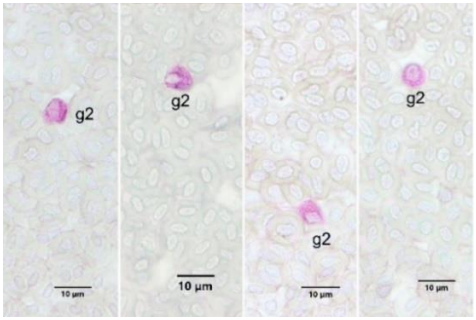

Figure 17B. PAS-positive cells in gilthead seabream blood fixed with methanol and anticoagulated with heparin. g2: Granulocyte type 2. PAS staining method.

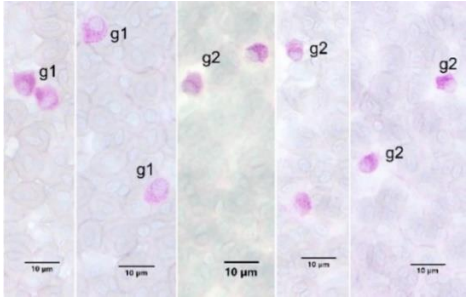

Figure 18. PAS-positive cells in gilthead seabream blood fixed with acetone and anticoagulated with heparin. g1: Granulocyte type 1, g2: Granulocyte type 2. PAS staining method.

3.2.3. *SB Staining Method*

Granulocytes showing positive reactions with SB stain were detected in gilthead seabream blood samples prepared from both EDTA (Fig. 19A, B) and heparin (Fig. 20A, B) fixed in formalin vapors. The granules were observed to be round and rod-shaped, with variations in cell size and granule density. Examination of the smears revealed large, round or oval-shaped cells with similarly round or oval nuclei, which were classified as granulocyte type 1. These cells were considered to be eosinophilic leukocytes. Smaller cells with oval or lobed nuclei were classified as granulocyte type 2. Granulocyte type 2 cells were mostly considered to be neutrophilic leukocytes, with the possibility of small eosinophilic or basophilic leukocytes.

No significant difference was observed between heparin and EDTA anticoagulants for SB staining in gilthead seabream blood, with SB-positive granules unaffected.

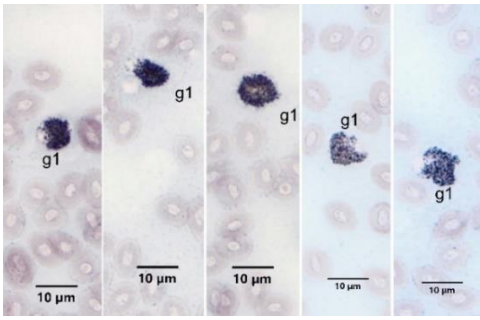

Figure 19A. SB-positive cells in gilthead seabream blood with EDTA. g1: Granulocyte type 1. SB staining method.

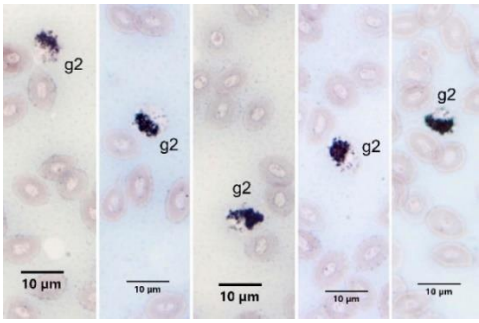

Figure 19B. SB-positive cells in gilthead seabream blood with EDTA. g2: Granulocyte type 2. SB staining method.

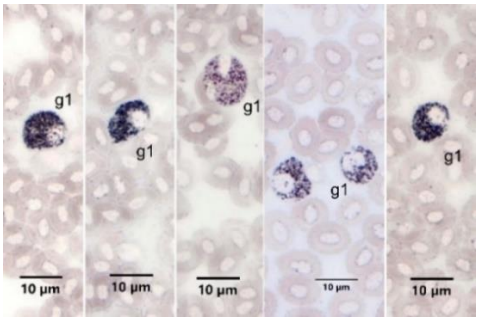

Figure 20A. SB-positive cells in gilthead seabream blood anticoagulated with heparin. g1: Granulocyte type 1. SB staining method.

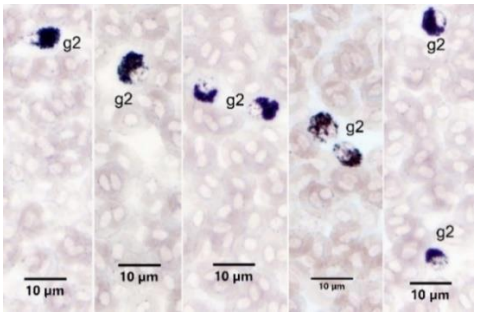

Figure 20B. SB-positive cells in gilthead seabream blood anticoagulated with heparin. g2: Granulocyte type 2. SB staining method.

3.2.4. *TB Staining Method*

After methanol fixation, TB pH 4 staining was applied to blood smears with EDTA and heparin anticoagulants. The nuclei of all cells and cytoplasm of some were basophilic. While classic blue-purple granule-containing cells were searched for, only a few cells with light blue cytoplasm and tiny blue granules were detected, which were considered basophilic leukocytes. No significant difference was observed between EDTA and heparin for TB staining in gilthead seabream blood smears (Fig. 21)

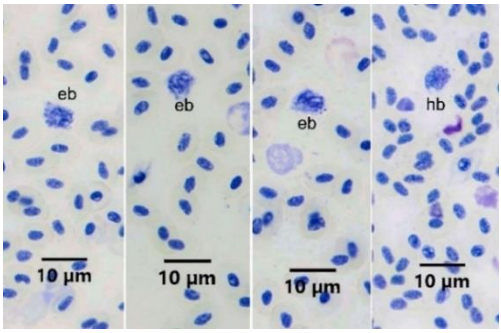

Figure 21. Basophilic leukocytes showing TB-positive reaction in gilthead seabream blood. eb: EDTA-treated blood, hb: Heparin-treated blood. TB pH 4 staining.

3.2.5. *MGG Staining Method*

After methanol fixation and May-Grünwald Giemsa staining, both anticoagulants showed results, with heparin blood smears providing more distinct staining, especially for eosinophils. Erythrocytes had a classical appearance, oval-shaped with pink cytoplasm and a central oval nucleus. Immature erythrocytes (rubricytes) had lighter cytoplasm and a round shape (Fig. 22A, B). Among the agranulocytes in gilthead seabream , lymphocytes had a round shape and their sparse cytoplasm appeared basophilic (Fig. 22A, B). Both small and large lymphocytes were observed, some of which had pseudopod-like cytoplasmic formations.

Monocyte leukocytes in gilthead seabream blood were observed in two forms: one with a kidney-shaped nucleus and small amounts of basophilic cytoplasm, and another with dark blue cytoplasm, oval or kidney-shaped nuclei, and cytoplasmic vacuoles (Fig. 23). The use of anticoagulants did not alter the appearance of monocytes (Fig. 23).

