CONTEMPORARY METHODOLOGICAL APPROACHES IN BIOLOGY

EDITOR
ASSOC. PROF. MENDERES ÇENET, PH.D.

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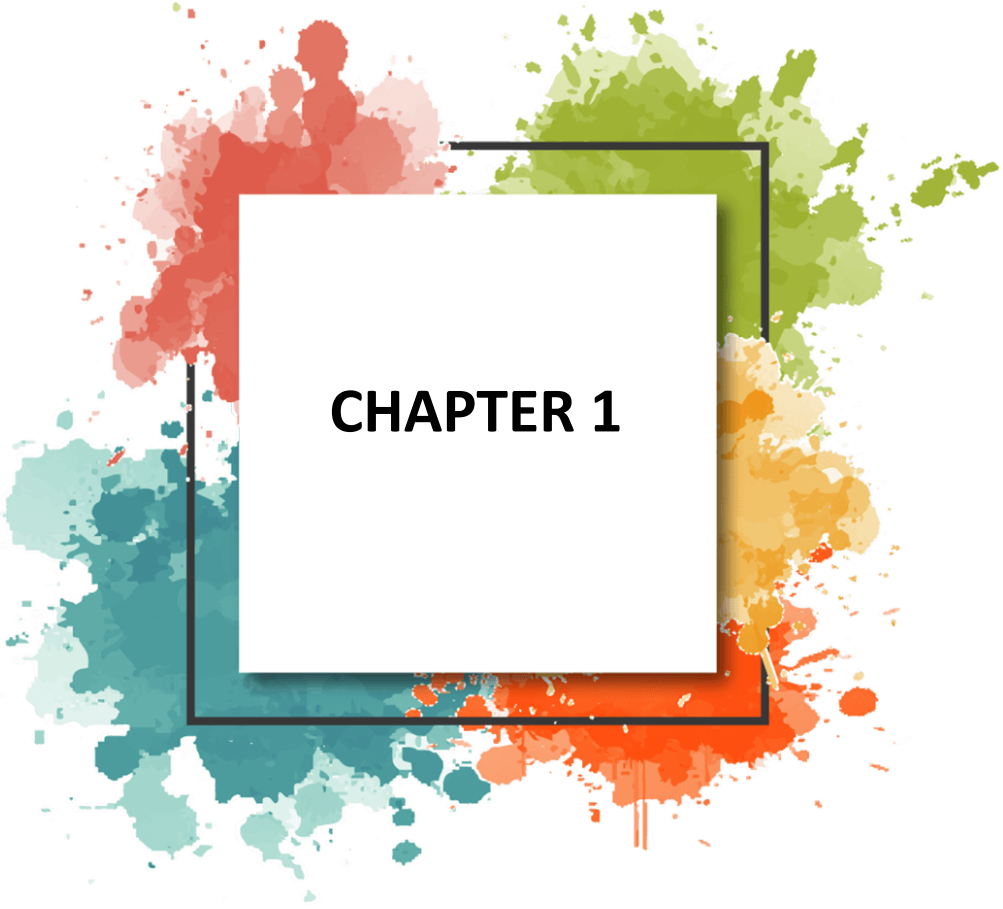
CHAPTER 1

The Effects of Olive Leaves on Health

***Hülya Yıldız*[1]**

Introduction

Olives have been considered a source of healing among the people; olive fruit, olive oil, leaves and shoots, and extracts obtained from them have been used since ancient times for pain relief, lowering blood pressure, lowering blood sugar, and treating many diseases, including cancer (Pieroni et al., 1996). A study on determining the antioxidant capacity of olive leaves showed that the dried extract of olive leaves has a higher antioxidant potential than the fresh leaf extract (Kamran et al., 2015). The olive tree, belonging to the Oleaceae family, is a long-lived, woody tree that always has green leaves. Olive leaves contain many biologically rich chemical components. Among these compounds, we can mention oleuropein, hydroxytyrosol, flavonoids, phenolic acids, and triterpeneids (Guerreiro et al., 2023, Pongrac et al 2022).

Chemical Composition of Olive Leaves

The ratio of chemical components in olive leaves varies depending on the geographical region, olive variety, leaf maturity, harvest time, and extraction method. Young leaves are generally richer in oleuropein, while the proportion of flavonoids and triterpenoids may increase in mature leaves (Lockyer et al., 2017, Pongrac et al 2022).

1. Polyphenol Compounds

Olive leaves contain the highest proportion of oleuropein, a phenolic glycoside. Oleuropein ($C_{25}H_{32}O_{13}$; ma: 540.51 g/mol) is a secoiridoid found in the fruit and especially the leaves of the olive tree (Duke, 1992). Oleuropein, which gives olives and olive oil their bitter-astringent taste, is a glycosidic ester of hydroxytyrosol and elenolic acid (Panizzi et al., 1960; Andrewes et al., 2003). Oleuropein's antioxidant, anti-inflammatory, antimicrobial, and cardioprotective properties are quite important for human health (Somova et al., 2003; de Bock et al., 2013). While the oleuropein content in olive oil ranges from 0.005-0.12%, in olive leaves it ranges from 1-14%. Oleuropein can be obtained in various proportions through leaf extraction (Yıldız & Uylaşer, 2011). Studies have investigated the effects of oleuropein, obtained from olive leaves, on oxidative damage, enzymatic and non-enzymatic antioxidant compounds in diabetic rabbits given alloxan. It was reported that oleuropein has the ability to inhibit

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hyperglycemia and oxidative damage associated with diabetes and is beneficial in preventing complications related to oxidative stress (Al-Azzawie et al., 2006).

