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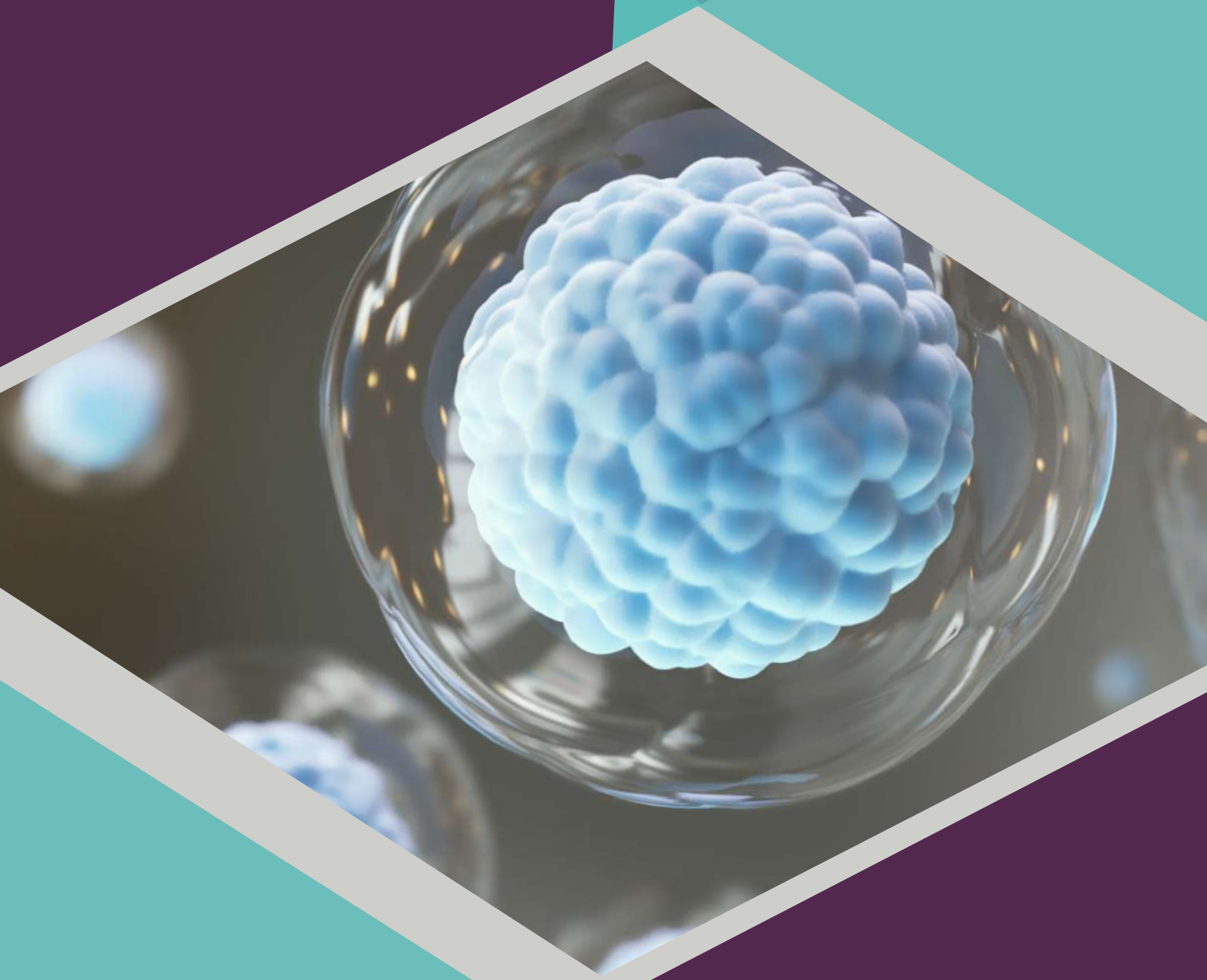

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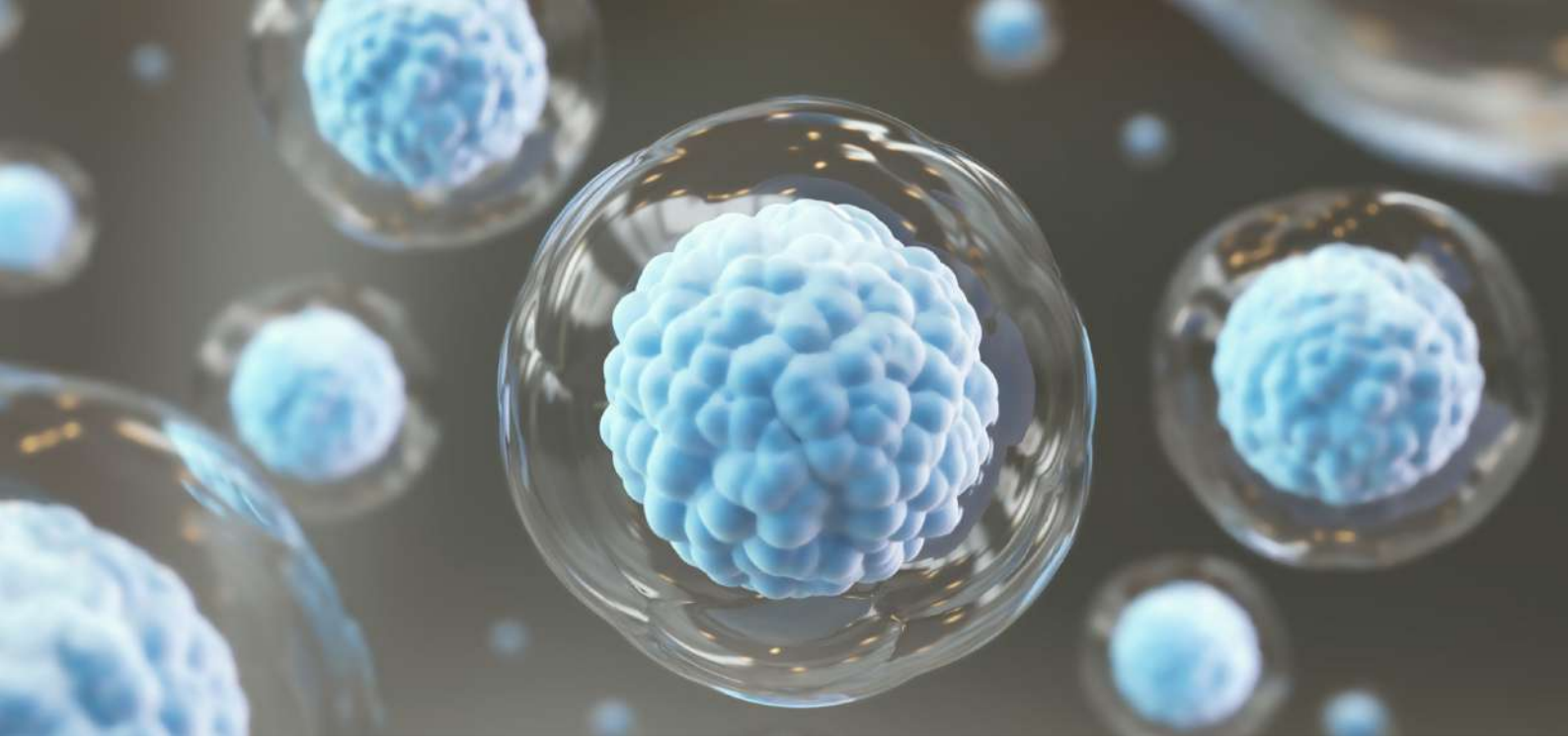

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Australian regulation of autologous human cell and tissue products: implications for commercial stem cell clinics

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In 2018, Australia's Therapeutic Goods Administration introduced regulatory reforms that set stricter criteria around the regulation of products derived from a patient's own cells and tissues, posing significant implications for clinics offering stem cell treatments. We review the regulatory framework and discuss its potential commercial implications, including the ambiguities that may arise from it in practice, as well as the likely impact it will have on product development and advertising practices in the future.

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In Australia, the *Therapeutic Goods Act 1989* (Cth) and several other legislative instruments, including regulations, determinations, specifications and orders, collectively determine whether different therapeutic goods require registration on the Australian Register of Therapeutic Goods (ARTG) [1]. Therapeutic goods listed on the ARTG can be lawfully supplied within Australia. Provisions also exist for patients to access products not listed in the ARTG under certain circumstances; however, these provisions cannot be used to facilitate commercial activities [2]. In 2011, Australia implemented a framework for the regulation of biologicals utilising a risk-based approach [3]. Under this framework, risk was assessed by a consideration of the degree of manipulation of the biological during manufacture and its intended use. However, a number of biologicals were excluded from regulation at the time, including autologous human cell and tissue (HCT) products that met certain conditions. The primary criterion for the exclusion of these HCT products was that their use and manufacture occurred under the supervision of a registered medical practitioner [4]. One of the primary justifications for this exclusion was that these HCT products were directed toward the legitimate purposes of medical practice and were therefore beyond the regulatory jurisdiction of the Therapeutic Goods Administration (TGA). While corporate entities regularly invest in establishing the safety and efficacy of therapeutic goods in order to register and sell them in the marketplace, excluded goods are not required to be registered. Consequently, the exclusion from regulation has contributed to the persistence of a poor evidence base about the therapeutic benefits of autologous HCT products globally [5]. The exclusion has also contributed to the proliferation of stem cell clinics that claim to offer therapeutic stem cell treatments with little evidence [6].

There have been several examples of harm arising from the use of autologous HCT products, both physical and financial, and this has made it apparent that existing regulations were not sufficient to protect the public. Incidents of death and harm were reported internationally [7,8]. In Australia cases of harm have also been reported, including the recent death of an elderly patient who was treated for dementia [8]. A review of websites offering stem cell therapies in Australia and Japan found that advertisements for stem cell therapies were often misleading, falsely portraying unproven autologous interventions as meeting high standards of scientific evidence [9].

In response to concerns relating to the lack of regulation of autologous HCT products, in 2015 and 2016, the Therapeutic Goods Administration, the Australian agency responsible for therapeutic goods including medical

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devices, pharmaceuticals and biological products, conducted public consultation in a broad review of the current regulation of autologous cellular therapies. This led to several amendments to the *Therapeutic Goods Regulations 1990* (Cth) (TG Act) [10], which introduced new conditions for *exempting* certain goods from TGA oversight; these new conditions were additional to changes around existing regulations that *excluded* certain goods from TGA regulation. These amendments to the regulation of autologous HCT products came into force on the 1 July 2018, with a transition period ending on 30 June 2019 [11].

The rapidly increasing number of commercial stem cell clinics offering autologous HCT products to the general public is a concern to regulators across the globe. In this paper we focus on autologous stem cell products that can be classified as biologicals as this covers those products commonly utilised by stem cell clinics (e.g., products derived from adipose tissue). Stem cell products derived from blood, such as hematopoietic progenitor cells, are classified as medicines rather than biologicals by the TGA [12]. While this paper focuses on the regulation of stem cell products in the Australian context, the approach to regulation described here, and the consequences that arise from it, are instructive for other jurisdictions tackling similar regulatory challenges.

Overview of regulation of autologous human cell and tissue products

Since the introduction of the biological framework in 2011, a number of determinations and orders have been made by the Commonwealth Government (the Commonwealth Department of Health) to specify which therapeutic goods are not regulated by the TGA (see Table 1 for a summary of these changes), as well as the conditions under which certain goods are exempted from some aspects of therapeutic goods regulation. The Therapeutic Goods (Excluded Goods) Determination 2018 was amended in 2019 to set out the criteria by which autologous HCT products will qualify for exclusion from TGA regulations. These criteria for exemptions from regulation are also set out in schedules 5, 5A, and 7 of the Therapeutic Goods Regulation 1990 (Cth) ('TG Regulation'). The relevant provisions came into force on 20 June 2019. From a broad regulatory perspective, these reforms have created three separate categories of autologous HCT products (Table 2), namely:

- products that are 'excluded' from regulation and not subject to the operation of the *TG Act*;
- products that are 'exempt' from some elements of the *TG Act*; and
- products that are fully regulated as biological products [12].

Critically, excluded and exempted autologous HCT products do not need to be included in the ARTG, and therefore are not assessed for safety and effectiveness by the TGA. By contrast, products that are fully regulated as biological products must be registered as biologicals under one of the classes specified in the biological framework (either class 1, 2, 3 or 4) [13]. Fully regulated biologicals must be assessed for safety and effectiveness by the TGA, except for Class 1 biologicals. These products are low risk and must only be utilized with an appropriate level of governance and oversight. For Class 1 biologicals, only a statement of compliance is needed, including confirmation that the product is safe [13,14]. Under the revised regulations, no autologous HCT products – whether they are 'excluded', 'exempted' or classified as biological products – can be advertised to consumers [15]. While health services in general can be advertised, the regulations prohibit the advertisement or promotion of any service that directly or indirectly refers to HCT products or uses common trade names for those products, such as 'stem cells'.

Below the criteria that these categories must satisfy are presented in more detail. A direct comparison of these different pathways is presented in simplified form in Table 2.

Excluded autologous human cell and tissue products

The exclusion category emphasizes the setting in which care is provided and where products are manufactured; it does not focus on the risk profile of the product itself. The current criteria for exclusion are specified in the Therapeutic Goods (Excluded Goods) Determination 2018 (see last row of Table 1). An excluded autologous HCT product must be manufactured and administered with a high degree of medical (or dental) practitioner oversight within a hospital setting. Specifically, the cells must be collected from a patient under the care of the registered medical (or dental) practitioner, and the product must be manufactured under the practitioner's supervision within the same hospital in which the patient is admitted. Storage and testing of the product may occur outside the hospital setting, but only where these services are provided by a person under contract with the hospital.

These exclusion criteria are set out in a determination of the Australian Federal Minister for Health, and this determination has been made on the basis that the Minister, on behalf of the Commonwealth government, accepts

Table 1. Evolution of exclusion criteria for autologous human cell and tissue products.

Legislation	Relevant text regarding excluded autologous HCT products	Note
Therapeutic Goods (Excluded Goods) Order No. 1 of 2011 (in force 30 May 2011)	4(q): Human cells and tissues that are: (i) collected from a patient who is under the clinical care and treatment of a medical practitioner registered under a law of a State or an internal Territory; and (ii) manufactured by that medical practitioner, or by a person or persons under the professional supervision of that medical practitioner, for therapeutic application in the treatment of a single indication and in a single course of treatment of that patient by the same medical practitioner, or by a person or persons under the professional supervision of the same medical practitioner.	The key principle is ensuring that a medical practitioner has adequate oversight of the collection, manufacture and administration of the HCT-product treatment.
Therapeutic Goods (things that are biologicals) specification 2017 (No. 1) (in force 2 May 2017)	This specification provides that "*things that comprise or contain live animal cells, tissues or organs*" are biologicals according under the *TG Act*.	This specification ensures that autologous HCT products that are not explicitly excluded under the 2011 Order are treated as biological products. The conditions for exclusion are modified by the following two instruments.
Amendment to the Therapeutic Goods (Excluded Goods) Order No. 1 of 2011 (in force 15 June 2018)	Removed human tissues and cells from the Therapeutic Goods (Excluded Goods) Order No. 1 of 2011.	This amendment removes autologous HCTs from the 2011 Order for the purpose of the 2018 Determination (below).
Therapeutic Goods (Human Cells, Tissues and Organs) Determination 2018 (in force 1 July 2018)	4(a): Therapeutic goods in relation to which the following apply: (a) the goods comprise, contain or are derived from human cells or human tissues collected from a patient who is under the clinical care of a medical or dental practitioner; (b) the goods were manufactured by, or under the professional supervision of, the practitioner in a hospital in a State or internal Territory for that patient who must be a patient of that hospital; (c) the practitioner is registered in a State or internal Territory; (d) the goods are not advertised directly to consumers.	This Determination replaces the 2011 Order. The main differences are: the inclusion of dental practitioners as overseers; the inclusion of the requirement that the HCT goods must be manufactured in a hospital in which the patient is admitted; and the inclusion of a clause prohibiting advertising. In addition, this determination removes the requirement that the product is for the 'treatment of a single indication and in a single course of treatment of that patient by the same medical practitioner'. This is now a criterion attaching to *exempt* products (rather than to 'excluded' products).
Therapeutic Goods (Excluded Goods) Determination 2018 (in force 20 June 2019)	Goods in relation to which the following paragraphs apply: (a) the goods comprise, contain or are derived from, human cells or human tissues collected from a patient (the relevant patient) who is under the clinical care of a medical or dental practitioner (the relevant practitioner); (b) the relevant practitioner is registered in a State or internal Territory; (c) subject to paragraph; (d) all steps in the manufacture of the goods are carried out by, or under the professional supervision of, the relevant practitioner in a hospital in a State or internal Territory (the relevant hospital); (d) if a step in the manufacture of the goods relating to the storage or testing of the goods is not carried out in the relevant hospital, it is carried out by a person under contract with the relevant hospital. In addition, the goods are: (a) used for the relevant patient, who is a patient of the relevant hospital; and (b) not advertised directly to consumers.	The Therapeutic Goods Amendment (Excluded Goods) Determination 2019 repeals the Therapeutic Goods (Human Cells, Tissues and Organs) Determination 2018 and amends the Therapeutic Goods (Excluded Goods) Determination 2018 to include new text to the left. The main change is that the new Determination allows some steps of manufacture relating to storage or testing of goods to occur outside of the hospital.

HCT: Human cell and tissue.

that regulations external to the *TG Act* sufficiently mitigate the risks associated with their use. As the TGA notes in respect of excluded goods, 'the Government has decided that there is appropriate external regulation for them'. [16] This includes the licensing and accreditation requirements imposed on private and public hospitals (standards that are prerequisites for government funding in some jurisdictions), as well as hospital administration and management structures at the state and national level. In Australia, all hospitals must be assessed against the National Safety and Quality Health Service Standards [17]. Moreover, the law regulating health practitioners, set out in various state Acts known as the Health Practitioner Regulation National Law, ensures that practitioners meet certain registration and accreditation standards. Depending on the state or territory jurisdiction, the Health Practitioner Regulation National Law also ensures that practitioners' health, performance and conduct is investigated, monitored or disciplined when appropriate by the professional regulator, such as Australian Health Practitioners Regulatory Authority (APHRA) or, in the case of medical practitioners, the Medical Board of Australia. Where a practitioner

Table 2. Three categories of autologous human cell and tissue products.

	Excluded	Exempted	Fully regulated
Setting for treatment	Patient admitted to a hospital.	Patient need not be admitted to a hospital.	N/A
Collection of material	Collected from hospital patient who is under the clinical care of a medical or dental practitioner.	Collected from a patient who is under the clinical care of a medical or dental practitioner.	N/A
Manufacture	Product must be manufactured in the hospital in which the patient is admitted. Storage and testing may be carried out by a person under contract with the hospital. There is no requirement to meet GMP standards.	Product may be manufactured outside a hospital. Product must be manufactured by the practitioner with primary responsibility for patient, or under the supervision of that practitioner. There is no requirement to meet GMP standards.	As per Australian code of GMP for human blood and blood components, human tissues and human cellular therapy products.
Intended use restrictions	None	For a single indication in a single procedure.	As per registration.
Risk profile	N/A	Restricted to minimal risk products in other words only minimally manipulated products for homologous use.	Products are classified according to the biologic framework: Class 1, 2, 3 or 4.
Advertising	No direct to consumer advertising permitted.	No direct to consumer advertising permitted.	No direct to consumer advertising.
Listing on Australian Register of Therapeutic Goods	No	No	Yes
Legislative exemptions	Therapeutic Goods Act 1989 (Cth) does not apply.	Parts 3-2 (Registration and Listing of Therapeutic Goods) and 3-2A (Biologicals) of the Therapeutic Goods Act does not apply as specified in Schedules 5, 5A, and 7 of the Therapeutic Goods Regulation 1990 (Cth). This is on condition that events and concerns about unintended harmful effects are reported to authorities.	None
Exemptions as per Therapeutic Goods Administration guidance	N/A	Requirements for accessing 'unapproved' products do not apply; evidence that manufacturing facility complies with GMP requirements is not required.	Manufacturers of Class 1 biologicals do not need a GMP manufacturing license or certificate; Class 1 biologicals do not require the Therapeutic Goods Administration to undertake a premarket assessment of supporting data.

GMP: Good manufacturing practice; N/A: Not applicable.

is in contravention of the TGA regulations, the TGA may provide a complaint or notification to AHPRA or the Medical Board of Australia, and that complaint may then result in the investigation and potentially the prosecution of a practitioner under the National Law.

Exempt autologous human cell and tissue products

The exemption category emphasizes the product's safety profile, which is based on how the product is manufactured, its intended use and how it is utilized. The criteria for exemption are specified in schedules 5, 5A and 7 of the Therapeutic Goods Regulation 1990 (Cth) (TG Regulation). An exempted autologous HCT product must be only minimally manipulated during manufacture and it must be intended only for homologous use – that is, the intended use must match the original biological function of the cells or tissues [18]. It must also be utilized for only a single indication in a single procedure. As per the exclusion category, the exempted autologous HCT product must be collected, manufactured and administered with a high degree of medical (or dental) practitioner oversight, which may occur outside of the hospital setting. It is therefore possible that unscrupulous practitioners may see an opportunity to profit by offering exempt HCT products to patients even when they are not appropriate or optimal for the patient's condition. Minimal manipulation is defined in the TG Regulation as any manufacturing process that does not alter the 'biological characteristics, physiological functions or structural properties of the original cells or tissues that are relevant to the purpose for which the manufacturer of the goods intends the goods to be used' [19]. Although not all cell functions need to be preserved, the manufacturer of a potentially exempt product must be able to show that selected characteristics related to the intended use have been sufficiently maintained. The TG Regulation defines homologous use as instances where the product is used to 'repair, reconstruct, replace or

supplement the cells or tissues of a person' where the therapeutic good will perform the same basic function in the recipient as was performed by the original cells or tissues [20]. More specifically, according to the TGA, isolating an autologous HCT product from adipose tissue via dissociating cell–cell contacts and isolating the cellular portion for the purpose of reinjecting the cells would be considered more than minimal manipulation, as the process used to isolate the cells would likely change the cellular product's properties. In addition, using stromal vascular fraction isolated from adipose tissue via liposuction for regenerating cartilage and tendons is interpreted as a nonhomologous use as this is not the basic function of cells isolated from adipose tissue [19,20]. This is significant, as adipose-tissue derived autologous HCTs are commonly offered by stem cell clinics [9]. In addition, an 'unproven clinical use' of an autologous HCT product is likely to be considered a non-homologous use by the TGA, and therefore treatments based on weak, preliminary, or uncertain evidence will likely not qualify for exemption (and therefore use) by independent stem cell clinics.

It is up to the manufacturer to hold information on compliance with applicable standards (such as labeling standards imposed by the TG Act and other instruments) and evidence demonstrating the safety and efficacy of the product if it manufactures and supplies a putatively exempt product. However, as the TGA guide notes, the TGA, under most circumstances, 'will not review such information [although] when a safety issue arises we may request it for review'. [18]. This indicates that the TGA has adopted a responsive rather than proactive response in relation to overseeing the safety of exempt biologicals.

As the TG Regulation specifies, criminal penalties may apply if the quality, safety or efficacy of the product is found to be unacceptable or does not comply with applicable standards. The TGA undertakes various compliance actions, ranging from warning letters to injunctions to applications for civil penalties [21]. Usually, only serious noncompliance will warrant criminal proceedings; however, in such cases, the TGA will prepare a brief of evidence to submit to the Commonwealth Director of Public Prosecutions. Till date, no criminal or enforcement actions have been taken against practitioners or clinics with reference to breaches of the amended TG Regulations.

Manufacturers and suppliers of exempt autologous medical and biological products are still required to report adverse events and are responsible for conducting a recall if applicable. However, they do not need to provide evidence of GMP.

Fully regulated autologous human cell and tissue products

Autologous HCT products that are biologicals and do not meet the requirements for governance, supervision, manufacture and use specified for excluded and exempt products are fully regulated under the biologicals framework. Fully regulated products must be registered in the ARTG and are classified as either Class 1, 2, 3 or 4 biologicals according to their risk profile [13]. Class 1 biologicals are those deemed to be low risk and that have appropriate oversight mechanisms in place. The TGA has recently stated that some fecal microbiota transplant products are classified as Class 1 [22], and the regulations have been amended to include these products. Class 2 biologicals are also considered low risk, but are defined more specifically as products that have only been minimally manipulated and are for homologous use – in this respect it overlaps with the definition for *exemption*. No Class 2 biologicals have been listed in the TG Regulations; however, platelet rich plasma products are likely to be classified as a Class 2 biologicals where the product is minimally manipulated and used for homologous use. Class 3 biologicals are considered medium risk, defined as any product that does not satisfy the Class 2 requirements for minimal manipulation and homologous use. Finally, Class 4 biologicals are considered high risk, and these products must be specifically mentioned in the TG Regulation.

Importantly, Class 1 biologicals are exempt from some requirements applicable to other fully regulated biologicals. Namely, they do not require a premarket assessment of supporting data, nor do manufacturers need to hold a GMP license or certificate, or clearances for the biological manufacture. Class 1 biologicals only require a submission of a statement of compliance in which the product sponsor confirms that the product satisfies the criteria for Class 1 classification, and is safe for the purposes for which it is used [14].

Possible uses of the Class 1 biologicals registration pathway by stem cell clinics

In theory (since no Class 1 biologicals have been registered to date), it appears that stem cell clinics offering products that do not satisfy all the criteria for exemption could apply for a Class 1 biologicals classification. Like exempted products, Class 1 biologicals can be manufactured without a GMP license, do not require a pre-assessment of evidence of safety and efficacy, and need adequate oversight. It appears that the Class 1 biological classification may be useful in cases where a stem cell clinic wishes to store and reuse a product (and so breaching the single indication

in a single procedure criterion) or if more flexible supervision requirements are sought. Alternatively, the Class 1 biological classification could be utilized by stem cell clinics that provide products that may be deemed safe but that do not necessarily meet the requirements for minimal manipulation or homologous use required for exempted products. As mentioned above, the TGA has interpreted minimal manipulation and homologous use in narrow terms, which would mean that the adipose tissue-derived autologous HCT products commonly offered by stem cell clinics would not be exempted. Registering products as Class 1 biologicals may provide a pathway for stem cell clinics wishing to continue their operations without ambiguity about the status of their practices.

The TGA has acknowledged that determining when a use is homologous may be complex, and that in cases of ambiguity, the manufacturer intending on registering their biological on the ARTG will generally need to provide evidence to support their claim through the registration process, outlined below. Unproven clinical uses are therefore likely to be treated as nonhomologous uses [20]. A stem cell clinic providing products satisfying all the other requirements for exemption could apply for Class 1 classification if they are confident that the product is nevertheless safe. To register a biological on the ARTG in Class 1, a manufacturer must submit a statement of compliance that names the sponsor of the product, and confirms that the product is safe, meets all relevant manufacturing standards, and is suitable for Class 1 classification. That statement is then reviewed by the TGA and may be subject to a comprehensive evaluation [14].

Implications & consequences of the regulatory changes

Commercial

The new regulatory framework for autologous HCT products makes it more difficult for stem cell clinics to offer unregulated products outside of a hospital setting. Independent stem cell clinics operating in Australia will have to either limit the range of services they provide or change the type of services they provide – which is likely unfeasible given the rather narrow interpretation for minimal manipulation and homologous use the TGA has presented. Clinics may also be able to partner with a hospital to qualify for exclusion from regulation, or register their products with the TGA. In these circumstances, it is possible that practitioner may continue to offer exempt HCT products to patients even when doing so is not in the a patient's best interest.

If a stem cell clinic decides that the best option is to register their products as biologicals, the most straightforward option is to apply for Class 1 classification as described above. Other options would require the submission of a comprehensive evidence dossier demonstrating the safety and efficacy of each product and therapeutic target and a manufacturing license. While demonstrating safety and efficacy is routine for research and development (R&D) focused pharmaceutical companies, stem cell clinics do not generally have the same R&D capacity required to meet the evidence requirements for registration of novel products in the ARTG. This is further complicated by the fact that the development of human-derived cell therapies present a range of novel research challenges [23,24], likely requiring even greater technical expertise than is usually required for other therapeutic goods.

Stem cell clinics could also maintain and extend their autologous services by partnering with a hospital that could provide the required oversight to exclude stem cell therapies from the TG regulation. Alternatively, the new regulations may provide hospitals with a lucrative competitive advantage and incentivize them to compete with established stem cell clinics. Precedents for these developments exists in the USA, where a number of reputable hospitals have started to offer stem cell services to the public [25]. One question that remains unanswered in this space is what constitutes a 'hospital'. While the original proposal suggested it meant 'accredited hospital', this has not been specified in any TGA guidance to date [26].

Product development & evidence

To date there have been relatively few stem cell autologous products that have been approved by regulators and fewer autologous stem cell products. These include Holoclar, a therapy based on autologous limbal stem cells, approved in Europe in 2015; ReliNethra, a similar animal origin-free product authorized for marketing in India in 2008; Cupistem, an autologous adipose-derived stem cell for the treatment of inflammation and regeneration of joint tissues and approved in South Korea in 2012; and a number of autologous bone marrow stem cell therapies [27]. The International Society for Cell and Gene Therapy has published a comprehensive list of cell, tissue and gene products with marketing authorization in various jurisdictions, including Australia [28]. Greater oversight of stem cell clinics may potentially incentivize industry to develop autologous HCT products, and thereby enhance the evidence base. Alternatively, if the new regulations incentivize stem cell clinics to partner with hospitals, particularly large teaching hospitals with strong research capabilities and ethics review processes, this development could be

advantageous for building the evidence base as well. On the other hand, if the stem cell clinics partner with small hospitals that provide inadequate research support or ethics review, this may perpetuate current concerns about poor epistemic standards.

Advertising

The new TGA regulations explicitly disallow direct-to-consumer advertising for autologous HCT products. This includes not only the advertisement of the products themselves but also health services that may refer or direct people to these products [15]. For instance, the TGA specifies that medical (and dental) practitioners or clinics advertising health services must not provide information or advice on their website regarding 'particular types of treatments involving biologicals or excluded autologous HCT products', and cannot utilize trade names or colloquial names – for example 'stem cells' – in advertising. The intention is that patients should be made aware of specific treatment options only within the context of a consultation with a medical professional.

However, there is no prohibition on education seminars where treatments for particular diseases can be explained to the audience, nor on associative advertising where the term 'stem cell' may form part of the name of a clinic promoting 'biological therapy'. Nor is there a prohibition on any indirect reference to therapies that are said to use patients' 'own biological process to treat' a disease [29]. The boundary between education and advertising has long been contentious [30], and these new regulations do not directly address this issue and are therefore unlikely to curb advertising of therapies to consumers in any meaningful way. Moreover, the new regulations do not address indirect methods of enhancing the credibility of unproven therapies via 'tokens of legitimacy' that can mislead consumers [9].

Conclusion

In Australia, stem cells products that are not blood, a blood product or derived from blood, are regulated as biologicals under the TGA's new three-category framework for the regulation of autologous HCT products. In this paper we have compared these three categories (excluded, exempt and fully regulated) and their implications for stem cell clinics. The new framework introduces stricter criteria that limits the range of services independent stem cell clinics can provide outside of a hospital setting, and that can be utilized without registration in the ARTG. These new regulations should incentivize stem cell clinics and hospitals to enter mutually beneficial partnerships, and to conduct more robust research that could enhance the evidence base for stem cell products being utilized in the marketplace. However, it also provides opportunities for business-as-usual. The Class 1 biological classification may in some instances provide an avenue that stem cell clinics can utilize to continue independent operations in cases where the treatments offered do not qualify for exemption. Stem cell clinics may continue their operations under the patronage of (or in partnership with) hospitals, particularly private hospitals, which could be profitable for both parties. Finally, although the new regulations do not permit direct-to-consumer advertising of any stem cell products defined as a biologic, this is unlikely to impact on current advertising practices in any meaningful way due to various advertising strategies utilized that can bypass the strict letter of the law.

Future perspective

Advances in technologies and their implementation in innovative medical practice will increasingly challenge regulators across the globe. Custom-designed medicines and devices, cellular therapies in which manufacture is an inherent part of the treatment procedure, or a hybrid of these, will not fit neatly into predefined categories of either a medicine or device. As technology becomes ever more intertwined with medical practice, new governance structures will need to be introduced that are capable of working across these historical distinctions to balance community demand for access to new therapeutics while ensuring that appropriate standards are met. The case of autologous stem cell therapies is a harbinger of the regulatory complexity we will likely face in the coming decade alongside technological developments. In particular the proliferation of digital marketing must be strictly monitored to ensure that the public is not misinformed about their healthcare choices. While any future regulatory regime must involve timely implementation of reforms to ensure safety and assessment of clinical performance, it must also include active enforcement of standards, together with effective communication to the public about the likely therapeutic benefits of any medical innovation.