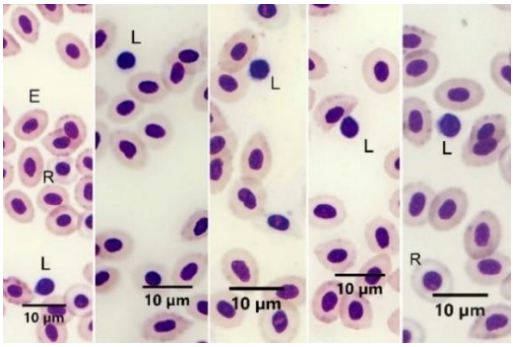

Figure 22A. Appearance of blood cells in gilthead seabream blood with EDTA anticoagulant. E: Erythrocyte, L: Lymphocyte. MGG staining method.

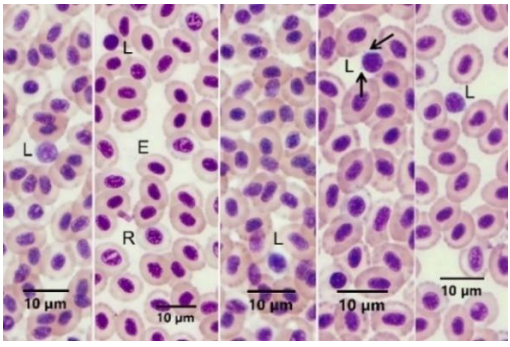

Figure 22B. Appearance of blood cells in gilthead seabream blood with heparin anticoagulant. E: Erythrocyte, R: Rubricyte, L: Lymphocyte. MGG staining method.

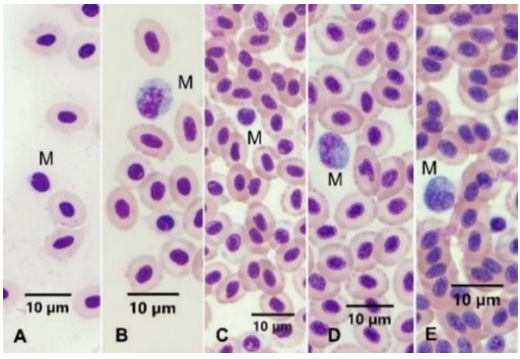

Figure 23. Monocytes in gilthead seabream blood. A-B: Monocytes in blood with EDTA. M: Monocyte. C-D-E: Monocytes in heparinized blood. M: Monocyte.

In MGG staining of gilthead seabream blood, eosinophils, neutrophils, and heterophil leukocytes were observed among granulocytes, but no basophils were identified. Eosinophils with round to oval-shaped nuclei and acidophilic granules were observed in both EDTA (Fig. 24A) and heparinized (Fig. 24B) smears. Occasionally, very small eosinophils were detected. It was also observed that EDTA anticoagulant caused eosinophil lysis in gilthead seabream blood (Fig. 24A).

In gilthead seabream, granulocytes, especially neutrophils, had basophilic cytoplasm and lobed, occasionally round or oval-shaped nuclei, but their granules were not visible (Fig. 25A, B). Since no acidic or basic granules were detected, these cells were identified as neutrophils. Some cells with lobed nuclei, similar

in size to neutrophils, were classified as heterophils due to the slight acidophilic appearance of their blue cytoplasm (Fig. 25A, B). The anticoagulant used did not affect the appearance of neutrophils or heterophils. In gilthead seabream , thrombocytes with a fusiform shape were observed to have round-oval nuclei and minimal bluish cytoplasm (Fig. 26). The choice of anticoagulant did not affect thrombocyte staining.

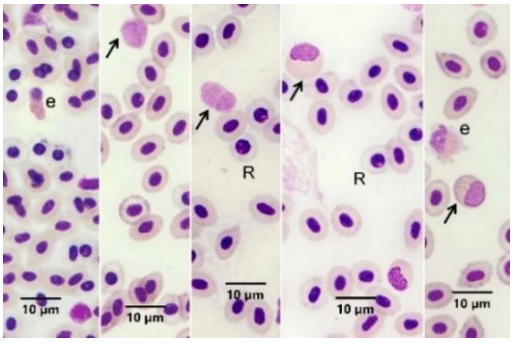

Figure 24A. Appearance of blood cells in gilthead seabream with EDTA. e: Eosinophil, Arrows: Lysed eosinophils, R: Rubricyte. MGG staining method.

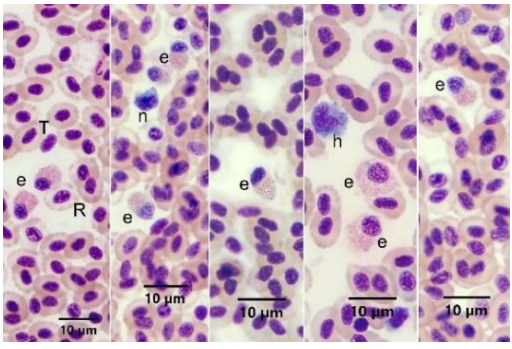

Figure 24B. Appearance of blood cells in gilthead seabream with heparin. e: Eosinophil, n: Neutrophil, h: Heterophil, T: Thrombocyte, R: Rubricyte. MGG staining method.

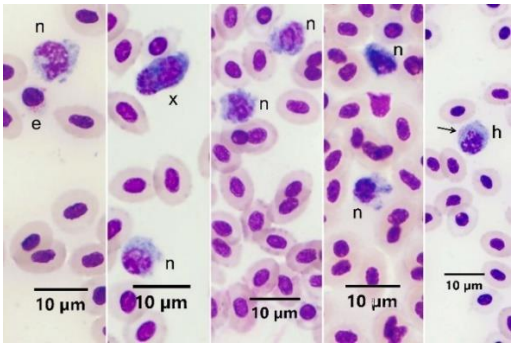

Figure 25A. Blood cells in gilthead seabream blood with EDTA. e: eosinophil, n: neutrophil, x: precursor cell, h: heterophil. Arrow: acidophilic part of heterophil. MGG staining.

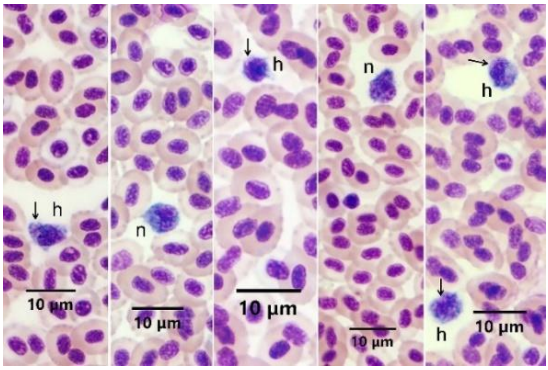

Figure 25B. Blood cells in gilthead seabream with heparin: n: neutrophil, h: heterophil, arrows: acidophilic part of heterophil. MGG staining.