2. Flavonoids

Olive leaves are known to contain flavonoid compounds such as luteolin, apigenin, and diosmetin. Although the flavonoid concentration in the leaf varies, these flavonoids increase the plant's antioxidant capacity. Along with antioxidant activity, these flavonoids play a role in eliminating free radicals and modulating cellular inflammatory responses (Visioli et al., 2002).

3. Hydroxytyrosol and Tyrosol

Hydroxytyrosol and tyrosol are powerful antioxidants. These antioxidant compounds are released during the hydrolysis of the flavonoid oleuropein. These released antioxidants are effective in protecting cellular DNA from oxidative damage by inhibiting ROS production, and also in supporting cardiovascular health by preventing lipid peroxidation (Corona et al., 2006).

4. Phenolic Acids

Olive leaves are also rich in phenolic acids. The main phenolic acids are caffeic acid, chlorogenic acid, and ferulic acid. These compounds have also been reported to be quite effective in antioxidant activity and in protecting health against metabolic diseases (Benavente-García et al., 2000).

5. Triterpenoids, Vitamins and Minerals

Olive leaves also contain triterpenoids such as maslinic acid and oleanolic acid. These terpenoid compounds have anti-inflammatory and hepatoprotective properties (Giner et al., 2007). Olive leaves also contain vitamin C, calcium, magnesium, and potassium, offering additional health benefits (Pongrac et al. 2022).

Biological Effects of Olive Leaves

It has been determined that phenolic compounds in olive fruit and leaves have inhibitory and delaying effects on the growth of microorganisms, that olive leaf extract has antioxidant properties (31), and that it is a potential source of antifungal substances and can be used as an additive in the food industry and in the pharmaceutical industry (Korukluoğlu et al. 2008).

1. Antioxidant Effect

Polyphenols, flavonoids, hydroxytyrosol and tyrosol, phenolic acids, and triterpenoids found in olive leaves exhibit antioxidant properties. These chemical components are effective in eliminating free radicals, known as reactive oxygen species. Thus, DNA damage caused by free radicals is prevented. Furthermore,

lipid peroxidation and protein oxidation are also reduced through antioxidant activity (Visioli et al., 2002; Corona et al., 2006). For example, one study determined that oleuropein and hydroxytyrosol, active ingredients in olive leaves, prevent lipid peroxidation. It was also observed that this prevention was more effective than trolox, a vitamin E analogue (Umeno et al. 2015). Another study indicated that hydroxytyrosol and oleuropein showed a greater antioxidant effect on DNA and lipid oxidation than natural and synthetic antioxidants. Studies have shown that olive leaves contain flavonoid structures with more free hydroxyl groups, in addition to the most effective phenolic compound antioxidants for radical cations (Benavente-Garcia et al., 2000). Another study examining the effect of olive leaves on pancreatic beta cells found a reduction in hydrogen peroxide (H2O2) toxicity in beta cells. Furthermore, this study compared the effect of olive leaf extract with only oleuropein and found that olive leaf extract had a greater antioxidant effect (Cumaoğlu et al. 2011). In addition to oleuropein, olive leaves also contain various compounds exhibiting antioxidant effects, such as vanillin, vanillic acid, apigenin, diosmetin, rutin, luteolin, and caffeic acid. Based on this information, the antioxidant effect of olive leaves has gained undeniable importance (Ryan et al., 2002; Skerget et al., 2005; Ok-Hwan et al., 2010).

2. Antimicrobial and Antiviral Effects

Oleuropein and hydroxytyrosol are effective against gram-positive and gram-negative bacteria. They have also shown in vitro activity against viral infections such as influenza and herpes simplex (Bisignano et al., 1999; Romani et al., 2017). In preclinical studies, their activity against Staphylococcus aureus, Escherichia coli, and some influenza and herpes viruses has been observed (Bisignano et al., 1999; Romani et al., 2017). It has been determined that the phenolic compounds in olive leaves, oleuropein and its degradation product hydroxytyrosol, exhibit anti-HIV properties, and that their use, separately and together, prevents the entry and integration of the virus into the cell, both intracellularly and extracellularly (Bao et al., 2007; Lee-Huang et al., 2007; Şekeroğlu & Gezici, 2020). Therefore, the use of olive leaf is recommended as an alternative approach to support immunity and reduce the risk of infection (Şekeroğlu & Gezici, 2020).

3. Cardiovascular Effect

Olive leaf supports cardiovascular health, especially thanks to its oleuropein and polyphenol content. It is suggested that the extract obtained from the fruit and leaves of the olive tree has vasodilatory, hypotensive, anti-rheumatic, diuretic, hypoglycemic, cholesterol-lowering, and cardioprotective effects (Pieroni et al. 1996). Oleuropein has been reported to prevent lipid peroxidation and cardiotoxicity induced by anthracyclines (Gürbüz & Öğüt, 2018). Clinical

