JOURNAL OF DENTISTRY 42 (2014) 761–768

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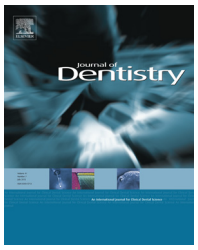

Review

Dental pulp stem cells: State of the art and suggestions for a true translation of research into therapy

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ARTICLE INFO

Article history:
Received 22 January 2014
Received in revised form
18 February 2014
Accepted 20 February 2014

Keywords:
Adult stem cells
Bone regeneration
Human grafts
Scaffold
Tissue engineering
Stem cell therapy

ABSTRACT

Objectives: Stem cells have the ability to rescue and/or repair injured tissue. In humans, it is possible to isolate different types of stem cells from the body. Among these, dental pulp stem cells (DPSCs) are relatively easily obtainable and exhibit high plasticity and multipotential capabilities. In particular they represent a gold standard for neural-crest-derived bone reconstruction in humans and can be used for the repair of body defects in low-risk autologous therapeutic strategies.

Sources: An electronic search was conducted on PubMed databases and supplemented with a manual study of relevant references.

Results: All research described in this review highlight that DPSCs are mesenchymal stem cells that could be used in clinical applications. Unfortunately, very few clinical trials have been reported. Major obstacles imposed on researchers are hindering the translation of potentially effective therapies to the clinic. Both researchers and regulatory institutions need to develop a new approach to this problem, drawing up a new policy for good manufacturing practice (GMP) procedures. We strongly suggest that only general rules be standardized rather than everything. Importantly, this would not have an effect on the safety of patients, but may very well affect the results, which cannot be identical for all patients, due to physiological diversity in the biology of each patient. Alternatively, it would be important to study the role of specific molecules that recruit endogenous stem cells for tissue regeneration. In this way, the clinical use of stem cells could be successfully developed.

Conclusions: DPSCs are mesenchymal stem cells that differentiate into different tissues, maintain their characteristics after cryopreservation, differentiate into bone-like tissues when loaded on scaffolds in animal models, and regenerate bone in human grafts. In summary, all data reported up to now should encourage the development of clinical procedures using DPSCs.

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http://dx.doi.org/10.1016/j.jdent.2014.02.018
0300-5712

1. Introduction

The stem cell field represents an area of particular interest for scientific research. The results so far obtained give good expectations for the use of stem cells in clinical trials. New therapeutic strategies have been made possible thanks to great advancements in stem cell biology, with the aim of regenerating tissues injured by disease.[1,2] Based on their ability to rescue and/or repair injured tissue and partially restore organ function, multiple types of stem/progenitor cells have been speculated. A primary goal is to identify how different tissues and organs can arise from undifferentiated stem cells.

Stemness is the capability of undifferentiated cells to undergo an indefinite number of replications (self-renewal) and give rise to specialized cells (differentiation). Therefore, stem cells differ from other types of cells in the body because they are capable of sustaining self-renewal, are unspecialized, and can give rise to differentiated cell types. Differentiation can be recognized by a change in the morphology of the cell and by the detection of tissue-specific proteins.[3] Stem cells may remain quiescent (non-dividing) for long periods of time until they are activated by a physiological need for more cells to maintain tissues, by disease, or by tissue injury. Thus, the primary role of adult stem cells is to maintain and repair the tissue in which they are found. They are thought to reside in specific areas termed stem cell niches.[4] Adult stem cells have been identified in many organs and tissues, including brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin, teeth, heart, gut, liver, ovarian epithelium, and testis.[5] Among these tissues, dental pulp is considered a rich source of mesenchymal stem cells suitable for tissue engineering applications and, for this reason, many studies are performed with the final aim of obtaining new bone.[6–8]

Tissue engineering is a multidisciplinary field that combines biology, engineering, and clinical science in order to generate new tissues and organs. This science involves different steps, such as the identification of appropriate cells, the development of scaffolds, and the study of morphogenic signals required to induce cells to regenerate a tissue or organ.[9] After having discussed the state of the art in the field of dental pulp stem cells research and their potential use in bone engineering, here we try to suggest how to overcome the problems limiting the translationability of research, with the aim of improving the health of patients.