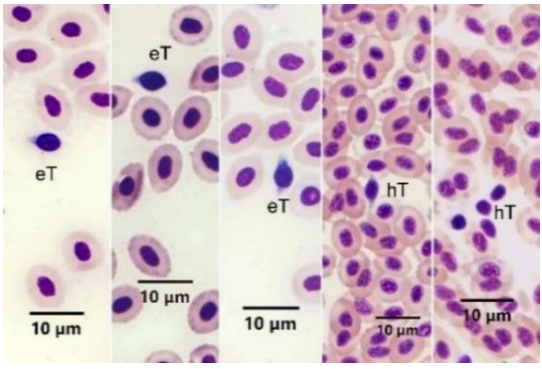

Figure 26. Thrombocytes in gilthead seabream blood with EDTA and heparin: eT: thrombocyte in EDTA, hT: thrombocyte in heparin. MGG staining.

3.2.6. ANAE pH 5.8 Staining Method

The application of the ANAE staining method to heparinized gilthead seabream blood revealed positive reactions in granulocytes, lymphocytes, and monocytes. Some lymphocytes and thrombocytes did not show any reaction (Fig. 27A, B).

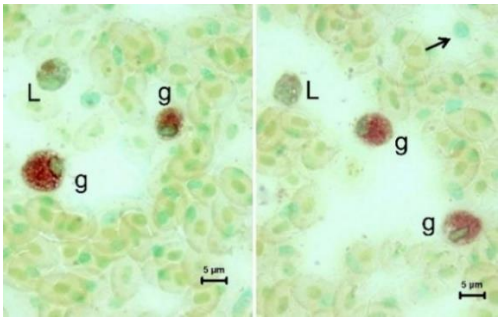

Figure 27A. ANAE-positive cells in heparinized gilthead seabream blood. L: ANAE-positive lymphocyte, g: granulocyte, arrow: ANAE-negative lymphocyte. ANAE pH 5.8 staining method.

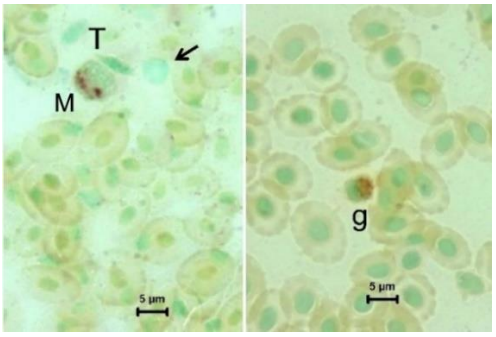

Figure 27B. ANAE-positive cells in heparinized gilthead seabream blood. M: monocyte, T: Thrombocyte, g: granulocyte, arrow: ANAE-negative lymphocyte. ANAE pH 5.8 staining method.

3.2.7. MGP Staining Method

After Carnoy fixation, the MGP staining method revealed very few MGP-positive cells. These cells exhibited various sizes and resembled the morphology of granulocytes (Fig. 28A, B). Cells with eccentric nuclei were likely plasma cells, and those with fragmented nuclei were presumed to be immature granulocytes. Positive cells were more visible in heparinized blood smears, with minimal difference compared to EDTA-treated smears.

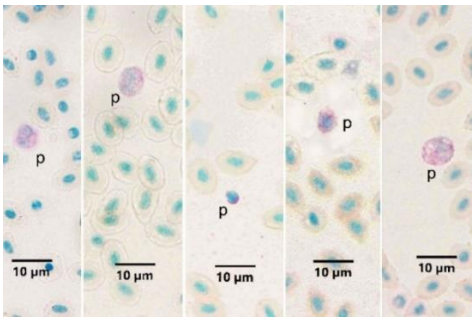

Figure 28A. MGP-positive cells in gilthead seabream blood treated with EDTA. p: MGP-positive cells. MGP staining method.

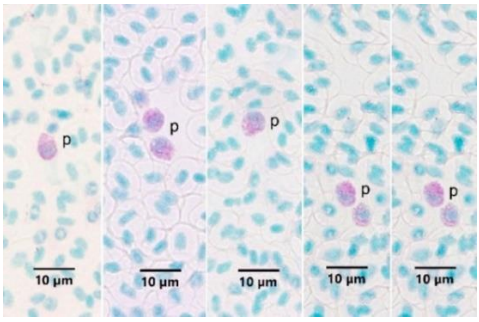

Figure 28B. Appearance of MGP-positive cells in heparinized gilthead seabream blood. p: MGP-positive cells. MGP staining method.

3.3. Rainbow Trout Blood

3.3.1. CR Staining Method

CR staining was applied to investigate eosinophilic leukocytes in rainbow trout blood, revealing CR (+) eosinophils (Fig. 29A, B). Their granules were orange and finely granular, while the cytoplasm appeared basophilic, more pronounced in heparinized smears. The nuclei were round-oval or lobulated. No difference in staining of eosinophil granules was observed between anticoagulants.

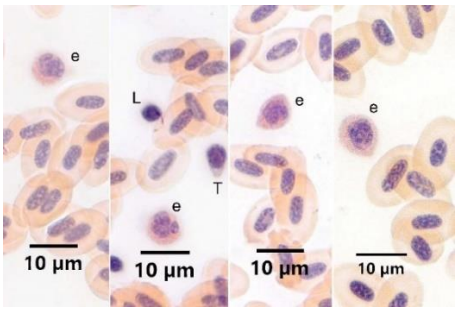

Figure 29A. Appearance of eosinophils in rainbow trout blood with EDTA anticoagulant. e: Eosinophil, L: Lymphocyte, T: Thrombocyte. CR staining method.

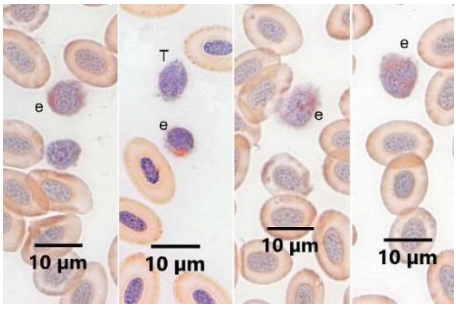

Figure 29B. Appearance of eosinophils in rainbow trout blood with heparin anticoagulant. e: Eosinophil, T: Thrombocyte. CR staining method.

3.3.2. *PAS Staining Method*

In smears prepared with EDTA anticoagulant and fixed with methanol (Fig. 30A) or acetone (Fig. 30B), PAS staining showed weak positivity. Positive cells had lobulated nuclei, indicating granulocytes, while thrombocytes showed minimal positivity. Smears prepared with heparin anticoagulant and fixed with methanol (Fig. 31A) or acetone (Fig. 31B) showed weak PAS positivity, similar to EDTA results. Granulocytes were identified by their lobulated nuclei, while thrombocytes exhibited minimal positivity.