Executive summary

- Regulatory reforms for autologous human cell and tissue (HCT) products came into force in Australia on the 1 July 2018, with a transition period ending on 30 June 2019.
- These reforms were motivated by concern about the proliferation of stem cell clinics offering unproven stem cell therapies, and emerging reports of harm.
- These reforms created three categories of autologous HCT products: exclude, exempted and fully regulated.
- Excluded products are not subject to therapeutic goods regulation.
- Exempted products are subject to some aspects of therapeutic goods regulation.
- No autologous HCT products may be advertised to consumers.

Excluded & exempted autologous HCT products

- Excluded products are those provided in a highly regulated environment. These products must be manufactured and administered in a hospital with a high degree of medical or dental practitioner oversight.
- Exempt products are those deemed to pose low-risk. These products must be only minimally manipulated, for homologous use, used for a single indication in a single procedure and administered with a high degree of medical or dental practitioner oversight.
- Manufacturers of exempt products must hold information on compliance, that the Therapeutic Goods Administration may review if a safety issue arises.
- No requirement to meet Good Manufacturing Practice (GMP) standards.

Fully regulated autologous HCT products

- Products not excluded or exempted are fully regulated by the Therapeutic Goods Administration as a Class 1, 2, 3 or 4 biological.
- Class 1 products are considered low risk and do not require a pre-assessment of safety and efficacy data, nor do manufacturers of these products require a GMP license.

Implications & consequences of the regulatory changes

- The new regulatory framework makes it more difficult for stem cell clinics to offer products outside of a hospital setting. These changes have the potential to enhance patient safety and to encourage the development of a stronger evidence base for putative stem cell therapies.
- Clinics currently using products that no longer satisfy the criteria for exclusion or exemption have a number of options available.
- Stem cell clinics could seek to partner with hospitals to qualify for the exclusion provisions. This would allow clinics to continue to provide, or even expand, their services.
- Stem cell clinics could shift their focus from service provision to R&D and product development.
- Alternatively, if satisfied that their products are safe, stem cell clinics could attempt to register their products as Class 1 biologicals.
- Challenges remain regarding the regulation of direct-to-consumer advertising.

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References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. The Therapeutic Goods Act 1989 and the Poisons Standard (2020). www.tga.gov.au/therapeutic-goods-act-1989-poisons-standard
2. Therapeutic Goods Administration. Australian register of therapeutic goods (2020). www.tga.gov.au/artg
3. Therapeutic Goods Administration. Regulatory framework for biologicals (2020). www.tga.gov.au/regulatory-framework-biologicals
4. Therapeutic goods (excluded goods) order no. 1 of 2011 (2020). www.tga.gov.au/therapeutic-goods-excluded-goods-order-no-1-2011
5. Marks PW, Witten CM, Califf RM. Clarifying stem-cell therapy's benefits and risks. *N. Engl. J. Med.* 376(11), 1007–1009 (2017).
6. Berger I, Ahmad A, Bansal A, Kapoor T, Sipp D, Rasko JEJ. Global distribution of businesses marketing stem cell-based interventions. *Cell Stem Cell* 19(2), 158–162 (2016).
7. Bauer G, Elsallab M, Abou-El-Enein M. Concise review: a comprehensive analysis of reported adverse events in patients receiving unproven Stem cell-based interventions. *Stem Cells Transl. Med.* 7(9), 676–685 (2018).

• **Offers a comprehensive review of scientific publications, case reports and mass media publications to assess the reported cases and safety incidents associated with unproved stem cell-based interventions.**

8. Lysaght T, Lipworth W, Hendl T *et al.* The deadly business of an unregulated global stem cell industry. *J. Med. Ethics* 43(11), 744–746 (2017).

•• **Important article outlining an adverse event in which a recipient of a stem cell-based intervention died in Australia in December 2013 and the need for strong regulation of innovative therapies.**

9. Munsie M, Lysaght T, Hendl T, Tan HL, Kerridge I, Stewart C. Open for business: a comparative study of websites selling autologous stem cells in Australia and Japan. *Regen. Med.* 12(7), 777–790 (2017).

•• **Key study of online marketing practices of Japanese and Australian clinics offering unproven autologous stem cell-based interventions in Australia and Japan.**

10. Therapeutic Goods (Human Cells, Tissues and Organs) Determination 2018 (2018). www.legislation.gov.au/Details/F2018L00785

11. Therapeutic Goods Administration. Communication to stakeholders: autologous human cells and tissues (HCT) products (2018). www.tga.gov.au/communication-stakeholders-autologous-human-cells-and-tissues-hct-products

12. Therapeutic Goods Administration. Autologous human cells and tissues products regulation. www.tga.gov.au/autologous-human-cells-and-tissues-products-regulation

13. Therapeutic Goods Administration. Classification of biologicals (2019). www.tga.gov.au/classification-biologicals

14. Therapeutic Goods Administration. Applying for inclusion of a class 1 biological in the ARTG (2020). www.tga.gov.au/applying-inclusion-class-1-biological-artg#supply-documentation-statement

15. Therapeutic Goods Administration. Australian regulatory guidelines for advertising therapeutic goods (2019). www.tga.gov.au/book-page/advertising-different-types-therapeutic-goods

16. Therapeutic Goods Administration. What is regulated as a biological (2020). www.tga.gov.au/what-regulated-biological

17. Australian Commission on Safety and Quality in Health Care. Assessment to the NSQHS standards (2019). www.safetyandquality.gov.au/standards/nsqhs-standards/assessment-nsqhs-standards

18. Therapeutic Goods Administration. Exempt autologous human cells and tissues (2018). www.tga.gov.au/sites/default/files/exempt-autologous-human-cells-and-tissues.pdf

19. Therapeutic Goods Administration. Method of preparation: interpretation of minimal manipulation (2019). www.tga.gov.au/method-preparation-interpretation-minimal-manipulation

20. Therapeutic Goods Administration. Intended use: interpretation of homologous use (2018). www.tga.gov.au/intended-use-interpretation-homologous-use

21. Therapeutic Goods Administration. Compliance actions and outcomes (2019). www.tga.gov.au/hubs/compliance-and-enforcement/compliance-actions-and-outcomes

22. Therapeutic Goods Administration. Faecal microbiota transplant (FMT) product regulation (2020). www.tga.gov.au/sites/default/files/faecal-microbiota-transplant-fmt-product-regulation.pdf

23. Petricciani J, Hayakawa T, Stacey G, Trouvin JH, Knezevic I. Scientific considerations for the regulatory evaluation of cell therapy products. *Biologicals* 50, 20–26 (2017).

• **Provides an overview of the current regulatory landscape for cell-based interventions, and outlines the need for a global effort to develop a set of international principles to facilitate regulatory evaluation of new products.**

24. Lysaght T, Munsie M, Castricum A *et al.* A roundtable on responsible innovation with autologous stem cells in Australia, Japan and Singapore. *Cytotherapy* 20(9), 1103–1109 (2018).

•• **Key report on a roundtable event in Singapore that identifies ethical and regulatory challenges in three areas of translating stem cell-based interventions, namely cell manufacturing and processing, innovative uses of autologous cells in clinical practice and standards of evidence.**

25. Szabo L. Swedish, Mayo Clinic among hospitals plunging into lucrative stem-cell treatments. *The Seattle Times.* (2019). www.seattletimes.com/seattle-news/health/hospitals-plunge-into-market-of-experimental-injections-as-patients-try-to-avoid-surgery/

26. Therapeutic Goods Administration. Including so called 'autologous stem cell' therapies (2017). www.tga.gov.au/media-release/regulation-autologous-cell-and-tissue-products

•• **Important media release from the TGA summarizing the changes to legislation that occurred after the 2016 and 2017 consultation processes.**

27. Alliance for Regenerative Medicine. Available products (2020). https://alliancerm.org/available-products/

28. International Society for Cell and Gene Therapy. List of cell/tissue/gene products with marketing authorization in Australia (2018). https://cdn.ymaws.com/isctglobal.org/resource/resmgr/ptf2019/patient_resource_tables_webs.pdf

29. Macquarie Stem Cells. Facebook website (2020). www.facebook.com/macquariestemcells/

30. Rodwin MA. Drug advertising, continuing medical education, and physician prescribing: a historical review and reform proposal. *J. Law Med. Ethics* 38(4), 807–815 (2010).

Review

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Regenerative Medicine

Autologous fat grafting for nerve regeneration and neuropathic pain: current state from bench-to-bedside

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Despite recent advances in microsurgical techniques, functional recovery following peripheral nerve injury remains slow and inadequate. Poor peripheral nerve regeneration not only leaves patients with significant impairments, but also commonly leads to the development of debilitating neuropathic pain. Recent research has demonstrated the potential therapeutic benefits of adipose-derived stem cells, to enhance nerve regeneration. However, clinical translation remains limited due to the current regulatory burdens of the US FDA. A reliable and immediately translatable alternative is autologous fat grafting, where native adipose-derived stem cells present in the transferred tissue can potentially act upon regenerating axons. This review presents the scope of adipose tissue-based therapies to enhance outcomes following peripheral nerve injury, specifically focusing on their role in regeneration and ameliorating neuropathic pain.

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Keywords: adipose-derived stem cells • ADSCs • allodynia • autologous fat • fat grafting • nerve regeneration • neuropathic pain • stromal vascular faction • SVF

Injuries to peripheral nerves present a serious clinical problem, affecting approximately 5% of all patients presenting to level I trauma centers and often cause profound morbidity, life-long disability and chronic neuropathic pain [1,2]. To reduce these adverse sequelae of peripheral nerve injuries, surgical interventions have focused on restoring nerve continuity [3]. The surgical method of choice is the primary end-to-end repair. This approach has the potential to provide the optimal functional outcome, as long as it can be performed with little or no tension [4,5]. However, when a nerve gap or a segmental defect exists, tensionless primary repair may not be possible and nerve bridging strategies must be performed [6,7]. Nerve autografts are superior to other bridging strategies such as nerve conduits because they provide the optimal biologic scaffold for regeneration and activated Schwann cells to support the axonal regeneration [8]. Despite the advantages of this technique, nerve autografts have the inherent disadvantage of donor site numbness, limited availability and nerve diameter discrepancy [9].

Schwann cells play a crucial role in the cellular response to nerve injury and secrete several cytokines such as TNF-α and IL-1 that recruit macrophages and other immune cells to initiate a regenerative response [10,11]. Schwann cells and macrophages subsequently phagocytize degenerating myelin sheaths to prepare the local environment for regenerating axons [11,12]. Activated Schwann cells rapidly proliferate following nerve injury and provide trophic factors, extracellular matrix molecules, cell-adhesion molecules and physical guidance cues to guide nascent axons to bridge the injury site and enter the viable distal stump [13,14]. Prolonged deprivation of neuronal contact gradually reduces both the proliferative and secretive capacity of Schwann cells (Schwann cell senescence) that may eventually result in apoptosis of these cells [15]. This is of particular importance in proximal nerve injuries, where regenerating nerve fibers need to regenerate over great distances before successfully reaching their end-organs [15]. It is not surprising that exogenous transferring of freshly activated Schwann cells to the regenerating axons can enhance neural regeneration [16]. However, obtaining Schwann cells requires sacrificing functional donor nerves and requires

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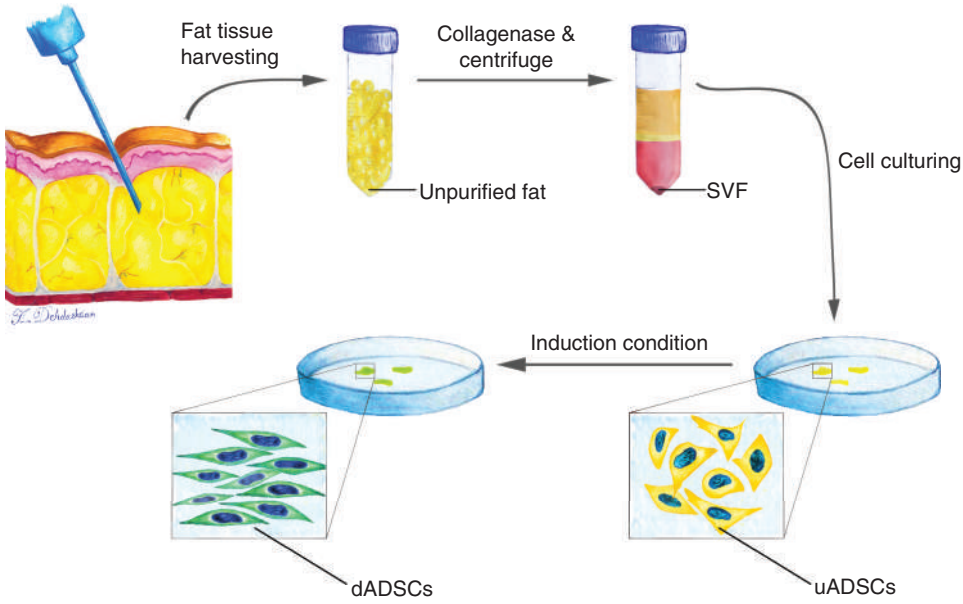

Figure 1. Subcutaneous adipose tissue is a rich source of adipose-derived stem cells that is easily accessible to harvest. Unpurified fat is used routinely in reconstructive surgeries. SVF is a concentrated product of ADSCs, which is obtained through collagenase digestion followed by centrifugation. Resuspension of SVF and then cell culture isolates purified uADSCs. Specific culture media can be used to induce phenotype change toward Schwann cell dADSCs. ADSC: Adipose-derived stem cell; dADSC: Differentiated adipose-derived stem cell; SVF: Stromal vascular fraction; uADSC: Undifferentiated adipose-derived stem cell.

extensive cell culturing process. Given the difficulty of *in vitro* cell expansion and the invasive nature of harvesting autologous Schwann cells, clinical translation of this promising technique has yet to be realized.

Over the past decade, cell therapy using mesenchymal stem cells has been shown to be a promising avenue of research to augment Schwann cell activation and enhance nerve regeneration [17,18]. Adipose-derived stem cells (ADSCs) are the most favorable mesenchymal stem cells to utilize due to both their abundancy and ease of harvest [19]. These cells can be purified, cultured and even genetically modified to enhance their efficacy for nerve regeneration [19,20]. However, one current limitation on the use of ADSCs is that the FDA classifies purified ADSCs as manufactured drugs/biologics subject to strict regulation [19,21]. Under the current guidelines, ADSCs can only be 'minimally manipulated', therefore excluding common experimental interventions such as enzymatic dissociation of adipose tissue [22]. In light of this limitation, one potential alternative is to perform autologous fat grafts to utilize native ADSCs within adipose tissue to improve nerve regeneration. In this review, we examine the fundamentals of ADSCs and fat grafting for both the enhancement of nerve regeneration and the mitigation of chronic neuropathic pain. We will further summarize the current state-of-the-art in the clinical care and the future of ADSC treatment for nerve injury.

Experimental application of adipose derived stem cells for peripheral nerve regeneration

Subcutaneous fat tissue is an abundant source of ADSCs that can be easily harvested and processed to obtain purified undifferentiated ADSCs (uADSCs) and be induced to transdifferentiate to Schwann cell-like cells (differentiated ADSCs [dADSCs]) (Figure 1) [19,23]. *In vitro* studies have suggested that dADSCs have an affinity for neuronal cells and are able to enhance neurite outgrowth and myelination [24–26]. The same is also true for uADSCs due to the constitutive secretion of a number of neurotrophic factors [27,28]. Several *in vivo* experiments in rodents have successfully demonstrated direct differentiation of ADSCs into myelinating Schwann cells with subsequent improved neuronal regeneration [19,29,30]. The engrafted dADSCs were shown to directly co-localize with regenerating axons and form myelin sheaths [29–32]. Even without direct trans-differentiation to Schwann cell phenotype, uADSCs can indirectly exert their beneficial effect on regenerating axons by secreting neurotrophic factors, angiogenic factors and immunomodulatory cytokines (Figure 2 & Table 1) [33–35].

Table 1. Experimental application of adipose-derived stem cells to enhance nerve regeneration.

Significance	Study type	Level of fat processing (animal)	Nerve injury model – repair method (animal)	Description	Specific outcome	Author (year)	Ref.
ADSCs can be induced to express Schwann cell phenotype and enhance neurite growth.	*In vitro*	dADSCs (rat)	N/A	Enhance nerve regeneration	• Myelin formation by dADSCs	Xu *et al.* (2008), Radtke *et al.* (2009), Wei *et al.* (2010), Tomita *et al.* (2013)	[24–26,28]
		uADSCs (human)			• Secretion of neurotrophic factors such as nerve growth factor, brain-derived neurotrophic factor and glial-derived neurotrophic factor.	Kalbermatten *et al.* (2011)	[27]
	In vivo	dADSCs (human) uADSCs (human)	Crushed tibial nerve – ADSC injection into the nerve(rat)		• Higher secretion of neurotrophic factor and superior neuronal survival and myelin formation following dADSCs transplantation.	Tomita *et al.* (2013)	[28]
dADSCs enhance nerve regeneration and preserve Schwann cell phenotype following transplantation	*In vivo*	dADSCs (rat) uADSCs (rat)	Facial nerve defect – conduit (rat)	Transplanted dADSCs improve: 1. Myelination and fiber diameter 2. Muscle fiber cross-sectional area 3. CMAP amplitude 4. Nerve conduction velocity 5. Functional motor outcome	• Restore vibrissae movements. • Outcomes were superior for dADSCs transplantation.	Sun *et al.* (2011)	[29]
		dADSCs (rat)	Delayed CP repair – ADSC injection into the nerve (rat)		• Re-myelination similar to the Schwann cell-transplanted group. • Superior gait outcome and CMAP amplitude compared with Schwann cell transplantation.	Tomita *et al.* (2012)	[30]
					• dADSCs increased motorneuron regeneration. • Outcomes were superior for dADSCs compared with Schwann cell-seeded conduits.	Di Summa *et al.* (2011)	[36]
						Hsieh *et al.* (2016), Di Summa *et al.* (2010)	[31,37]
					• Comparable results with Schwann cell transplantation	Schaakxs *et al.* (2015), Wang *et al.* (2012)	[38,39]
		dADSCs (human)				Syu *et al.* (2019), Hsueh *et al.* (2014)	[36,40]
		dADSCs (rat)	Sciatic nerve defect – augmented nerve allograft (rat)		• Increased survival of DRG neurons. • Enhanced sciatic nerve re-vascularization.	Masgutov *et al.* (2018)	[41]
		dADSCs (mice)	Sciatic nerve defect – conduit (mice)			Sowa *et al.* (2016)	[35]

ADSC: Adipose-derived stem cell; CMAP: Compound muscle action potential; CP: Common peroneal; dADSC: Differentiated adipose-derived stem cell; DRG: Dorsal root ganglion; FPS: Facial palsy scoring; SFI: Sciatic function index; SVF: Stromal vascular fraction; uADSC: Undifferentiated adipose-derived stem cell; VEGF: Vascular endothelial growth factor.

Table 1. Experimental application of adipose-derived stem cells to enhance nerve regeneration (cont.).

Significance	Study type	Level of fat processing (animal)	Nerve injury model – repair method (animal)	Description	Specific outcome	Author (year)	Ref.
uADSCs are the same effective as uADSCs to enhance nerve regeneration		uADSCs (rat)	Sciatic nerve defect – conduit (rat)	Transplanted uADSCs improve: 1. Myelination and fiber diameter 2. Muscle fiber cross-sectional area 3. CMAP amplitude 4. Nerve conduction velocity 5. Functional sensory-motor outcome	• Significant recovery of sensory (pinch test) and motor (toe spread score) functions.	Carriel *et al.* (2013)	[42]
		uADSCs (rat) dADSCs (rat)			Outcomes were more prominent for dADSCs transplantation.	Liu *et al.* (2020)	[43]
		uADSCs (rat) dADSCs (rat)			There were no significant differences between uADSCs and dADSCs nerve regeneration capacity.	Kim *et al.* (2014)	[44]
			Facial nerve defect – conduit (rat)		• Similar efficacy between uADSCs, dADSCs and Schwann cell-seeded conduits.	Watanabe *et al.* (2017)	[45]
		uADSCs (rat)	Sciatic nerve defect – augmented nerve allograft (rat)			Liu *et al.* (2011), Saller *et al.* (2018)	[42,46]
Similar to purified ADSCs, lower processed SVF also improves nerve regeneration	*In vivo*	SVF (rat) uADSCs (rat)	Facial nerve defect – conduit (rat)	SVF treatment improved: 1. Functional motor outcomes 2. Preserved muscle mass 3. Increased re-myelination	• Higher axon and fiber diameter in SVF treated group, whereas myelin thickness was superior in uADSC treated rats. SVF, similar to uADSCs, Increased CMAP and reduced latency.	Shimizu *et al.* (2018)	[47]
		SVF (rat)	Facial nerve defect – conduit (rat)		SVF significantly improved FPS to the level of nerve autograft.	Matsumine *et al.* (2017)	[48]
			Sciatic nerve defect – conduit (rat)		• Augmented nerve outgrowth without direct transdifferentiation of ADSCs to Schwann cells. Increased expression of Neuregulin-1 and VEGF.	Suganuma *et al.* (2012) Mohammadi, Azizi *et al.* (2012), Mohammadi, Sanaei *et al.* (2014), Mohammadi, Assadollahi *et al.* (2014)	[49–52]
Minimally manipulated fat grafting benefits nerve regeneration	*In vivo*	Pedicled fat pad (rat)	Sciatic nerve crush – fat pad wrapping (rat)	Fat grafting following nerve injury improves re-myelination, fiber density and axon count.	• Greater muscle force compared with nonfat grafted controls.	Kilic *et al.* (2013)	[53]
		Fat graft (rat)	Sciatic nerve defect – conduit (rat)		• Fat grafting enhanced sensory (pinprick) and motor recovery (SFI). • Restore nerve microtubular structure.	Tuncel *et al.* (2015)	[54]
		Fat graft (rat)	Transection of sural nerve – neurorrhaphy and fat grafting		Fat grafting reduced inflammation	Bloancă *et al.* (2017)	[55]

ADSC: Adipose-derived stem cell; CMAP: Compound muscle action potential; CP: Common peroneal; dADSC: Differentiated adipose-derived stem cell; DRG: Dorsal root ganglion; FPS: Facial palsy scoring; SFI: Sciatic function index; SVF: Stromal vascular fraction; uADSC: Undifferentiated adipose-derived stem cell; VEGF: Vascular endothelial growth factor.

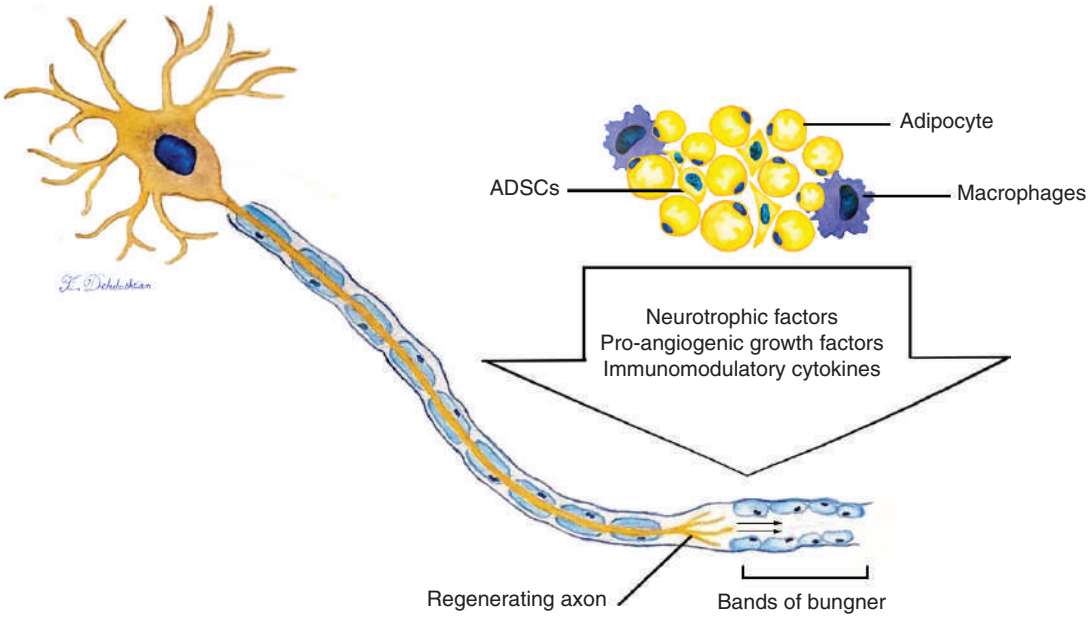

Figure 2. Schwann cells are major players in the nerve regeneration process by providing both trophic and structural support for sprouting axons. ADSCs not only supplement proliferating Schwann cells by mimicking their phenotype, but also release several trophic factors that enhance axonal growth, cellular survival and revascularization.
ADSC: Adipose-derived stem cell.

Transplantation of dADSCs in rats has been shown to have a positive impact on neural regeneration comparable to purified Schwann cells [31,36]. Researchers combined dADSCs with different conduits and acellular nerve allografts to show improved axonal regeneration, enhanced functional outcomes on walking track analysis and reduced fibrosis following repair of murine sciatic nerve defects [19,37–40,56]. In a recent study, Masgutov *et al.* augmented nerve autografts with dADSC to repair rat sciatic nerve defects and demonstrated increased neuronal survival, axonal regeneration and myelination [41]. In addition, enhanced restoration of blood flow to the nerve injury area was shown using laser Doppler microcirculation imaging.

Similar to dADSCs, the combination of both nerve gap repair and uADSC transplantation also enhances peripheral nerve regeneration [19,46]. Liu *et al.* injected uADSCs into 15 mm acellular nerve allografts to repair rat sciatic nerve defects and showed significantly improved sciatic functional index (SFI) values, sciatic nerve conduction velocity, gastrocnemius muscle mass and myelinated fiber counts [57]. Carriel *et al.* entubulated 10 mm rat sciatic nerve gaps with collagen conduits containing uADSC-hydrogel and found that this protocol significantly improved pinch test scores (sensory function), toe spread scores (motor function) and electromyographic outcomes versus acellular conduit controls [42]. Immunohistochemically, uADSCs improved Schwann cell migration, axon regeneration, remyelination and extracellular matrix organization. Saller *et al.* wrapped 20 mm autografts with uADSC-loaded hydrogels and demonstrated improved functional SFI values and preserved gastrocnemius muscle mass at 16 weeks in rats [58]. Histomorphometric analysis (myelinated fiber count, g-ratio) demonstrated improved axon regeneration and remyelination. Although some authors reported significantly better neuroregenerative results with dADSCs compared with uADSCs [29,43], others showed no significant differences in nerve regeneration or functional recovery [32,44,45]. Watanabe *et al.* compared Schwann cell-, dADSC- and uADSC-filled silicon conduits to repair 7 mm facial nerve defects in rats and found similar pro-regenerative potential with improved regeneration and remyelination in all cell-treated groups [45]. Facial palsy scoring increased longitudinally displaying a significant functional improvement at 6 and 13 weeks postoperatively.

Investigators also examined the neuroregenerative effects of adipose tissue that did not undergo intensive processing [19]. Liposuction is a common and safe procedure that can be followed by simple collagenase digestion and centrifugation of the lipoaspirate to yield stromal vascular fraction (SVF), which has a concentration of approximately 30% uncultured uADSCs [49,59]. The acquisition of SVF through this straightforward process is less time-consuming and addresses some of the safety concerns compared with purified ADSCs [19]. To examine

the neuroregenerative effects of SVF on axonal regeneration, Suganuma *et al.* entubulated 10 mm rat sciatic nerve gaps with silicone conduits and luminal SVF-hydrogel [49]. Significant increases were demonstrated in Schwann cell migration and axon regeneration versus either saline or collagen filled conduits. However, immunohistochemistry evaluation showed insignificant overlapping of newly migrated Schwann cells and transplanted ADSCs implying the paracrine effect to be responsible for the observed neuroregenerative benefits of ADSCs instead of direct Schwann cell-like differentiation. In addition, quantitative reverse transcription PCR (qRT-PCR) analysis showed significant increases in NRG1 and VEGF mRNA expression, which have both been previously shown to be involved in the remyelination process. A series of studies combined SVF therapy with 10 mm vein, artery, chitosan, silicone and allograft conduits to repair rat sciatic nerve defects [50–52]. The combination repairs improved walking track SFI, preserved gastrocnemius mass and increased myelinated fiber counts. Matsumine *et al.* bridged 7-mm rat facial nerve gaps with SVF-infused silicone conduits and showed the hybrid conduits significantly improved facial palsy scoring at 13 weeks, similar to positive control reversed nerve autografts [48]. Shimizu *et al.* expanded upon these results and added a uADSC group for comparison [47]. Both uADSCs and SVF treatment groups significantly improved histologic and physiologic parameters of neuronal regeneration compared with empty conduit controls. While the SVF treated group displayed significantly higher axon and fiber diameters in histomorphometric analysis, the uADSC treated group had higher myelin thickness and lower g-ratio. Facial nerve electrostimulation and vibrissal muscle recording demonstrated a nonsignificant higher compound muscle action potential amplitude (suggesting reinnervation) and lower latency (suggesting remyelination) in SVF treated rats, suggesting SVF to be as effective as uADSCs to enhance nerve regeneration. Nevertheless, obtaining SVF is more feasible in clinical practice without any further need of complex and time consuming cell culturing which can be performed while the patient is under the surgery.

Studying minimally manipulated adipose tissue is important for quick translation to clinical use [19]. Autologous fat grafting is a common clinical procedure for volume augmentation, contouring and tissue rejuvenation [59]. Until now, limited studies have examined the efficacy of minimally processed adipose tissue to enhance nerve regeneration. Using a rat nerve injury model, Kilic *et al.* managed sciatic nerve crush (axonotmetic) injuries with pedicled inguinal fat pad wrappings [53]. The flaps successfully increased axon regeneration, remyelination and tibialis anterior muscle force versus untreated controls at 4 weeks. While the exact mechanism was not studied, the authors attribute improved outcomes to ADSC secretions, vascular nourishment and macrophage immunomodulation. Tuncel *et al.* bilaterally operated on 10-mm rat sciatic nerve gaps and placed 3 ml of centrifuged minced fat around the surgical sites on one side [54]. The authors sub-grouped different repair methods (sham control, primary neurorrhaphy, autograft, acellular nerve allograft, conduit entubulation) and compared the fat treated side with the contralateral hind paw as control. Autologous fat grafting significantly improved pinprick sensation, walking track SFI and the damaged neuronal cytoskeleton at 12 weeks. The study similarly attributed positive outcomes to ADSC neurotrophic, pro-angiogenic and immunomodulatory signaling. Results from a more recent study combining primary neurorrhaphy with surgical site fat grafting also described improved nerve regeneration, Schwann cell migration and decreased inflammation [55].

In summary, ADSCs can be induced to express a Schwann cell phenotype and also secrete high quantities of several neurotrophic factors. Although highly processed ADSCs exert superior support for nerve regeneration, current evidence shows that residing ADSCs in the minimal manipulation of fat tissue can also benefit regenerating nerves. However, additional preclinical basic science experiments are needed to fully delineate the neuroregenerative benefits and safety information of ADSCs for possible clinical application.