2. Dental pulp stem cells (DPSCS)

Dental pulp, a soft connective tissue within the dental crown, is an interesting source of adult stem cells because of the large amount of cells present and the non invasiveness of the isolation methods compared to other adult tissue sources.[8,10,11] Dental pulp contains mesenchymal stem cells defined as dental pulp stem cells (DPSCs). DPSCs are obtained from human permanent and primary teeth, human wisdom teeth, human exfoliated deciduous teeth (SHEDs), and apical papilla.[7,12–14] Moreover, DPSCs can be also isolated from supernumerary teeth, which are generally discarded.[15] Other sources of dental stem cells are the periodontal ligament, which houses periodontal ligament stem cells (PDLSCs),[16] and the dental follicle, which contains dental follicle progenitor cells (DFPCs).[17,18] DPSCs have been isolated from different organisms, including humans, mouse, rat, sheep, chimpanzee, and pig.[19,21–23]

DPSCs differentiate into different kinds of cells and tissues[24–28] and their multipotency has been compared to those of bone marrow stem cells (BMSCs). It has been demonstrated that proliferation, availability, and cell number of DPSCs are greater than BMSCs.[20]

There are two widely used methods for the isolation of dental pulp stem cells: the explant method (DPSC-OG) and the enzymatic digestion method of the pulp tissue (DPSC-EZ) (Fig. 1). The explant method is based on outgrowth of cells from tissue fragments and subsequent adherence on a plastic surface.[29,30] The second technique consists of sterile surgical removal of dental pulp, digestion in collagenase/dispase, characterization, and screening through the use of specific markers.[13] It has been demonstrated that the outgrowth method allows DPSCs to differentiate into skeletal muscle fibres.[29] Isolation by enzymatic digestion is based on the use of flow-cytometers with a fluorescent activated cell sorter (FACS)

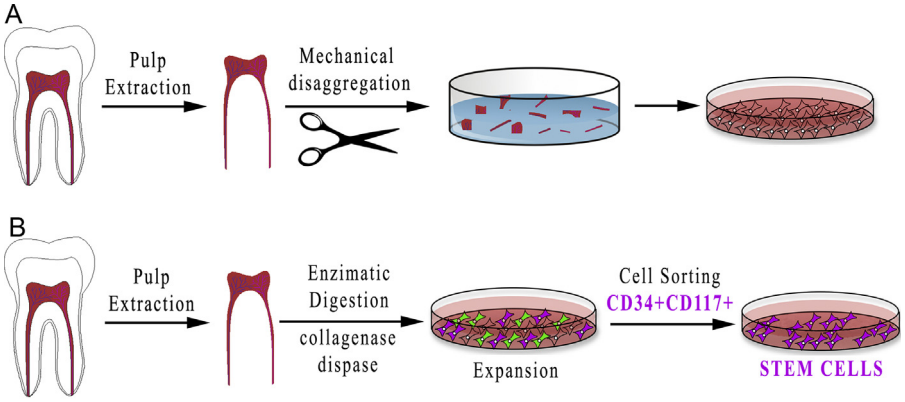

Fig. 1 – (A) Explant method by which dental pulp is fragmented into pieces and cultured in medium; (B) enzymatic digestion method by which dental pulp is digested in collagense/dispase solution and then the cell suspension obtained is screened for expression of stemness markers by flow cytometry.

by which DPSCs are isolated using both morphological (complexity and size of cells) and antigenic characteristics.[13] Different mesenchymal stem cell markers are used to select different subsets of DPSCs showing diverse biological behaviours.[31] Yang and colleagues[32] demonstrated that STRO1 identifies a subgroup of DPSCs with odontogenic and osteogenic properties.[32] Another DPSC population is positive for CD34 and CD117 and negative for CD45.[13] This population has great self-expansion and osteogenic differentiation capabilities and produces a living autologous fibrous bone (LAB) tissue *in vitro*[13] and bone tissue when implanted in mice. Such DPSCs can be expanded for long periods and split for up to 80 passages while still showing plasticity and the capacity for nodule and bone chip formation *in vitro*.[13,33] This capability occurs if cells are not detached from their substrate, avoiding the loss of cell to cell interactions, fundamental for extracellular matrix secretion.[28]