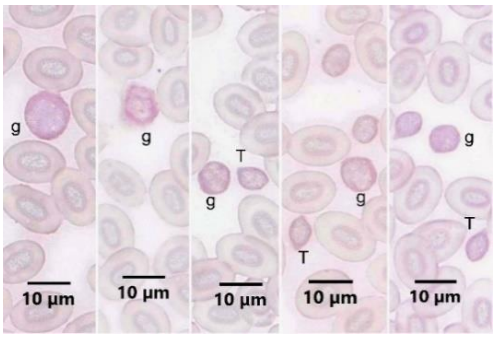

Figure 30A. PAS-positive cells in rainbow trout blood with EDTA and methanol fixation. g: Granulocyte, T: Thrombocyte. PAS staining method.

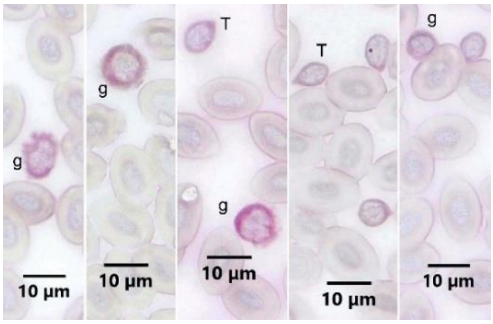

Figure 30B. PAS-positive cells in rainbow trout blood with EDTA and acetone fixation. g: Granulocyte, T: Thrombocyte.PAS staining method.

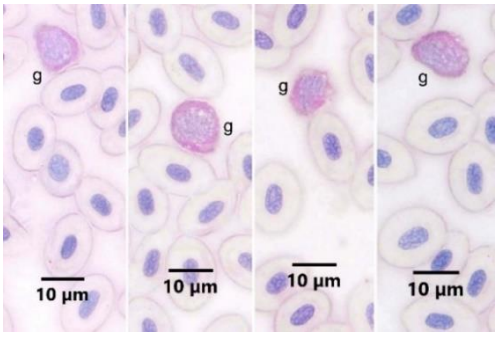

Figure 31A. PAS-positive cells in rainbow trout blood with heparin anticoagulant, methanol fixation. g: Granulocyte. PAS staining method.

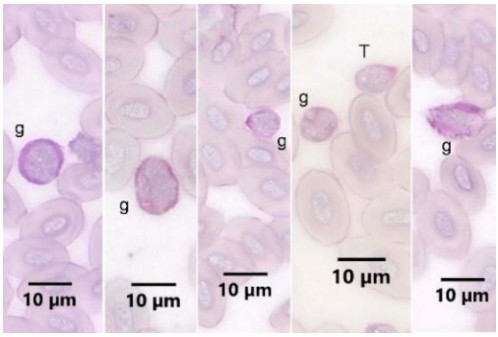

Figure 31B. PAS-positive cells in rainbow trout blood with heparin anticoagulant, acetone fixation. g: Granulocyte, T: Thrombocyte. PAS staining method.

3.3.3. *SB Staining Method*

When the SB staining method was applied to rainbow trout blood with EDTA or heparin anticoagulant, no differences were observed in cell positivity (Fig. 32A, B). The granules of SB-positive cells were round and rod-shaped. Larger cells with more prominent nuclei were classified as granulocyte type 1 (Fig. 32A, B). The nuclei of type 1 granulocytes appeared round-oval or lobulated. Smaller cells with lobulated nuclei were identified as granulocyte type 2 (Fig. 32A, B).

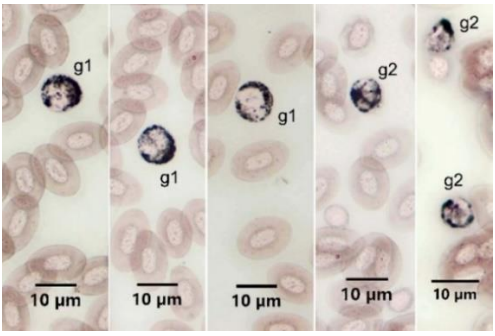

Figure 32A. SB-positive cells in rainbow trout blood with EDTA anticoagulant. g1: Granulocyte type 1, g2: Granulocyte type 2. SB staining method.

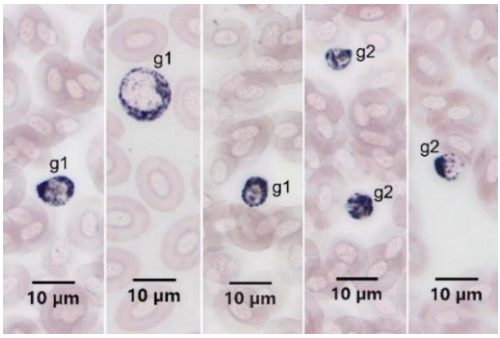

Figure 32B. SB-positive cells in rainbow trout blood with heparin anticoagulant. g1: Granulocyte type 1, g2: Granulocyte type 2. SB staining method.

3.3.4. *TB Staining Method*

When the Toluidine blue pH 4 staining method was applied (Fig.33), the nuclei of all cells and the cytoplasm of some cells were basophilically stained. A small number of cells with light blue cytoplasm and tiny blue granules were detected and identified as basophils (Fig. 33). No significant differences were observed between EDTA and heparin in TB staining of rainbow trout blood smears.

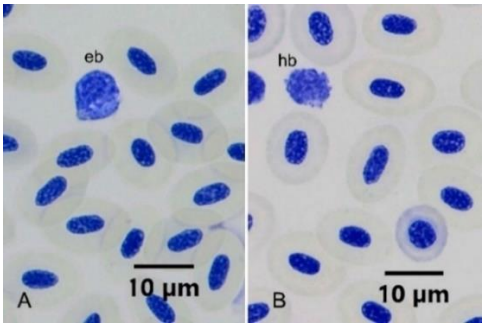

Figure 33. A: Basophil leukocytes in rainbow trout blood with EDTA (eb). **B:** Basophil leukocytes in rainbow trout with heparin anticoagulant (hb). TB (pH 4) staining method.

3.3.5. *MGG Staining Method*

Heparin and EDTA blood smears were methanol-fixed and stained using the May-Grünwald Giemsa method. Erythrocytes were oval-shaped with pink cytoplasm and central oval nuclei. Immature erythrocytes (rubricytes) had lighter cytoplasm and appeared oval or roundish (Fig. 34A, B).

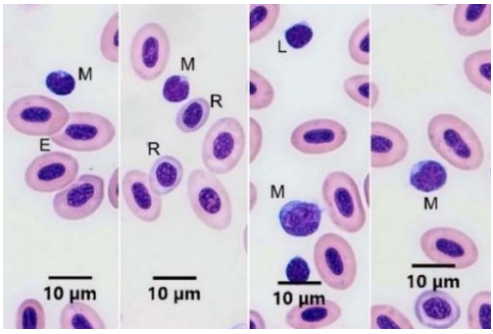

Figure 34A. Blood cells in rainbow trout blood with EDTA. E: Erythrocyte, R: Rubricyte, L: Lymphocyte, M: Monocyte. MGG staining method.