Experimental application of adipose-derived stem cells for neuropathic pain

Inflammation is a prerequisite for the removal of myelin debris following a nerve injury. This robust inflammatory process eventually subsides once the Schwann cell-axon homeostatic state has been achieved [60]. A proportion of axons within the nerve trunk that do not reach their end-organs continue to express inflammatory cytokines and trophic factors for a prolonged duration [61]. This situation is dramatically increased in major extremity injuries such as amputations where the original physiologic target is no longer available [62,63]. In fact, the nerve regeneration process can be a double-edged sword in which incessant inflammatory status and upregulated neurotrophins lead to changes in both nerve fiber density and neuronal excitability, promoting the establishment of chronic neuropathic pain [60,64–66]. Enhancement of nerve regeneration following ADSC administration may not only increase the number of nerve fibers that successfully reach their targets, but may also simultaneously reduce the incidence of neuropathic pain development. In this regard, Haselbach *et al.* repaired rat sciatic nerve defects using fibrin

conduits seeded with dADSCs [67]. Autotomy, which is a hallmark behavior trait of neuropathic pain in rodents, was significantly reduced when compared with animals receiving either empty or Schwann cell-filled conduits. Similar results were shown by Lee *et al.*, who investigated the effect of intraneural and epineural injection of undifferentiated human adipose-derived stem cells (hADSC) on nerve crush injury in rats [68]. Enhanced nerve regeneration was shown in the epineurally treated nerves with a higher remyelination rate, near-normal locomotion and faster electrophysiological recovery. Notably, enhanced regeneration was associated with significantly lower sensitivity to mechanical stimuli that was correlated with a lower expression of the pain marker calcitonin gene-related peptide in the L4 dorsal root ganglion (DRG). The authors found that neuropathic pain attenuation for intraneural hADSCs injection was not as effective as epineural injection because the needle insertion created a secondary damage that was not resolved for the duration of the study.

Hyperalgesia begins with peripheral sensitization following peripheral nerve injuries. Pro-inflammatory mediators such as IL-1β and TNF-α alter ion channel expression in injured nerves, resulting in a lower depolarization threshold and ectopic neuronal discharge [69–72]. The entrapment of sprouting axons into the local fibrosis and scar tissue further aggravates the frequency of these spontaneous firings due to continuous mechanical stimulation [73–76]. Emerging evidence suggests that the inflammatory cascade following nerve injury is not confined to the local injury site, but also expands to the neuronal cell bodies in the DRGs and their secondary neurons in the dorsal horn of the spinal cord [65,77]. Rampant proliferation of macrophages in the corresponding DRGs [78] and the pathologic gliosis (both astrocytes and microglia) in the spinal cord [77,79] leads to central sensitization and enduring neuropathic pain [77]. Microglia and astrocytes actively maintain central neuro-inflammation by upregulating the expression of several pro-inflammatory genes such as *COX-2*, *iNOS*, *IL-1*, *IL-6* and *TNF-α* [77,80–82]. A recent *in vitro* study showed that ADSCs that secrete exosomes (vesicles that contain bioactive molecules) are able to suppress microglial activation and downregulate pro-inflammatory mediators with subsequent protection of neuronal cells against apoptosis and cytotoxicity [61]. ADSCs can modulate the immune response to nerve injury not only through their exosomes, but also directly through cell contact (Figure 3 & Table 2) [61,83–86]. In fact, due to the expression of specific chemokine receptors, ADSCs can selectively migrate toward the injury sites and positively impact damaged cells [20,87,88]. It has been shown that both thermal and mechanical allodynia was attenuated in murine sciatic chronic constriction injury models when ADSCs are applied directly to the nerve or given intravenously [20,89,90]. Treated animals had lower levels of IL-1β [89,90], IL-6 [90] and a higher level of IL-10 (a major anti-inflammatory cytokine) in their injured sciatic nerve [89,90]. Treatment also effectively alleviated central neuro-inflammation by reducing TNF-α, iNOS, neuronal apoptosis and astrocyte expansion in the spinal cord [20,89]. Using a mouse model of streptozotocin-induced diabetic neuropathy, Brini and colleagues showed that intravenous injection of either hADSC or purified exosomes significantly reduced thermal and mechanical allodynia within hours of a single injection [83]. Although the effect was more prominent for hADSC compared with the purified exosomes alone, booster exosome injections were shown to enhance the anti-allodynic effect to almost the same level as the hADSCs. While transplanted ADSCs were initially sequestered in filtering organs such as the lungs and liver, they migrated to the injured pancreas and sciatic nerve after 1 week. As a result, the levels of IL-1β, TNF-α and IL-6 was reduced in the sciatic nerve, L4-6 DRGs and the lumbar spinal cord. Simultaneously, decreased IL-10 levels were restored in these areas. Furthermore, calcitonin gene-related peptide expression that was previously upregulated in the DRGs, decreased significantly following ADSC therapy. Based on these studies, it can be implicated that purified ADSCs can effectively attenuate central and peripheral neuro-inflammation and therefore, mitigate neuropathic pain.

A series of *in vivo* experiments have been performed to determine if unpurified fat grafts, which contain ADSCs, can be used effectively to treat burn-induced neuropathic pain [75,91]. Third-degree hindpaw burn injury in rats causes neuropathic pain with concomitant central and peripheral sensitization [75,91,92]. In two consecutive studies [75,91], Huang *et al.* transferred 0.4 ml of autologous fat to the hind paw of rats 4 weeks after burn injury and compared results to saline-injected controls. At 1 week following administration, a significant decrease in mechanical allodynia was seen in fat grafted animals compared with the saline-injected rats. Histological analysis demonstrated successful fat grafting in this model with incorporated fat droplets within the dermis of the hind limb. In addition, the fat grafted animals had better skin texture and lower collagen density that hypothetically could reduce nerve entrapment leading to peripheral sensitization. In addition, reduced expression of iNOS, neuronal NO synthase and COX-2 were displayed in both the skin and subdermal tissue in the fat grafted animals. A further notable observation was the ability of the treatment to modulate central neuro-inflammation by suppressing microglial activation associated with downregulation of iNOS, COX-2, IL-1β and TNF-α levels. Investigators later reproduced the same results after application of purified uADSCs [92].

Table 2. Experimental application of adipose-derived stem cells for neuropathic pain conditions.

Significance	Study type	Level of fat processing (animal)	Nerve injury model – repair method (animal)	Outcomes	Specific findings	Author (year)	Ref.
ADSCs secrete bioactive molecules that modulate central neuro-inflammation	*In vitro*	N/A	N/A	Decrease activation of NF-κB and therefore reduce expression of TNF-α, IL-6, IL-1β, iNOS and COX-2	• ADSCs secrete exosomes that can inhibit microglial activation and neuronal apoptosis	Feng *et al.* (2019)	[61]
Effective nerve regeneration as a result of ADSC transplantation simultaneously reduce the development of neuropathic pain	*In vivo*	dADSCs (rat)	Sciatic nerve defect – conduit (rat)	Improved nerve regeneration subsides the inflammatory status and decreases neuronal hypersensitization	• Decreased autotomy as an index of neuropathic pain.	Haselbach *et al.* (2016)	[67]
		uADSCs (human)	Sciatic nerve crush injury – ADSCs injection into the nerve		• Lower mechanical allodynia • Lower expression of CGRP in L4 DRGs	Lee *et al.* (2015)	[68]
ADSCs Reestablish the balance between pro-inflammatory and anti-inflammatory cytokines and prevent neuropathic pain development.		uADSCs (rat)	CCI – systemic iv. injection (rat)	Due tropism toward the injured tissue and paracrine effect, ADSCs mitigate inflammation in the central and peripheral nervous system. As a result, ADSCs alleviate thermal and mechanical hypersensitivity.	• Decreased TNF-α, GFAP and apoptotic cells in the spinal cord	Forouzanfar *et al.* (2018)	[20]
			CCI – systemic ip. injection and local injection near the injury (rat)		• Increased sciatic IL-10, while decrease IL-6 and IL-1b	Mert *et al.* (2017)	[90]
		uADSCs (human)	CCI – systemic iv. injection (mice)		In the sciatic nerve: • Reduction in IL-1β within hours • Restoration of IL- 10 levels In the serum: • Increased IL-10 In the spinal cord: • Decrease iNOS expression	Sacerdote *et al.* (2013)	[89]
		uADSCs (human) ADSC secretome (human)	STZ-induced diabetic neuropathic pain – systemic iv. injection		• Both uADSCs and their secretomes were effective in alleviating neuropathic pain. • Decreased the levels of IL-1β, TNFα and IL-6, while increased IL-10 in the sciatic nerve, L4-6 DRGs and the lumbar spinal cord. • Modulated CGRP expression in the DRGs	Brini *et al.* (2017)	[83]
Minimally manipulated fat grafting express the same benefits in neuropathic pain alleviation		Fat graft (rat) uADSCs (rat)	Burn-induced neuropathic pain – fat grafting and subcutaneous cell injection (rat)	Residing ADSCs within lipoaspirate reduce central and peripheral neuroinflammation and mitigate mechanical allodynia	In the skin: • Reduced COX-2, iNOS and nNOS • Improved scar texture In the dorsal horn of the spinal cord: • Reduced iNOS, nNOS, COX2, IL-1b and TNFa • Decreased microglial activation • Attenuated apoptosis	Huang *et al.* (2014) Huang *et al.* (2015) Lin *et al.* (2018)	[75,91,92]

ADSC: Adipose-derived stem cell; CCI: Chronic constriction injury; dADSC: Differentiated adipose-derived stem cell; DRG: Dorsal root ganglion; ip.: Intraperitoneal; iv.: Intravenous; STZ: Streptozotocin; uADSC: Undifferentiated adipose-derived stem cell.

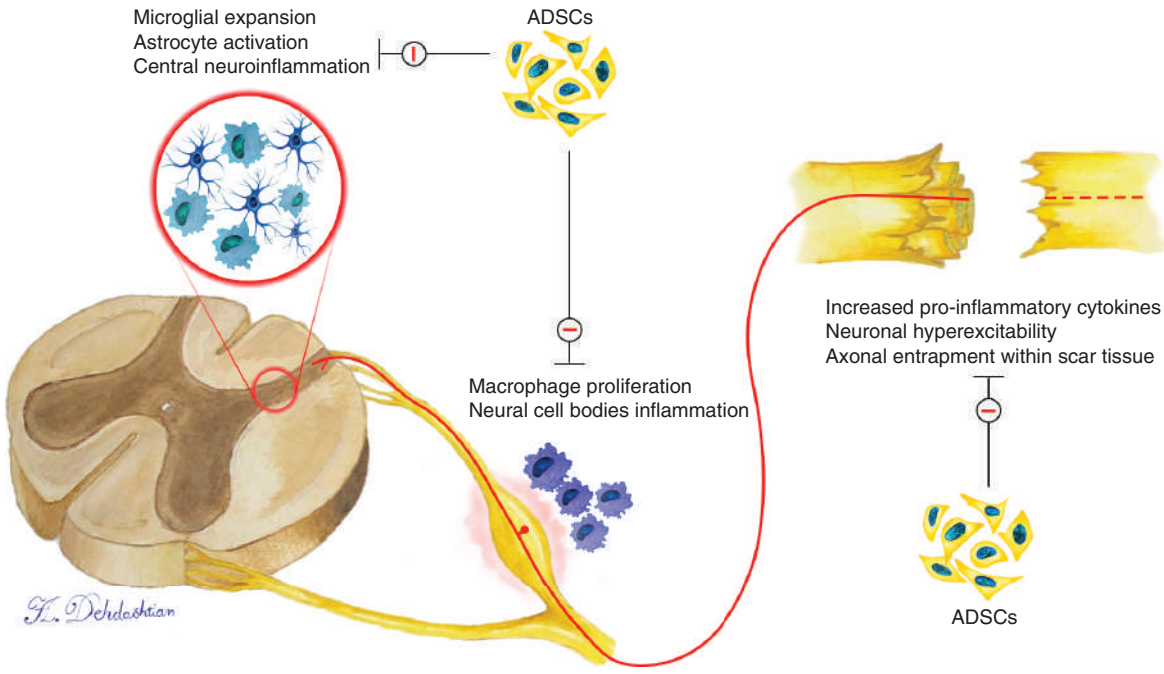

Figure 3. Profound disability and the development of neuropathic pain are two main consequences of peripheral nerve injuries. Upregulation of pro-inflammatory cytokines changes the expression of cation channels in terminal neurons that leads to ectopic neuronal discharges and peripheral hypersensitivity. Local scar tissue contraction may chronically stimulate sensitized axons and increase the frequency of ectopic discharges. Ascending inflammatory responses involve DRGs and the dorsal horn of the spinal cord with subsequent glial activation and central neuroinflammation. ADSCs not only reduce ectopic neuronal discharges by softening scar tissue but also reduce both peripheral and central neuroinflammation. ADSCs mediate enhanced nerve regeneration and secrete several immunomodulatory macromolecules to suppress the chronic inflammatory status. Immunomodulation can also suppress glial cell (astrocytes and microglia) activation, which is essential for the maintenance of neuropathic pain treatment.
ADSC: Adipose-derived stem cell; DRG: Dorsal root ganglia.

Despite limited evidence, ADSCs have shown promising results in mitigating neuropathic pain. Due to the intrinsic tropism toward the injured tissue, enhancing nerve regeneration and secreting immunomodulating factors, ADSCs can re-establish the balance between pro-inflammatory and anti-inflammatory cytokines and reduce neuronal hypersensitization. Considering the current limitation of the clinical application of highly manipulated ADSCS, the efficacy of autologous fat in this context needs to be more extensively examined.

Clinical application of adipose tissue derivatives for nerve regeneration

Despite the encouraging results of experimental studies, there have not been any Phase I clinical trials that have specifically evaluated the application of purified ADSCs for either nerve repair or neuropathic pain. While ADSC-based treatments have never been studied for any specific peripheral nerve injuries in human, Haahr *et al.* (2016) examined the use of SVF to treat erectile dysfunction (ED) following prostatectomy (RP) [93]. ED can occur following RP when the periprostatic nerves are injured or completely resected for oncologic reasons. The incidence of ED has been reported to be as high as 86% and its treatment has been suboptimal utilizing current medical therapies [94]. In this study, 17 patients with post-RP ED received SVF injection into their corpus cavernosum. Outcomes were measured at 1, 3 and 6 months postoperatively using the erectile hardness score and the international index of erectile function-5 questionnaire. Following the SVF treatment, eight patients (47%) regained erectile function sufficient for penetration during intercourse. Among the patients who did not improve, six (35%) of them had urinary incontinence indicating either more advanced pre-operative neurovascular degeneration or more extensive neurovascular injury. Following these results, the same group of researchers recruited four more patients and extended the study to 12 months [95]. Neither newly recruited patients nor patients with urinary incontinence showed any marked improvement after 12 months. Although newly recruited patients demonstrated urinary continence, they had a history of ED before RP surgery and the authors therefore suggested that the treatment

failure in this group was due to their underlying medical condition. Despite these promising results, some caveats need to be considered. First, the study was not blinded and the control group was not present for an accurate comparison since the spontaneous recovery of ED is rarely achieved. Second, all measurements were conducted by subjective tools, which could be biased depending on patients and investigators expectations for the cellular therapy. Indeed, any conclusion from this data warrants a more extensive randomized controlled trial.

Clinical application of adipose tissue derivatives for neuropathic pain

Chronic neuropathic pain affects 7–10% of the world's population and is unfortunately refractory to the majority of current medical therapies [62,96]. The neuroregenerative and immunomodulatory effects of ADSCs in animal studies has been nicely demonstrated. Based on this, translational scientists and surgeons have explored the use of SVF and autologous fat grafting to treat neuropathic pain conditions (Table 3) [97]. Postmastectomy pain syndrome (PMPS) is a chronic neuropathic pain condition that is thought to arise from damage to the breast nerve fibers during mastectomy, lumpectomy or adjuvant radiation therapy [98]. Caviggioli *et al.* assessed pain using the visual analog scale (VAS) and showed that autologous fat grafting successfully reduced PMPS in 63 mastectomy patients after 1 year (VAS reduction from 3.23 ± 2.96 to 1.04 ± 2.71) compared with 35 medically managed controls (VAS reduction of 1.04 ± 2.71, mean difference of 2.19, $p = 0.0005$). The improvement in pain was sufficient to allow 28 patients (44%) to stop using any pain medications [99]. In a second study, they examined the impact of fat grafting on pain in patients with a prior history of lumpectomy and radiotherapy [100]. At 10 months postoperatively on average, all 57 patients treated with fat grafting experienced significant pain alleviation (VAS reduction 3.1 ± 2.7) compared with the 35 control patients (VAS reduction of 0.9 ± 0.6, mean difference = 2.2, $p \leq 0.005$). However, the randomization process for both studies was ambiguous, which may potentially bias the results due to confounding-by-indication. While each study may not separately provide an unbiased answer, the same authors later published a review paper using the pooled data of both studies and clarified that the pain differences between the treatment and the control groups was not statistically significant before lipotransfering [101].

The first randomized controlled clinical trial assessing the efficacy of fat grafting for PMPS was conducted by Juhl *et al.* [102]. A total of 15 patients with a prior history of unilateral mastectomy without reconstruction were enrolled in the study. Patient reported outcomes were measured at 3 and 6 months following the initial intervention and included the VAS pain score, neuropathic pain symptom inventory and DoloTest (to assess the quality of life during pain). Fat grafted patients (n = 8) displayed a significant improvement in their pain (average VAS reduction of 54.9%) and quality of life measures 3 and 6 months after the intervention ($p \leq 0.001$ for all measurements). However, several limiting factors of this study must be considered, including both the limited number of patients and the study being nonblinded. Cogliandro *et al.* later studied PMPS in postmastectomy implant-based breast reconstruction in 46 patients who underwent fat grafting and 24 nonfat grafted controls with an average follow-up of 2.5 years [103]. Using the BREAST-Q questionnaire, a validated survey instrument that is specifically designed to measure patient-reported outcomes after breast surgery, investigators showed that 1 year following the treatment, the shooting neuropathic pain was subsided in 33 (72%) fat-grafted patients and seven (29%) controls ($p < 0.001$). However, the treatment did not significantly affect the pain of the arm, shoulder, upper back or neck.

Fat grafting has also been examined in the management of symptomatic end-neuromas. In 2013, Vaienti and colleagues published a retrospective case series showing a modest beneficial effect of fat grafting in painful neuromas of the upper extremity [104]. Eight patients whose neuroma pain was not responsive to medical therapy or had relapsed despite prior surgery were treated with a combination of neuroma excision and fat grafting. Outcomes were measured using the disabilities of the arm shoulder and hand (DASH) questionnaire, as well as VAS for pain at baseline, 2, 6 and 12 months postsurgery. 2 months postoperatively pain started to decrease while both DASH and VAS scores were significantly reduced after 6 months (-47%, $p = 0.028$; and -50%, $p = 0.017$, respectively). Surprisingly, pain relapsed at 12 months follow-up. Most recently, the same group published an extended case series of 26 patients who were evaluated for 24 months [105]. In addition to VAS and DASH scores, quality of life was also evaluated using the WHO Quality of Life (WHOQOL-BREF) assessment tool. They observed a significant decrease in VAS score (4 points, 95% CI 1.4–4.1, $p < 0.001$), an improvement in DASH score (15 points, 95% CI 8.2–27.7, $p < 0.001$) and also an improvement in every index of quality-of-life for the duration of the study. Pain was completely resolved in two patients (VAS = 0), although it was shown to become worse in three patients. While the authors attributed the relapse of neuroma pain in their previous study to fat resorption, the conclusion regarding the efficacy of this approach is limited. In a retrospective study, Calcagni *et al.* examined the use of SVF-enriched fat grafting in the management of end-neuroma of the superficial branch of the radial

Table 3. Clinical application of adipose-derived stem cells for nerve regeneration and neuropathic pain.

	Author (year)	Study design	Indication	n of subjects	Fat treatment	Description	Follow-up (months)	Outcomes	Ref.
Nerve regeneration	Haahr *et al.* (2016)	CT (single arm)	Post-RP ED	17 (T = 17, C = 0)	SVF	Injection into the corpus cavernosum	6	Sufficient erection in eight (47%) Six patients (35%) had pre-RP urinary incontinency indicating an extensive neurovascular injury	[93]
	Haahr *et al.* (2018)			21 (T = 21, C = 0)		Recruitment of four more patients and extension of the study to 12 months	12	Sufficient erection in eight (38%) Newly recruited patients had pre-RP ED	[95]
Neuropathic pain	Caviggioli *et al.* (2011)	CT (nonrandomized)	PMPS	98 (T = 63, C = 35)	AF	Medical management in controls	12	VAS was decreased 68% in T group (3.23 ± 2.96 to 1.04 ± 2.71, $p = 0.0005$) and 28 patients (44%) stopped analgesic use	[99]
	Maione *et al.* (2014)	CT (nonrandomized)	PMPS	92 (T = 57, C = 35)	AF	Patients had prior history of lumpectomy and radiation	10	Significant VAS reduction in T group compared with the C group (3.1 ± 2.7 vs 0.9 ± 0.6, $p \leq 0.005$)	[100]
	Caviggioli *et al.* (2016)	CT	PMPS	190 (T = 120, C = 70)	AF	Pooled data from both previous studies	12	VAS reduction of 3.19 ± 2.86. There was no significant difference in pretreatment VAS between T and C groups 48 patients (40%) stopped analgesic use	[101]
	Juhl *et al.* (2016)	RCT	PMPS	15 (T = 8, C = 7)	AF	Control group did not receive any surgical intervention	6	54% VAS reduction ($p < 0.001$), 54% NPSI reduction ($p < 0.001$), 49% DoloTest reduction ($p < 0.001$)	[102]
	Cogliandro *et al.* (2017)	CT (nonrandomized)	PMPS	70 (T = 46, C = 24)	AF	Pain was assessed using BREAST-Q	30	Significant pain reduction in the muscles of the chest and breast area sharp and Shooting pains (all with a $p < 0.01$) Treatment did not benefit the pain of the neck, upper back, arm, shoulder and rib sites	[103]
	Vaineti *et al.* (2013)	CS (retrospective)	Neuroma	8	AF	Neuroma excision followed by fat grafting	12	At 6 months: • VAS reduction of 47% ($p = 0.028$) • DASH reduction of 50 % ($p = 0.017$) Pain relapsed at 12 months	[104]

ADSC: Adipose-derived stem cell; AF: Autologous fat; AKA: Above-knee amputations; C: Control; CS: Case series; CT: Clinical trial; DASH: Disabilities of the arm, shoulder and hand; ED: Erectile dysfunction; NPSI: Neuropathic pain symptom inventory; PMPS: Postmastectomy pain syndrome; PROMIS: Patient-Reported Outcomes Measurement Information System; RCT: Randomized controlled trial; RP: Radical prostatectomy; SVF: Stromal vascular fraction; T: Treatment; VAS: Visual analogue scale; WHOQOL-BREF: WHO Quality of Life: Brief Version.

Table 3. Clinical application of adipose-derived stem cells for nerve regeneration and neuropathic pain (cont.).

Author (year)	Study design	Indication	n of subjects	Fat treatment	Description	Follow-up (months)	Outcomes	Ref.
Vaienti *et al.* (2020)	CS (retrospective)	Neuroma	26	AF	Neuroma excision followed by fat grafting	24	VAS reduction of 50% ($p < 0.001$) DASH improvement of 31% ($p < 0.001$) Improvement in all domains of WHOQOL-BREF VAS score deteriorated in three patients (12%)	[105]
Calcagni *et al.* (2016)	CS (retrospective)	Neuroma	5	SVF enriched AF	5 ml of SVF and 2 ml of the lipid fraction Neuroma excision followed by fat grafting	36	36% VAS reduction in all patients ($p = 0.104$) No relapse of pain at any study time points	[106]
Zimmermann *et al.* (2018)	CS (retrospective)	Neuroma	10 (T = 5, C = 5)	SVF enriched AF	Control group received intramuscular transposition instead of fat grafting	36	At 2 months: • 50% VAS reduction in T ($p = 0.102$) • 93% VAS reduction in C ($p = 0.034$) At 6 months: • 57% VAS reduction in T ($p = 0.102$) • 28% VAS reduction in C ($p = 1.02$), showing a relapsed pain At 36 months: • 50% VAS reduction in T ($p = 0.066$) • 42% VAS reduction in C ($p = 0.194$) Pain relapse in C can be due to neuroma regrowth	[107]
Bourne *et al.* (2018)	CT (single arm)	Neuroma	5	SVF enriched AF	Patients with history of AKA and refractory pain	24	50% decrease in VAS score ($p = 0.18$) Improved skin quality and $61.5 \pm 24.1\%$ graft volume retention	[108]
Fredman *et al.* (2016)	CS (retrospective)	Burn-induced neuropathic pain	7	AF	Pain was refractory to the previous surgical and medical treatments	12	Six patients showed significant improvement in pain (59% PROMIS score reduction, $p < 0.001$) Tinel signs were disappeared	[109]

ADSC: Adipose-derived stem cell; AF: Autologous fat; AKA: Above-knee amputations; C: Control; CS: Case series; CT: Clinical trial; DASH: Disabilities of the arm, shoulder and hand; ED: Erectile dysfunction; NPSI: Neuropathic pain symptom inventory; PMPS: Postmastectomy pain syndrome; PROMIS: Patient-Reported Outcomes Measurement Information System; RCT: Randomized controlled trial; RP: Radical prostatectomy; SVF: Stromal vascular fraction; T: Treatment; VAS: Visual analogue scale; WHOQOL-BREF: WHO Quality of Life: Brief Version.

nerve [106]. Although not statistically significant, all five patients who underwent neuroma excision combined with SVF-enriched fat grafting displayed a continuous attenuation of pain for 36 months without relapses at any follow-up time points (VAS reduction from 2.2 ± 0.97 to 1.4 ± 1.26, p = 0.104). This group recently published a study comparing these patients to another group of patients who underwent neuroma excision and intramuscular transposition [107]. 2 months following surgeries, investigators observed that pain was significantly reduced in the transposition group (VAS score from 2.8 ± 0.45 to 0.2 ± 0.45, p = 0.034) while the fat grafted group showed a nonsignificant reduction in pain (VAS score from 2.8 ± 0.45 to 1.4 ± 1.52, p = 0.102). Most interestingly, at 6-month follow-up, pain had relapsed in the transposition group, which was possibly a consequence of recurrent neuroma formation within the muscle. Thus, the authors suggested that the sustained mitigation of neuroma pain for 36 months in the SVF-enriched fat grafted group is not only due to mechanical padding on the neuroma bulb, but was also related to the biologic immunomodulatory effect of ADSCs.

One of the main reasons that patients with major limb amputations abandon their prosthetic limbs is neuroma-related residual limb pain [110]. In addition, patients can have unstable scars, thin soft tissues and inadequate bone coverage of their residual limb that can lead to erosions, ulcerations and wound breakdown on their residual limb [108]. Fat grafting can be very helpful for these patients with poorly fitting prostheses, insufficient soft tissue and prominent bone by adding additional soft tissue volume in the desired areas [111]. Recently, Bourne *et al.* described a prospective pilot cohort study using SVF-enriched fat grafting to restore soft tissue volume and reduce residual limb pain in five military personnel with prior bilateral above-knee amputations [108]. At the 2-year follow-up, the authors found that mean VAS scores for pain decreased from 3.0 ± 2.5 to 1.2 ± 1.6 (p = 0.18). All patients had improvement in the skin quality and soft tissue volume showing 61.5 ± 24.1% volume retention of grafts in CT scan assessments. Therefore, they suggested that both the biologic and cushioning effects of grafted fat may reduce overall residual limb pain.

Neuroma formation, as well as nerve entrapment within fibrotic tissue, have been suggested as possible mechanisms of burn-induced neuropathic pain [112,113]. Autologous fat grafting has been previously shown to be a safe and effective modality to improve burn scar quality [114]. In 2016, Fredman *et al.* performed two sessions of fat grafting, separated by 2 months, on seven burn patients whose scar pain persisted despite prior medical and surgical interventions [109]. Pain intensity was assessed using the Patient-Reported Outcomes Measurement Information System (PROMIS) on a scale of 1–5, in which a higher number represents more severe pain. All patients, with the exception of one, had a significant improvement in pain intensity 1 year after the fat grafting procedure (PROMIS score decreased from 3.4 ± 1.3 to 1.4 ± 1.3, $p < 0.001$). Multiple Tinel signs (suggestive of painful neuromas) that were present pre-operatively disappeared in all seven patients. However, Tinel test has a widely variable sensitivity (49–84%) [115] and the low number of patients enrolled in the study can bias the study.

Conclusion

Autologous unpurified adipose tissue grafts innately possess native ADSCs, whose differentiative and proliferative qualities may enhance nerve regeneration and attenuate subsequent neuropathic pain. Minimally invasive structural fat grafting is commonly used in different reconstructive surgeries, rejuvenation, and more recently, in healing of radiotherapy-induced tissue damage and other restrictive scar formation. The aforementioned clinical studies presented in this review have provided preliminary insight regarding the benefits of fat grafting as a potential treatment for neuropathic pain. Although these results are encouraging, future randomized clinical trials are required to fully elucidate the efficacy of both SVF transfer and fat grafting for neuropathic pain.

Future perspective

Significant research needs to be conducted in order to understand whether the clinical application of purified ADSCs in humans is safe and effective. Since the discovery of ADSCs, many private clinics worldwide have used ADSCs to treat a wide variety of medical conditions. Although most of these procedures have been performed with limited complications, there have unfortunately been reports of blindness, teratoma formation and even death [116]. Subsequently, purified stem cell therapy is highly regulated by the FDA and its application is prohibited except for specific conditions such as hematopoiesis after chemotherapy [22]. Because of a shift toward SVF application in US private clinics, the FDA explicitly declared that the enzymatic disruption or ultrasonic cavitation of fat tissue for obtaining SVF is considered as 'more than minimal manipulation', which makes it unapproved for human use [22,117]. It is worth mentioning that different countries have different regulatory standpoints. Similar to the FDA, the EMA considers ADSCs and nonhomologous application of SVF as advanced therapy medicine products

and is therefore not approved for current clinical use [118]. Fortunately, several clinical trials have already begun to evaluate the safety of different mesenchymal stem cells and their long term results may eventually change the paradigm of the current treatment for incurable diseases [119–122]. In addition, advancements in the science and technology with subsequent development of newer tissue processing techniques will eventually lead to safer isolation of stem cells for clinical application. However, in the present state, only fat grafting with minimal manipulation is compatible with the FDA guidelines.

When Zuk and colleagues originally described the presence of ADSCs in lipoaspirate, it led to a new interpretation of fat grafting as a vehicle for stem cell transplantation [123]. Currently, fat grafting is performed not only for aesthetic purposes but also as a regenerative tool at the cellular level [124]. Adipose tissue yields 5000 ADSCs per each gram, which is almost tenfold higher than the number of mesenchymal stem cells residing in the same amount of bone marrow [125]. Whether autologous fat grafting can be as effective as purified ADSCs to enhance nerve regeneration has yet to be answered. Furthermore, different methods of preparation of unpurified fat grafts are associated with varying levels of cell survival and volume retention [59]. Due to cellular apoptosis after transplantation, it also needs to be clarified whether or not ADSCs that survive are sufficient to exert any beneficial effects on nerve regeneration.

Current clinical studies focusing on ADSCs are mostly retrospective, underpowered, single centered and lack randomization. Although many have shown the potential benefits of autologous fat grafting, the lack of a placebo-treated control group limits the value of the studies. While it might be ethically challenging to perform placebo based surgery, comparing fat grafting with conventional care may be a reasonable approach. Also, as described earlier in this review, suboptimal nerve regeneration leads to neuropathic pain development, whereas, augmented regenerative response may be able to prevent neuropathic pain. Therefore, neuropathic pain and nerve regeneration are two intertwined subjects that should be addressed together. Nevertheless, many of the current published studies have predominantly focused on one aspect and neglected to comment on the other. Hypothetically, ADSCs can secrete trophic factor and several anti-inflammatory mediators to simultaneously enhance neural regeneration and prevent neuropathic pain. In addition, the variation of timing, animal injury models and the route of administration of ADSCs makes the inferences drawn extremely difficult to interpret. In our opinion, it is a reasonable strategy if future research efforts simultaneously aim to: provide sufficient safety evidence for clinical application of purified ADSCs, and investigate the efficacy of alternative approaches that are already proven to be safe, to enhance nerve regeneration and reduce the development of neuropathic pain. Although limited, the current evidence is promising to show that fat grafting can be a safe alternative for stem cell transplantation with the major advantage of being immediately translatable to the clinic. However, comprehensive experimental studies are required to indicate its benefits to be comparable to that of transplanted purified ADSCs.

Executive summary

Peripheral nerve injury consequences

- Despite the intrinsic capacity of injured peripheral nerves to regenerate, functional recovery is often slow, incomplete and associated with chronic neuropathic pain development.
- Slow neuro-regeneration process leaves distal targets to be denervated for a great length of time and eventually, the optimum period for reinnervation will be lost.
- Suboptimal neuronal regeneration leads to an incessant inflammatory status that initiates peripheral and central neurosensitization. As a result, neuropathic pain develops and is maintained over time.