studies have shown that olive leaf extract lowers systolic and diastolic blood pressure in hypertensive patients (Susalit et al., 2011). Furthermore, it can reduce LDL cholesterol while increasing HDL cholesterol, which lowers the risk of atherosclerosis (de Bock et al., 2013). These mechanisms are also known to be supported by antioxidant and anti-inflammatory effects. Literature indicates that the phenolic compounds found in olive leaves minimize the harmful effects of free radicals in the body through their antioxidant properties. This effect has also been observed to be significant in protecting against cardiovascular diseases. Polyphenol-rich olive leaf extracts have been shown to reduce lipogenesis and lipid accumulation. In animal models and preclinical studies, olive leaf supplementation reduced fatty liver and serum triglyceride levels (de Bock et al., 2013). In another study by Eidi et al., serum cholesterol, triglyceride, urea, uric acid, creatinine, aspartate aminotransferase, and alanine aminotransferase levels were significantly reduced in diabetic rats given 0.5 g/kg of olive leaf (2009). Andreadou et al. In a study conducted by Susalit et al. (2009), hypercholesterolemic rabbits that received 10-20 mg/kg of oleuropein for 6 weeks showed a significant decrease in total cholesterol and triglyceride levels at the end of the study. In a study by Susalit et al. (2011), it was determined that patients with stage 1 hypertension who received 500 mg/day of olive leaf extract twice a day for 8 weeks had lower systolic and diastolic blood pressure. Similarly, in another study of 60 prehypertensive male individuals with an average age of 45, a significant decrease in systolic and diastolic blood pressure was observed in patients given olive leaf extract. Furthermore, both studies indicated a positive effect on lipid profiles in the patients. In a study conducted on hypertensive volunteers, it was observed that 1,600 mg of oleuropein intake also reduced systolic and diastolic blood pressure (Lockyer et al. 2017; Cabrera et al. 2015). In a study investigating the cardioprotective effect of oleuropein in rats, it was stated that oleuropein treatment was both protective against myocardial infarction and protective against heart failure developing due to myocardial infarction (Janahmadi et al., 2015).

4. Effect on Type 2 Diabetes and Metabolic Syndrome

Studies have shown that polyphenols in olive leaves exhibit a hypoglycemic effect, and this effect is mainly due to the oleuropein component. In a study conducted with diabetic rats, blood glucose levels were found to be significantly lower in groups receiving 16 mg/kg and 8 mg/kg of oleuropein and hydroxytyrosol. Furthermore, the hypoglycemic effect was found to be more effective in the group receiving oleuropein and hydroxytyrosol at a level of 16 mg/kg compared to the group receiving it at a level of 8 mg/kg. Moreover, this study also showed that the group given oleuropein and hydroxytyrosol had higher hepatic glucose concentrations compared to the control group (Jemai et al. 2009).

Olive leaf extracts can increase insulin sensitivity and improve glucose metabolism. In clinical trials, significant reductions in HbA1c and fasting blood glucose levels were observed (Katsarou et al., 2015). Polyphenols support insulin secretion by protecting pancreatic β-cells from oxidative stress. In a study comparing diabetic rats given olive leaf extract at doses of 0.1, 0.25, and 0.5 g/kg respectively for 14 days via intragastric gavage with healthy rats given the same doses, serum glucose levels decreased significantly in the diabetic rat group receiving 0.5 mg/kg of olive leaf extract daily, while glucose levels remained unchanged in healthy rats (Eidi et al. 2009). In a 12-week study of 46 overweight middle-aged participants, olive leaf extract increased insulin sensitivity by 15% and pancreatic β-cell response by 28% (de Bock et al., 2013).

5. Neurodegenerative Diseases

Olive leaf polyphenols show protective potential in Alzheimer's and Parkinson's diseases by reducing oxidative stress and neuroinflammation (Rigacci & Stefani, 2016). Cellular studies have shown that they prevent β-amyloid accumulation and synaptic damage.

6. Anti-inflammatory and Oxidative Stress-Related Diseases

Olive leaf extracts reduce inflammation by acting on the NF-κB pro-inflammatory signaling pathway and suppressing the expression of pro-inflammatory cytokines TNF-α and IL-6. With these effects, it is shown to be a potential supportive treatment in diseases associated with chronic inflammation (metabolic syndrome, obesity, cardiovascular diseases) (Hadrich et al., 2015).

It has been determined that hydroxytyrosol, released by the hydrolysis of oleuropein found in olive leaves, suppresses the expression of pro-inflammatory cytokines, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in a dose-dependent manner, thus reporting that hydroxytyrosol has a strong anti-inflammatory property (Zhang et al., 2009).

7. Cancer

Olive leaf extract and polyphenols derived from olive leaves have been shown to exhibit antiproliferative and apoptosis-inducing effects in various cancer types, including breast, bladder, mesothelioma, and leukemia (Goulas et al., 2009; Fares et al., 2011; Samet et al., 2014). In a study by Goulas et al. (2009) using olive leaf extract, an antiproliferative effect was observed in MCF-7 and T-24 bladder cancer cells. Furthermore, another study found that olive leaf extract induced apoptosis in K562 cells and initiated the differentiation process of the cells into monocytes/macrophages (Samet et al., 2014). Oleuropein has been found to cause cell cycle arrest in cancer cells by downregulating cyclin D1, cyclin D2, cyclin D3, CDK4, and CDK6, and upregulating p53, CDKN2A, CDKN2B, and

CDKN1A. It has also been reported that oleuropein induces apoptosis by inhibiting Bcl-2 and activating Bax, caspase-9, and caspase-3 gene expression (Seçme et al., 2016; Sirianni et al., 2010). Furthermore, oleuropein has been shown to induce estrogen and inhibit proliferation in MCF-7 cells (Sirianni et al., 2010).

Conclusion

The health benefits of olives, known for centuries, are increasingly supported by scientific studies, including the benefits of their leaves. Data obtained from studies show that olive leaf has antioxidant, antihypertensive, hypocholesterolemic, hypolipidemic, hypoglycemic, antimicrobial, anti-inflammatory, antithrombotic, and cardioprotective effects. These effects are currently observed primarily due to the strong antioxidant properties of olive leaf, particularly the compounds oleuropein and hydroxytyrosol. The increasing number of studies on olive leaf is crucial for research into its effects in treating new diseases, achieving optimal benefits, and determining the appropriate dosage and method of use. More detailed research on olive leaf will shed light on this subject for current and future researchers.