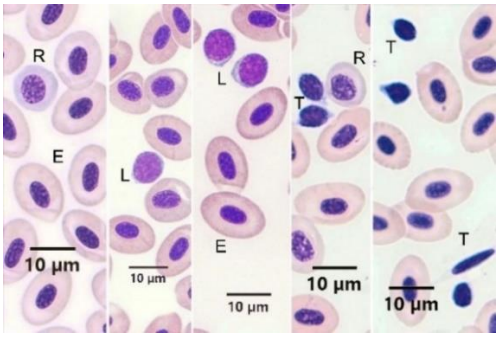

Figure 34B. Blood cells in rainbow trout blood with heparin. E: Erythrocyte, R: Rubricyte, L: Lymphocyte, T: Thrombocyte. MGG staining method.

In rainbow trout, lymphocytes, observed as round-shaped, exhibited minimal basophilic cytoplasm (Fig. 34A, B). Both small and large lymphocytes were present. Monocyte leukocytes in rainbow trout blood appeared in two distinct forms. One group showed kidney-shaped nuclei and minimal basophilic cytoplasm, while a larger group had darker blue cytoplasm, oval or kidney-shaped nuclei, and cytoplasmic vacuoles (Fig. 34A, Fig.35).

In rainbow trout, neutrophils exhibited slightly basophilic cytoplasm and nuclei that were mostly lobed, sometimes horseshoe-shaped. The presence of granules in the cytoplasm was not observed (Fig. 36A, B). Since the granules were neither acidic nor basic, these cells were classified as neutrophils. Alongside neutrophils, eosinophils, another type of granulocyte, were also observed in rainbow trout blood.

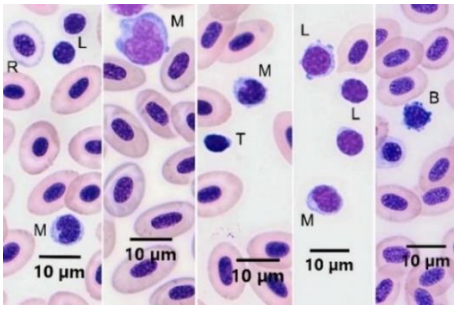

Figure 35. Blood cells in rainbow trout with heparin R: Rubricyte, L: Lymphocyte, M: Monocyte, T: Thrombocyte, B: Basophil. MGG staining method.

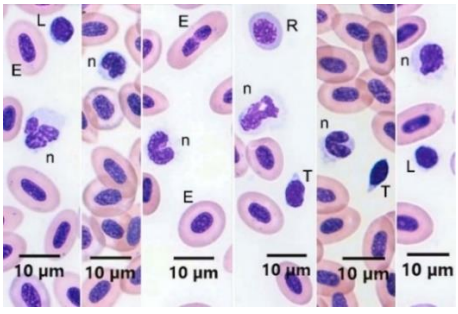

Figure 36A. Blood cells in rainbow trout blood with EDTA. E: Erythrocyte, R: Rubricyte, L: Lymphocyte, n: Neutrophil, T: Thrombocyte. MGG staining method.

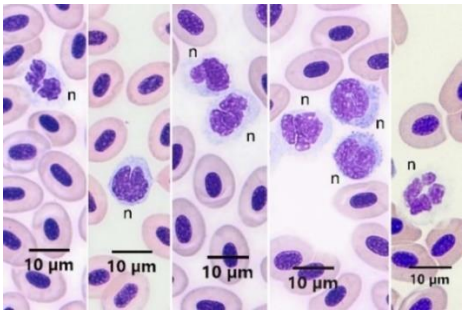

Figure 36B. Blood cells in rainbow trout with heparin anticoagulant. n: Neutrophil. MGG staining method.

In rainbow trout, very few eosinophils were observed in EDTA anticoagulant-treated blood, and those that were seen appeared lysed (Fig. 37A). Eosinophils were more clearly visible in blood samples treated with heparin (Fig. 37B). In blood with heparin anticoagulant, eosinophil granulocytes were distinguished by their basophilic cytoplasm and acidophilic granules. Their nuclei appeared flattened oval or lobed

Basophilic granulocytes were observed in both heparinized (Fig. 35) and EDTA-treated blood smears (Fig. 37A) of rainbow trout. Thrombocytes in rainbow trout were spindle-shaped, with oval nuclei that fit the cell shape, and a very faint bluish cytoplasm (Fig.34B, Fig. 36A). The use of anticoagulants did not affect thrombocyte staining.

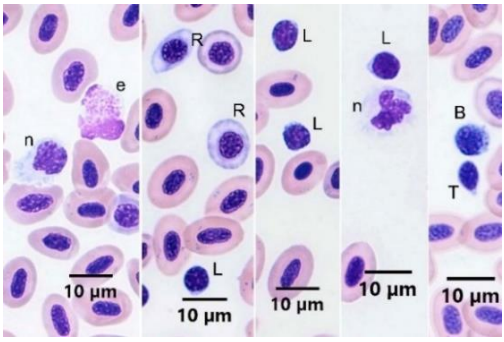

Figure 37A. Blood cells in rainbow trout with EDTA. e: Eosinophil, n: Neutrophil, R: Rubricyte, L: Lymphocyte, B: Basophil. MGG staining method.

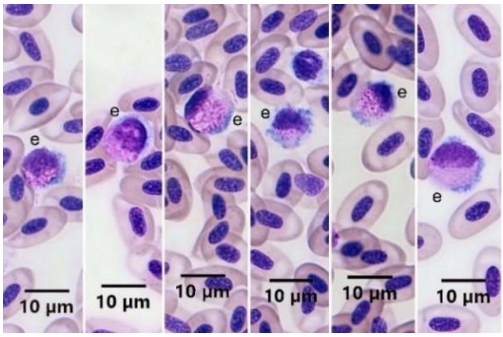

Figure 37B. Blood cells in rainbow trout with heparin anticoagulant. e: Eosinophil. MGG staining method.

3.3.6. *ANAE PH 5.8 Staining Method*

Upon applying the ANAE staining method to heparinized rainbow trout blood, positive reactions were observed in certain granulocytes (Fig. 38A), monocytes, and some lymphocytes (Fig. 38B, C). Notably, some lymphocytes (Fig. 38B) and thrombocytes showed no reaction. The nuclear morphology and abundant granules in the ANAE-positive granulocytes (g1) suggested they might be eosinophils.

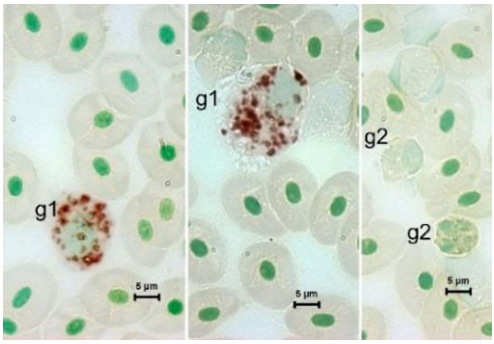

Figure 38A. ANAE positivity in heparinized rainbow trout blood. g1: ANAE positive granulocytes, g2: ANAE negative granulocytes. ANAE pH 5.8 Staining Method.