Application of ADSCs as a strategy to combat nerve injury sequelae in experimental studies

- Adipose-derived stem cells (ADSCs) are potentially capable to acquire mesenchymal-lineaged Schwann cell phenotype and provide trophic and structural support for regenerating axons.
- Either through direct cell contact or secreting immunomodulatory macromolecules, ADSCs can suppress peripheral and central neuroinflammation and attenuate neuropathic pain.

Challenges for clinical translation & possible solutions

- Due to uncertainties regarding the safety of ADSCs, their clinical application is strictly controlled by the US FDA. However, minimally manipulated fat tissue is currently exempted from FDA regulatory burdens.
- Autologous fat grafting is known as a safe vehicle to effectively transfer ADSCs in reconstructive surgeries.

Experimental & clinical evidence regarding the application of minimally manipulated adipose tissue to enhance nerve regeneration & mitigate neuropathic pain

- Although limited, fundamental studies have shown that supplementing a nerve repair site with lipoaspirates or simply wrapping a fat pad around crushed nerves can enhance nerve regeneration in rodents. It has also been reported that fat grafting of burn scars in rats successfully reduced both neuronal inflammation and neuropathic pain. Unfortunately, despite the close association of nerve regenerative processes and neuropathic pain development, most studies only focused on one aspect and neglected the other.
- There have been reports showing that the clinical application of stromal vascular fraction significantly improves radical prostatectomy induced erectile dysfunction resulting from disruption of periprostatic nerve fibers.
- Surgeons have also clinically utilized fat grafting in several neuropathic pain conditions and have demonstrated satisfactory mitigation of neuropathic pain in postmastectomy pain syndrome, symptomatic end-neuromas and burn-induced neuropathic pain.
- Limitations of current studies include the retrospective nature of the report, low power, poor randomization and the lack of placebo controls. Extensive randomized controlled clinical trials are therefore required to substantiate the current literature.

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The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Foster CH, Karsy M, Jensen MR, Guan J, Eli I, Mahan MA. Trends and cost-analysis of lower extremity nerve injury using the National Inpatient Sample. *Neurosurgery* 85(2), 250–256 (2019).
2. Taylor CA, Braza D, Rice JB, Dillingham T. The incidence of peripheral nerve injury in extremity trauma. *Am. J. Phys. Med. Rehabil.* 87(5), 381–385 (2008).
3. Dahlin L. Techniques of peripheral nerve repair. *Scand. J. Surg.* 97(4), 310–316 (2008).
4. Schmidhammer R, Zandieh S, Hopf R *et al.* Alleviated tension at the repair site enhances functional regeneration: the effect of full range of motion mobilization on the regeneration of peripheral nerves—histologic, electrophysiologic and functional results in a rat model. *J. Trauma Acute Care Surg.* 56(3), 571–584 (2004).
5. Yi C, Dahlin LB. Impaired nerve regeneration and Schwann cell activation after repair with tension. *Neuroreport* 21(14), 958–962 (2010).
6. Dahlin LB. The role of timing in nerve reconstruction. *Int. Rev. Neurobiol.* 109, 151–164 (2013).
7. Grinsell D, Keating C. Peripheral nerve reconstruction after injury: a review of clinical and experimental therapies. *BioMed Res. Int.* 2014, 13 (2014).

8. Fowler JR, Lavasani M, Huard J, Goitz RJ. Biologic strategies to improve nerve regeneration after peripheral nerve repair. *J. Reconstr. Microsurg.* 31(04), 243–248 (2015).
9. Kubiak CA, Kung TA, Brown DL, Cederna PS, Kemp SW. State-of-the-art techniques in treating peripheral nerve injury. *Plast. Reconstr. Surg.* 141(3), 702–710 (2018).
10. Wei Z, Fei Y, Su W, Chen G. Emerging role of Schwann cells in neuropathic pain: receptors, glial mediators and myelination. *Front. Cell. Neurosci.* 13, 116 (2019).
11. Dubový P. Wallerian degeneration and peripheral nerve conditions for both axonal regeneration and neuropathic pain induction. *Ann. Anat.* 193(4), 267–275 (2011).
12. Kuffler DP. Mechanisms for reducing neuropathic pain. *Mol. Neurobiol.* 57(1), 67–87 (2020).
13. Frostick SP, Yin Q, Kemp GJ. Schwann cells, neurotrophic factors and peripheral nerve regeneration. *Microsurgery* 18(7), 397–405 (1998).
14. Gordon T. Nerve regeneration: understanding biology and its influence on return of function after nerve transfers. *Hand Clin.* 32(2), 103–117 (2016).
15. Sulaiman OA, Gordon T. Role of chronic Schwann cell denervation in poor functional recovery after nerve injuries and experimental strategies to combat it. *Neurosurgery* 65(Suppl. 4), A105–A114 (2009).
16. Lopez-Verrilli MA, Picou F, Court FA. Schwann cell-derived exosomes enhance axonal regeneration in the peripheral nervous system. *Glia* 61(11), 1795–1806 (2013).
17. Li R, Liu Z, Pan Y, Chen L, Zhang Z, Lu L. Peripheral nerve injuries treatment: a systematic review. *Cell Biochem. Biophys.* 68(3), 449–454 (2014).
18. Kubiak CA, Grochmal J, Kung TA, Cederna PS, Midha R, Kemp SW. Stem-cell–based therapies to enhance peripheral nerve regeneration. *Muscle Nerve* 61(4), 449–459 (2020).
19. Walocko FM, Khouri Jr RK, Urbanchek MG, Levi B, Cederna PS. The potential roles for adipose tissue in peripheral nerve regeneration. *Microsurgery* 36(1), 81–88 (2016).
20. Forouzanfar F, Amin B, Ghorbani A *et al.* New approach for the treatment of neuropathic pain: fibroblast growth factor 1 gene-transfected adipose-derived mesenchymal stem cells. *Eur. J. Pain* 22(2), 295–310 (2018).
21. Faroni A, Terenghi G, Reid AJ. Adipose-derived stem cells and nerve regeneration: promises and pitfalls. *Int. Rev. Neurobiol.* 108, 121–136 (2013).
22. Bora P, Majumdar AS. Adipose tissue-derived stromal vascular fraction in regenerative medicine: a brief review on biology and translation. *Stem Cell. Res. Ther.* 8(1), 145 (2017).
23. Dezawa M, Takahashi I, Esaki M, Takano M, Sawada H. Sciatic nerve regeneration in rats induced by transplantation of *in vitro* differentiated bone-marrow stromal cells. *Eur. J. Neurosci.* 14(11), 1771–1776 (2001).
24. Xu Y, Liu Z, Liu L *et al.* Neurospheres from rat adipose-derived stem cells could be induced into functional Schwann cell-like cells *in vitro*. *BMC Neurosci.* 9(1), 21 (2008).
25. Radtke C, Schmitz B, Spies M, Kocsis J, Vogt P. Peripheral glial cell differentiation from neurospheres derived from adipose mesenchymal stem cells. *Int. J. Dev. Neurosci.* 27(8), 817–823 (2009).
26. Wei Y, Gong K, Zheng Z *et al.* Schwann-like cell differentiation of rat adipose-derived stem cells by indirect co-culture with Schwann cells *in vitro*. *Cell Prolif.* 43(6), 606–616 (2010).
27. Kalbermatten DF, Schaakxs D, Kingham PJ, Wiberg M. Neurotrophic activity of human adipose stem cells isolated from deep and superficial layers of abdominal fat. *Cell Tissue Res.* 344(2), 251–260 (2011).
28. Tomita K, Madura T, Sakai Y, Yano K, Terenghi G, Hosokawa K. Glial differentiation of human adipose-derived stem cells: implications for cell-based transplantation therapy. *Neuroscience* 236, 55–65 (2013).
29. Sun F, Zhou K, Mi W-J, Qiu J-H. Combined use of decellularized allogeneic artery conduits with autologous transdifferentiated adipose-derived stem cells for facial nerve regeneration in rats. *Biomaterials* 32(32), 8118–8128 (2011).
30. Tomita K, Madura T, Mantovani C, Terenghi G. Differentiated adipose-derived stem cells promote myelination and enhance functional recovery in a rat model of chronic denervation. *J. Neurosci. Res.* 90(7), 1392–1402 (2012).
31. Di Summa PG, Kingham PJ, Raffoul W, Wiberg M, Terenghi G, Kalbermatten DF. Adipose-derived stem cells enhance peripheral nerve regeneration. *J. Plast. Reconstr. Aesthet. Surg.* 63(9), 1544–1552 (2010).
32. Orbay H, Tobita M, Mizuno H. Mesenchymal stem cells isolated from adipose and other tissues: basic biological properties and clinical applications. *Stem Cells Int.* 2012, 461718 (2012).
33. J Salgado A, L Reis R, Sousa N, M Gimble J. Adipose tissue derived stem cells secretome: soluble factors and their roles in regenerative medicine. *Curr. Stem Cell Res. Ther.* 5(2), 103–110 (2010).
34. Kapur SK, Katz AJ. Review of the adipose derived stem cell secretome. *Biochimie* 95(12), 2222–2228 (2013).

35. Sowa Y, Kishida T, Imura T *et al.* Adipose-derived stem cells promote peripheral nerve regeneration *in vivo* without differentiation into Schwann-like lineage. *Plast. Reconstr. Surg.* 137(2), 318e–330e (2016).

36. Di Summa PG, Kalbermatten DF, Pralong E, Raffoul W, Kingham PJ, Terenghi G. Long-term *in vivo* regeneration of peripheral nerves through bioengineered nerve grafts. *Neuroscience* 181, 278–291 (2011).

37. Hsieh S-C, Chang C-J, Cheng W-T, Tseng T-C, Hsu S-H. Effect of an epineurial-like biohybrid nerve conduit on nerve regeneration. *Cell Transplant.* 25(3), 559–574 (2016).

38. Schaakxs D, Kalbermatten DF, Pralong E, Raffoul W, Wiberg M, Kingham PJ. Poly-3-hydroxybutyrate strips seeded with regenerative cells are effective promoters of peripheral nerve repair. *J. Tissue Eng. Regen. Med.* 11(3), 812–821 (2017).

39. Wang Y, Zhao Z, Ren Z *et al.* Recellularized nerve allografts with differentiated mesenchymal stem cells promote peripheral nerve regeneration. *Neurosci. Lett.* 514(1), 96–101 (2012).

40. Syu W-Z, Hueng D-Y, Chen W-L, Chan JY-H, Chen S-G, Huang S-M. Adipose-derived neural stem cells combined with acellular dermal matrix as a neural conduit enhances peripheral nerve repair. *Cell Transplant.* 28(9-10), 1220–1230 (2019).

41. Masgutov R, Masgutova G, Mukhametova L *et al.* Allogenic adipose derived stem cells transplantation improved sciatic nerve regeneration in rats: autologous nerve graft model. *Front. Pharmacol.* 9, 86 (2018).

42. Carriel V, Garrido-Gómez J, Hernández-Cortés P *et al.* Combination of fibrin-agarose hydrogels and adipose-derived mesenchymal stem cells for peripheral nerve regeneration. *J. Neural Eng.* 10(2), 026022 (2013).

43. Liu Y, Dong R, Zhang C *et al.* Therapeutic effects of nerve leachate-treated adipose-derived mesenchymal stem cells on rat sciatic nerve injury. *Exp. Ther. Med.* 19(1), 223–231 (2020).

44. Kim D-Y, Choi Y-S, Kim S-E *et al. In vivo* effects of adipose-derived stem cells in inducing neuronal regeneration in Sprague-Dawley rats undergoing nerve defect bridged with polycaprolactone nanotubes. *J. Korean Med. Sci.* 29(Suppl. 3), S183–S192 (2014).

45. Watanabe Y, Sasaki R, Matsumine H, Yamato M, Okano T. Undifferentiated and differentiated adipose-derived stem cells improve nerve regeneration in a rat model of facial nerve defect. *J. Tissue Eng. Regen. Med.* 11(2), 362–374 (2017).

46. Zhang R, Rosen JM. The role of undifferentiated adipose-derived stem cells in peripheral nerve repair. *Neural Regen. Res.* 13(5), 757 (2018).

47. Shimizu M, Matsumine H, Osaki H *et al.* Adipose-derived stem cells and the stromal vascular fraction in polyglycolic acid-collagen nerve conduits promote rat facial nerve regeneration. *Wound Repair Regen.* 26(6), 446–455 (2018).

48. Matsumine H, Numakura K, Climov M, Watanabe Y, Giatsidis G, Orgill DP. Facial-nerve regeneration ability of a hybrid artificial nerve conduit containing uncultured adipose-derived stromal vascular fraction: an experimental study. *Microsurgery* 37(7), 808–818 (2017).

- **While SVF contains a lower concentration of ADSCs, it was still able to improve nerve regeneration similar to the purified and cultured ADSCs in rats.**

49. Suganuma S, Tada K, Hayashi K *et al.* Uncultured adipose-derived regenerative cells promote peripheral nerve regeneration. *J. Orthop. Sci.* 18(1), 145–151 (2013).

50. Mohammadi R, Azizi S, Delirezh N, Hobbenaghi R, Amini K. Transplantation of uncultured omental adipose-derived stromal vascular fraction improves sciatic nerve regeneration and functional recovery through inside-out vein graft in rats. *J. Trauma Acute Care Surg.* 72(2), 390–396 (2012).

51. Mohammadi R, Sanaei N, Ahsan S, Rostami H, Abbasipour-Dalivand S, Amini K. Repair of nerve defect with chitosan graft supplemented by uncultured characterized stromal vascular fraction in streptozotocin induced diabetic rats. *Int. J. Surg.* 12(1), 33–40 (2014).

52. Mohammadi R, Asadollahi A, Amini K. Uncultured undifferentiated adipose-derived nucleated cell fractions combined with inside-out artery graft accelerate sciatic nerve regeneration and functional recovery. *Int. J. Oral Maxillofac. Surg.* 43(9), 1161–1168 (2014).

53. Kilic A, Ojo B, Rajfer RA *et al.* Effect of white adipose tissue flap and insulin-like growth factor-1 on nerve regeneration in rats. *Microsurgery* 33(5), 367–375 (2013).

54. Tuncel U, Kostakoglu N, Turan A *et al.* The effect of autologous fat graft with different surgical repair methods on nerve regeneration in a rat sciatic nerve defect model. *Plast. Reconstr. Surg.* 136(6), 1181–1191 (2015).

- **Fat grafting enhanced nerve regeneration with subsequent improvement in motor and sensory functions in a rat model of nerve injury.**

55. Bloancă V, Ceauşu AR, Jitariu AA *et al.* Adipose tissue graft improves early but not late stages of nerve regeneration. *In Vivo* 31(4), 649–655 (2017).

56. Hsueh Y-Y, Chang Y-J, Huang T-C *et al.* Functional recoveries of sciatic nerve regeneration by combining chitosan-coated conduit and neurosphere cells induced from adipose-derived stem cells. *Biomaterials* 35(7), 2234–2244 (2014).

57. Liu G-B, Cheng Y-X, Feng Y-K *et al.* Adipose-derived stem cells promote peripheral nerve repair. *Arch. Med. Sci.* 7(4), 592 (2011).

58. Saller MM, Huettl R-E, Mayer JM *et al.* Validation of a novel animal model for sciatic nerve repair with an adipose-derived stem cell loaded fibrin conduit. *Neural Regen. Res.* 13(5), 854 (2018).

59. Strong AL, Cederna PS, Rubin JP, Coleman SR, Levi B. The current state of fat grafting: a review of harvesting, processing and injection techniques. *Plast. Reconstr. Surg.* 136(4), 897 (2015).

60. Kuffler DP. Injury-induced effectors of neuropathic pain. *Mol. Neurobiol.* 57(1), 51–66 (2020).

61. Feng N, Jia Y, Huang X. Exosomes from adipose-derived stem cells alleviate neural injury caused by microglia activation via suppressing NF-kB and MAPK pathway. *J. Neuroimmunol.* 334, 576996 (2019).

62. Xie W, Strong JA, Zhang J-M. Active nerve regeneration with failed target reinnervation drives persistent neuropathic pain. *eNeuro* 4(1), 1–16 (2017).

•• **The incomplete process of nerve regeneration can be directly associated with neuropathic pain development.**

63. Davies AJ, Kim HW, Gonzalez-Cano R *et al.* Natural killer cells degenerate intact sensory afferents following nerve injury. *Cell* 176(4), 716–728.e718 (2019).

64. Latremoliere A, Woolf CJ. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J. Pain* 10(9), 895–926 (2009).

65. Meacham K, Shepherd A, Mohapatra DP, Haroutounian S. Neuropathic pain: central vs. peripheral mechanisms. *Curr. Pain Headache Rep.* 21(6), 28 (2017).

66. Marcol W, Kotulska K, Larysz-Brysz M, Kowalik JL. BDNF contributes to animal model neuropathic pain after peripheral nerve transection. *Neurosurg. Rev.* 30(3), 235–243 (2007).

67. Haselbach D, Raffoul W, Larcher L, Tremp M, Kalbermatten DF, Di Summa PG. Regeneration patterns influence hindlimb automutilation after sciatic nerve repair using stem cells in rats. *Neurosci. Lett.* 634, 153–159 (2016).

68. Lee HY, Lee H-L, Yun Y *et al.* Human adipose stem cells improve mechanical allodynia and enhance functional recovery in a rat model of neuropathic pain. *Tissue Eng. Part A* 21(13-14), 2044–2052 (2015).

69. Noh M-C, Stemkowski PL, Smith PA. Long-term actions of interleukin-1β on K+, Na+ and Ca2+ channel currents in small, IB4-positive dorsal root ganglion neurons; possible relevance to the etiology of neuropathic pain. *J. Neuroimmunol.* 332, 198–211 (2019).

70. Stemkowski PL, Noh MC, Chen Y, Smith PA. Increased excitability of medium-sized dorsal root ganglion neurons by prolonged interleukin-1β exposure is K+ channel dependent and reversible. *J. Physiol.* 593(16), 3739–3755 (2015).

71. Czeschik JC, Hagenacker T, Schäfers M, Büsselberg D. TNF-α differentially modulates ion channels of nociceptive neurons. *Neurosci. Lett.* 434(3), 293–298 (2008).

72. Ratté S, Prescott SA. Afferent hyperexcitability in neuropathic pain and the inconvenient truth about its degeneracy. *Curr. Opin. Neurobiol.* 36, 31–37 (2016).

73. Yan H, Gao W, Pan Z, Zhang F, Fan C. The expression of α-SMA in the painful traumatic neuroma: potential role in the pathobiology of neuropathic pain. *J. Neurotrauma* 29(18), 2791–2797 (2012).

74. Onode E, Uemura T, Takamatsu K *et al.* Nerve capping with a nerve conduit for the treatment of painful neuroma in the rat sciatic nerve. *J. Neurosurg.* doi:10.3171/2018.10.JNS182113 (2019) (Epub ahead of print).

75. Huang S-H, Wu S-H, Chang K-P *et al.* Autologous fat grafting alleviates burn-induced neuropathic pain in rats. *Plast. Reconstr. Surg.* 133(6), 1396–1405 (2014).

76. Strong AL, Agarwal S, Cederna PS, Levi B. Peripheral neuropathy and nerve compression syndromes in burns. *Clin. Plast. Surg.* 44(4), 793–803 (2017).

77. Ji R-R, Donnelly CR, Nedergaard M. Astrocytes in chronic pain and itch. *Nat. Revi. Neurosci.* 20(11), 667–685 (2019).

78. Yu X, Liu H, Hamel KA *et al.* Dorsal root ganglion macrophages contribute to both the initiation and persistence of neuropathic pain. *Nat. Commun.* 11(1), 1–12 (2020).

79. Inoue K, Tsuda M. Microglia in neuropathic pain: cellular and molecular mechanisms and therapeutic potential. *Nat. Rev. Neurosci.* 19(3), 138 (2018).

80. Suter MR, Wen Y-R, Decosterd I, Ji R-R. Do glial cells control pain? *Neuron Glia Biol.* 3(3), 255–268 (2007).

81. Miyoshi K, Obata K, Kondo T, Okamura H, Noguchi K. Interleukin-18-mediated microglia/astrocyte interaction in the spinal cord enhances neuropathic pain processing after nerve injury. *J. Neurosci.* 28(48), 12775–12787 (2008).

82. Shih R-H, Wang C-Y, Yang C-M. NF-kappaB signaling pathways in neurological inflammation: a mini review. *Front. Mol. Neurosci.* 8, 77 (2015).

83. Brini AT, Amodeo G, Ferreira LM *et al.* Therapeutic effect of human adipose-derived stem cells and their secretome in experimental diabetic pain. *Sci. Rep.* 7(1), 1–15 (2017).

• **ADSCs and their secretomes mitigated central and peripheral neuronal inflammation and subsequently improved neuropathic pain in diabetic rats.**

84. Da Silva Meirelles L, Fontes AM, Covas DT, Caplan AI. Mechanisms involved in the therapeutic properties of mesenchymal stem cells. *Cytokine Growth Factor Rev.* 20(5-6), 419–427 (2009).

▶85. Yang J, Zhang X, Chen X, Wang L, Yang G. Exosome mediated delivery of miR-124 promotes neurogenesis after ischemia. *Mol. Ther. Nucleic Acids* 7, 278–287 (2017).

▶86. Han Y, Seyfried D, Meng Y *et al.* Multipotent mesenchymal stromal cell–derived exosomes improve functional recovery after experimental intracerebral hemorrhage in the rat. *J. Neurosurg.* 131(1), 290–300 (2018).

87. Kang SK, Shin IS, Ko MS, Jo JY, Ra JC. Journey of mesenchymal stem cells for homing: strategies to enhance efficacy and safety of stem cell therapy. *Stem Cells Int.* 2012, 342968 (2012).

▶88. Erba P, Terenghi G, J Kingham P. Neural differentiation and therapeutic potential of adipose tissue derived stem cells. *Curr. Stem Cell Res. Ther.* 5(2), 153–160 (2010).

▶89. Sacerdote P, Niada S, Franchi S *et al.* Systemic administration of human adipose-derived stem cells reverts nociceptive hypersensitivity in an experimental model of neuropathy. *Stem Cells Dev.* 22(8), 1252–1263 (2013).

▶90. Mert T, Kurt AH, Altun İ, Celik A, Baran F, Gunay I. Pulsed magnetic field enhances therapeutic efficiency of mesenchymal stem cells in chronic neuropathic pain model. *Bioelectromagnetics* 38(4), 255–264 (2017).

▶91. Huang S-H, Wu S-H, Lee S-S *et al.* Fat grafting in burn scar alleviates neuropathic pain via anti-inflammation effect in scar and spinal cord. *PLoS ONE* 10(9), 1–13 (2015).

▶92. Lin C-H, Wu S-H, Lee S-S *et al.* Autologous adipose-derived stem cells reduce burn-induced neuropathic pain in a rat model. *Int. J. Mol. Sci.* 19(1), 34 (2018).

▶93. Haahr MK, Jensen CH, Toyserkani NM *et al.* Safety and potential effect of a single intracavernous injection of autologous adipose-derived regenerative cells in patients with erectile dysfunction following radical prostatectomy: an open-label Phase I clinical trial. *EBioMedicine* 5, 204–210 (2016).

•• **SVF injection into the corpus cavernosum improved erectile dysfunction in patients whose neurovascular bundle had been severed due to the radical prostatectomy surgery.**

▶94. Salonia A, Burnett AL, Graefen M *et al.* Prevention and management of postprostatectomy sexual dysfunctions part 1: choosing the right patient at the right time for the right surgery. *Eur. Urol.* 62(2), 261–272 (2012).

95. Haahr MK, Jensen CH, Toyserkani NM *et al.* A 12-month follow-up after a single intracavernous injection of autologous adipose-derived regenerative cells in patients with erectile dysfunction following radical prostatectomy: an open-label Phase I clinical trial. *Urology* 121, 203.e206–203.e213 (2018).

96. Van Hecke O, Austin SK, Khan RA, Smith B, Torrance N. Neuropathic pain in the general population: a systematic review of epidemiological studies. *PAIN*® 155(4), 654–662 (2014).

97. Alessandri-Bonetti M, Egro FM, Persichetti P, Coleman SR, Rubin JP. The role of fat grafting in alleviating neuropathic pain: a critical review of the literature. *Plast. Reconstr. Surg.Global Open* 7(5), 1–8 (2019).

▶98. Stevens PE, Dibble SL, Miaskowski C. Prevalence, characteristics and impact of postmastectomy pain syndrome: an investigation of women's experiences. *Pain* 61(1), 61–68 (1995).

▶99. Caviggioli F, Maione L, Forcellini D, Klinger F, Klinger M. Autologous fat graft in postmastectomy pain syndrome. *Plast. Reconstr. Surg.* 128(2), 349–352 (2011).

▶100. Maione L, Vinci V, Caviggioli F *et al.* Autologous fat graft in postmastectomy pain syndrome following breast conservative surgery and radiotherapy. *Aesthetic Plast. Surg.* 38(3), 528–532 (2014).

101. Caviggioli F, Maione L, Klinger F, Lisa A, Klinger M. Autologous fat grafting reduces pain in irradiated breast: a review of our experience. *Stem Cells Int.* 2016, 1–5 (2016).

▶102. Juhl AA, Karlsson P, Damsgaard TE. Fat grafting for alleviating persistent pain after breast cancer treatment: a randomized controlled trial. *J. Plast. Reconstr. Aesthet. Surg.* 69(9), 1192–1202 (2016).

• **Autologous fat grafting reduced post-mastectomy neuropathic pain and enhanced the quality of life.**

▶103. Cogliandro A, Barone M, Tenna S, Coppola MM, Persichetti P. The role of lipofilling after breast reconstruction: evaluation of outcomes and patient satisfaction with BREAST-Q. *Aesthetic Plast. Surg.* 41(6), 1325–1331 (2017).

▶104. Vaienti L, Merle M, Battiston B, Villani F, Gazzola R. Perineural fat grafting in the treatment of painful end-neuromas of the upper limb: a pilot study. *J. Hand Surg.* 38(1), 36–42 (2013).

105. Vaienti L, Amendola F, Borelli F, Zaccaria G, Cottone G. Perineural fat grafting in end-neuroma pain treatment: long-term outcomes. *Eur. J. Plast. Surg.* 37, 1–6 (2020).

▶106. Calcagni M, Zimmermann S, Scaglioni MF, Giesen T, Giovanoli P, Fakin RM. The novel treatment of SVF-enriched fat grafting for painful end-neuromas of superficial radial nerve. *Microsurgery* 38(3), 264–269 (2016).

▶107. Zimmermann S, Fakin RM, Giovanoli P, Calcagni M. Outcome of stromal vascular fraction-enriched fat grafting compared to intramuscular transposition in painful end-neuromas of superficial radial nerve: preliminary results. *Frontiers Surg.* 5, 10 (2018).

▶108. Bourne DA, Thomas RD, Bliley J *et al.* Amputation-site soft-tissue restoration using adipose stem cell therapy. *Plast. Reconstr. Surg.* 142(5), 1349–1352 (2018).

▶109. Fredman R, Edkins RE, Hultman CS. Fat grafting for neuropathic pain after severe burns. *Ann. Plast. Surg.* 76, S298–S303 (2016).

- **Autologous fat grafting significantly reduced pain in burn patients with refractory neuropathic pain to the previous medical and surgical interventions.**

110. Mcfarland LV, Hubbard Winkler SL, Heinemann AW, Jones M, Esquenazi A. Unilateral upper-limb loss: satisfaction and prosthetic-device use in veterans and servicemembers from Vietnam and OIF/OEF conflicts. *J. Rehabil. Res. Dev.* 47(4), 299–316 (2010).
111. Bavikatte G, Kulkarni J, Choukairi F, Lees V. Use of lipomodeling to forearm residuum to assist fitting of below-elbow prosthesis. *J. Prosthetics Orthotics* 24(1), 50–51 (2012).
112. Hamed K, Giles N anderson J *et al.* Changes in cutaneous innervation in patients with chronic pain after burns. *Burns* 37(4), 631–637 (2011).
113. Summer GJ, Dina OA, Levine JD. Enhanced inflammatory hyperalgesia after recovery from burn injury. *Burns* 33(8), 1021–1026 (2007).
114. Klinger M, Caviggioli F, Klinger FM *et al.* Autologous fat graft in scar treatment. *J. Craniofac. Surg.* 24(5), 1610–1615 (2013).
115. Lifchez SD, Means Jr KR, Dunn RE, Williams EH. Intra-and inter-examiner variability in performing Tinel's test. *J. Hand Surg.* 35(2), 212–216 (2010).
116. Knoepfler PS, Turner LG. The FDA and the US direct-to-consumer marketplace for stem cell interventions: a temporal analysis. *Regen. Med.* 13(1), 19–27 (2018).
117. Tocco I, Widgerow AD, Lalezari S, Banyard D, Shaterian A, Evans GR. Lipotransfer: the potential from bench to bedside. *Ann. Plast. Surg.* 72(5), 599–609 (2014).
118. Dykstra JA, Facile T, Patrick RJ *et al.* Concise review: fat and furious: Harnessing the full potential of adipose-derived stromal vascular fraction. *Stem Cells Transl. Med.* 6(4), 1096–1108 (2017).

- **At the current state, only autologous fat grafting is considered safe and applicable to the clinical practice.**

119. Berry JD, Cudkowicz ME, Windebank AJ *et al.* NurOwn, phase 2, randomized, clinical trial in patients with ALS: safety, clinical and biomarker results. *Neurology* 93(24), e2294–e2305 (2019).
120. Cohen JA, Imrey PB, Planchon SM *et al.* Pilot trial of intravenous autologous culture-expanded mesenchymal stem cell transplantation in multiple sclerosis. *Mult. Scler.* 24(4), 501–511 (2018).
121. Levi AD, Okonkwo DO, Park P *et al.* Emerging safety of intramedullary transplantation of human neural stem cells in chronic cervical and thoracic spinal cord injury. *Neurosurgery* 82(4), 562–575 (2018).
122. Levy ML, Crawford JR, Dib N, Verkh L, Tankovich N, Cramer SC. Phase I/II study of safety and preliminary efficacy of intravenous allogeneic mesenchymal stem cells in chronic stroke. *Stroke* 50(10), 2835–2841 (2019).
123. Zuk PA, Zhu M, Ashjian P *et al.* Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell* 13(12), 4279–4295 (2002).
124. Rigotti G, Marchi A, Galiè M *et al.* Clinical treatment of radiotherapy tissue damage by lipoaspirate transplant: a healing process mediated by adipose-derived adult stem cells. *Plast. Reconstr. Surg.* 119(5), 1409–1422 (2007).
125. Strem BM, Hicok KC, Zhu M *et al.* Multipotential differentiation of adipose tissue-derived stem cells. *Keio J. Med.* 54(3), 132–141 (2005).

Research Article

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Regenerative Medicine

Autologous plasma rich in growth factors technology for isolation and *ex vivo* expansion of human dental pulp stem cells for clinical translation

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Aim: This study investigated the use of the autologous technology of plasma rich in growth factors (PRGF) as a human-based substitute to fetal bovine serum (FBS) in the culture of human dental pulp stem cells. **Materials & methods:** Stem cell characterization was performed. Analysis of isolation, proliferation, migration, trilineage differentiation, senescence and cryopreservation were compared between FBS and PRGF. **Results:** Human dental pulp stem cell cultures isolated and maintained with PRGF showed a significantly higher number of cells per explant than FBS cultures. Cell proliferation, migration, osteogenic mineralization and adipogenic differentiation were found to be significantly higher in PRGF than FBS. **Conclusion:** The autologous PRGF technology could be a suitable and safer substitute for FBS as a culture medium supplement for clinical translation of cell therapy.

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Keywords: autologous cell therapy • dental pulp stem cells • fetal bovine serum • nonanimal alternatives • plasma rich in growth factors • stem cell therapy • xeno-free culture

Stem cell therapy has emerged as an alternative approach for the repair of damaged or lost tissues in a wide variety of diseases. In particular, mesenchymal stem cells (MSCs) are the most promising candidates among adult stem cells due to their accessibility and lack of ethical concerns [1–3]. They are typically obtained from many organs and tissues including, bone marrow, adipose tissue, brain, skin, teeth, skeletal muscle and heart [2,4,5]. Among these tissues, dental pulp is a cranial neural crest-derived tissue enclosed into a dental cavity surrounded by mineralized dentin [6]. In 2000, Gronthos *et al.* [7] isolated for the first time, human dental pulp stem cells (hDPSCs) from the pulp tissue of third molars. hDPSCs have an easy surgical access as they can be collected from discarded permanent teeth and harvested following noninvasive isolation methods thus, representing a valuable source of readily accessible stem cells with special biological properties of MSCs and neural crest stem cells [8–10]. Since MSCs occur scarcely in tissues, an *ex vivo* expansion following Good manufacturing practices (GMP) guidelines is required to yield a suitable dose in order to achieve therapeutic outcomes [2,11,12]. Currently, many experimental concerns are raised for human cell therapies related to the establishment of standardized xeno-free culture protocols in order to improve safety and reproducibility [13].