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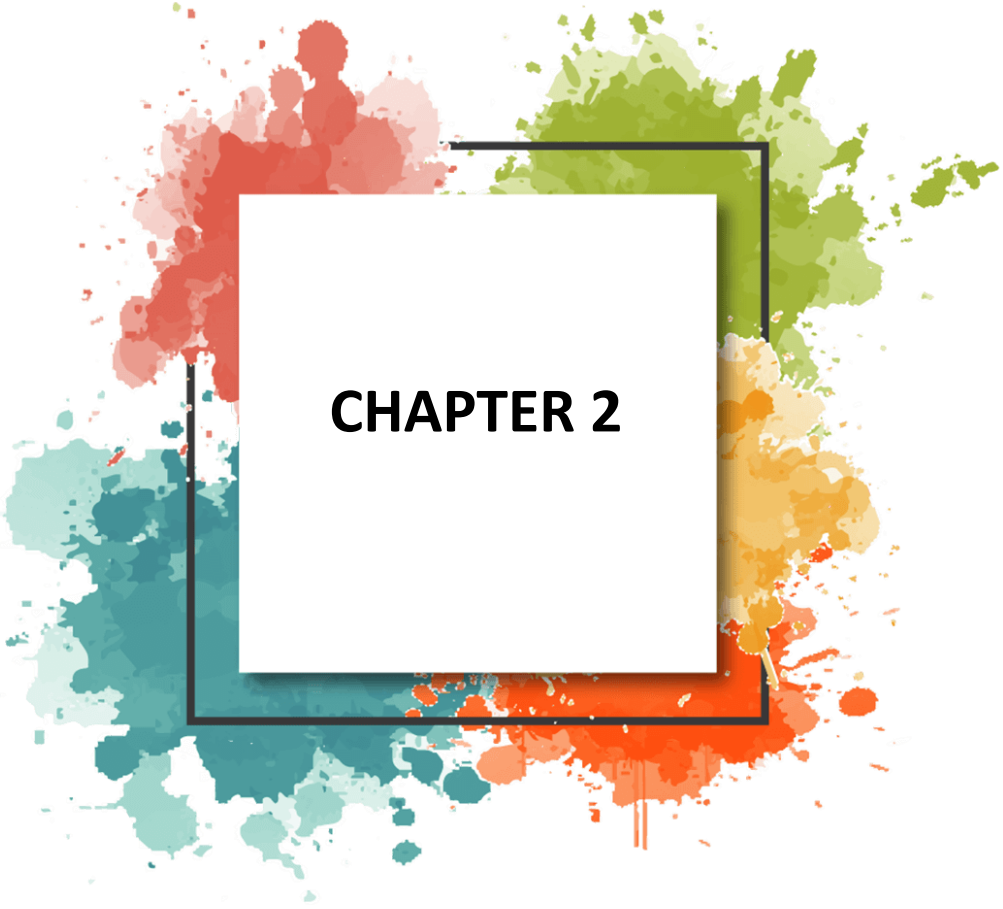
CHAPTER 2

Single-Cell Gel Electrophoresis (Comet Assay) and Dna Repair Capacity

Salih Özden[1] & Meltem Özden[2]

1. The Paradigm Shift in Genotoxicity Testing

Genotoxicity evaluation has long relied on "static" endpoints (DNA strand breaks, micronuclei, chromosomal aberrations, etc., measured at a single time point). However, it has become increasingly evident that the risk of carcinogenesis, aging, and chronic diseases depends not only on the extent of DNA damage but also on how rapidly and accurately such damage is repaired. Consequently, the focus in genotoxicity biomarkers is shifting from static measurements of DNA damage toward the phenotypic assessment of dynamic DNA repair capacity (DRC) (Azqueta et al., 2014; Voděnková et al., 2020).

DNA repair can be broadly categorized into four major pathways: (i) base excision repair (BER; responsible for oxidative base lesions, and single-base modifications), (ii) nucleotide excision repair (NER; involved in the removal of bulky adducts, UV-induced pyrimidine dimers), (iii) mismatch repair (MMR; correcting replication errors), and (iv) double-strand break (DSB) repair (via homologous recombination (HR) and non-homologous end joining, NHEJ). A common feature of these pathways is the generation of transient single-strand breaks (SSBs) and/or alkali-labile sites as repair intermediates in many types of lesions. This characteristic underlies the suitability of the Comet assay as a phenotypic window for monitoring DNA repair kinetics (OECD, 2016).

[1] Assistant Professor, Sabuncuoğlu Şerefeddin Health Services Vocational School, Department of Medical Services and Techniques, Medical Laboratory Techniques Program, Amasya University, Ipekköy, 05100, Amasya, Türkiye
ORCID: 0000-0002-2248-825X
[2] Lecturer, Sabuncuoğlu Şerefeddin Health Services Vocational School, Department of Medical Services and Techniques, Medical Laboratory Techniques Program, Amasya University, Ipekköy, 05100, Amasya, Türkiye
ORCID: 0000-0002-1815-2577

2. Single-Cell Gel Electrophoresis (Comet Assay): Principles and Advances

2.1. Physical and Chemical Basis of the Methodology

The Comet Assay was first introduced in 1984 by Ostling and Johanson under neutral pH conditions to detect DNA double-strand breaks. Later, in 1988, Singh and colleagues developed the alkaline version of the method, enabling the detection of single-strand breaks (SSBs) and alkali-labile sites. The comet assay (single-cell gel electrophoresis) is based on the analysis of DNA from individual cells or nuclei embedded in agarose, followed by cell lysis, DNA unwinding (particularly under alkaline conditions), electrophoresis, and fluorescent imaging. Damaged DNA fragments or relaxed DNA loops migrate further in the electrophoretic field, forming the "tail" region, whereas the "head" represents relatively intact DNA. OECD Test Test Guideline 489 (TG 489) defines the methodological steps and interpretative framework, particularly for in vivo alkaline comet applications (OECD, 2016).