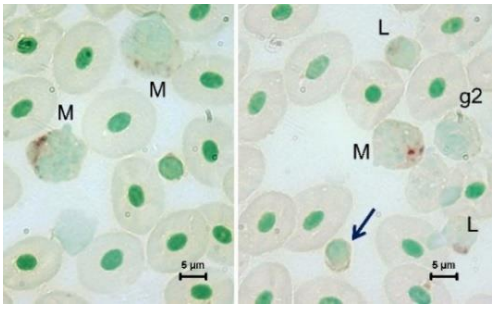

Figure 38B. ANAE positivity in heparinized rainbow trout blood. M: Monocyte, L: ANAE positive lymphocyte, g2: ANAE negative granulocyte, Arrow: ANAE negative lymphocyte. ANAE pH 5.8 Staining Method

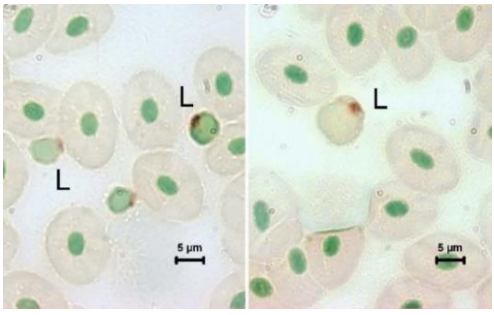

Figure 38C. ANAE positivity in heparinized rainbow trout smears. L: ANAE positive lymphocyte. ANAE pH 5.8 Staining Method.

3.3.7. *MGP Staining Method*

After Carnoy fixation, only a few MGP-positive cells were observed in the smears. These cells varied in size and resembled the morphology of granulocytes (Fig. 39A, B). In EDTA-treated blood, a few cells similar to thrombocytes also showed positivity (Fig. 39A), although this was not observed in heparinized smears. It was suggested that these cells might be plasma cells or immature granulocytes.

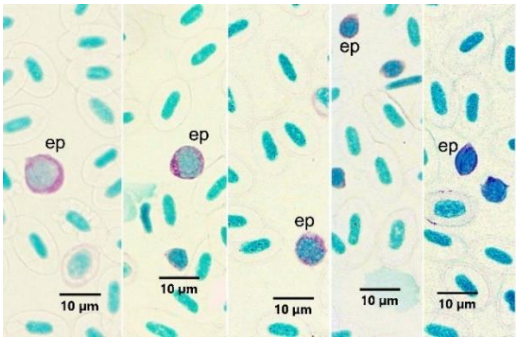

Figure 39A. Appearance of MGP - positive cells in EDTA anticoagulated rainbow trout blood. ep: MGP-positive cells. MGP Staining Method.

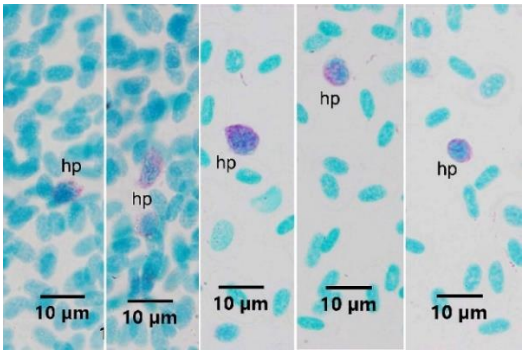

Figure 39B. Appearance of MGP-positive cells in heparin anticoagulated rainbow trout blood. hp: MGP-positive cells. MGP Staining Method.

4. DISCUSSION

Fish hematology terminology is largely derived from mammalian hematology (28). This study examines the morphology and cytochemical characteristics of peripheral blood cells in marine (sea bass, gilthead seabream) and freshwater (rainbow trout) carnivorous fish. Erythrocytes, rubricytes, lymphocytes, monocytes, and thrombocytes were identified in all three species, showing similarities to birds and mammals. The most common cell type observed in fish blood was the nucleated erythrocyte. Fish erythrocytes are generally oval with round to oval nuclei (14). In this study, all three species exhibited erythrocytes with pink cytoplasm and centrally located oval nuclei. Similar features were reported in sea bass by Esteban et al. (11). Immature erythrocytes, distinguished by lighter cytoplasm and rounder shape compared to mature erythrocytes, were also observed. In A. nobilis, A. ocellatus, H. malabaricus, and A. bimaculatus, erythrocytes stained with brilliant cresyl blue or methylene blue revealed dark inclusions and granular material, indicating residual RNA, and were identified as reticulocytes (29). In fish, erythropoiesis is influenced by anemia, temperature, seasons, and bleeding, affecting circulating reticulocyte numbers. Reticulocyte count may reflect a compensatory response to environmental factors such as

anemia, temperature, and oxygen availability (30, 31). One of the most notable differences in fish blood cells is that thrombocytes are nucleated and larger than those in birds (28, 32, 33). In some fish species, thrombocytes are difficult to distinguish from small lymphocytes when stained with Romanowsky-type dyes (28, 34-36). Lymphocytes, more numerous than thrombocytes, have lightly basophilic cytoplasm and a less dense nucleus, while thrombocytes are fewer with clear cytoplasm. Despite these differences, distinguishing them in fish blood can be challenging (34).

In this study, erythrocytes, rubricytes, lymphocytes, monocytes, and thrombocytes were easily differentiated in all three fish species using the MGG method. Due to difficulties in distinguishing granulocytes with conventional MGG staining, cytochemical features were first identified and then combined with morphological characteristics. After revealing granulocyte properties, May-Grünwald Giemsa staining results were examined.

4.1. CR Staining Method

In the study, eosinophilic granulocytes in sea bass and rainbow trout were characterized by lightly basophilic cytoplasm and CR-positive, fine granules. In gilthead seabream, eosinophils exhibited slightly basophilic cytoplasm but denser and larger granules compared with those of the other species. A range in eosinophil sizes was observed, including some very small eosinophils. While eosinophilic granulocytes in sea bass and rainbow trout were unaffected by anticoagulant differences, EDTA caused lysis in gilthead seabream.

A literature review found no previous use of CR staining in fish blood. This study is the first to demonstrate eosinophils in fish using CR. Meseguer et al. (37) reported acidophilic granulocytic cells in the anterior kidney of seabream, supporting the CR findings for gilthead seabream in this study.