Other markers expressed by DPSCs are CD29 and CD44[34] as well as CD73 and CD105[35], all markers of mesenchymal stemness. Despite this, these markers are not used for isolating DPSCs; however, they are used for characterization. DPSCs are reported to express also OCT4 and Nanog, transcriptional factors involved in pluri/multipotency maintenance.[36] Another technique used to isolate stem cells is based on the efflux of Hoechst33342 dye. Stem cells are able to exclude Hoechst dye through the membrane efflux pumps of ATP-binding cassette transporters, such as ABCG2.[37] Iohara et al. isolated porcine and canine DPSCs by their side population profile.[21,38]

Numerous studies have evaluated the multipotency of DPSCs. Various studies have demonstrated that DPSCs are able to differentiate into osteoblasts when cultured in osteogenic medium supplemented with dexamethasone, beta-glycerophosphate, and ascorbic acid.[39,40] However, Laino and colleagues showed that $CD34^{+}CD117^{+}$ DPSCs can be differentiated into osteoblasts with standard medium supplemented with 20% FBS and without osteogenic inducers.[13] Recently, it has been also demonstrated[41] that the specific suppression of individual histone deacetylases (HDACs) by RNA interference could enhance osteoblast differentiation, and that HDAC1 as well as HDAC2 are critical enzymes for osteoblast differentiation. In particular, treatment of DPSCs with VPA significantly improves mineralized matrix formation, enhancing expression of bone glycoproteins, such as osteopontin and bone sialoprotein—both involved in the formation of the mineralized matrix—but negatively affecting the expression of osteocalcin, a late-stage marker of differentiation.[41] Different culture conditions have been found to improve osteogenic lineage differentiation: for example, with the use of a piastrinic lysate as an alternative to FBS.[42]

Overall, DPSCs are more suitable than BM-MSCs for mineralized tissue regeneration.[20] Other studies highlighted the ability of DPSCs to express chondrogenic markers.[43,44]

DPSC differentiate also into other cell types, including smooth muscle cells, adipocyte-like cells, and neurons.[45,26,46] It was recently demonstrated that DPSCs express proteins involved in melanogenesis at different points during their differentiation *in vitro*, even when they are not stimulated by a selective differentiating medium for melanocytes, demonstrating for the first time that DPSCs are capable of spontaneously differentiating into mature melanocytes[47]. In addition, DPSCs are able to form capillary-like structures when cultured with VEGF.[48] It has been demonstrated[28] that human DPSCs synergistically differentiate into osteoblasts and endothelial cells. Great attention has also been focused on the ability of DPSCs to differentiate into odontoblast-like cells, characterized by polarized cell bodies and accumulation of mineralized nodules.[25,49,50]

Another fundamental aspect investigated is the maintenance of cell stemness. Human DPSCs exposed to optimal concentrations of small molecules (Pluripotin (SC1), 6-bromoindirubin-3-oxime, and rapamycin) showed increased expression of STRO-1, NANOG, OCT4, and SOX2, but diminished differentiation into odonto/osteogenic, adipogenic, and neurogenic lineages *in vitro*.[51]

Another aspect to be considered is the ability of DPSCs to maintain their characteristics after cryopreservation for years. Osteoblasts differentiated from DPSCs, as well as DPSCs themselves, are still capable of re-starting proliferation, differentiation, and producing a mineralized matrix.[33,52]

3. DPSCS and perspectives for tissue engineering

In recent years, studies have focused on the possible application of stem cells and tissue engineering to mend and regenerate body structures.[6,8,33,53] This new idea of therapy, known as regenerative medicine, may be ready for clinical applications in the future. It has been hypothesized that stem cells could play a key role in future medical treatments because they can be readily grown and induced to differentiate into different cell types in culture.[53] The general aim of tissue engineering is to restore the vitality and function of diseased and traumatized tissue. Several approaches, already studied in oral and maxillofacial surgery, can be used for bone repair, including: (1) autologous grafts using cells and growth factors with osteogenic properties[54]; (2) allogenic and xenogenic grafts, using portions of bone with osteoinductive properties from other humans or animals[55]; (3) osteoinductive biomaterials that serve as carriers for osteogenic growth factors[56]; (4) synthetic materials able to regenerate bone with or without partial resorption.