2.2. Standard Protocol and Current Modifications of the Alkaline and Neutral Comet Assay

The classical alkaline comet assay (pH > 13) is sensitive to single-strand breaks (SSBs), alkali-labile sites, and certain repair intermediates. In contrast, the neutral comet assay is primarily sensitive to double-strand breaks (DSBs) and is therefore employed as a complementary variant in mechanistic contexts such as ionizing radiation exposure, topoisomerase inhibition, or replication stress–induced DNA breakage.

Current methodological developments have concentrated along three major axes:

1. Throughput and standardization:

The adoption of 96/384-well formats has increased assay throughput while reducing process-related variability through simultaneous handling. For instance, the 384-well CometChip platform has demonstrated suitability for large-scale screening and epidemiological studies (Ge et al., 2014; Li et al., 2022).

2. Lesion-specificity:

The lesion specificity of the comet assay has been substantially enhanced through modifications integrated into standard protocols. As emphasized by Tice et al. (2000), enzyme-modified comet approaches employing lesion-specific DNA repair enzymes (e.g., formamidopyrimidine DNA glycosylase (FPG),

endonuclease III (ENDOIII)) enable the conversion of oxidized bases and other non–strand-breaking lesions into detectable strand breaks. Furthermore, as validated by Muruzabal et al. (2021), the detection of DNA cross-links—lesions that restrict electrophoretic DNA migration—has been achieved through optimization of the standard comet assay to encompass this lesion class. These refinements have expanded the analytical scope of the assay beyond simple strand breaks, rendering a broader spectrum of genotoxic lesions measurable (Tice et al., 2000; Muruzabal et al., 2021).

3. Reporting quality:

The Minimum Information for Reporting Comet Assay (MIRCA) consensus aims to enhance transparency and inter-laboratory comparability of comet assay data by providing a comprehensive reporting framework designed to minimize methodological variability. These standards require detailed documentation of all critical technical parameters, including physical matrix properties (e.g., gel dimensions and agarose concentration), lysis conditions (duration and composition), electrophoresis parameters (voltage gradient, current, and duration), scoring methodology (software-based algorithms or visual classification), and quality control measures ensuring data reliability (Møller et al., 2020).

2.3. Next-Generation Software and Parameters for Image Analysis

For the quantitative analysis of comet images and reliable scoring of DNA damage, traditional parameters such as % tail DNA (tail intensity), tail moment, and olive tail moment have been widely employed as primary indicators. However, manual or semi-automated analysis methods are both time-consuming and susceptible to subjective interpretation by researchers, which can lead to user-dependent errors (inter-observer variability). To address this limitation, both open-source and commercial solutions have been developed to standardize the analysis workflow and provide more objective results. In this context, OpenComet, developed as a plugin for the ImageJ platform, provides high-accuracy analysis of images obtained from both alkaline and neutral comet assay protocols. Using advanced image processing algorithms, the software automatically detects comets, distinguishes between the intact DNA region (head) and the damaged DNA region (tail), and computes critical parameters such as % DNA in tail, tail moment, and olive tail moment without requiring user intervention. As a result, OpenComet provides a high-throughput solution that increases speed while minimizing human bias, representing a prominent open-source tool for comet assay analysis (Győri et al., 2014).

In recent years, deep learning–based segmentation approaches have emerged in comet analysis, offering improved automation and reliability, particularly for datasets with low signal-to-noise ratios or complex backgrounds. DeepComet, developed by Hong et al. (2020) and based on the Mask R-CNN architecture, can accurately separate comets from complex backgrounds and from neighboring cells in contact, unlike traditional thresholding methods. One of the principal advantages of this model lies in its ability to accurately identify the "ghost cell" phenomenon—characterized by extremely high levels of DNA damage and often overlooked by conventional software—and thereby prevent these highly damaged comets from being excluded from analysis. When compared to existing tools such as Comet Assay IV, DeepComet demonstrates high correlation with manual scoring for critical parameters including tail length, % tail DNA, and tail moment, indicating excellent agreement with standard scoring methods (Hong et al., 2020).

3. Measurement of DNA Repair Capacity (DRC) by the Comet Assay: Methodological Approaches

The fundamental rationale for using the comet assay to measure DNA repair capacity (DRC) is to monitor the time-dependent kinetics of DNA damage reduction or to capture the activity of lesion-specific DNA repair enzymes as a phenotypic readout. In this context, two principal approaches have emerged. In the first approach, living cells are exposed to a DNA-damaging agent, and the rate at which the induced damage is removed by the cell (cellular repair kinetics) is quantified following defined incubation periods. The second, more specific approach—namely the in vitro repair assay—utilizes damaged nucleoids as substrates, which are subsequently incubated with protein extracts derived from the cells under investigation. Through this method, the ability of enzymes present in the extract (particularly those involved in the base excision repair (BER) and nucleotide excision repair (NER) pathways) to recognize and incise DNA lesions within the substrate is quantified by measuring the newly generated DNA strand breaks. Accordingly, these modified comet assay protocols provide a powerful phenotypic tool for evaluating the functional DNA repair response of individuals or cell lines to genetic damage (Azqueta et al., 2014; Voděnková et al., 2020).