4.2. PAS Staining method

In the study, two PAS-positive granulocytes (Granulocyte 1 and Granulocyte 2) were identified in sea bass blood, indicating glycogen or neutral mucosubstances. The two anticoagulants did not affect PAS positivity, but fixative solutions influenced the results. Acetone fixation yielded better PAS staining than methanol, especially with heparin, while acetone was preferred over methanol when EDTA was used (Table 1). Zinkl et al. (34) reported PAS-positive neutrophils in striped sea bass, and Esteban et al. (11) observed glycogen accumulation in acidophils under electron microscopy. Meseguer et al. (37) reported the presence of ß-glycogen particles in mature heterophils and

acidophilic promyelocytes in sea bass anterior kidney but did not find glycogen in basophilic cells. Do Vale et al. (38) identified glycogen in sea bass neutrophils. In the present study, PAS-positive granulocyte 1s in sea bass are likely eosinophils, while granulocyte 2s are predominantly neutrophils. Both cell types appear to contain glycogen and other neutral mucosubstances.

In gilthead seabream blood, PAS staining revealed two types of granulocytes (granulocyte 1 and 2) based on cell size and nuclear characteristics. Heparin anticoagulant provided better PAS staining results than EDTA. EDTA notably affected granulocyte 1s negatively. When PAS staining was performed on gilthead seabream blood, methanol fixation yielded better results than acetone. No reports of PAS-positive granulocytes in gilthead seabream blood were found in the literature. In this study, large granulocyte type 1 cells were identified as eosinophils, while type 2 cells were primarily neutrophils, with some small eosinophils or basophils present. Estensoro et al. (39) did not find PAS-positive granulocytes in intestinal tissue, and Zuasti and Ferrer (40) reported glycogen in platelets, but no PAS positivity was observed in platelets here.

In the present study, PAS staining of rainbow trout blood samples with heparin or EDTA and fixed with acetone or methanol revealed weak positivity in granulocytes and thrombocytes. Afonso et al. (41) observed glycogen particles in neutrophils in the peritoneal exudate of rainbow trout under electron microscopy. Additionally, Suzuki (42) reported phagocytosis by neutrophils and thrombocytes in rainbow trout (formerly Salmo gairdneri). However, no reports of PAS-positive granulocytes in rainbow trout blood were found in the literature. Cytoplasmic PAS-positive granules have been observed in neutrophil-like granulocytes of many fish species, including catfish and turbot (43, 44). PAS positivity, indicating the presence of polysaccharides like glycogen, was shown in the cytoplasm of turbot thrombocytes (44). Tavares-Dias (45) suggested that eosinophils in the freshwater fish Astronotus ocellatus are PAS-positive and contain glycogen. When applying PAS to fish blood cells, testing alternative anticoagulants and fixatives is essential for accurate results. In this study, PAS positivity was generally observed in granulocytes. Future studies should confirm glycogen presence through enzyme digestion. No sources were found in the literature discussing the effect of anticoagulant differences on PAS positivity in fish blood cells.

4.3. *SB Staining Method*

A literature review revealed no previous data on SB staining in sea bass and gilthead seabream blood. The SB positivity findings in this study are the first for

these fish species. In sea bass blood, cells with rod-shaped granules were detected, suggesting granulocytes as these cells varied in size and exhibited different nuclear features. In gilthead seabream blood, SB staining identified granulocytes 1 and 2 cells as SB-positive, similar to the PAS staining results. Granulocyte type 1 was likely eosinophils, while type 2 predominantly consisted of neutrophils, with small eosinophils or basophils also possible. Ueda et al. (46) demonstrated SB-positive phospholipids in both eosinophils and neutrophils of the teleost fish Oreochromis niloticus (tilapia). Havixbeck et al. (47) showed SB-positive neutrophils in the teleost Carassius auratus. SB staining in rainbow trout blood revealed SB-positive cells, which were classified as granulocytes 1 and 2 based on their morphological characteristics. Similar findings were reported by Plytycz et al. (48) and Sasaki et al. (49), who observed SB-positive polymorphonuclear leukocytes and SB-negative monocytes in the rainbow trout head kidney.

In this study, SB staining revealed positivity in granulocytes across all three fish species, with no effect from anticoagulant differences on SB positivity. Flerova and Balabanova (50) reported that in various phylogenetic groups of bony fish, neutrophils contained long granules or fibrillar granules with crystalline material, while eosinophil granules were large, electron-dense, and homogeneous. At the light microscopic level, the study found that SB-positive granules were rod-shaped in sea bass and both rod and round-shaped in gilthead seabream and rainbow trout. Hayhoe (20) noted that granules showing sudanophilia in neutrophils and eosinophils were also peroxidase-positive, indicating their specificity. Peroxidase activity was not examined in the present study. The SB positivity is likely due to phospholipid molecules found in primary and secondary (specific) granules, potential secretory granules, and the membrane structure of mitochondria in granulocytes (51, 52). Additionally, it can be suggested that cells with similar morphology that show positivity in both PAS and SB staining may also contain glycolipids

4.4. *TB Staining Method*

In the blood of sea bass, gilthead seabream, and rainbow trout, all cells' nuclei and the cytoplasm of some basophilic cells were stained with toluidine blue at pH 4. Among them, a few cells, thought to be basophils, were identified with light blue cytoplasm and very fine blue granules (orthocromatic granules). No metachromasia was observed. The difference in anticoagulants did not affect the TB staining properties.

In the blood of sea bass, gilthead seabream, and rainbow trout, all cells' nuclei and the cytoplasm of some basophilic cells were stained with toluidine blue at pH 4. Among them, a few cells, thought to be basophils, were identified with light blue cytoplasm and very fine blue granules (orthocromatic granules). No metachromasia was observed. The difference in anticoagulants did not affect the TB staining properties. Meseguer et al. (37) reported basophilic granular cells in the anterior kidney of sea bass, supporting our finding of basophils in their peripheral blood. López-Ruiz et al. (53) couldn't detect basophils in gilthead seabream with Giemsa staining but identified basophilia at the electron microscopic level. Campbell (54) reported that basophilic granules in lower vertebrates are weakly stained with ethanol-soluble dyes, such as Wright's stain. Tavares-Dias (21) detected basophils in only four out of 15 freshwater teleosts using toluidine blue, with metachromasia observed in granules of basophils from tilapias, I. punctatus, and L. macrocephalus. Suzuki (55) found that carp basophils were not stained by Astra blue or Alcian blue, and metachromasia was not seen with TB pH 3. He suggested that fish lack sulfate radicals in their acid mucopolysaccharide molecules, as basophilic material could be separated in diluted electrolyte solutions. In this study, orth chromatic staining indicates that TB-positive cells contain non-sulfated mucopolysaccharides. Further studies are needed to explore alternative detection methods for fish basophils and granule content.

4.5. *MGG Staining Method*

In sea bass blood, MGG staining using both heparin and EDTA yielded results. Some erythrocytes exhibited crescent-shaped, spiral, or lobed nuclei. Researchers (56, 57) propose these nuclear anomalies as responses to chemical contamination and toxicity. Further studies are needed for clarification.