Fetal bovine serum (FBS) has long been employed as a widely accepted standard cell culture supplement for both research and clinical use [14]. However, the use of this animal-derived product entails several safety and regulatory concerns. FBS poses a great batch to batch variability, hence hampering reproducibility and making standardization of the production process difficult. Another issue is the risk of contamination with harmful pathogens such as viruses, mycoplasms or prions among others. In addition, limited availability, ethical issues regarding animal welfare or the induction of host immunologic reactions have also been raised [12,13,15–17]. To address all these risks, regulatory authorities, industry and the research community demand suitable alternatives to provide safe, regulated

Future Medicine

Table 1. Characterization of the human dental pulp stem cells' donors.

Cell line	Age	Gender	Smoker	Impacted wisdom teeth	Assay
A	14	Male	No	38	Proliferation, migration, differentiation, senescence and cryopreservation
B	14	Female	No	48	Proliferation, migration, differentiation, senescence and cryopreservation
C	16	Female	No	48	Isolation and cryopreservation
D	16	Male	No	28	Isolation
E	20	Female	No	48	Isolation
F	18	Female	No	38	Isolation
G	16	Female	No	38	Isolation

and effective cell therapy products to patients [18]. Currently, many autologous or allogenic human blood-derived products such as human serum [19,20], platelet-rich plasma (PRP) [21], platelet lysate [14,22–24] or umbilical cord blood serum [25,26] are being explored as potential FBS replacement.

Regarding PRPs, diverse protocols are being performed leading to multiple commercially available products, which methodological and composition variability contribute to the heterogeneity of the final therapeutic outcomes. Therefore, it is necessary to evaluate each product individually for providing precise results according to the composition of that specific PRP. In this sense, the technology of plasma rich in growth factors (PRGF) is the pioneering technology for the use of autologous growth factors, proteins and biomaterials from human plasma and platelets for healing purposes [27,28]. PRGF is a platelet-rich plasma mainly characterized by the absence of leukocytes, thus avoiding the promotion of inflammation. In addition, calcium chloride is used to activate PRGF rather than bovine thrombin, thus leading to a more biocompatible and safe technology. Multiple formulations can be obtained from this autologous technology [29,30] that confers a great versatility to be widely applied in different medical fields (maxillofacial surgery, dermatology, ophthalmology and orthopedics [28,31]), as well as to be used for *ex vivo* stem cell expansion [32].

The development of standardized xeno-free culture protocols that maintain cell viability, phenotype and the potential of differentiation, represents one of the main milestones of cell therapies for clinical translation. In this study, the use of PRGF as a human-based substitute to FBS in the isolation, expansion, senescence, cryopreservation and differentiation potential of hDPSCs populations has been investigated.

Materials & methods

Cell isolation & culture

The study was performed following the principles of the Declaration of Helsinki, as revised in 2013. Normal impacted wisdom teeth were obtained from 14 to 16 year old healthy patients by senior surgeons [33], after written informed consent of donor parents was given (Table 1). Three primary hDPSC cultures were isolated using the explant method, which is briefly described below. Tooth surfaces were cleaned and mechanically fractured around the cementum–enamel junction. After gently separating, the pulp tissue from the crown and the root was minced into fragments of 1–2 mm^3 that were placed into six-well plates and cultured in Dulbecco's modified eagle's medium (DMEM)/F-12 (1:1 volume; Gibco–Invitrogen, NY, USA) with 2 mM glutamine, 50 μg/ml gentamicin and 2.5 μg/ml amphotericin B (Sigma–Aldrich, MO, USA; from here on referred as isolation medium) supplemented with 10% FBS (Biochrom AG, Leonorenstr, Berlin, Germany). Cells growing out from the pulp explants were maintained at 37°C in a humidified, 5% CO_2 atmosphere and medium was changed every 2–3 days. When reaching 70–80% confluence, cells were detached with animal origin-free trypsin-like enzyme (TrypLE select enzyme; Gibco–Invitrogen) and cryopreserved or subcultured for further experiments. Cell viability was assessed by trypan blue dye exclusion. Since then, cell cultures were maintained with isolation medium without amphotericin (onward, culture medium) supplemented with 10% FBS. Except for senescence assays, cells between third and sixth passages were used in the experiments.

hDPSCs characterization

hDPSC populations before passage 4 were characterized by flow cytometric analysis by the expression of cell surface antigens following the standard criteria established for the International Society for Cell Therapy (ISCT) [34]. Concisely, detached cells were placed in 5 ml tubes (2.5×10^5 per antibody) and washed twice with stain

buffer (Becton, Dickinson and Company, NJ, USA). After finishing the blocking time, cells were incubated with fluorescinated antibody in the same blocking buffer at 4°C for 1 h and protected from light. Antibodies against CD73, CD90, CD14, CD19, CD45 and IgG1 (all of them FITC-conjugated), CD105, CD34 and IgG1 (APC-conjugated) and HLA-DR and IgG2a (PE-Cy5-conjugated) were used to phenotypic identification. All antibodies were purchased from Becton, Dickinson and Company. After washing twice with stain buffer, hDPSCs were fixed in 1% paraphormaldehyde in stain buffer and they were preserved in this solution and protected from light until their analysis by flow cytometry. The expression profile was analyzed by a Gallios flow cytometer (Beckman–Coulter, Buckinghamshire, UK).

Preparation of PRGF

After written informed consent was provided, blood from five donors was collected into 9 ml tubes with 3.8% (wt/v) sodium citrate. Citrated venous blood was centrifuged at 580 g for 8 min (Endoret Dentistry, BTI Biotechnology Institute, S.L., Álava, Spain) at room temperature (RT) and the plasma column just above the buffy coat was collected. Platelets, erythrocytes and leukocytes counts were performed with a hematology analyzer (Micros 60, Horiba ABX, Occitania, France). Plasma preparations were activated following the manufacturer's instructions and PRGF supernatants were collected by aspiration after centrifugation at 1000 g for 10 min at RT. After being filtered, obtained PRGF supernatants were stored in aliquots at -80°C until use.

PRGF effectiveness in hDPSC isolation

To evaluate the efficiency of PRGF supernatant in the isolation process, primary hDPSC cultures were obtained from normal impacted wisdom teeth from five healthy patients (16–20 years of age), after written informed consent was provided (Table 1). The protocol described in 'Cell isolation and culture' section was followed except for that only one fragment of pulp tissue was placed into each well of 48-well plate. Therefore, eight pulp tissue pieces of similar size from each donor were achieved and maintained in culture medium supplemented with 10% FBS (four fragments) or 10% PRGF (the other four fragments). In this last case, primary cultures were incubated with PRGF obtained from different donors.

Explant cultures were followed-up in order to determine the day that cells were observed for the first time in each well and the number of explants from which cells grew out. When hDPSC cultures of one of the eight wells reached subconfluence stage, cells from all wells were harvested and counted in a hemocytometer.

Analysis of the migration potential

Passage 3 human DPSCs obtained from two donors were seeded at high density (21,000 cell/cm^2) in culture inserts (Ibidi GmbH, Planegg/Martinsried, Munich, Germany) previously placed on a 24-well plate. Inserts comprise two cell growth areas and a gap between them. Cell cultures were maintained with culture medium supplemented with 10% FBS until confluence. Insert devices were then carefully removed and two cell monolayers, leaving a cell-free gap of 500 $\pm$ 50 µm, were created.

Cells were washed with phosphate-buffered saline (PBS) and incubated with culture medium supplemented with either 10% FBS or 10% PRGF from three donors and in triplicate. After 24 h, treatments were removed and cells nuclei were stained with 1/500 Hoechst 33342 (Molecular Probes-Thermo Fisher Scientific, MA, USA) in PBS for 10 min. Phase contrast images of the central part of the gap were captured after removing the insert devices just before adding the treatments, and phase contrast and fluorescence images of the same area were captured after 24 h of incubation using a digital camera (Leica DFC300 FX, Leica Microsystems, Wetzlar Hesse, Germany) coupled to an inverted microscope (Leica DM IRB). The gap area was measured and migratory cells were counted using the Image J Software (NIH, MD, USA). Results were expressed as the number of cells migrated per mm^2 of area.

Cell proliferation assay

Cell proliferation was evaluated by Cyquant cell proliferation assay (Molecular Probes-Thermo Fisher Scientific). hDPSCs at passage 3 from two primary cultures were seeded on 96-well optical-bottom black plates at a density of 4000 cells/cm^2 and they were maintained with cultured medium supplemented with either 10% FBS or 10% PRGF from three different donors for 24, 48, 72 and 96 h. Five replicates of each primary cell culture for each time and treatment were assayed according to manufacturer's instructions.

Briefly, after removing the treatments, wells were carefully washed with PBS. To promote cell lysis, the microplates were then frozen at -80°C until assayed. After thawing the plates, RNase A (1.35 Ku/ml) were added into wells and

cell lysates were incubated with enzymatic solution for 1 h at room temperature. Then, the same volume of 2x GR dye/cell-lysis buffer was added to each sample and incubated at room temperature for 5 min with gently agitation and protected from light. Sample fluorescence was measured with a fluorescence microplate reader (Twinkle LB 970, Berthold Technologies GmbH&Co, Bad Wildbad, Baden-Wurtemberg, Germany). Results were transformed into DNA concentration using a DNA standard curve, which was included in each assay.

Trilineage differentiation

Osteogenic differentiation

Osteogenic differentiation was induced on two hDPSCs primary cultures. Cells at passage 3 were seeded on 48-well plates at a density of 4000 cells/cm^2 and maintained in culture medium with 10% FBS. After reaching 50–70% confluence, the medium was switched to different treatments, using the reagents supplied in the human mesenchymal stem cell functional identification kit (R&D Systems, MN, USA) and following manufacturer's instructions. To compare the osteogenic differentiation potential of FBS versus PRGF, cells were incubated with culture medium supplemented with 10% FBS or 10% PRGF from three donors, plus 1x osteogenic supplements containing dexamethasone, ascorbate–phosphate and β-glycerolphosphate. The culture medium was changed every 3–4 days and all treatments were assayed in triplicate.

After 28 days, mineralization was visualized by Alizarin red staining of calcium deposits. Briefly, cells were fixed with 4% paraphormaldehyde in PBS for 30 min at RT. After washing twice with ultrapure water, cells were incubated with 1.4% alizarin red solution (pH 4.1 ± 0.1; Millipore Corporation, MA, USA) for 5 min. Finally, cells were washed three times with ultrapure water. Before and after staining, phase contrast images of cell cultures were captured using a digital camera (Leica DFC300 FX) coupled to an inverted microscope (Leica DM IRB).

To quantify calcium deposition by differentiated cells, cetilpyridinium chloride (Sigma–Aldrich) was added into wells and incubated in gently agitation until the monolayer was colorless. After achieving a complete elution, 100 µl of this solution from each well were transferred into wells of 96-well plates. Absorbance at 540 nm was measured in an iEMS Reader (Thermo Labsystems-Thermo Fisher Scientific). Measurements were made in duplicate.

Adipogenic differentiation

For adipogenic differentiation, cells at passage 3 from two hDPSC primary cultures were seeded on 96-well optical-bottom black plates and on 24-well plates at a density of 10,000–15,000 cells/cm^2, respectively. Cells were maintained in culture medium with 10% FBS until reaching 100% confluence. Then, the medium was replaced with the different treatments analyzed using the reagents supplied in the human mesenchymal stem cell functional identification kit. Cells were incubated for 35 days with Minimum essential medium eagle-alpha modification (αMEM; Sigma–Aldrich) with 2 mM glutamine, 50 µg/ml gentamicin, 1x adipogenic inductors (containing hydrocortisone, isobutylmethylxanthine and indomethacin) and supplemented with 10% FBS or 10% PRGF as appropriate, being the latter obtained from three donors. The culture medium was changed every 3–4 days and all treatments were assayed in triplicate.

After adipogenic differentiation in 24-well plates, the intracellular accumulation of neutral lipids was confirmed by HCS LipidTOX red staining. Briefly, after fixing cells with 4% paraphormaldehyde in PBS, 1:100 LipidTox solution (Molecular Probes-Thermo Fisher Scientific) was added and incubated for 30 min. Cells nuclei were stained with 1/500 Hoechst 33342 in PBS for 10 min.

FABP4 was also detected in adipogenic-differentiated hDPSCs using reagents that were included in the differentiation kit (human mesenchymal stem cell functional identification kit) and following the manufacturer's indications. Finally, Hoechst 33342 was used to stain cells nuclei following the previously described protocol.

After adipogenic differentiation in 96-well optical-bottom black plates, total cellular concentrations of triglycerides were determined by a coupled enzyme assay (adipogenesis assay kit, Sigma–Aldrich) in accordance with the manufacturer's instructions. The resulting fluorimetric products were measured with a fluorescence microplate reader (Twinkle LB 970).

Chondrogenic differentiation

Chondrogenic differentiation was induced in two hDPSC primary cultures by using the 'pellet culture' technique that was described in the human mesenchymal stem cell functional identification kit. In brief, 2.5×10^5 cells at passage 5 were placed in each 15 ml tube and pelleted into micromasses by centrifugation. Then, 0.5 ml of treatment was added into them. Pellets were incubated with culture medium supplemented with ITS (insulin,

transferring, selenious acid, bovine serum albumin and linoleic acid), chondrogenic supplements (dexamethasone, ascorbate–phosphate, proline, pyruvate and recombinant transforming growth factor beta 3) and with or without 10% PRGF from three different donors. The culture medium was changed every 2–3 days. Four replicates of treatments and controls were assayed.

After 42 days of culture, one pellet of each treatment was harvested, fixed in 4% paraformaldehyde, dehydrated with ethanol, cleared with xylene substitutes and finally embedded in paraffin. Chondrogenic differentiation was confirmed by alcian blue staining of 5 μm thickness sections followed by nuclear fast red counterstaining.

To quantitatively assess the success of the chondrogenic differentiation, an assay kit for measurement of sulfated glycosaminoglycans (sGAGs; AMS Biotechnology [Europe] Limited, Abingdon, Oxfordshire, UK) was used in the remaining pellets. For this purpose, the pellets were digested with an enzymatic solution containing 300 μg/ml of papain during 1 h and 20 min at 60°C, following manufacturer's instructions for the papain digestion procedure. Finally, samples were transferred into a 96-well microplate, and assayed in duplicate. After adding the same volume of 1,9-dymethylmethylene blue (DMB) dye, absorbance was measured at 540 nm in an iEMS Reader.

In the three differentiation assays, hDPSCs cultured without differentiation inducers in the corresponding medium were included as negative controls.

Cellular senescence assays

To compare the effect of PRGF versus FBS on the cellular senescence, passage 4 hDPSCs were cultured in parallel until passage 11 with culture medium supplemented with either 10% FBS or PRGF obtained from three donors. For this purpose, primary cultures obtained from two donors were included. SA-β-gal activity and the relative amounts of telomerase were measured in the cultured cells of that replicative age.

For SA-β-gal activity detection, cells were seeded on 48-well plates at a density of 10,000 cells/cm^2. After 24 h, the Senescence cells histochemical staining kit (Sigma–Aldrich) was used to identify blue-stained senescent cells following manufacturer's instructions. The different cellular conditions of the two hDPSC primary cultures were assayed on triplicate.

40,000 cells/cm^2 hDPSCs that were maintained from passage 4 to passage 11 with either FBS or PRGF and hDPSCs at passage 4 were seeded on 48-well plates. After culturing for 24 h, the colorimetric cell-based ELISA CytoGlow™ kit (AssaybioTech, CA, USA) was used to detect telomerase protein, following manufacturer's indications. Briefly, after fixing, cells were incubated with the antitelomerase antibody overnight at 4°C and then for 1.5 h with the HRP-conjugated antimouse IgG antibody. A colored solution was generated after the incubation with the substrate in which absorbance was measured in an iEMS Reader (OD_{450}). Results were normalized with the crystal violet cell staining of those same wells, proportional to cell counts. For this purpose, the die was extracted with a SDS solution and absorbance at 540 nm was measured (OD_{540}). The measured OD_{450} readings were normalized using OD_{540} values via the proportion OD_{450}/OD_{540}. Cell conditions were assayed in triplicate.

hDPSCs cryopreservation

The effectiveness of the cryopreservation medium was assessed by the evaluation of two key aspects: the percentage of living cells immediately after thawing and the percentage of adherent cells in culture after 24 h from defrosting. The effect of PRGF versus FBS in these two processes was tested. With this intention, hDPSCs obtained from three donors and between fourth and fifth passage were included in the assay.

In brief, subconfluent cultures were harvested and identical volume (125 μl) and cell concentration (285 cells/μl) were gradually frozen with different cryopreservation media consisting of 40% culture medium, 50% FBS or PRGF obtained from three donors and 10% dimethyl sulfoxide (DMSO). Four replicates of cryopreserved cells with each freezing medium were stored in liquid nitrogen. After 24 h and 1 month, two cryovials were thawed at 37°C for 1 min. First, three aliquots of the frozen cells suspension were reserved for living cell counting by trypan blue dye exclusion in a hemocytometer. Living and dead cells were counted and results were expressed by percentage of living cells. Identical remaining volumes of cells were immediately seeded into 24-well plates and maintained in culture medium supplemented with 10% FBS for 24 h. Then, hDPSCs were detached and the number of adherent cells was counted. Taking into account the seeding volume, results were expressed by percentage of living cells that were able to adhere.

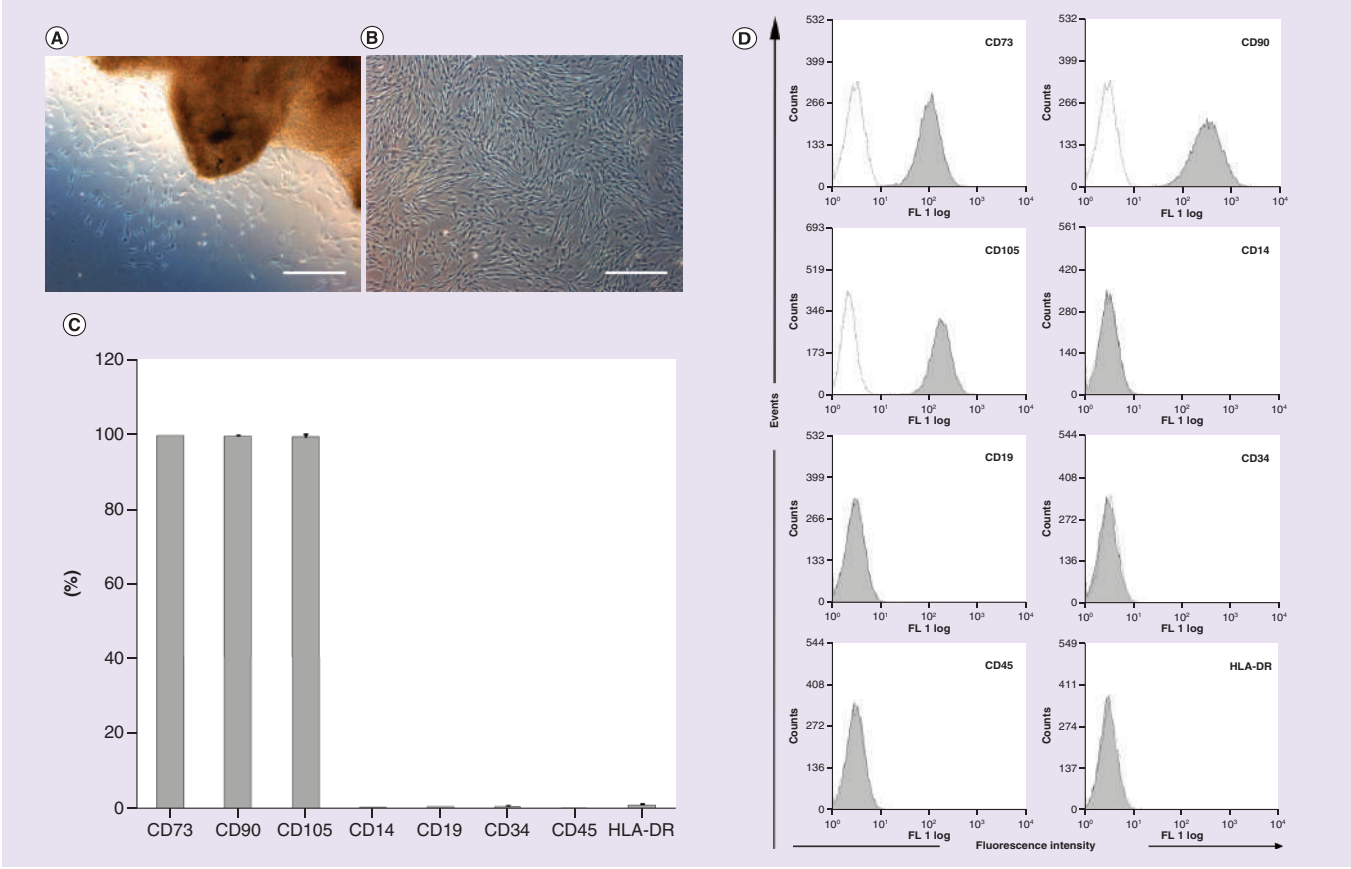

Figure 1. Isolation and culture of human dental pulp stem cells (A & B, respectively). Immunophenotype analysis of primary human dental pulp stem cells by flow cytometry **(C & D)**. Staining with isotype controls (open histograms) and with specific antibodies (filled histograms). Scale bars = 500 μm.

Statistical analysis

The results were presented as mean ± standard deviations. After checking the normal distribution (the Shapiro–Wilk test) and homoscedasticity (the Levene test), differences were analyzed using either the Student's t-test or the analysis of variance test, with Bonferroni-corrected post hoc test, as appropriate. In those cases in which parametric tests were not applicable, significant differences were analyzed using Mann-Whitney or Kruskal-Wallis tests. Significance was assigned at the $p < 0.05$ level.

Results

Immunophenotypic characterization

Isolated dental pulp stem cells (DPSCs) exhibited a spindle-shaped morphology in culture (Figure 1A & B). Flow cytometric analysis was conducted to confirm the mesenchymal surface marker expression. The DPSC cultures were positive for MSCs markers CD73 (99.9 ± 0.1%), CD90 (99.6 ± 0.3%) and CD105 (99.5 ± 0.6%). Cells were also negative for CD14 (0.4 ± 0.1%), CD19 (0.6 ± 0.1%), CD34 (0.6 ± 0.1%), CD45 (0.2 ± 0.1%) and HLA-DR (1.0 ± 0.1%; Figure 1C & D).

Comparative isolation

Isolation capabilities of both supplemented media were evaluated. No significant differences were found regarding the number of days that the cells took to leave the explants (13 ± 5 and 11 ± 3 days for FBS-supplemented medium and PRGF-supplemented medium, respectively). There were also no differences in the percentage of explants from which cells were obtained. However, dental pulp cultures supplemented with PRGF showed a significant increase in the number of cells that were obtained per explant (9458 ± 7370 and 19520 ± 5534 cells/explant for FBS and PRGF-supplemented medium, respectively; Figure 2).

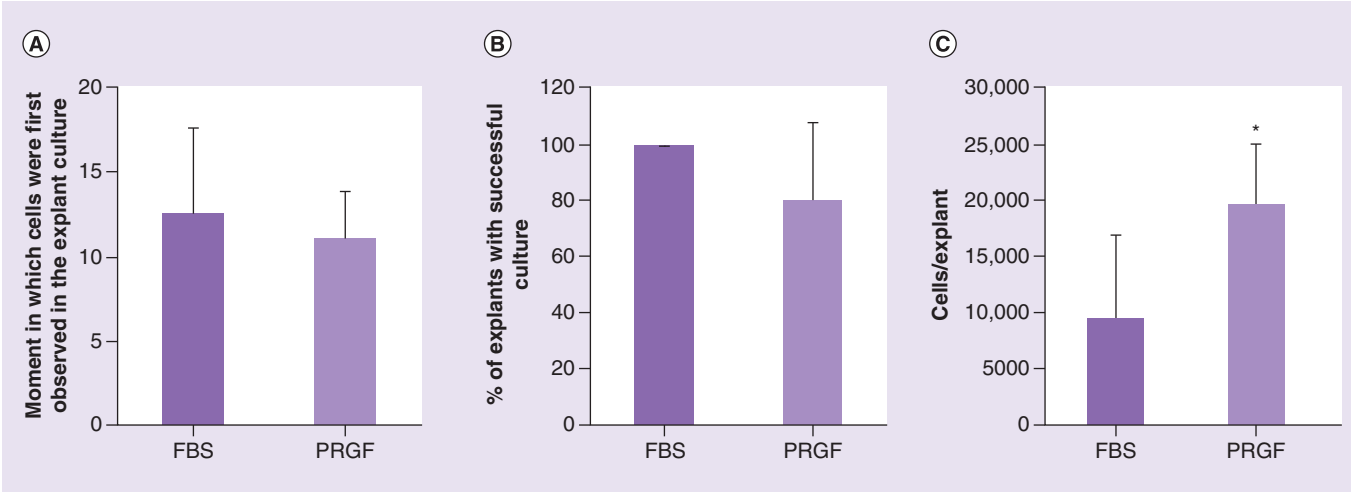

Figure 2. Comparative evaluation of the plasma rich in growth factors versus fetal bovine serum effectiveness in the human dental pulp stem cells isolation process. (A) The first day in which cells emerged from the explants, **(B)** the percentage of explants from which cells emerged and **(C)** the number of cells counted per well at the end of the culture time.
*Statistically significant differences compared with FBS group ($p < 0.05$).
FBS: Fetal bovine serum; PRGF: Plasma rich in growth factor.

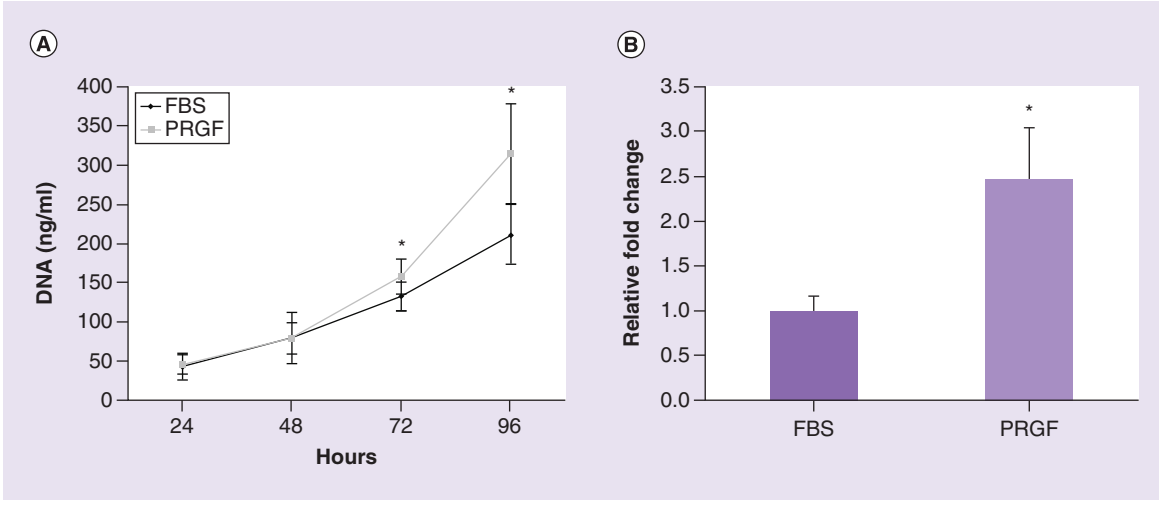

Figure 3. Proliferative and migratory capacity of human dental pulp stem cells. Proliferation **(A)** and migration **(B)** of primary human dental pulp stem cells cultured with either FBS or PRGF-supplemented medium.
*Statistically significant differences compared with FBS group ($p < 0.05$).
FBS: Fetal bovine serum; PRGF: Plasma rich in growth factor.

Proliferation & migration assay

Cell proliferation analysis of primary hDPSC cultures was performed to determine the influence of the supplement protocols. There was no significant difference in cell proliferation after treatment with both culture supplements (FBS and PRGF) for 24 and 48 h. However, after 72 and 96 h, statistically significant differences were observed. hDPSCs cultured with PRGF-supplemented medium showed a statistically higher DNA concentration than hDPSCs cultured with FBS (133 ± 18 vs 158 ± 23 ng/ml and 211 ± 38 vs 314 ± 64 ng/ml for FBS and PRGF-supplemented medium for 72 and 96 h, respectively). Plateau phase was not reached within the experimental assay time (Figure 3A).

Figure 3B shows the migratory capacity of hDPSCs after treatment with both supplements for 24 h. PRGF-supplemented medium enhanced significantly cell migration 2.5 ± 0.6-folds with respect to cells cultured with FBS as supplement (Figure 3B).

Trilineage differentiation

hDPSCs cultured with differentiation media and supplemented with either FBS or PRGF demonstrated differentiation into the three lineages, as confirmed by positive alizarin red S (osteogenic), LipidTOX and FABP4 (adipogenic) and alcian blue staining (chondrogenic; Figure 4B, D, E & G, respectively). Noninduced cells were negative for the aforementioned staining (data not shown).

Osteogenic differentiation

An *in vitro* osteogenic differentiation assay was performed for 4 weeks to determine the effects of both culture supplements (FBS and PRGF). Visible differences were observed in the extracellular matrix mineralization (Figure 4A). The alizarin red S staining confirmed that mineral deposition was more prominent in the hDPSCs cultured with the osteogenic medium supplemented with PRGF (Figure 4B). Alizarin red elution revealed that calcium deposits significantly increased when hDPSCs were cultured with the osteogenic medium supplemented with PRGF up to 1.731 ± 0.742-folds more than when cultured with FBS-supplemented medium (Figure 4C).

Adipogenic differentiation

hDPSCs were induced toward the adipogenic lineage. After 35 days of treatment, the neutral lipid stain LipidTox revealed the formation of cytoplasmic lipid droplets with both culture supplements (FBS and PRGF; Figure 4D). Immunohistochemical staining also detected FABP4 expression in both treatments (Figure 4E). Quantitative analysis by a fluorimetric assay showed a significantly higher triglycerides concentration in hDPSCs cultured with the adipogenic medium supplemented with PRGF with respect to the cells cultured with FBS as supplement (2.29 ± 1.03-folds; Figure 4F).

Chondrogenic differentiation

An *in vitro* chondrogenic differentiation assay was performed for 42 days to determine the effects of PRGF addition. The alcian blue staining confirmed the chondrogenic induction with differentiation media both with and without PRGF addition (Figure 4G). No significant differences were detected in the quantitative analysis of sGAGs in hDPSCs cultured with the chondrogenic medium supplemented with PRGF with respect to the cells cultured without PRGF as supplement (1.89 ± 1.65 and 1.00 ± 0.33, respectively; Figure 4H).

Cell senescence

At early passages, all DPSCs were morphologically similar with a high proliferation rate. Conversely, as cells were increasing in passages, proliferation decreased and changes in cellular size and morphology were detected. High passage cells were larger with a flat and stellate appearance. In addition, SA-β-gal staining revealed low levels of cell senescence in hDPSCs at fourth passage. However, a significant increase in the number of positive cells for SA-β-gal were observed in hDPSCs at 11th passage regardless of the supplement used (Figure 5B & C). Moreover, these results were in line with those obtained after telomerase quantification. Significantly higher telomerase values were detected in hDPSCs at fourth passage with respect to hDPSCs values at 11th passage cultured with either FBS or PRGF (1.845 ± 0.247 vs 1.072 ± 0.384 and 0.936 ± 0.098 for hDPSCs at fourth passage and hDPSCs at 11th passage cultured with FBS-supplemented medium and PRGF-supplemented medium, respectively; Figure 5A). In fact, no significant differences were observed between the cells maintained until passage 11 with culture medium supplemented with either FBS or PRGF.