3.1. In Vitro Repair Assays: Treatment of Cells with Genotoxic Agents and Time-Dependent Monitoring

In this approach, designed to evaluate cellular DNA repair capacity, cells are first exposed for a short duration to a selected genotoxic agent (e.g., ionizing radiation or H_2O_2) to induce DNA damage. The damaging agent is subsequently

removed, and a time-dependent incubation period is initiated to allow the cells to repair the induced lesions. Samples collected at defined time points are used to construct a "repair curve" (e.g., % tail DNA plotted against time), which quantitatively reflects repair efficiency.

The accuracy of this curve and its comparability across studies are directly dependent on several critical components:

- **Initial Damage Load:** The type of inducing agent, its dose, and the duration of exposure determine whether DNA repair systems reach saturation; excessive damage may overwhelm or mask repair mechanisms. Therefore, dose and exposure duration must be carefully optimized to avoid masking functional repair capacity.
- **Cell Cycle Phase and Proliferation Status:** The phase of the cell cycle (G1, S, or G2/M) and the proliferation rate influence which repair pathway (e.g., NHEJ or HR) is predominantly active, thereby affecting the slope of the repair curve.
- **Cytotoxicity and Secondary Strand Breaks:** Cytotoxic effects induced by the genotoxic agent may trigger apoptosis or necrosis. These processes can lead to DNA fragmentation (secondary strand breaks) independent of enzymatic repair mechanisms, which may confound the interpretation of the repair curve.
- **Technical Standardization:** Lysis duration, buffer composition, and particularly electrophoresis conditions (voltage, duration, and temperature) determine assay sensitivity and influence the detectability of even minor differences in repair capacity.

Comprehensive and standardized reporting of these variables is essential for ensuring that comet assay data are considered biologically meaningful and reliable (Azqueta et al., 2014; Møller et al., 2020; Voděnková et al., 2020).

3.2. Ex vivo Repair Assays: Induction of Repair in Isolated Lymphocytes Using Lesion-Specific Enzymes

In human biomonitoring studies, peripheral blood lymphocytes (or peripheral blood mononuclear cells) constitute the most commonly used model system due to their easy accessibility and their potential to reflect the overall genetic damage status of the organism. In frequently preferred ex vivo study designs, viable cells isolated from individuals are exposed under controlled laboratory conditions to a defined genotoxic stressor (e.g., ionizing radiation or alkylating agents). Following this damage induction step, the cellular repair process is monitored at defined time intervals using the Comet Assay, enabling the generation of an

individual-specific “repair kinetics” profile. This approach allows not only the assessment of existing DNA damage but also the evaluation of the individual’s DNA repair capacity as a phenotypic biomarker in the context of environmental or occupational exposures, genetic polymorphisms, lifestyle factors, and various clinical conditions (e.g., cancer susceptibility). Therefore, ex vivo DRC analysis represents a powerful diagnostic tool in population-based risk assessment and in the identification of susceptible subgroups (Azqueta et al., 2014).

In the determination of DNA repair capacity, the incorporation of lesion-specific enzymes into the standard Comet Assay protocol (enzyme-modified comet assay) enhances the sensitivity of the method, thereby enabling the detection of specific classes of DNA lesions. In this approach, following the lysis step, nucleoids are treated with specific DNA glycosylases that convert base lesions into excision breaks. For example, EndoIII is used for the detection of oxidized pyrimidines, whereas FPG is traditionally employed to identify oxidized purines (particularly 8-oxoG). However, Smith et al. (2006) demonstrated that the human 8-hydroxyguanine DNA glycosylase (hOGG1) enzyme exhibits substantially higher specificity than FPG in recognizing 8-oxoG lesions. The study also provided an important technical caution: FPG and EndoIII may display cross-reactivity not only toward oxidative lesions but also toward certain alkylation products. This may lead to misinterpretation of lesion type, particularly when investigating novel genotoxic agents with incompletely characterized mechanisms of action. Consequently, conclusions regarding lesion specificity require careful evaluation, taking into account the broad substrate spectrum of these enzymes (Smith et al., 2006).

3.3. In Vitro Acellular Repair Assays: Use of Cell Extracts and Substrate DNA

In the “comet-based in vitro DNA repair assay” approach, substrate nucleoids or DNA carrying specific lesions are incubated with the cell extract to be tested; repair enzymes present in the extract introduce breaks at lesion sites, which are then measured using the comet assay. This approach provides a robust phenotypic readout of repair capacity at the enzymatic activity level rather than the transcript level (Azqueta et al., 2014; Voděnková et al., 2020). Optimized protocols presented as methodological references describe in detail the precise measurement of BER and NER activities using comet-based in vitro repair assays. This standardized approach governs each step of the process, which is based on the incubation of substrate nucleoids containing specific DNA lesions (e.g., photosensitizer/light-induced lesions for BER; UV-C-treated cells for NER) with cell extracts. To enhance inter-experimental and inter-laboratory

comparability, critical quality control steps are recommended, including standardization of protein concentration, the use of negative and positive controls, and optimization of the enzyme-to-substrate ratio. These optimized approaches allow not only the reliable assessment of total repair capacity but also the phenotypic evaluation of the individual contributions of distinct repair pathways, facilitating their integration into large-scale molecular epidemiology studies (Voděnková et al., 2020).

4. Detection of Specific DNA Damage and Repair

4.1. Use of Lesion-Specific Glycosylases (FPG, EndoIII, hOGG1)

In the enzyme-modified Comet Assay, following lysis, nucleoids are incubated with lesion-specific DNA glycosylases/endonucleases. These enzymes convert the corresponding base lesions into breaks, thereby enhancing the sensitivity of the alkaline comet assay.