After distinguishing erythrocytes, lymphocytes, monocytes, and thrombocytes in sea bass, it was observed that the remaining cells' cytoplasmic granules were not stained by MGG method. Since the presence of granules in these cells was demonstrated cytochemically in the present study, they were generally referred to as granulocytes. Esteban et al. (11) identified granulocytes, particularly heterophils with fibrous cytoplasmic granules, using electron microscopy. Pavlidis et al. (58) reported no eosinophils in sea bass blood; however, eosinophils were demonstrated in this study using CR. The researchers also noted that sea bass neutrophils had large, lymphocyte-like nuclei with little cytoplasm. In this study, neutrophil nuclei were oval, horseshoe-shaped, or lobed, with lobes sometimes overlapping. Both EDTA and heparin anticoagulants

provided results for MGG staining of gilthead seabream blood, although cells, particularly eosinophils, stained better with heparin. In the MGG staining of gilthead seabream blood, eosinophils, neutrophils, and heterophils were observed among granulocytic leukocytes, but no basophils were found. López-Ruiz et al. (53) reported eosinophils in gilthead seabream with round or bilobed nuclei, and eosin-stained granules in pale blue or pink cytoplasm. The present study found that EDTA caused turgor and lysis in eosinophils, and it was concluded that EDTA is unsuitable for MGG staining of gilthead seabream blood. Estensoro et al. (39) demonstrated eosinophils in gilthead seabream intestines using Giemsa staining with Bouin's solution. In this study, in contrast to López-Ruiz et al. (53), both neutrophils and heterophils were identified in gilthead seabream blood. Milas et al. (59) also observed neutrophils and acidophilic granulocytes in gilthead seabream, classifying the acidophilic cells as eosinophils and heterophils.

In the present study, both EDTA and heparin anticoagulants yielded results in rainbow trout blood MGG staining, but eosinophils were only fully observed with heparinised blood. Eosinophils were observed with acidophilic granules in a basic cytoplasm. In MGG staining, basophils were identified in smears with both EDTA and heparin anticoagulants. However, when EDTA was used, eosinophils exhibited undesirable changes such as turgor and lysis. Maqbool et al. (60) reported that in their study on rainbow trout, EDTA anticoagulant caused erythrocyte swelling in MGG staining, but did not induce any morphological changes in leukocytes, regardless of whether EDTA or heparin was used. Contrary to these findings, Cretu et al. (61) reported the absence of eosinophils or basophils in rainbow trout blood using MGG staining, while Nabi et al. (62) also found no eosinophils or basophils in Giemsa-stained smears. However, in the present study, the morphology of CR-positive eosinophils, especially in heparinized blood, resembled that of eosinophils observed in MGG staining. Barber and Westermann (63) identified eosinophils in catfish blood using the MGG staining technique. Rainbow trout neutrophils among granulocytes in our study were observed to possess lightly basophilic cytoplasm and a mostly lobed, sometimes horseshoe-shaped nucleus, with no visible granules in the cytoplasm. Van et al. (64) demonstrated that trout neutrophils form extracellular traps. MGG staining allowed the differentiation of erythrocytes, rubricytes, lymphocytes, monocytes, and thrombocytes, similar to the differentiation observed in mammalian and avian cells, in all three fish species

4.6. *ANAE Boyama Metodu*

Scapigliati et al. (65) produced and characterized monoclonal antibodies against sea bass thymocytes. In the present study, T lymphocytes in sea bass were identified using both the T lymphocyte antibody DLT15 and the ANAE staining method.

In this study, non-specific esterases were detected in lymphocytes and granulocytes of gilthead seabream blood using the ANAE method. No similar data was found in the literature. However, Meseguer et al. (66) reported acid phosphatase, alkaline phosphatase, and peroxidase in sea bream eosinophils at both light and electron microscope levels. The ANAE staining method in our study revealed positive reactions in eosinophils, monocytes, and some lymphocytes of rainbow trout blood. Plytycz et al. (48) also identified non-specific esterases in the monocytes of rainbow trout anterior kidney. Blaxhall and Hood (67) showed ANAE-positive T lymphocytes in the peritoneal exudates of brown trout (Salmo trutta L), while Afonso et al. (41) reported similar findings in rainbow trout. A literature review found no ANAE positivity in rainbow trout eosinophils. However, Dönmez et al. (26) demonstrated ANAE positivity in Kangal fish blood cells. In this study, non-specific esterase was detected in lymphocytes, granulocytes, and monocytes, potentially localized in the cell membrane, cytoplasmic vesicles, lysosomes, or cytosol. Ultrastructural examination (68) could help determine the exact localization of the enzyme in fish blood cells.

4.7. *MGP Staining Method.*

Meseguer et al. (69) identified plasma cells in the head kidney of sea bass, and Romano et al. (70) confirmed this in graft studies. Eren and Bozkurt (2017a; b) demonstrated plasma cells in the head kidney and spleen using MGP staining. Esteban et al. (11) used Giemsa to identify plasma cells in sea bass blood, with oval, reddish nuclei and dark blue cytoplasm. In this study, a small number of MGP-positive cells were observed in sea bass blood, showing varying sizes. López-Ruiz et al. (53) reported finding a few plasma cells in gilthead seabream blood, which they characterized with round, reddish nuclei and dark blue cytoplasm using Giemsa staining. In the present study, MGP staining revealed a few MGP-positive cells in gilthead seabream blood, exhibiting varying sizes and granulosite-like morphology. For rainbow trout, MGP staining was also applied to identify plasma cells, and a few MGP-positive cells of varying sizes and nuclear morphologies were observed. Bromage et al. (71) also noted plasma cells in rainbow trout.

In sea bass, sea bream, and rainbow trout blood, pyrinophilic-positive cells with eccentric nuclei and dense cytoplasmic staining resembled plasma cells. Pyrinophilic cells with lobed nuclei may be immature granulocytes, while some thrombocytes in rainbow trout contained pyrinophilic material. These cells, likely plasmablasts, plasma cells, or immature granulocytes, indicate high RNA activity, as supported by their morphology. The PAS and MGP positivity in rainbow trout thrombocytes further suggests RNA activity. While anticoagulant differences had little effect in sea bass and sea bream, EDTA was more suitable for MGP staining in rainbow trout.

CONCLUSION

The present study is a descriptive investigation of the morphology and cytochemical properties of peripheral blood cells in marine carnivorous fish species, Sea bass (Dicentrarchus labrax) and gilthead seabream (Sparus aurata), as well as in the freshwater species rainbow trout (Oncorhynchus mykiss). Upon reviewing the results, it was concluded that the morphological and cytochemical properties of the blood cells are likely influenced by the taxonomic family differences rather than environmental habitat variations. Furthermore, it was suggested that alternative methods for the use of anticoagulants and detection procedures should be considered when performing cytochemical studies on fish blood. This study provides valuable information that will serve as a foundation for future cytochemical and ultrastructural research. Based on these findings, examining the granular content of granulocytes in different fish species using enzyme-histochemical methods at light and electron microscopic levels will contribute to a better understanding of the evolutionary development of non-specific and specific immunity in fish.

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