Cryopreservation

Both FBS and PRGF were also investigated as a supplement for hDPSCs cryopreservation. Cells were stored up to 1 month prior to recovery and evaluation. Upon thawing, no differences were detected in the mean hDPSC viability, as determined by trypan blue staining. After 24h of freezing, the percentage of live cells was 76 ± 15% and 77 ± 16% for FBS and PRGF-supplemented medium, respectively. Viability did not decrease with the freezing time with none of the supplements (79 ± 10% and 86 ± 6% for FBS and PRGF-supplemented medium, respectively, after 1 month of storage in liquid nitrogen; Figure 6A). After viability determination, cells were cultured overnight to assess the ability of cells to adhere after cryopreservation. No statistically significant differences were detected in the percentage of adherent cells regardless of time and freezing supplement (76 ± 13% vs 70 ± 21% and 99 ± 19% vs 77 ± 26% for FBS and PRGF-supplemented medium and 24 h and 1 month of cryopreservation, respectively; Figure 6B).

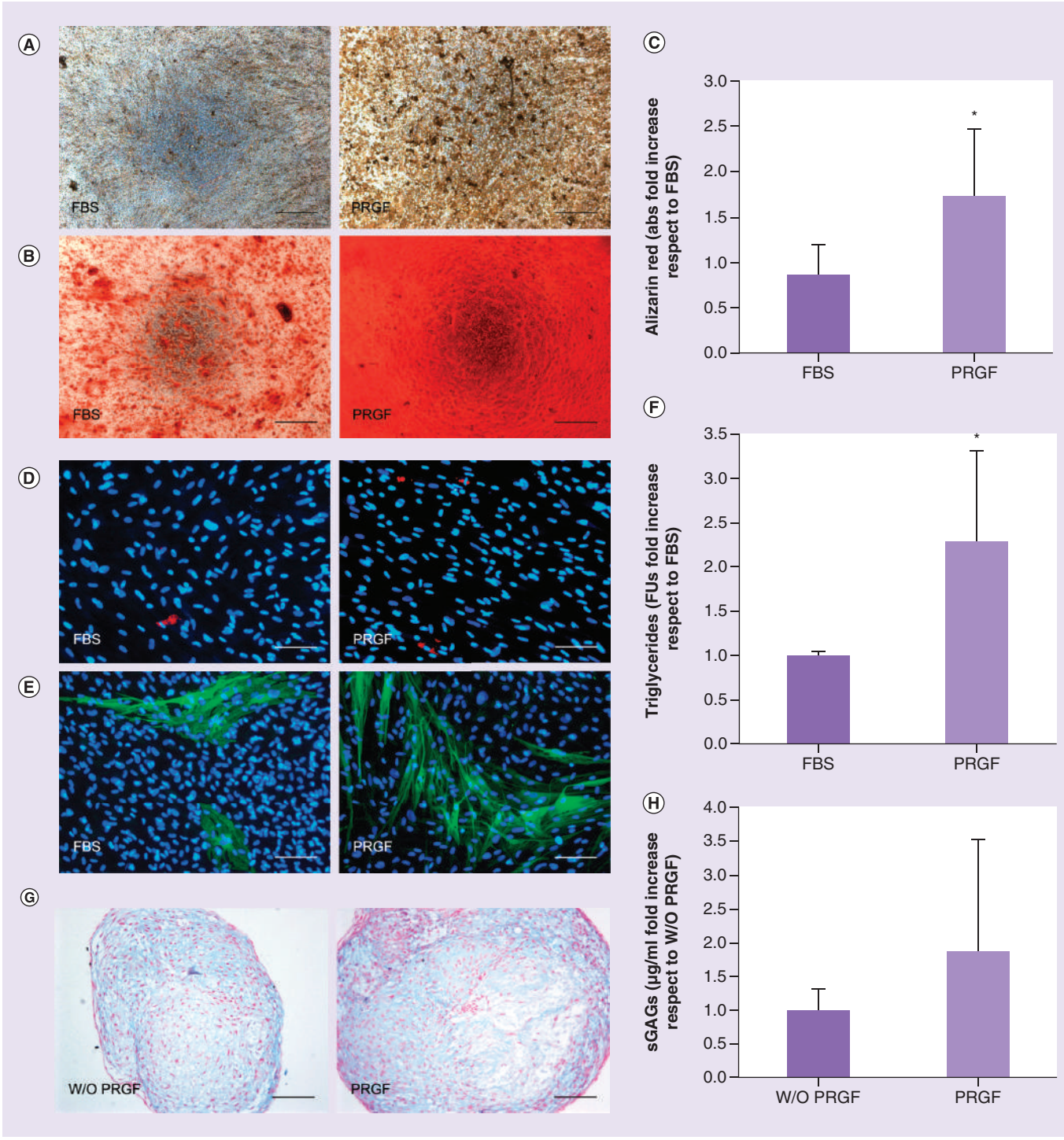

Figure 4. Multineage differentiation capacity of human dental pulp stem cells. Osteogenic **(A–C)**, adipogenic **(D–F)** and chondrogenic **(G & H)** differentiations. Phase contrast images showing the appearance of the mineralized matrix obtained after 28 days of culture with osteogenic inductors and FBS or PRGF supplements **(A)**. Alizarin red staining of the calcium deposits **(B)**. Alizarin red elution with cetilpyridinium chloride to quantify calcium deposition by differentiated cells **(C)**. *Statistically significant differences compared with FBS group ($p < 0.05$). FABP4 detection by immunofluorescence in the hDPSCs differentiated toward the adipogenic lineage with FBS or PRGF supplements **(D)**. HCS LipidTOX red staining of the intracellular accumulation of neutral lipids after adipogenic differentiation **(E)**. Total cellular concentrations of triglycerides determined in hDPSCs after 35 days of adipogenic differentiation. *Statistically significant differences compared with FBS group ($p < 0.05$). Alcian blue staining of hDPSCs pellet sections after 42 days of chondrogenic differentiation **(G)**. sGAGs measurement in papain digested pellets to quantitatively assess the level of the chondrogenic differentiation **(H)**. Scale bars = 400 µm **(A, B & G)** and 100 µm **(D & E)**.
Abs: Absorbance; FBS: Fetal bovine serum; FU: Fluorescence units; hDPSC: Human dental pulp stem cell; PRGF: Plasma rich in growth factor; sGAG: Sulfated glycosaminoglycan.

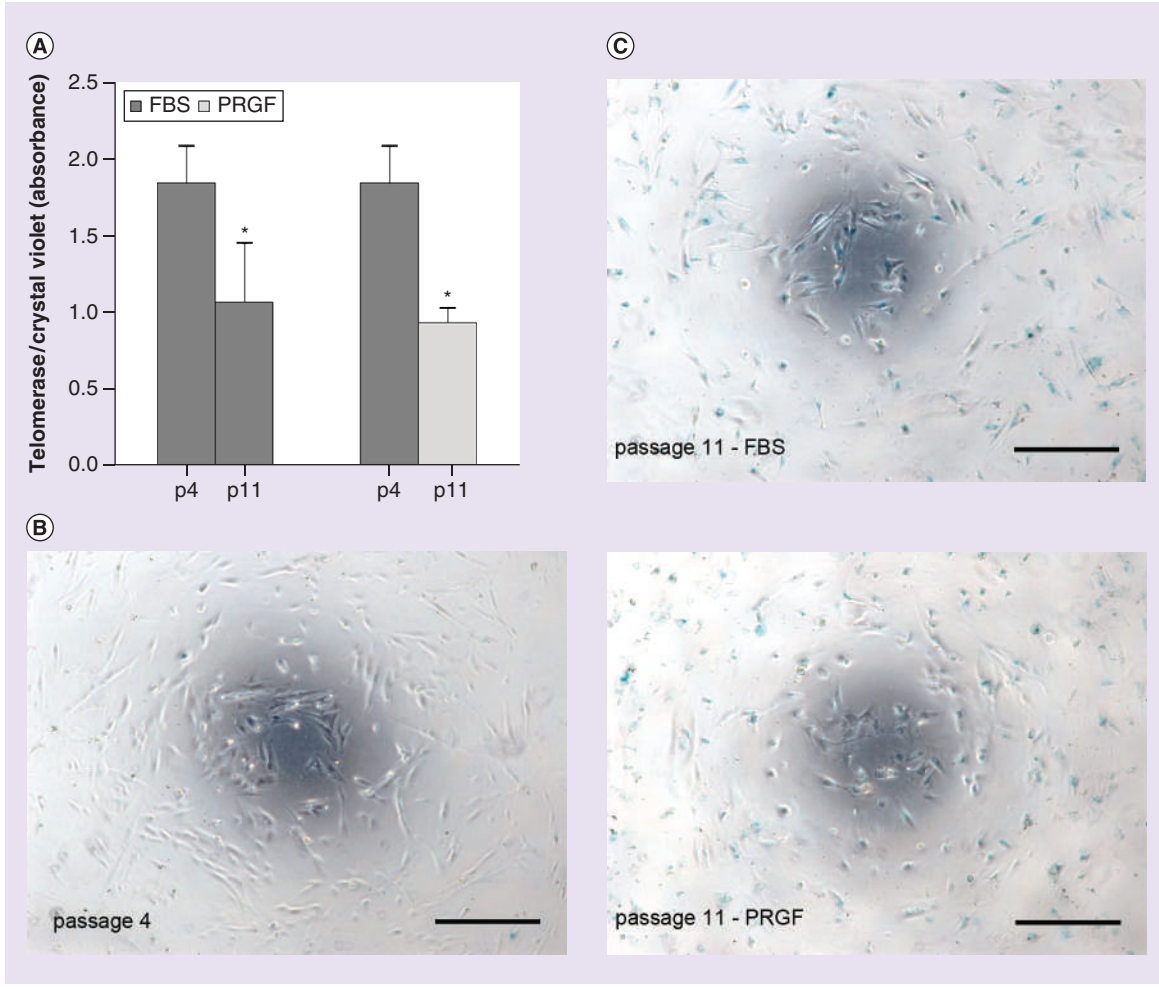

Figure 5. Effects of prolonged *in vitro* culture expansion on primary human dental pulp stem cells. Telomerase activity at both early (p4) and later passages (p11; **A)**. Representative images of senescence-associated β-galactosidase staining **(B & C)**. Scale bars = 500 μm.
*Statistically significant differences compared with human dental pulp stem cells at passage 4 ($p < 0.05$).
FBS: Fetal bovine serum; PRGF: Plasma rich in growth factors.

Discussion

In recent years, mesenchymal stem cell-based therapies have been developed to improve surgical techniques aimed at repairing human tissues. In this context, hDPSCs represent a valuable source of these progenitor cells due to the noninvasive nature of the isolation methods and to their high plasticity and multipotential capabilities [10]. Administering therapeutically meaningful numbers of hDPSCs in patients requires extensive *in vitro* cell propagation while preserving their functional properties. One of the main challenges to achieve a safer therapy is the use of animal-derived free products to avoid undesirable complications. In this sense, the objective of the present work is to assess the use of PRGF as a FBS substitute in the culture media for isolation, expansion, stimulation of the specific phenotype differentiation and finally, cryopreservation of hDPSC cells.

In previous studies, donor age-related biological properties have been described [35,36]. To avoid possible divergences in the different primary hDPSC cultures responses nonattributable to the culture medium supplements, similar aged donors were selected. On the other hand, impacted teeth were chosen to minimize the risk of pulp tissue contamination by oral microorganism, enabling to skip additional steps to samples disinfection with chemicals. In addition to this, in line with GMP guidelines, the explant method was used for cell isolation as the obtained cells are considered 'minimally manipulated' by the US FDA. Moreover, this technique would be easier, faster, safer and cheaper than enzymatic dissociation.

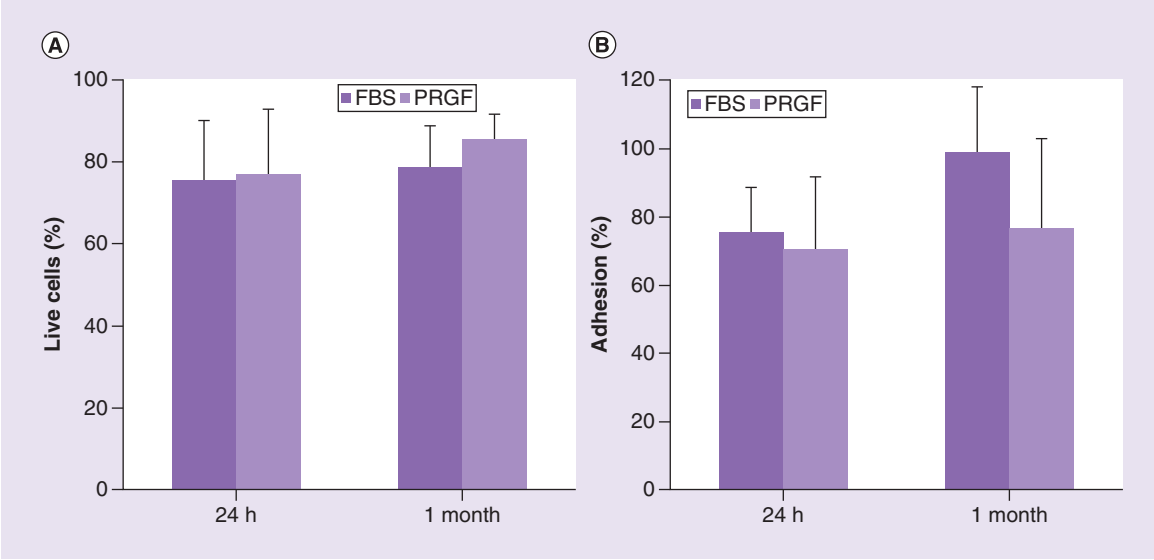

Figure 6. Effect of both culture supplements (fetal bovine serum and plasma rich in growth factors) on cryopreservation for at least 1 month. Determination of cell viability **(A)** and cell adhesion **(B)** after thawing and plating, respectively.
FBS: Fetal bovine serum; PRGF: Plasma rich in growth factor.

There are multiple studies comparing the safety and efficacy of PRP versus FBS as a stem cell culture media supplement for *ex vivo* procedures. Among the most recent ones, it should be mentioned the promising PRGF technology as an alternative to the actual xenogeneic supplementation in the cell therapy for corneal diseases [32]. The results of that study are in line with those obtained in the present work. Our study showed that PRGF supplement could be used as FBS substitute in the culture medium to successfully isolate hDPSCs, since no differences were found in the percentage of explants from which cells grew out. Differences were neither detected in the moment in which cells were first observed in the explant culture. Conversely, the number of cells per explant that were recovered when PRGF was included in the culture medium was significantly higher than in the case of FBS. This fact was in agree with the outcomes obtained in the proliferation assay. Our results correlate with previous works in which PRPs were proved like suitable substitutes for FBS for long-term maintenance of hDPSCs [37,38]. In fact, Muraglia *et al.* confirmed an increased cell proliferation of several primary cell cultures promoted by PRP-derived supplements, as well as stem cell cultures maintaining their biological stemness properties [24]. Similar effects have been described in DPSC cultures after treating with PRPs [38,39]. In particular, the stimulation of cell proliferation by PRGF was also described in stem cells obtained from several sources like bone marrow, eye or adipose tissue [32,40,41]. PRGF offers an autologous source of biologically active molecules being able to synergistically stimulate not only proliferation but also cell motility. In this respect, an increase of 2.5-folds in the number of migratory cells per mm^2 was achieved in the presence of PRGF in comparison with the same percentage of FBS in the culture medium. Supporting these results, a highly increased cell migration capability of epidermal stem cells incubated with PRP has been recently observed [42], the same effect has been described in adipose-derived stem cells after treating with PRGF [41].

The PRGF potential to stimulate hDPSCs differentiation was assessed as a quality control of the *in vitro* expanded cells. Osteogenic, adipogenic and chondrogenic differentiation were accomplished. Moreover, osteogenic and adipogenic differentiation capability was even improved in the presence of PRGF compared to FBS. As chondrogenic differentiation can be achieved in absence of FBS [43–45], the addition of PRGF was assessed to determine a possible improvement in this differentiation capability. Regardless of PRGF inclusion, results showed that no statistically significant differences were found in the potential of chondrogenic differentiation.

Numerous studies have also confirmed the contribution of PRPs in the differentiation potential of several human stem cells phenotypes to be applied in different regenerative therapies. In this sense, adipose-derived stem cell differentiation into myofibroblast-like cells [46] as well as toward chondrogenic and osteogenic phenotypes [47,48] have been induced with differentiation culture medium containing PRP. Another clear example is the chondrogenic

induction of bone marrow-derived stem cells by PRP [49]. Takahashi *et al.* [40] recently described an increased ALP activity with a stronger Alizarin red S staining in bone marrow-derived stem cells when PRGF was added.

Concerning to DPSCs, the potential of PRP to improve the differentiation processes has been assessed in multiple experimental works. Vasanthan *et al.* already showed the expression of several hepatic markers after substitution of FBS by PRP derivates in the differentiation culture medium of DPSC cultures [50]. Similarly, the efficacy of PRP on promoting osteogenic differentiation of DPSCs has been proved [22,38,39]. Recently, Otero *et al.* have quantified a higher DPSCs osteogenic induction by PRP in comparison with other differentiation reagents [51].

Another possible risk to keep in mind with respect to stem cell therapies is the cellular alteration that could be observed because of expanding cell cultures for a long time, in particular, those related to mitotic arrest. One of the main causes for cellular senescence has been attributed to progressive telomere shortening [52,53]. Detectable amounts of telomerase in hDPSC cultures have been quantified in our assays, but probably they are not sufficient to fully maintain telomere length in order to avoid the decrease of proliferation capability and cellular senescence. Moreover, lower amounts of this protein have been detected in hDPSCs at late passages. According to these results, higher telomere lenghts in DPSCs with high proliferative capacity compared with DPSCs with low proliferative rate have been recently described by Alraies *et al.* [19,54]. In our cellular senescence assays, we did not find differences between high passages DPSCs that were maintained with PRGF or those cultured with FBS as culture medium supplement. Taking into account the improvement in the proliferation capacity of hDPSCs when PRGF is added to the culture medium, it would not be necessary to expand cells until advanced passages for obtaining the appropriate number of cells.

In the case of hDPSCs are not to be used immediately, cryopreservation could be chosen as another option. There are previous reports on hDPSCs cryopreservation for different periods of time using DMSO as cryoprotectant agent [55,56]. Results in the present work confirmed that the use of either PRGF or FBS in the freezing medium did not show differences with respect to the cell viability after thawing. Moreover, their capability to adhere to culture flasks was neither altered. In this sense, Alsulaimani *et al.* showed the successful cryopreservation of hDPSCs maintaining their stemness properties for 2 years but using FBS in the freezing medium [57].

Conclusion

To the best of our knowledge, this is the first study where the autologous PRGF technology has been investigated as a human-based substitute to FBS in the whole process of culture of hDPSCs, from the isolation to the cryopreservation process. Taking all these results together, PRGF represents a promising alternative to FBS for isolate, culture and cryopreserve stem cells from dental pulp. Even more, PRGF would be a potent stimulator of hDPSC capacities to proliferate, migrate and differentiate, aimed at a specific therapeutic therapy. Therefore, the autologous PRGF technology could be a suitable and safer substitute for FBS as a culture medium supplement for clinical translation of cell therapy.

Translational perspective

Considering the current limitations of available therapeutic approaches, research efforts are being directed toward tissue engineering. This alternative approach focuses on overcoming the shortcomings of conventional treatment modalities to restore the original architecture and functionality. In this sense, stem cell therapy represents an emerging and promising tool because of its great therapeutic potential in regenerative medicine. DPSCs are obtained by noninvasive isolation methods, thus representing a valuable source of easy access. Nevertheless, *ex vivo* cell expansion is needed to obtain functional *in vitro* tissue substitutes prior to implantation. Despite FBS has been used for a long time as a widely accepted standard cell culture supplement, several concerns have led to explore other alternatives to provide safer and regulated cell therapy products. In recent years, PRP products have emerged as new tools to be used for their properties in tissue repair. The use of human PRPs as effective cell culture additive would minimize the risk of immunological reactions or infections. However, there are numerous protocols for the preparation of PRPs with multiple commercially available products. This methodological and composition variability contribute to the heterogeneity of the final therapeutic outcomes often described in the literature. In our findings, the autologous PRGF technology (a specific PRP product) that has been widely applied in different medical fields, has demonstrated its ability as a substitute for FBS in the culture medium. The implementation of human autologous PRGF into standard culture media supplement leads to personalized cellular therapies to facilitate the translation of stem cell therapy for clinical application. Further *in vivo* research should be performed to confirm these results that enable its regular use as an autologous standard culture medium supplement.

Summary points

- Stem cell therapy represents an emerging alternative approach because of its great therapeutic potential in regenerative medicine.
- Human dental pulp stem cells (hDPSCs) are obtained by noninvasive isolation methods, thus representing a valuable source of easy access with high plasticity and multipotential capabilities.
- *Ex vivo* cell expansion is required to achieve therapeutic outcomes.
- Fetal bovine serum (FBS) has been used as a widely accepted standard cell culture supplement however several safety and regulatory concerns have led to establish standardized xeno-free culture protocols.
- Platelet-rich plasma (PRP) products have emerged as a new alternative to be used. However, the variability in the PRP production contributes to the heterogeneity of the final therapeutic outcomes what makes it essential to evaluate each product individually.
- Plasma rich in growth factors (PRGF) is a great versatile PRP mainly characterized by the absence of leukocytes and by the use of calcium chloride to activate it, thus leading to a more biocompatible and safe technology avoiding inflammation.
- hDPSCs isolation, proliferation, migration, osteogenic mineralization and adipogenic differentiation were found to be significantly higher in cultures supplemented with PRGF than in cultures supplemented with FBS.
- The autologous PRGF technology has demonstrated its ability as a substitute for FBS supplement in the hDPSCs culture medium, thus enabling the clinical translation of stem cell therapy.

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Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

References

1. Anitua E, Prado R, Orive G. Endogenous morphogens and fibrin bioscaffolds for stem cell therapeutics. *Trends Biotechnol.* 31(6), 364–374 (2013).
2. Oikonomopoulos A, Van Deen WK, Manansala A-R *et al.* Optimization of human mesenchymal stem cell manufacturing: the effects of animal/xeno-free media. *Sci. Rep.* 5(1), 16570 (2015).
3. Herberts CA, Kwa MS, Hermsen HP. Risk factors in the development of stem cell therapy. *J. Transl. Med.* 9(1), 29 (2011).
4. La Noce M, Paino F, Spina A *et al.* Dental pulp stem cells: state of the art and suggestions for a true translation of research into therapy. *J. Dent.* 42(7), 761–768 (2014).
5. Pisciotta A, Carnevale G, Meloni S *et al.* Human dental pulp stem cells (hDPSCs): isolation, enrichment and comparative differentiation of two sub-populations. *BMC Dev. Biol.* 15(1), (2015).
6. Bonnamain V, Thinard R, Sergent-Tanguy S *et al.* Human dental pulp stem cells cultured in serum-free supplemented medium. *Front. Physiol.* 4, 357 (2013).
7. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. *Proc. Natl Acad. Sci. USA* 97(25), 13625–13630 (2000).
8. Maioli M, Basoli V, Santaniello S *et al.* Osteogenesis from dental pulp derived stem cells: a novel conditioned medium including melatonin within a mixture of hyaluronic, butyric, and retinoic acids. *Stem Cells Int.* 2016, 2056416 (2016).

9. Nakashima M, Iohara K, Murakami M. Dental pulp stem cells and regeneration. *Endodontic Topics* 28(1), 38–50 (2013).
10. Anitua E, Troya M, Zalduendo M. Progress in the use of dental pulp stem cells in regenerative medicine. *Cytotherapy* 20(4), 479–498 (2018).
11. Bieback K. Platelet lysate as replacement for fetal bovine serum in mesenchymal stromal cell cultures. *Transfus. Med. Hemother.* 40(5), 326–335 (2013).
12. Burnouf T, Strunk D, Koh MBC, Schallmoser K. Human platelet lysate: Replacing fetal bovine serum as a gold standard for human cell propagation? *Biomaterials* 76, 371–387 (2016).
13. Tekkatte C, Gunasingh GP, Cherian KM, Sankaranarayanan K. 'Humanized' stem cell culture techniques: the Animal Serum Controversy. *Stem Cells Int.* 2011, 1–14 (2011).
14. Wu RX, Yu Y, Yin Y, Zhang XY, Gao LN, Chen FM. Platelet lysate supports the *in vitro* expansion of human periodontal ligament stem cells for cytotherapeutic use. *J. Tissue Eng. Regen. Med.* 11(8), 2261–2275 (2017).
15. Rauch C, Feifel E, Amann E-M *et al.* Alternatives to the use of fetal bovine serum: human platelet lysates as a serum substitute in cell culture media. *ALTEX* 28(4), 305 (2011).
16. Jung S, Panchalingam KM, Rosenberg L, Behie LA. *Ex vivo* expansion of human mesenchymal stem cells in defined serum-free media. *Stem Cells Int.* 2012, 1–21 (2012).
17. Verbeek R. Generation of mesenchymal stem cells as a medicinal product in organ transplantation. *Curr. Opin. Organ Transplant.* 18(1), 65–70 (2013).
18. Dessels C, Potgieter M, Pepper MS. Making the switch: alternatives to fetal bovine serum for adipose-derived stromal cell expansion. *Front. Cell Dev. Biol.* 4, 115 (2016).
19. Ferro F, Spelat R, Beltrami AP, Cesselli D, Curcio F. Isolation and characterization of human dental pulp derived stem cells by using media containing low human serum percentage as clinical grade substitutes for bovine serum. *PLoS ONE* 7(11), e48945 (2012).
20. Witzeneder K, Lindenmair A, Gabriel C *et al.* Human-derived alternatives to fetal bovine serum in cell culture. *Transfus. Med. Hemother.* 40(6), 417–423 (2013).
21. Fernandes G, Yang S. Application of platelet-rich plasma with stem cells in bone and periodontal tissue engineering. *Bone Res.* 4, 16036 (2016).
22. Abuarqoub D, Awidi A, Abuharfeil N. Comparison of osteo/odontogenic differentiation of human adult dental pulp stem cells and stem cells from apical papilla in the presence of platelet lysate. *Arch. Oral Biol.* 60(10), 1545–1553 (2015).
23. Matthyssen S, Ni Dhubhghaill S, Van Gerwen V, Zakaria N. Xeno-free cultivation of mesenchymal stem cells from the corneal stroma. *Invest. Ophthalmol. Vis. Sci.* 58(5), 2659–2665 (2017).
24. Muraglia A, Todeschi MR, Papait A *et al.* Combined platelet and plasma derivatives enhance proliferation of stem/progenitor cells maintaining their differentiation potential. *Cytotherapy* 17(12), 1793–1806 (2015).
25. Rungsiwiwut R, Ingrungruanglert P, Numchaisrika P, Virutamasen P, Phermthai T, Pruksananonda K. Human umbilical cord blood-derived serum for culturing the supportive feeder cells of human pluripotent stem cell lines. *Stem Cells Int.* 2016, 1–12 (2016).
26. Shetty P, Bharucha K, Tanavde V. Human umbilical cord blood serum can replace fetal bovine serum in the culture of mesenchymal stem cells. *Cell. Biol. Int.* 31(3), 293–298 (2007).
27. Anitua E. Plasma rich in growth factors: preliminary results of use in the preparation of future sites for implants. *Int. J. Oral Maxillofac. Implants* 14(4), 529–535 (1999).
28. Anitua E, Alkhraisat MH, Orive G. Perspectives and challenges in regenerative medicine using plasma rich in growth factors. *J. Control. Rel.* 157(1), 29–38 (2012).
29. Anitua E, Pino A, Troya M, Jaén P, Orive G. A novel personalized 3D injectable protein scaffold for regenerative medicine. *J. Mater. Sci. Mater. Med.* 29(1), 7 (2018).
30. Anitua E, Troya M, Zalduendo M, Tejero R, Orive G. Progress in the use of autologous regenerative platelet-based therapies in implant dentistry. *Curr. Pharm. Biotechnol.* 17(5), 402–413 (2016).
31. Anitua E, Sánchez M, Orive G, Andía I. The potential impact of the preparation rich in growth factors (PRGF) in different medical fields. *Biomaterials* 28(31), 4551–4560 (2007).
32. Riestra AC, Vazquez N, Chacon M *et al.* Autologous method for *ex vivo* expansion of human limbal epithelial progenitor cells based on plasma rich in growth factors technology. *Ocul. Surf.* 15(2), 248–256 (2017).
33. Marrelli M, Gentile S, Palmieri F, Paduano F, Tatullo M. Correlation between Surgeon's experience, surgery complexity and the alteration of stress related physiological parameters. *PLoS ONE* 9(11), e112444 (2014).
34. Dominici M, Le Blanc K, Mueller I *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8(4), 315–317 (2006).
35. Kellner M, Steindorff MM, Strempel JF, Winkel A, Kühnel MP, Stiesch M. Differences of isolated dental stem cells dependent on donor age and consequences for autologous tooth replacement. *Arch. Oral Biol.* 59(6), 559–567 (2014).

36. Wu W, Zhou J, Xu CT, Zhang J, Jin YJ, Sun GL. Derivation and growth characteristics of dental pulp stem cells from patients of different ages. *Mol. Med. Rep.* 12(4), 5127–5134 (2015).
37. Kleplová TS, Soukup T, Řeháček V, Suchánek J. Human plasma and human platelet-rich plasma as a substitute for fetal calf serum during long-term cultivation of mesenchymal dental pulp stem cells. *Acta Medica (Hradec Kralove)* 57(3), 119–126 (2014).
38. Saeed MA, El-Rahman MA, Helal ME, Zaher AR, Grawish ME. Efficacy of human platelet rich fibrin exudate vs fetal bovine serum on proliferation and differentiation of dental pulp stem cells. *Int. J. Stem Cells* 10(1), 38–47 (2017).
39. Wen J, Li HT, Li SH, Li X, Duan JM. Investigation of modified platelet-rich plasma (mPRP) in promoting the proliferation and differentiation of dental pulp stem cells from deciduous teeth. *Braz. J. Med. Biol. Res.* 49(10), e5373 (2016).
40. Takahashi K, Okada H, Eda T *et al.* Plasma rich in growth factors stimulates proliferation and mineralization in mesenchymal stem cells from human bone marrow. *Int. J. Oral-Med. Sci.* 16(1), 1–7 (2017).
41. Mellado-López M, Griffeth RJ, Meseguer-Ripolles J, Cugat R, García M, Moreno-Manzano V. Plasma rich in growth factors induces cell proliferation, migration, differentiation, and cell survival of adipose-derived stem cells. *Stem Cells Int.* 2017, 5946527 (2017).
42. Wu Y, Fan J, Zhang B, Tang J, Li Q. Enhanced proliferation and migration capability of epidermal stem cells by PRP and PDGF stimulation. *Int. J. Clin. Exp. Pathol.* 10(8), 8804–8812 (2017).
43. Westin CB, Trinca RB, Zuliani C, Coimbra IB, Moraes AM. Differentiation of dental pulp stem cells into chondrocytes upon culture on porous chitosan–xanthan scaffolds in the presence of kartogenin. *Mater. Sci. Eng. C Mater. Biol. Appl.* 80, 594–602 (2017).
44. Zhang W, Walboomers XF, Shi S, Fan M, Jansen JA. Multilineage differentiation potential of stem cells derived from human dental pulp after cryopreservation. *Tissue Eng.* 12(10), 2813–2823 (2006).
45. Struys T, Moreels M, Martens W, Donders R, Wolfs E, Lambrichts I. Ultrastructural and immunocytochemical analysis of multilineage differentiated human dental pulp- and umbilical cord-derived mesenchymal stem cells. *Cells Tissues Organs* 193(6), 366–378 (2011).
46. Chignon-Sicard B, Kouidhi M, Yao X *et al.* Platelet-rich plasma respectively reduces and promotes adipogenic and myofibroblastic differentiation of human adipose-derived stromal cells via the TGFβ signalling pathway. *Sci. Rep.* 7(1), 2954 (2017).
47. Scioli MG, Bielli A, Gentile P, Cervelli V, Orlandi A. Combined treatment with platelet-rich plasma and insulin favours chondrogenic and osteogenic differentiation of human adipose-derived stem cells in three-dimensional collagen scaffolds. *J. Tissue Eng. Regen. Med.* 11(8), 2398–2410 (2017).
48. Xu FT, Li HM, Yin QS *et al.* Effect of activated autologous platelet-rich plasma on proliferation and osteogenic differentiation of human adipose-derived stem cells *in vitro*. *Am. J. Transl. Res.* 7(2), 257–270 (2015).
49. Wang S-Z, Chang Q, Kong X-F, Wang C. The chondrogenic induction potential for bone marrow-derived stem cells between autologous platelet-rich plasma and common chondrogenic induction agents: a preliminary comparative study. *Stem Cells Int.* 2015, 589124 (2015).
50. Vasanthan P, Gnanasegaran N, Govindasamy V *et al.* Comparison of fetal bovine serum and human platelet lysate in cultivation and differentiation of dental pulp stem cells into hepatic lineage cells. *Biochem. Eng. J.* 88, 142–153 (2014).
51. Otero L, Carrillo N, Calvo-Guirado JL, Villamil J, Delgado-Ruiz RA. Osteogenic potential of platelet-rich plasma in dental stem-cell cultures. *Br. J. Oral Maxillofac. Surg.* 55(7), 697–702 (2017).
52. Turinetto V, Vitale E, Giachino C. Senescence in human mesenchymal stem cells: functional changes and implications in stem cell-based therapy. *Int. J. Mol. Sci.* 17(7), 1164 (2016).
53. Flores I, Blasco MA. The role of telomeres and telomerase in stem cell aging. *FEBS Lett.* 584(17), 3826–3830 (2010).
54. Alraies A, Alaidaroos NY, Waddington RJ, Moseley R, Sloan AJ. Variation in human dental pulp stem cell ageing profiles reflect contrasting proliferative and regenerative capabilities. *BMC Cell Biol.* 18(1), 12 (2017).
55. Woods EJ, Perry BC, Hockema JJ, Larson L, Zhou D, Goebel WS. Optimized cryopreservation method for human dental pulp-derived stem cells and their tissues of origin for banking and clinical use. *Cryobiology* 59(2), 150–157 (2009).
56. Conde MCM, Chisini LA, Grazioli G *et al.* Does cryopreservation affect the biological properties of stem cells from dental tissues? A systematic review. *Braz. Dent. J.* 27(6), 633–640 (2016).
57. Alsulaimani RS, Ajlan SA, Aldahmash AM, Alnabaheen MS, Ashri NY. Isolation of dental pulp stem cells from a single donor and characterization of their ability to differentiate after 2 years of cryopreservation. *Saudi Med. J.* 37(5), 551–560 (2016).