- **Formamidopyrimidine DNA glycosylase (FPG):** FPG, widely employed in enzyme-modified Comet Assay protocols, is primarily used to detect oxidized purines such as 8-oxoguanine and formamidopyrimidine derivatives that cause destabilization of the DNA helix. However, specificity studies have revealed that the substrate range of this enzyme is broader than previously assumed, and under certain experimental conditions, it can also respond to alkylation-related products (e.g., ring-opened purine lesions). This has prompted reconsideration of interpretations suggesting that observed DNA breaks are solely due to "oxidative stress" when working with genotoxic agents with unknown mechanisms of action. Due to these potential cross-reactions, it is recommended either to use enzymes with higher specificity, such as hOGG1, or to interpret the resulting data with rigorous methodological scrutiny (Smith et al., 2006).

- **Endonuclease III (EndoIII):** Recognizes oxidized pyrimidines, including thymine glycol and uracil glycol.

- **Human 8-oxoguanine DNA glycosylase 1 (hOGG1):** In enzyme-modified Comet Assay protocols, lesion specificity of the enzymes used is critical to increase diagnostic accuracy for the type of damage. The use of hOGG1 enables a more specific readout, particularly for 8-oxoG-focused measurements. Comparative studies have shown that, unlike other commonly used enzymes such as FPG or EndoIII, hOGG1 does not produce significant responses to non-oxidative lesions (e.g., alkylation-induced damage). This high selectivity allows oxidative DNA damage to be clearly distinguished from other types of lesions in

studies investigating complex genotoxic mechanisms, thereby providing more reliable biomarker data (Smith et al., 2006).

This approach effectively converts the measurement of "total breaks" into a mechanistic biomarker, such as "oxidative base damage." However, standardization of parameters, including enzyme titration, incubation time, buffer composition and temperature, is critical (Møller et al., 2020).

4.2. Dynamics of Oxidative Stress-Induced DNA Damage Repair

The intracellular dynamics of oxidative DNA damage are shaped by the complex interplay between exposure duration and intensity, cellular antioxidant defense mechanisms, mitochondrial stress levels, inflammatory responses, and BER capacity. In this context, the enzyme-modified Comet Assay provides a highly sensitive analytical window for assessing the "repair or processing" rates of oxidative lesions, particularly on short time scales ranging from minutes to hours, in contrast to standard protocols.

Monitoring the time-dependent decrease of base lesions in nucleoids using specific glycosylases (e.g., FPG, hOGG1) directly reflects the cell's performance in dealing with genotoxic stress. These dynamic data significantly contribute to clarifying, at the molecular level, individual differences in responses to environmental pollutants or pharmacological treatments (i.e., inter-individual susceptibility) in both clinical and field studies. Consequently, enzyme-assisted approaches go beyond static measurements of damage, enabling the characterization of individual DNA repair capacity as a functional phenotypic biomarker (Azqueta et al., 2014).

5. Analysis of Individual Variability and Threshold Discussions

Comet assay data, particularly in human biomonitoring studies, can exhibit high inter-individual variability as well as laboratory-specific effects. This emphasizes the importance of within-study controls, historical control distributions, and laboratory-specific quality criteria, rather than relying on a single absolute threshold for decision-making. While OECD TG 489 highlights the importance of statistical evaluation and historical control data, it does not define a universal numerical threshold for a positive response (OECD, 2016).

The reliability of comet assay data depends not only on intra-laboratory technical precision but also directly on how the raw data obtained are processed statistically. A study analyzing extensive historical control data collected from five different laboratories strikingly demonstrated the magnitude of inter-laboratory variation (laboratory effect) on the results. The research showed that

the method used to summarize single-cell raw data (DNA % in tail) at the sample level—whether arithmetic mean, median, or geometric mean—can significantly affect the final results and their statistical significance. In particular, examination of the distribution of control data highlighted that selecting the appropriate summary statistic is a critical step for distinguishing biological variability from technical sources of variation when determining historical control ranges and interpreting positive/negative outcomes. These findings clearly underscore the importance of reporting not only the result itself but also the mathematical method used to summarize the data in comet assay studies (Tug et al., 2024).

6. Application Areas in Clinical and Field Studies

6.1. The Importance of DNA Repair Capacity in Occupational Exposure Groups

In occupational exposures (e.g., solvents, polycyclic aromatic hydrocarbons [PAHs], metals, pesticides, radiation), individuals may exhibit different DRC profiles under the same damage burden, which can contribute to inter-individual variability in risk. Comet-based DRC measurements can complement exposure biomarkers by providing insights into both the "effect" and "susceptibility" dimensions of exposure (Azqueta et al., 2014).

6.2. DRC in Cancer Risk Assessment and Personalized Medicine Applications

Precisely measuring DNA repair capacity at the individual level plays a critical role in cancer risk analysis and the development of personalized medicine strategies today. Determining the functional response of individuals to genetic damage provides strategic contributions in areas such as risk categorization, prediction of treatment response, and toxicity management (Azqueta et al., 2014; Nagel et al., 2017). Specifically, the assessment of individuals' functional responses to genetic damage offers strategic insights in three key domains within clinical practice:

- **Risk Categorization:** Identification of individuals with impaired DNA damage response (DDR) mechanisms, allowing for the early classification of high-risk groups in terms of cancer susceptibility.
- **Prediction of Treatment Response:** Personalized estimation of cellular responses to chemotherapeutic and radiotherapeutic agents, particularly those acting via double-strand breaks or BER/NER pathways.
- **Toxicity Management:** Evaluation of individual sensitivity to potential side effects and tissue damage arising during treatment.