Case Series

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Regenerative Medicine

Evaluation of autologous adipose-derived mesenchymal stem cell therapy in focal chondral defects of the knee: a pilot case series

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Aim: To evaluate the safety, pain, functional and structural improvements after autologous adipose-derived mesenchymal stem cell (ADMSC) therapy in combination with arthroscopic abrasion arthroplasty in focal chondral defects of the knee. **Methods:** Eight patients with a focal full thickness chondral defect of the knee underwent arthroscopic abrasion arthroplasty followed by postoperative intra-articular injections of autologous ADMSCs (50 x 10^6 ADMSCs at baseline and 6 months). Clinical outcome was assessed using numeric pain rating scale, Knee Injury and Osteoarthritis Outcome Score and the Western Ontario and McMaster Universities Osteoarthritis Index. Structural outcome was determined by magnetic resonance imaging. Outcome was assessed over 24 months. **Results:** No serious adverse events occurred. Participants observed clinically significant improvement in pain and function. Magnetic resonance imaging analysis showed cartilage regeneration with T2 mapping values comparable to hyaline cartilage. **Conclusion:** Arthroscopic abrasion arthroplasty in combination with intra-articular ADMSC therapy results in reproducible pain, functional and structural improvements with regeneration of hyaline-like cartilage. **Trial registration number:** ACTRN12617000638336

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Keywords: cartilage • chondral defect • mesenchymal stem cells • osteoarthritis • regeneration

It is recognized that articular cartilage has a limited capacity to heal with several studies indicating that isolated chondral defects predispose patients to later development of early onset and progressive osteoarthritis (OA) [1–4]. The management of focal chondral defects of the knee can be particularly challenging and is influenced by factors including patient age, partial or full thickness chondral loss and the site of the lesion.

Pain and functional limitation as a consequence of a chondral defect and the additional concern regarding later development of early onset OA has meant surgical intervention is often considered. These techniques include, but are not limited to, arthroscopic debridement/chondroplasty, bone marrow stimulating techniques including microfracture/microdrilling and arthroscopic abrasion arthroplasty and graft techniques such as autologous chondrocyte implantation (ACI) or osteochondral autograft transplantation. However, none of these surgical interventions guarantee a long-term sustained improvement in pain, function and structural improvement of the affected joint.

Microfracture was developed during the early 1980s and has become a commonly used surgical technique in the treatment of an isolated chondral defect. The technique involves the drilling or punching of holes through the

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subchondral plate and is thought to encourage migration of bone marrow derived pluripotent cells to the articular surface with later cartilage repair. More recently however, some clinicians have questioned the ongoing validity of this operative technique, arguing that it destroys the gross structure of the subchondral plate, may promote subchondral cyst formation, is associated with increased pain in a significant portion of patients and stimulates only fibrocartilage, with observed decrease in clinical outcome within 2 years [5–8].

Arthroscopic abrasion arthroplasty is a modification of the open Magnusson debridement arthroplasty and was pioneered by Lanny Johnson [9]. The use of abrasion arthroplasty for full thickness cartilage lesions has been associated with prolonged functional improvement with Sansone and colleagues noting sustained positive functional outcome at follow-up of 20 years in 89.5% of patients under the age of 50 years and in 55.7% of patients aged 50 or older [10]. Results were significantly better in patients with lesions less than 4 cm^2. This observation has led some authors to postulate that arthroscopic abrasion is a preferred bone marrow stimulation technique over microfracture [11]. Such outcomes however are not consistent with other articles citing less reproducible outcome with progression of disease within 3 years of follow-up and early conversion to total knee replacement [12].

Past research has shown histopathology of fibrocartilage formation post-microfracture or abrasion arthroplasty at second-look arthroscopy and subsequent biopsy [9,13,14]. As fibrocartilage lacks the load bearing properties of hyaline cartilage it is postulated that it may fail to significantly alleviate pain and delay progressive degeneration. The reduced load bearing properties of fibrocartilage and often observed inadequate defect filling have been postulated as a cause for poor long-term outcome.

Chondrocyte transplantation techniques including ACI and matrix induced ACI (MACI) have shown encouraging results in both preclinical and clinical trials with hyaline-like cartilage formation and observed long-term durability [15–18]. Such interventions however are technically difficult, are additionally limited by observed chondral donor site morbidity and at times show poor integration with surrounding native cartilage, early degeneration and inconsistent functional outcome.

An improved understanding of the pathology of chondral defects and the limitations observed in current surgical management techniques has seen renewed interest in the area of regenerative medicine. The ability of mesenchymal stem cells (MSCs) to differentiate along a mesodermal lineage including into bone and cartilage has led to interest in their potential role in assisting cartilage repair [19–21]. It is however their observed immunomodulatory role and expression of anabolic cytokines through both paracrine and cell-to-cell interaction that is now considered their primary mechanism of action and likely role in tissue repair [22–26].

MSCs may directly modulate the cytokines responsible for progressive cartilage degeneration seen in focal chondral lesions through the suppression of inflammatory T-cell proliferation, inhibition of monocyte maturation and resultant inhibition of the inflammatory cascades involving cytokines including IL-1, TNF-α and matrix metalloproteinases [27]. In addition to inhibition of inflammatory pathways, MSCs secrete several anabolic cytokines including TGF-β, VEGF and EGF, and may lead to local tissue repair through stimulation of trophic pathways [23,24,28].

Clinically, Gobbi and colleagues have shown the successful application of bone marrow derived MSCs within a collagen matrix in the treatment of full-thickness cartilage lesions of the knee [29]. Magnetic resonance imaging (MRI) indicated complete healing in 80% of trial participants with later histological analysis showing type II collagen and hyaline-like cartilage morphology. In addition to this some authors have suggested that the application of cellular therapies including MSCs may assist in the conversion of regenerative fibrocartilage following bone marrow stimulation techniques such as microfracture or abrasion arthroplasty to mature hyaline-like cartilage [30]. Both preclinical and early clinical trials have supported this hypothesis with intra-articular injections of MSCs in conjunction with surgical microfracture/microdrilling showing significant improvement in articular cartilage repair with hyaline-like cartilage regeneration and type II collagen shown on histopathology [31,32].

The aim of this case series is to assess both the safety/tolerability and efficacy of adipose-derived MSCs (ADMSCs) therapy in conjunction with arthroscopic abrasion arthroplasty in the treatment of focal full thickness chondral defects of the knee.

Materials & methods

Trial design

This prospective case series forms part of a broader case series approved by the Human Research Ethics Committee of Charles Sturt University. The case series is registered on the Australian New Zealand Clinic Trial Registry

Table 1. Eligibility criteria.

Inclusion criteria
1. Isolated full thickness (Grade IV) chondropathology of the medial or lateral femoral condyle as determined by the modified ICRS score†
2. Primary conservative treatment already undertaken including analgesia/anti-inflammatory medication, an attempted prescribed exercise and weight management program, and biomechanical adjustment including bracing if appropriate as prescribed by a physiotherapist, podiatrist or medical practitioner
3. Sufficient English skills to complete the questionnaires required for the study
Exclusion criteria
1. Age <18 years
2. Pregnancy
3. Breast feeding
4. Have any other causes of their symptoms suspected to be due to serious pathology such as tumor or referral from the spine
5. Current cancer
6. History of significant organ impairment/failure (i.e., renal failure)
7. History of allergy to any substances used within ADMSC preparation and treatment

†Specific criteria for this sub-group case series within the broader case series.
ADMSC: Adipose-derived mesenchymal stem cell; ICRS: International Cartilage Repair Society.

(ACTRN12617000638336). All participants underwent an eligibility screening process and completed a formal written informed consent form.

All participants underwent arthroscopic abrasion arthroplasty and intra-articular autologous ADMSC therapy. Intra-articular injections of ADMSCs were performed within a private practice setting and within 1 week of knee arthroscopy and again at 6 months. A total of 8 participants were enrolled. Participants were followed up for a period of 24 months.

Participant selection

As indicated, this prospective case series represents a subgroup of participants in a broader case series assessing the outcome of ADMSC therapy in arthritis/chondropathology. The participants in the case series met the inclusion criteria for the broader encompassing case series but specifically had MRI confirmed areas of focal isolated full thickness chondropathology (rather than diffuse arthritic change) which was assessed as amenable to arthroscopic abrasion arthroplasty (see Table 1). Grading of chondropathology was assessed according to modified International Cartilage Repair Society (ICRS) grading (see Outcome Measures) [33].

Baseline assessment included a musculoskeletal examination and diagnostic imaging (i.e., MRI) to determine eligibility. All participants received written information regarding the trial and completed a formal written informed consent form.

Autologous ADMSC preparation

Harvest procedure

Adipose tissue was chosen as a source of MSCs due to the ease of harvest, the relative abundance of MSCs (up to 10% of total stromal vascular fractions of the digested adipose tissue) and the observed chondrogenic potential of ADMSCs [34,35].

A minimally invasive liposuction (lipoharvest) was performed under local anesthetic tumescence control as previously described in past publications [36–41]. Lipoaspirate was collected using either a manual syringe suction technique or via mechanical suction into a sterile single use container (Shippert Medical, CO, USA). Lipoaspirate was transferred directly after collection to a clean room laboratory facility (Magellan Stem Cells, Victoria Australia) for further processing.

Isolation & expansion of MSCs

ADMSCs were isolated and expanded using previously published protocols [32–37]. All processing was performed within a clean room facility with ISO5 air quality or greater and within Class II Biological Safety Cabinets. ADMSCs were cyropreserved within cryovials using a validated control rate freezing method and stored in liquid nitrogen until required [42,43].

Isolated cell populations were confirmed as ADMSCs in accordance with standards established by the International Society of Cellular Therapy [44]. Using flow cytometry with Fluorescence-Activated Cell Sorting, isolated

Table 2. Fluorescence-activated cell sorting surface marker analysis.

	Histograms – positive markers (%)			Histograms – negative markers (%)			
	CD90 +ve	CD73 +ve	CD105 +ve	CD14 +ve	CD19 +ve	CD34 +ve	CD45 +ve
Mean (standard devitaion)	96.39 (2.83)	97.17 (4.33)	95.22 (5.28)	0.74 (0.3)	0.14 (0.2)	0.75 (0.56)	0.35 (0.1)

Mean data (n = 8 patients) is presented with standard deviation in brackets. See Supplementary Table 1 for individual participant data.

cells were assessed for the presence of MSC specific surface markers CD90, CD73 and CD105 and the absence of hematopoietic surface makers CD14, CD19, CD34 and CD45 (Table 2).

In addition to Fluorescence-Activated Cell Sorting analysis, all isolated ADMSC participant samples underwent independent sterility testing for microbial growth/contamination.

Carrier media

MSCs were suspended in an autologous biological carrier media which was either autologous conditioned serum (ACS) or platelet-rich concentrate (PRC; Supplementary Table 2). As participant recruitment occurred over considerable time, ACS or PRC was chosen dependent upon availability of resources.

ACS preparation involved the withdrawal of a total of 27 ml of whole blood via venipuncture and collected within 3 × 9 ml sterile S-Monovette® clotting activator tubes (Starstedt, Numbrecht, Germany). The tubes were placed in an incubator at 38° Celsius for 24 h and later centrifuged at 1000 rpm for 5 min with resultant separation of the whole blood and the serum. The serum layer was removed and filtered through a 0.2 micron syringe filter (PALL, NY, USA) producing an acellular sample of ACS.

PRC preparation involved the withdrawal of 25.5 ml of whole blood via venipuncture and collected within 3 × 8.5 ml ACD (trisodium citrate 22.0 g/l, citric acid 8.0 g/l and dextrose 24.5 g/l) BD Vacutainers (BD, NJ, USA). The blood underwent an initial soft spin at 1000 rpm for 10 min using a bench top centrifuge. The separated plasma containing the platelets was then transferred to a sterile tube which subsequently underwent a second hard spin at 3500 rpm for a total of 5 min resulting in formation of a platelet concentrate plug and platelet poor plasma (PPP). Excess PPP was removed and discarded leaving only 6 ml of PPP and platelet concentrate. Using gentle manual agitation the platelet plug was reconstituted within the PPP creating a platelet-rich plasma (PRP) preparation. The PRP was activated using the addition of 0.2 ml of calcium chloride (10%) and incubated at 37°C for 10 min, creating a fibrin clot. This was allowed to retract and resorb over time. The final preparation was passed through a 0.2 micro syringe filter (PALL, NY, USA) producing an acellular and sterile PRC.

Both autologous media preparations were stored at -20°C for later use at time of MSC therapy.

Intervention

Arthroscopic abrasion arthroplasty

Surgery was performed by an experienced orthopedic knee specialist under general anesthetic and lower limb vascular tourniquet control. An arthroscopic examination was performed with chondroplasty to areas of unstable cartilage and limited resection/debridement of displaced or complex meniscal tears, if present, using a 3 mm arthroscopic shaver. Arthroscopic abrasion arthroplasty was undertaken following methods previously described by Johnson *et al.* [9]. Abrasion was achieved using a 4–4.5 mm burr with areas of exposed bone eburnated down to the subchondral plate until capillary bleeding was observed (Figure 1).

In the immediate postoperative period, participants were placed in a continuous passive motion device until discharge on the following day. The continuous passive motion was set at 0° to 90° and participants were allowed to increase the passive ROM based on symptoms.

Injection method

At the time of injection, the cryopreserved cells were thawed in a prewarmed sterile water bath, with cryoprotectant removed through centrifugation and washing in chilled sterile phosphate buffered saline. The resultant cell pellet was resuspended in 3 ml of autologous carrier media. Cell number and viability was assessed using a Muse Cell Analyzer (Millipore Sigma, MA, USA; Table 3).

Participants underwent an initial intra-articular injection of ADMSCs within 1 week of their arthroscopic surgery and again at 6 months. At the time of injection, the participant's knee was prepared using standard sterility protocols

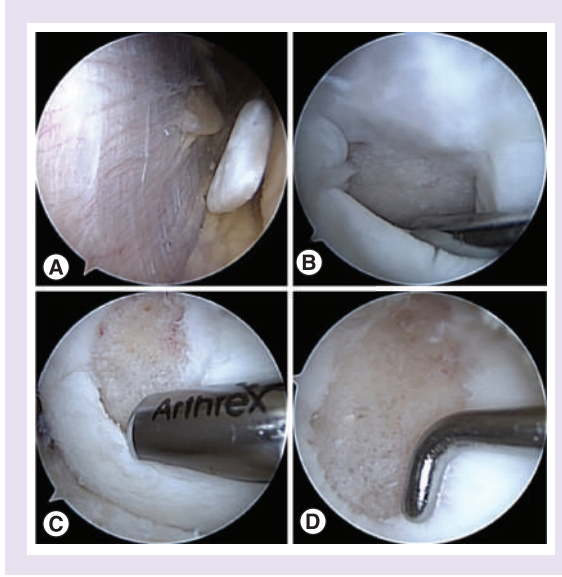

Figure 1. Arthroscopic abrasion arthroplasty. Intraoperative arthroscopic pictures showing **(A)** loose chondral body, **(B)** a full thickness chondral defect – modified International Cartilage Repair Society Grade IV – with unstable chondral border, **(C)** arthroscopic chondroplasty of unstable margins, **(D)** final area of abrasion arthroplasty after use of a 4 mm burr with exposure of subchondral bone.

Table 3. Average cell count and viability confirmed using a Muse Cell Analyzer.

	Baseline ADMSC injection		6 Month ADMSC Injection	
	Cell count (million)	Cell viability (%)	Cell count (Million)	Cell viability (%)
Mean (Standard deviation)	51.5 (2.9)	97.3 (1.2)	52.6 (5)	96.1 (2.5)

See Supplementary Table 3 for individual participant cell count and viability data.
ADMSC: Adipose-derived mesenchymal stem cell.

and 2 ml of 1% lidocaine infiltrated superficially to the joint capsule. Using a superolateral patella approach and under ultrasound guidance the resuspended autologous ADMSCs were injected into the intra-articular knee space. Prior to the first injection up to 15 ml of hemarthrosis effusion, a result of the arthroscopic abrasion arthroplasty, was aspirated and discarded.

Postoperative rehabilitation

Following arthroscopic abrasion arthroplasty the participants were limited to touch weight bearing with the use of crutches for a period of 4 weeks. During this time all participants were advised on a lower limb muscular activation program and commenced active range of motion using a stationary bike at minimal or no resistance. Participants were advised to complete 30–60 min of active range of motion exercises at least twice per day.

After 4 weeks, all participants were allowed to transition to partial weight bearing and then to weight bearing as tolerated. All participants were fitted with a uni-compartmental unloading knee brace (Ossur Unloader One, Ossur, Reykjavik, Iceland) and instructed to wear the brace for all partial to full weight bearing activities during the first 3 months postoperatively.

Outcome measures

Primary outcome aims of this trial were to assess the safety/tolerability of ADMSCs in combination with arthroscopic abrasion arthroplasty and also pain and functional changes as measured by the numeric pain rating scale (NPRS), the Knee Injury and Osteoarthritis Outcome Score (KOOS) and the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC). A secondary outcome of structural improvement was assessed using MRI with observation of modified ICRS grading at site of abrasion, MRI Observation of Cartilage Repair Tissue (MOCART) score and use of the validated noninvasive method of T2 relaxation time for cartilage quality (Table 4).

Questionnaires

Outcome questionnaires were recorded using the software program Clinical Intelligence (Clinical Intelligence, Melbourne, Australia) and were recorded at baseline, 1, 6, 12, 18 and 24 months.

Table 4. Outcome measures.

Outcome measure	Measurement point (months)
Primary outcome measure	
1. Adverse events	0, 1, 6, 12, 18, 24
2. Numeric Pain Rating Scale	0, 1, 6, 12, 18, 24
3. Knee Injury and Osteoarthritis Outcome Score	0, 1, 6, 12, 18, 24
4. Western Ontario and McMaster Universities Osteoarthritis Index	
Secondary outcome measure	
1. MRI analysis	0, 12, 24
– Modified ICRS grade	0, 12, 24
– MOCART score	0, 24
– T2 cartilage mapping	

ICRS: International Cartilage Repair Society; MOCART: MRI Observation of Cartilage Repair Tissue.

- NPRS – participants were asked to rate their average pain intensity over the past week on an 11 point scale of 0–10 [45].
- KOOS – consisting of 5 subscales – pain, symptoms, function in daily living, function in sport and recreation and knee related quality of life. A score is allocated to all subscales with 100 indicating no symptoms and 0 indicating maximal symptoms [46].
- WOMAC – a validated quality of life score in patients with symptomatic OA [47]. The Global WOMAC score is presented as an inverse percentage so to be comparable to the KOOS subscales.

Magnetic resonance imaging

MRI was performed with a dedicated knee coil on a 1.5 T or greater MRI. Proton density and proton density fat saturated (PDFS) images were taken in coronal, sagittal and axial planes. The area of chondropathology prior to, at 12 months and after 24 months (average 29 months) post-treatment was visually assessed by MRI using the modified ICRS grade and also the validated MOCART score [33,48]. The MOCART score assesses the area of cartilage repair and surrounding tissue over nine categories with a maximal total score of 100 indicating complete hyaline like cartilage repair. The modified ICRS grade was allocated using the below criteria -

– Grade 0: normal cartilage
– Grade 1: focal blistering and intra-cartilaginous low-signal intensity area with an intact surface and bottom
– Grade 2: irregularities on the surface or bottom and loss of thickness of less than 50%
– Grade 3: deep ulceration with loss of thickness of more than 50%
– Grade 4: full thickness cartilage wear with exposure of subchondral bone.

In addition to semi-quantitative assessment, cartilage quality at the site of abrasion was quantitatively assessed using the validated technique of MRI T2 relaxation time cartilage mapping [49,50]. T2 relaxation time values were compared against an area of native cartilage within the contralateral femoral condyle of the joint.

Sample size

This study represents a limited pilot case series to assess the safety/tolerability and efficacy of arthroscopic abrasion arthroplasty in combination with ADMSCs. No sample size calculation was performed.

Adverse events

An adverse event (AE) was defined as an undesirable clinical occurrence in a participant which was not present prior to treatment or which increased in severity after treatment and was observed during the period of follow-up. AEs may be related or unrelated to the treatment and may be expected or unexpected. AEs were assessed in regards to severity:

Mild: a transient symptom or clinical sign that does not interfere with the subject's usual activity and is resolved with use of simple interventions including simple analgesia.

Moderate: a symptom which limits the subject's usual daily activity and/or requires symptomatic treatment including regular analgesia (i.e., opiate analgesia).

Severe: a symptom which causes severe discomfort and/or significantly impacts on the subject's usual activity and extends for >2 weeks.

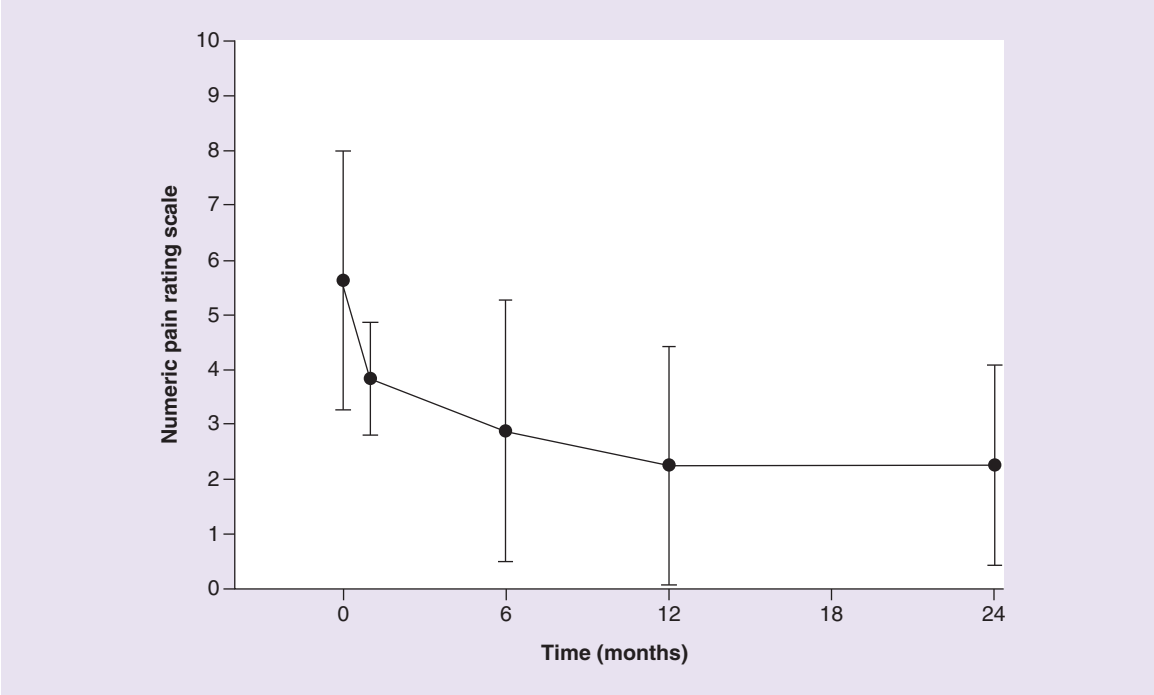

Figure 2. Numeric Pain Rating Scale.
Error bars represent 95% CI.

Results

Demographic characteristics

The case series consisted of four females and four males aged between 23 and 52 years of age (mean 36 years). Three participants had a past history of anterior cruciate ligament (ACL) rupture and had undergone prior ACL reconstruction. All knees were clinically stable on examination. Participants had a mean BMI of 25.24 with no participants categorized as obese (see Supplementary Table 4 for individual participant BMI data). All patients were otherwise well with no history of diabetes or smoking. A single patient had hypertension for which they took an antihypertensive and their blood pressure was well controlled. All participants used simple analgesia and/or oral nonsteroidal anti-inflammatories as required.

Site of the chondral lesion was evenly distributed between the medial compartment and lateral compartment with a single lesion involving the lateral tibial plateau. Average size of the Grade IV chondral lesion was 17.1 mm (length/sagittal plane) × 9.5 mm (width/coronal plan).

Pain & function

Consistent and progressive improvement in pain – as measured by NPRS – was documented over the period of follow-up with a mean (standard deviation) pain score of 5.6 (2.8) improving to 2.3 (2.2) at 24 months equating to a 60% reduction in pain levels (Figure 2). Variability among participants was observed in clinical recovery time following arthroscopic abrasion arthroplasty with 1 participant recording increased pain from baseline during the first 6 months of follow-up. This patient had an additional comorbidity of a past anterior cruciate ligament rupture with subsequent ligament reconstruction and incomplete recovery which may have influenced their expected outcome.

KOOS subscale analysis indicated consistent improvement in all subscales with values continuing to improve until final follow-up at 24 months (Figure 3). Sport and recreational activity subscale improved by an average of 111% with quality of life (QoL) increasing by over 50%.

Global WOMAC score expressed as an inverse percentage reflected the improvements seen in KOOS (Figure 4).

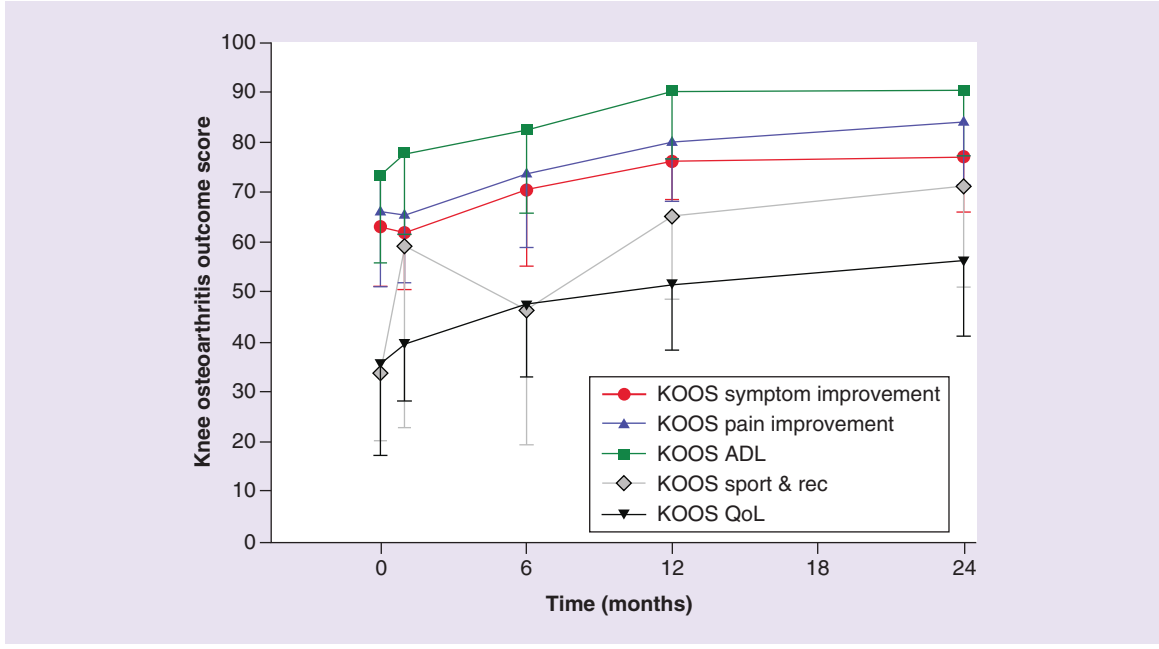

Figure 3. Knee Injury and Osteoarthritis Outcome Score.
Unidirectional error bars represent 95% CI.
KOOS: Knee Injury and Osteoarthritis Outcome Score; QoL: Quality of life.

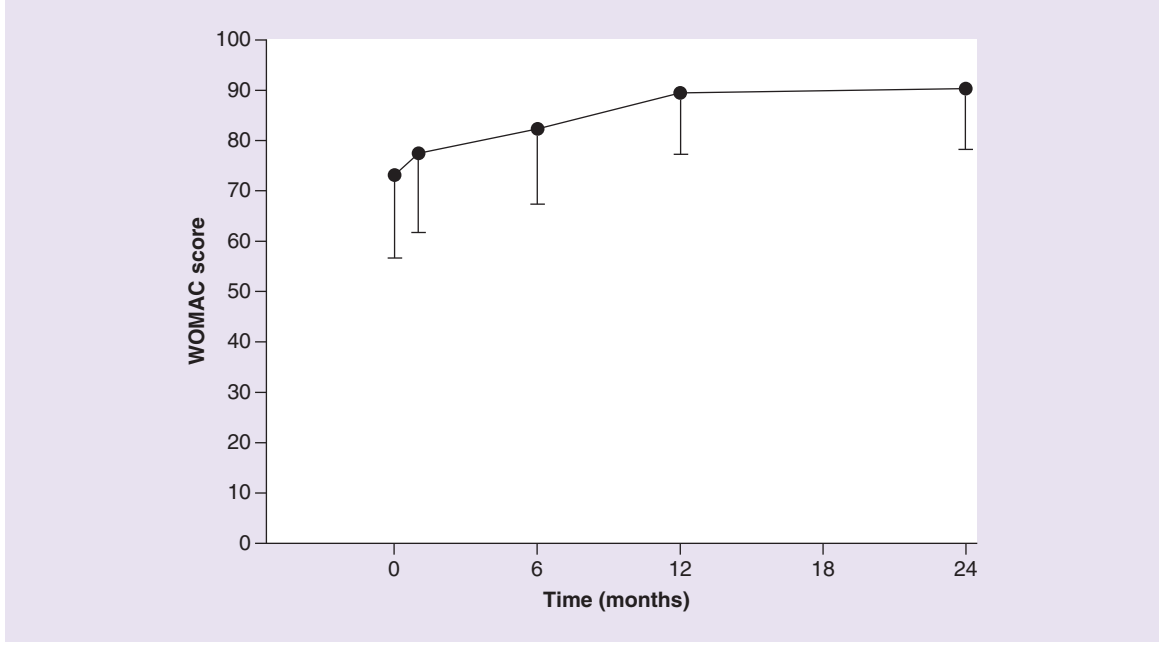

Figure 4. Global Western Ontario and McMaster Universities Osteoarthritis Index Score. Unidirectional error bars represent 95% CIs. Western Ontario and McMaster Universities Osteoarthritis Index is represented as an inverse score to allow direct comparison to Knee Injury and Osteoarthritis Outcome Score analysis.
WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index.