The implementation of these clinical objectives has been facilitated by high-throughput technologies that overcome the limitations of traditional methods. The 384-well CometChip platform, utilizing microfabrication techniques, enables the simultaneous analysis of thousands of cells in a standardized format. Its success in assessing cellular responses to chemotherapeutic agents such as etoposide indicates that DRC measurements can be translated into large datasets with high accuracy. Such innovative approaches accelerate drug screening processes and ultimately facilitate the integration of the Comet Assay into routine cancer diagnosis and treatment monitoring protocols. Consequently, they provide a data-driven foundation for personalized medicine applications (Li et al., 2022).

6.3. Repair Assays as a Decision-Support Tool in Environmental Biomonitoring

In environmental exposures (e.g., air pollution, water/soil contaminants, industrial mixtures), assessing not only the level of damage but also the dynamics of repair can aid in distinguishing "transient effects" from "persistent risk signals." When integrated into multi-biomarker panels, this approach can enhance the decision-support value of environmental biomonitoring (Azqueta et al., 2014; Møller et al., 2020).

7. Methodological Challenges, Limitations, and Standardization

Although the Comet Assay is popular due to its high sensitivity and low cost, minimizing inter-laboratory variability and standardizing protocols are critical for its recognition as a "contemporary methodological approach." Since the measurement of DNA repair capacity involves considerably more variables than standard damage detection, methodological rigor must be maintained at the highest level.

7.1. Protocol Standardization and Inter-Laboratory Variability

One of the greatest challenges affecting Comet Assay results is the variability in protocols applied across different laboratories. Factors such as agarose concentration, lysis duration, voltage in the electrophoresis tank, and even the temperature of the buffer solution can have dramatic effects on the outcomes. Given that variability in test conditions directly impacts experimental results, efforts to establish standardized procedures are essential.

The use of frozen reference standard cells—prepared from a single large cell line and with predefined damage levels (e.g., positive and negative control cells)—is recommended for each experiment. These reference cells are treated with DNA-damaging agents at concentrations expected to yield moderate comet

scores and then cryopreserved. Their use allows monitoring of intra-laboratory performance and provides comparability across different experiments (hCOMET Network, n.d.).

7.2. "Hedgehog" Cells and the Data Analysis Problem

Cells exhibiting extreme damage, with tails appearing completely detached from the nucleus (termed "Hedgehog" cells), represent a major methodological debate in analyses. These cells may reflect early stages of apoptosis. However, interpreting them directly as indicators of apoptosis or cytotoxicity can be misleading. Including these cells in repair capacity studies may cause repair data to appear misleadingly high or low. Therefore, it is recommended that Hedgehog cells be identified and reported in studies. Contemporary guidelines also advise treating these cells as a separate category for repair kinetics calculations or excluding them from analysis altogether (Azqueta & Collins, 2013; Møller et al., 2020).

7.3. Enzyme Calibration and Reagent Stability

The use of lesion-specific enzymes (e.g., FPG, endonuclease III) adds significant methodological power by allowing the differentiation of distinct types of damage and the separate tracking of their repair (Collins et al., 1997). However, the activity levels of these enzymes may vary between production batches (batch-to-batch). Excessive concentration of the enzyme leads to non-specific incisions, while insufficient concentration causes the damage to appear less severe than it actually is. Therefore, titration of each new enzyme batch and calibration using substrate DNA is a critical step to ensure the accuracy of the method.

7.4. Integration of High-Throughput Systems

The classical slide-based Comet Assay is relatively slow for epidemiological studies involving hundreds of samples. Manual steps in slide preparation and staining are vulnerable to human error. Mini-gel formats (12 or 96-well) and the CometChip technology provide physical standardization and reduce experimental time. However, the high cost of these systems and the requirement for specialized equipment limit their widespread adoption (Ge et al., 2014).

8. Future Perspectıves: New Horizons in Molecular Biology

8.1. AI-Assisted Automated Comet Assay Analysis

One of the most promising areas of near-term development is end-to-end automation (image quality control → segmentation → parameter extraction →

statistical analysis using Bayesian/mixed-effects models). Deep learning–based approaches may provide substantial performance improvements, particularly for challenging images (e.g., low signal intensity, irregular background, ghost cells). However, the need for multicenter datasets and standardized labelling is evident to ensure generalizability (Hong et al., 2020; Møller et al., 2020).

8.2. Integration of DNA Repair Capacity with Other "Omics" Data

Combining the DRC phenotype with transcriptomic, proteomic, metabolomic, or epigenomic data has the potential to close the gap between "pathway activity" and "phenotypic output." For example, BER/NER gene expression alone may not consistently reflect actual enzyme activity. Therefore, phenotypic measurements, such as comet-based in vitro repair assays, may provide significant value when integrated with multi-omics data to construct causal network models. In this context, studies focusing on methodological optimization, particularly protocols aimed at increasing the sensitivity and comparability of the comet assay, contribute to more reliable integration with omics datasets (Azqueta et al., 2014; Voděnková et al., 2020; Zheng et al., 2023).

8.3. Point-of-Care Applications

Standardization of the Comet test and its transformation into semi-automated systems capable of delivering rapid results, such as fast DNA repair kits that can determine patients' sensitivity to treatments like radiotherapy within 24 hours in hospital settings, would represent a major clinical advancement of the methodology. Such developments could enable point-of-care assessment of genotoxicity and DRC sensitivity in the future.

In conclusion, the Comet Assay plays a central role in the paradigm shift from classical genotoxicity testing toward the dynamic measurement of repair capacity. Advances such as standardization initiatives (MIRCA, OECD TG 489), high-throughput platforms (CometChip), and artificial intelligence–based image analysis support the evolution of DRC into a more reliable and scalable biomarker for clinical, occupational, and environmental applications.

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