Structural outcome

MRI assessment at 12 and 24+ months showed successful regeneration of articular cartilage in all 8 participants (Figure 5). Modified ICRS score assessment showed improvement from Grade IV to Grade 0 or 1 in all participants at 12 and 24+ months of follow-up. Where abrasion arthroplasty was performed good integration was observed

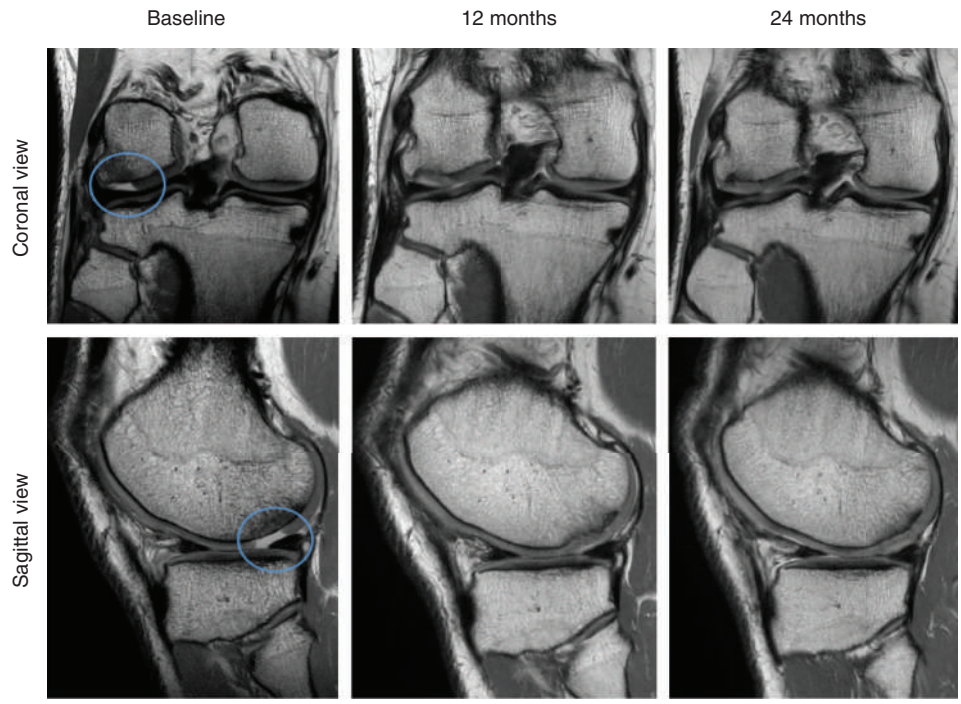

Figure 5. Sequential coronal and sagittal magnetic resonance imaging proton density images at baseline, 12 and 24 months of a participant. The focal chondral defect (blue circle) improves progressively over time with modified International Cartilage Repair Society score improving from Grade 1 at 12 months to Grade 0 at 24 months. Smooth integration is observed between regenerative and native cartilage.

between regenerated cartilage and native cartilage. MOCART score showed considerable cartilage repair at 12 months with a mean score of 79 out of a maximal 100. Additional improvement was observed after 24 months with a mean MOCART score of 89 (see Supplementary Table 5 for itemized MOCART score allocations). Results were comparable in lesions of the medial femoral condyle, lateral femoral condyle and lateral tibial plateau (LTP).

MRI T2-weighted imaging was performed after 24 months. Average T2 scores in the deep, intermediate and superficial zones compared favorably withchosen sample area of native cartilage of the contralateral femoral condyle (see Table 5 & Figure 6). Two participants had T2-weighting imaging also performed at 12 months. Comparison of average 12 month and 24+ month values indicated progressive maturation of regenerative cartilage (average 44.6 and 42.7 ms comparatively).

Table 5. Magnetic resonance imaging T2 relaxation time values. Native cartilage values were recorded over the central weight bearing region of the contralateral femoral condyle. Average values are in milliseconds with standard deviations in brackets.

Cartilage zone	MRI T2 relaxation time values	
	24 months	Native cartilage at 24 months
Deep zone	35.9 (15.9)	33.3 (12.6)
Intermediate zone	40.3 (9.5)	38.4 (11.2)
Superficial zone	45.4 (16.9)	47.0 (15.1)
Overall	40.6 (14.1)	39.6 (13.0)

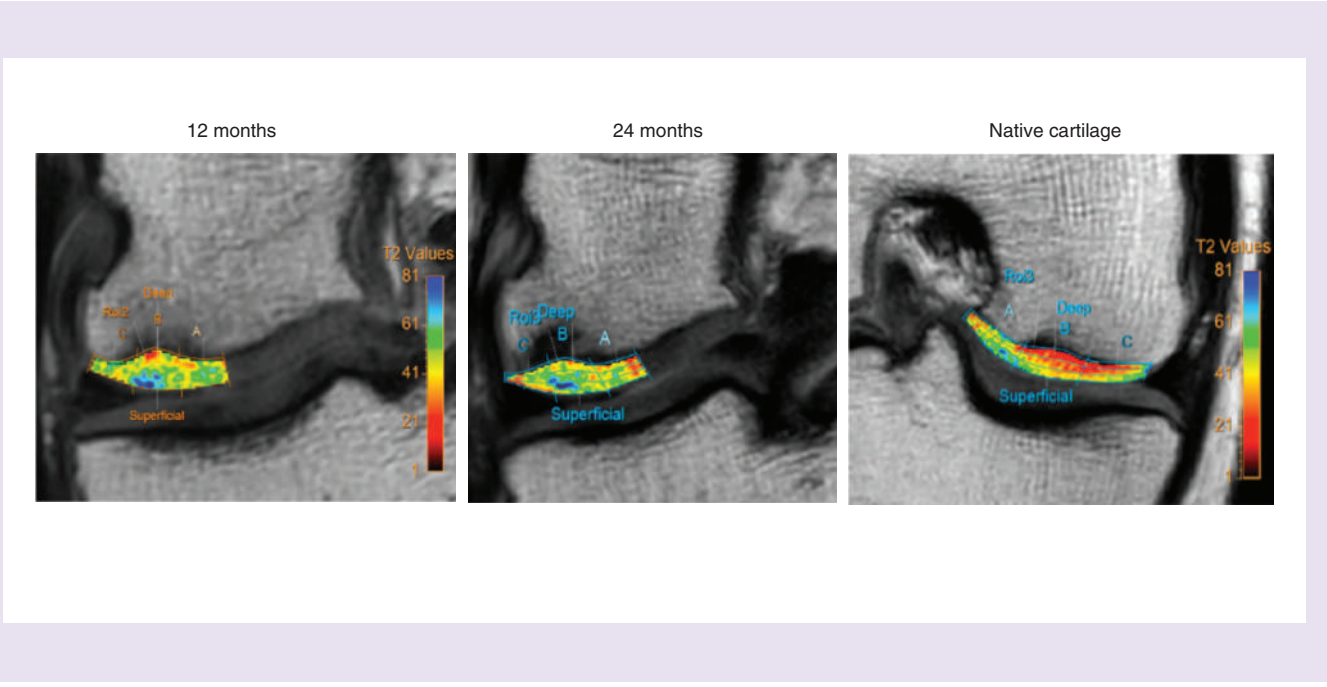

Figure 6. Example of sequential magnetic resonance imaging T2 mapping of the area of cartilage regeneration at 12 and 24 months. Formal T2 mapping was done over a number of slices to include the entire lesion and area of regeneration. This analysis indicated improved maturation over time with comparable T2 values to native cartilage.

Complications & AEs

Participants experienced expected pain and limitation in daily activities due to the postoperative period of touch weight bearing and use of crutches. A self-limiting flare of pain and swelling following ADMSC injections was observed which responded to conservative management including ice and simple analgesia including oral nonsteroidal anti-inflammatories. One participant noted discomfort and swelling lasting 3 weeks which was recorded as a severe AE. No serious AEs were observed. See Supplementary Table 6 for a complete list of graded AEs.

Discussion

The management of isolated chondral defects remains difficult. Current surgical techniques such as microfracture are associated with fibrocartilage formation and result in inconsistent pain, functional and structural results with anticipated progression to early degenerative OA. Other interventions involving graft techniques such as MACI, while showing promising long-term follow-up, are limited by technical difficulty, donor site morbidity and inconsistent integration with surrounding native cartilage.

In this case series, arthroscopic abrasion arthroplasty in combination with ADMSC therapy was observed to be a safe and well tolerated procedure. While participants experienced a self-limiting period of discomfort and swelling

following ADMSC therapy this was an expected AE and has been documented in past trials [32,37,38,40,41]. No serious AEs were observed.

Participants showed improvement across the course of follow-up as measured by quantitative pain and functional outcome scores. Variability was seen in recovery from arthroscopic abrasion arthroplasty with one participant recording worse pain and functional scores during the first 6 months of follow-up. The observed variability in recovery may relate to the size and position of the focal chondral lesion, additional intervention at arthroscopy (i.e., partial meniscal resection/debridement) and other pre-existing comorbidities (i.e., ACL reconstruction).

Improvement in pain and function was mirrored in structural improvement with chondral regeneration observed at 12 and 24+ months follow-up time points. All patients had improvement of a modified ICRS score from 4 to 1 or 0 at both 12 and after 24+ months. MOCART assessment indicated progressive improvement until final follow up. T2-weighted imaging values performed after 24 months were comparable to values taken in an area of central weight bearing native cartilage of the contralateral femoral condyle. In two patients who had T2 mapping performed at 12 and after 24 months, T2 mapping values indicated progressive cartilage maturation with reduced water content and improved collagen anisotrophy. This observation is not dissimilar to ACI techniques whereby long-term follow up has shown greater type II collagen and hyaline-like properties at 24 versus 12 months.

Final T2 mapping values after 24 months of follow-up compared favorably against studies assessing alternative surgical techniques. In a recent randomized controlled trial assessing the role of microfracture versus microfracture in combination with a biological scaffold (BST-CarGel) T2 mapping values at 12 months in both groups showed greater difference to native cartilage values than that observed in this case series [51]. Results in this trial were most comparable to past research involving MACI techniques with T2 mapping values approaching that of native cartilage [52].

In this case series the MSCs were suspended in an autologous biological carrier media of either ACS or PRC. A blood derived biological carrier media was chosen due to previously published studies indicating observed benefits in increased expression by MSCs of anti-inflammatory cytokines including IL-A receptor antagonist (IL-1ra) and anabolic growth factors such as TGF-β [53–55]. TGF-β has been shown to reduce expression of collagen type I, upregulate expression of collagen type II and also assist with migration of stromal cells to site of injury [55]. As arthroscopic abrasion arthroplasty results in a post-operative haemarthrosis – with release of blood derived growth factors including TGFβ at the site of abrasion/bleeding – the use of a biological carrier media may not have been of greater benefit over an isotonic electrolyte solution. It is noted that both ACS and PRC therapy have been documented in past studies to improve pain and function in the treatment of knee OA [55,56]. In this case series participants only received a single injection at baseline and again at 6 months, whilst treatment protocols of ACS and PRC typically involve multiple injections over weeks. It is however accepted that both ACS and PRC may have had additional effect to that of ADMSC therapy in regards to participant outcome and observed pain and functional improvements.

This trial is limited by its nature as a case series. A randomized controlled trial design with comparison of arthroscopic abrasion arthroplasty against arthroscopic abrasion arthroplasty in combination with MSC therapy would be more definitive. In addition, it is accepted that cartilage morphology would be best assessed by histopathology. As histopathology analysis would require repeat arthroscopy and chondral biopsy this could not be clinically justified. Importantly T2-weighted imaging allows a validated noninvasive quantitative assessment of regenerative cartilage against native cartilage with comparable values indicating similar cartilage anisotrophy and hyaline-like properties.

Whether ADMSCs directly integrated into the repair site and/or differentiate along a chondrocyte lineage is debatable. While some preclinical trials have shown successful engraftment of MSCs at the site of chondral repair this has not been consistently observed with some trials indicating reduction in MSC number over time [57,58]. It is proposed that the release of chemotactic cytokines at the site of abrasion arthroplasty – including TGF and CXCL chemokines – may assist in the migration of intra-articular injected MSCs to the site of abrasion with subsequent integration within the repaired tissue together with the secretion of a plethora of growth factors by these cells [55,59]. Use of cell labeling techniques including magnetic tagging would allow for additional assessment of cell migration, integration and retention and would be of benefit in future formal trials [60,61].

Conclusion

This pilot case series illustrates the novel and successful treatment of isolated focal chondral lesions of the knee with arthroscopic abrasion arthroplasty in combination with ADMSCs. Treatments were well tolerated with no serious AEs and participants experienced clinically significant pain, functional and structural improvement. Structural

analysis was comparable to more complicated graft techniques such as MACI with improved regenerative cartilage quality in comparison to other surgical techniques including microfracture.

Translational perspective

The use of MSC therapy in the management of chondropathology and OA is an emerging field in regenerative medicine. The pilot study is the first to show the successful use of ADMSCs in conjunction with a current surgical technique, arthroscopic abrasion arthroplasty, to enhance healing and result in hyaline-like cartilage formation. MSC therapy promises to improve the surgical management of musculoskeletal complaints with improved long-term outcome. The results of this pilot case series highlighting safety and preliminary efficacy mean that additional well-structured research with direct comparison against conventional treatment controls is justified.

Summary points

- The management of isolated focal chondral defects pose a challenge to treating clinicians.
- Isolated focal chondral defects are associated with significant pain and functional limitation and predispose patients to early onset degenerative osteoarthritis.
- Current surgical management options including microfracture and arthroscopic abrasion arthroplasty are limited due to fibrocartilage formation, poor defect filling, early degeneration and at times persistent pain and functional limitation.
- Chondral graft techniques such as autologous chondrocyte implantation can be associated with improved long-term results but are technically difficult, require multiple invasive surgeries and may result in donor site morbidity and poor integration with native cartilage.
- Previous preclinical and early clinical studies have supported the use of mesenchymal stem cell therapy in conjunction with past surgical management techniques including microfracture/microdrilling.
- This is the first case series to assess the use of adipose-derived mesenchymal stem cell (ADMSC) therapy in conjunction with arthroscopic abrasion arthroplasty.
- Clinically significant pain and functional improvement was observed over a 24 months follow-up period.
- Quantitative MRI analysis using validated T2-weighted imaging showed hyaline-like regenerative cartilage with values approaching that of native cartilage.
- No serious adverse events were observed and ADMSC therapy was well tolerated.
- Autologous ADMSC therapy represents an exciting development which may increase the success of existing surgical management of isolated focal chondral defects.

Author contributions

J Freitag, K Shah, J Wickham, D Li, C Norsworthy, A Tenen were involved in conception of the study. J Freitag, K Shah, J Wickham, D Li, C Norsworthy, A Tenen designed the study protocol.

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Financial & competing interests disclosure

J Freitag, A Tenen are clinic partners within Melbourne Stem Cell Centre. J Freitag, D Li, A Tenen are associated with Magellan Stem Cells and are part of its Clinical and Scientific Advisory Committee. K Shah is the Chief Scientific Officer at Magellan Stem Cells. The study was co-sponsored by Melbourne Stem Cell Centre and Magellan Stem Cells. Members of their Clinical and Scientific Advisory Board have been involved in the study conception and design and are listed as co-authors of this paper. Interpretation of results, and subsequent submission and publication decisions have been made independent of the sponsors. Mesenchymal stem cell therapy was performed within a private medical facility and funded by the patients/participants. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

Ethics approval was given for the study by the Human Research Ethics Committee of Charles Sturt University and registered prospectively on the Australian and New Zealand Clinic Trial Registry (Trial ID: ACTRN12617000638336). In addition, informed consent has been obtained from the participants involved.

Data sharing statement

Individual de-identified participant data which underlie results reported in this article will be available upon publication. The study protocol will also be available upon publication. Material will be accessible to investigators whose proposed use of data has been approved by an independent review committee and for data meta-analysis. Requests are to be directed to the corresponding author of the article.

References

1. Buckwalter JA, Mankin HJ. Articular cartilage. Part II: degeneration and osteoarthritis, repair, regeneration and transplantation. *J. Bone Joint Surg. Am.* 79(4), 612–632 (1997).
2. Farnworth L. Osteochondral defects of the knee. *Orthopedics* 23(2), 146–157 (2000).
3. Prakash D, Learmonth D. Natural progression of osteo-chondral defect in the femoral condyle. *Knee* 9(1), 7–10 (2002).
4. Bhosale AM, Richardson JB. Articular cartilage: structure injuries and review of management. *Br. Med. Bull.* 87(1), 77–95 (2008).
5. LaPrade RF, Bursch LS, Olson EJ, Havlas V, Carlson CS. Histologic and immunohistochemical characteristics of failed articular cartilage resurfacing procedures for osteochondritis of the knee: a case series. *Am. J. Sport Med.* 36(2), 360–368 (2008).
6. Mithoefer K, Williams III RJ, Warren RF *et al.* The microfracture technique for the treatment of articular cartilage lesions in the knee: a prospective cohort study. *J. Bone Joint Surg. Am.* 87(9), 1911–1920 (2005).
7. Bert JM. Abandoning microfracture of the knee: has the time come? *Arthroscopy* 31(3), 501–505 (2015).
8. Hunt S, Sherman O. Arthroscopic treatment of osteochondral lesions of the talus with correlation of outcome scoring systems. *J. Arthro. Rel. Surg.* 19(4), 360–367 (2003).
9. Johnson LL. Arthroscopic abrasion arthroplasty historical and pathologic perspective: present status. *Arthroscopy* 2(1), 54–69 (1986).
10. Sansone V, de Girolamo L, Pascale W, Melato M, Pascale V. Long-term results of abrasion arthroplasty for full-thickness cartilage lesions of the medial femoral condyle. *Arthroscopy* 31(3), 396–403 (2015).
11. Lubowitz JH. Arthroscopic microfracture may not be superior to arthroscopic debridement, but abrasion arthroplasty results are good, although not great. *Arthroscopy* 31(3), 506 (2015).
12. Bert JM, Maschka K. The arthroscopic treatment of unicompartmental gonarthrosis: a five-year follow-up study of abrasion arthroplasty plus arthroscopic debridement and arthroscopic debridement alone. *Arthroscopy* 5(1), 25–32 (1989).
13. Jakobsen RB, Engebtretsen L, Slauterbeck JR. An analysis of the quality of cartilage repair studies. *J. Bone Joint Surg. Am.*, 87(10), 2232–2239 (2005).
14. Magnussen RA, Dunn WR, Carey JL, Spindler KP. Treatment of focal articular cartilage defects in the knee: a systematic review. *Clin. Orthop. Relat. Res.* 466(4), 952–962 (2008).
15. Brittberg M, Lindahl A, Nilsson A. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N. Engl. J. Med.* 331, 889–895 (1994).
16. Peterson L, Minas T, Brittberg M. Two- to 9-year outcome after autologous chondrocyte transplantation of the knee. *Clin. Orthop. Relat. Res.* 374, 212–234 (2000).
17. Brittberg M, Nilsson A, Lindahl A. Rabbit articular cartilage defects treated with autologous cultured chondrocytes. *Clin. Orthop. Relat Res.* 326, 270–283 (1996).
18. Chiang H, Kuo TK, Tsai CC *et al.* Repair of porcine articular cartilage defect with autologous chondrocyte transplantation. *J. Orthop. Res.* 23, 584–593 (2005).
19. Barry FP, Murphy JM. Mesenchymal stem cells: clinical applications and biological characterization. *Int. J. Biochem. Cell Biol.* 36(4), 568–584 (2004).
20. Arinzeh TL. Mesenchymal stem cells for bone repair: preclinical studies and potential orthopaedic applications. *Foot Ankle Clin.* 10(4), 651–665 (2005).
21. Noel D, Djouad F, Jorgense C. Regenerative medicine through mesenchymal stem cells for bone and cartilage repair. *Curr. Opin. Investig. Drugs* 3(7), 1000–1004 (2002).

22. Djouad F, Bouffi C, Ghannam S. Mesenchymal stem cell: innovative therapeutic tools for rheumatic diseases. *Nat. Rev. Rheumatol.* 5, 392–399 (2009).
23. Caplan AI, Correa D. The MSC: an injury drugstore. *Cell Stem Cell.* 9(1), 11–15 (2011).
24. Nakagami H, Morishita R, Maeda K, Kikuchi Y, Ogihara T, Kaneda Y. Adipose tissue-derived stromal cells as a novel option for regenerative cell therapy. *J. Atheroscler. Thromb.* 13(2), 77–81 (2006).
25. Caplan AI. Mesenchymal stem cells. *J. Orth. Res.* 9(5), 641–650 (1991).
26. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J. Cell. Physiol.* 213, 341–347 (2007).
27. Goldring MB. Osteoarthritis and cartilage: the role of cytokines. *Curr. Rheumatol. Rep.* 2(6), 459–465 (2000).
28. Wu L, Leijten JC, Georgi N, Post JN, van Blitterswijk CA, Karperien M. Trophic effects of mesenchymal stem cells increase chondrocyte proliferation and matrix formation. *Tissue Eng.* 17(9-10), 1425–1436 (2011).
29. Gobbi A, Karnatzikos G, Sankineani SR. One-step surgery with multipotent stem cells for the treatment of large full-thickness chondral defects of the knee. *Am. J. Sports Med.* 42(3), 648–57 (2014).
30. Johnson LL. Arthroscopic abrasion arthroplasty: a review. *Clin. Orthop. Relat. R.* 391, S306–S317 (2001).
31. Saw KY, Hussin P, Loke SC *et al.* Articular cartilage regeneration with autologous marrow aspirate and hyaluronic acid: an experimental study in a goat model. *Arthroscopy* 25(12), 1391–1400 (2009).
32. Saw KY, Anz A, Jee CSK *et al.* Articular cartilage regeneration with autologous peripheral blood stem cells versus hyaluronic acid: a randomized controlled trial. *Arthroscopy* 29(4), 684–694 (2013).
33. Brittberg M, Winalski CS. Evaluation of cartilage injuries and repair. *J. Bone Joint Surg. Am.* 85(Suppl. 2), 58–69 (2003).
34. Baer PC, Geiger H. Adipose-derived mesenchymal stromal/stem cells: tissue localization, characterization, and heterogeneity. *Stem Cells Int.* 2012, 812693 (2012).
35. Im GI, Shin YW, Lee KB. Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells? *Osteoarthr. Cartilage* 13, 845–853 (2005).
36. Freitag J, Ford J, Bates D *et al.* Adipose derived mesenchymal stem cell therapy in the treatment of isolated knee chondral lesions: design of a randomised controlled pilot study comparing arthroscopic microfracture versus arthroscopic microfracture combined with postoperative mesenchymal stem cell injections. *BMJ Open* 5(12), e009332 (2015).
37. Freitag J, Li D, Wickham J, Shah K, Tenen A. Effect of autologous adipose-derived mesenchymal stem cell therapy in the treatment of a post-traumatic chondral defect of the knee. *BMJ Case Rep.* 2017 (doi:10.1136/bcr-2017-220852) (2017).
38. Freitag J, Shah K, Wickham J, Boyd R, Tenen A. The effect of autologous adipose derived mesenchymal stem cell therapy in the treatment of a large osteochondral defect of the knee following unsuccessful surgical intervention of osteochondritis dissecans – a case study. *BMC Musculoskelet. Disord.* 18(1), 298 (2017).
39. Freitag J, Wickham J, Shah K, Tenen A. Effect of autologous adipose-derived mesenchymal stem cell therapy in the treatment of acromioclavicular joint osteoarthritis. *BMJ Case Rep.* 12(e227865), doi:10.1136/bcr-2018-227865 (2019).
40. Freitag J, Norsworthy C, Wickham J, Shah K, Tenen A. High tibial osteotomy in combination with arthroscopic abrasion arthroplasty and autologous adipose-derived mesenchymal stem cell therapy in the treatment of advanced knee osteoarthritis. *BMJ Case Rep.* 12(e228003), doi:10.1136/bcr-2018-228003 (2019).
41. Freitag J, Bates D, Wickham J *et al.* Adipose-derived mesenchymal stem cell therapy in the treatment of knee osteoarthritis: a randomized controlled trial. *Regen. Med.* 14(3), 213–230 (2019).
42. Martinello T, Bronzini I, Maccatrozzo L *et al.* Canine adipose-derived mesenchymal stem cells do not lose stem features after a long-term cryopreservation. *Res. Vet. Sci.* 91(1), 18–24 (2011).
43. Goh BC, Thirumala S, Kilroy G, Devireddy RV, Gimble JM. Cryopreservation characteristics of adipose-derived stem cells: maintenance of differentiation potential and viability. *J. Tissue Eng. Regen. Med.* 1(4), 322–324 (2007).
44. Dominici M, Blanc K, Mueller I *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy* 8(4), 315–317 (2006).
45. Dworkin RH, Turk DC, Farrar JT *et al.* Core outcome measures for chronic pain clinical trials: IMMPACT recommendations. *Pain* 113(1–2), 9–19 (2005).
46. Roos EM, Roos HP, Lohmander LS, Ekdahl C, Beynnon BD. Knee Injury and Osteoarthritis Outcome Score (KOOS)-development of a self-administered outcome measure. *J. Orthop. Sports Phys. Ther.* 28(2), 88–96 (1998).
47. Bellamy N, Buchanan WW, Goldsmith CH, Campbell J, Stitt LW. Validation study of WOMAC: a health status instrument for measuring clinically important patient relevant outcomes to antirheumatic drug therapy in patients with osteoarthritis of the hip or knee. *J. Rheumat.* 15(12), 1833–1840 (1988).
48. Marlovits S, Singer P, Zeller P *et al.* Magnetic resonance observation of cartilage repair tissue (MOCART) for the evaluation of autologous chondrocyte transplantation: determination of inter-observer variability and correlation to clinical outcome after 2 years. *Eur. J. Radiol.* 57(1), 16–23 (2006).

49. Crema MD, Roemer FW, Marra MD *et al.* Articular cartilage in the knee: current MR Imaging techniques and applications in clinical practice and research. *Radiographics.* 31(1), 37–61 (2011).

50. Mamisch TC, Trattnig S, Quirbach S, Marlovits S, White LM, Welsch GH. Quantitative T2 mapping of knee cartilage: differentiation of healthy control cartilage and cartilage repair tissue in the knee with unloading – initial results. *Radiology* 254(3), 818–826 (2010).

51. Stanish WD, McCormack R, Forriol F *et al.* Novel scaffold-based BST-CarGel treatment results in superior cartilage repair compared with microfracture in a randomized controlled trial. *J. Bone Joint Surg.* 95(18), 1640–1650 (2013).

52. Trattnig S, Mamisch TC, Welsch GH *et al.* Quantitative T2 mapping of matrix-associated autologous chondrocyte transplantation at 3 Tesla: an *in vivo* cross-sectional study. *Invest. Radiol.* 42(6), 442–448 (2007).

53. Mifune Y, Matsumoto T, Takayama K *et al.* The effect of platelet-rich plasma on the regenerative therapy of musclederived stem cells for articular cartilage repair. *Osteoarthritis Cartilage.* 21(1), 175–185 (2013).

54. Weiss S, Hennig T, Bock R *et al.* Impactof growth factors and PTHrP on early and late chondrogenic differentiation ofhuman mesenchymal stem cells. *J Cell Physiol.* 223, 84–93 (2010).

55. Zhu Y, Yuan M, Meng H *et al.* Basic science and clinical application of platelet-rich plasma for cartilage defects and osteoarthritis: a review. *Osteoarthr. Cartilage* 21(11), 1627–1637 (2013).

56. Baltzer AW, Moser C, Jansen SA, Krauspe R. Autologous conditioned serum (Orthokine) is an effective treatment for knee osteoarthritis. *Osteoarthr. Cartilage* 17(2), 152–60 (2009)

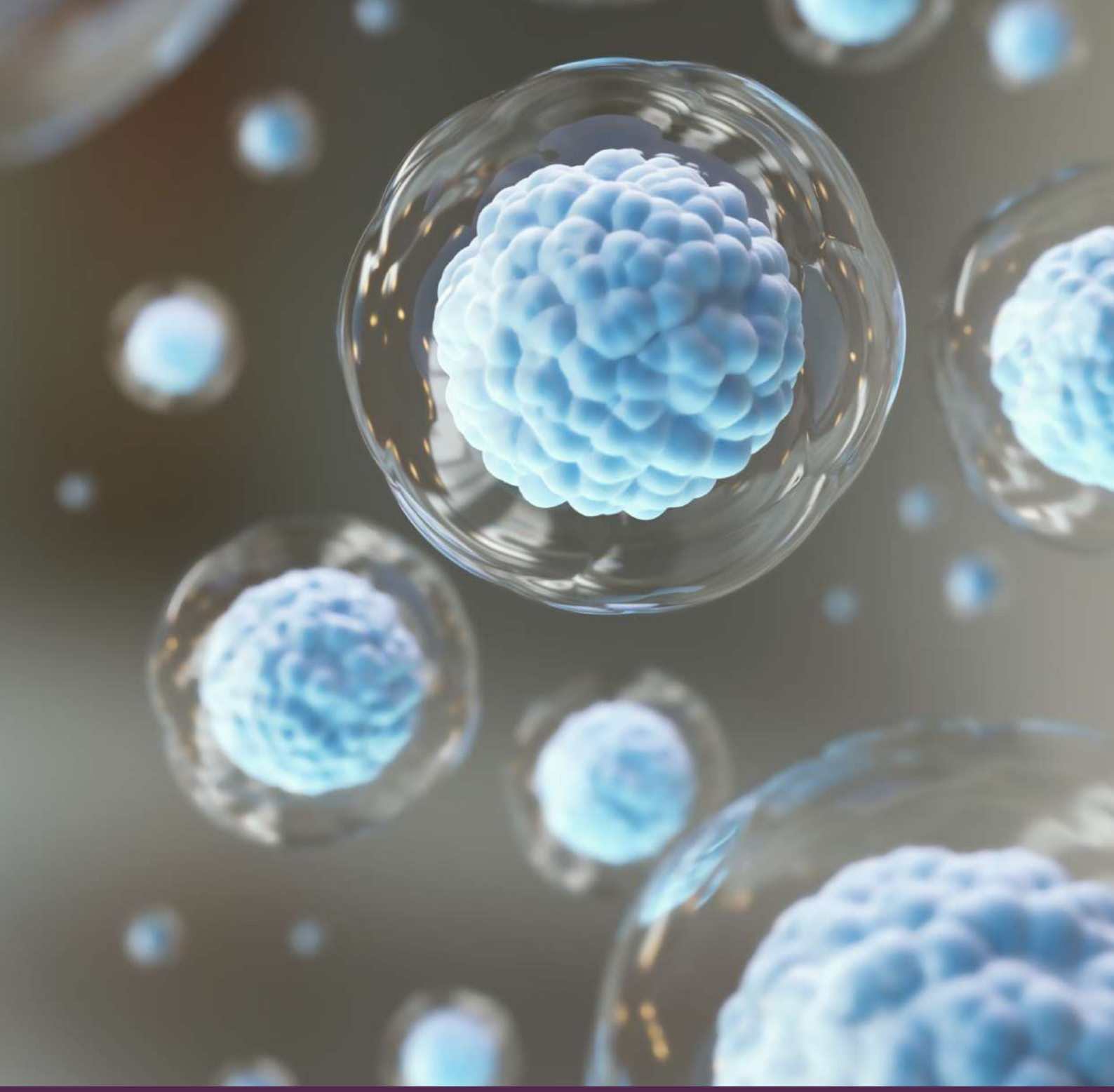
57. Lee KB, Hui JH, Song IC, Ardany L, Lee EH. Injectable mesenchymal stem cell therapy for large cartilage defects – a porcine model. *Stem Cell.* 25, 2964–2971 (2007).

58. Quintavalla J, Uziel-Fusi S, Yin J *et al.* Fluorescently labeled mesenchymal stem cells (MSCs) maintain multilineage potential and can be detected following implantation into articular cartilage defects. *Biomaterials* 23(1), 109–119 (2002).

59. Kalwitz G, Andreas K, Endres M *et al.* Chemokine profile of human serum from whole blood: migratory effects of CXCL-10 and CXCL-11 on human mesenchymal stem cells. *Connect. Tissue Res.* 51(2), 113–122 (2010).

60. Henning TD, Gawande R, Khurana A *et al.* Magnetic resonance imaging of ferumoxide-labeled mesenchymal stem cells in cartilage defects: *in vitro* and *in vivo* investigations. *Mol. Imaging* 11(3), 197–209 (2012).

61. Budde MD, Frank JA. Magnetic tagging of therapeutic cells for MRI. *J. Nucl. Med.* 50(2), 171–174 (2009).



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