The creation of a medical device for use in oral tissue engineering requires accessible sources of cells and biocompatible materials that can also carry signalling molecules. Many strategies used in tissue engineering depend on the use of scaffolds, and those for bone regeneration are highly sophisticated structures. These scaffolds are designed to support the adhesion, cell spreading, and deposition of new tissue, avoiding the need for artificial implants. An ideal scaffold should have specific chemical and physical characteristics: the main properties are biocompatibility, to optimize tissue regeneration without side effects from the immune response; biodegradability, so as to be absorbed simultaneously with cell growth; and specific mechanical properties, so as to be stable and to resist the *in vivo* stresses.

Numerous studies have been performed using DPSCs loaded onto scaffolds of collagen sponges (Fig. 2), hydroxyapatite (HA), chitosan, biocoral, or PLGA.[57,32,58–64] Yang and

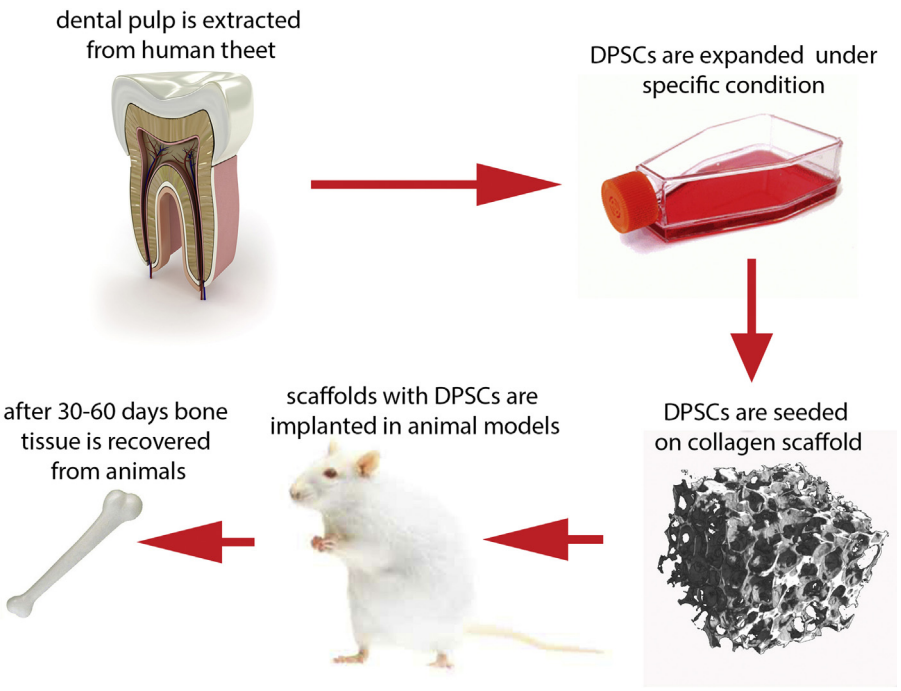

Fig. 2 – Diagram of bone tissue engineering from dental pulp stem cells.

colleagues[57] demonstrated that DPSCs seeded on a chitosan/collagen complex formed a dentine-pulp complex.[57] The same group reported also that DPSCs loaded on HA/TCP disks formed bone.[32] In contrast, Zhang and colleagues[58] reported that DPSCs loaded onto spongeous collagen and transplanted into mice were not able to form hard tissues. They hypothesized that the micro-structure and characteristics of the collagen might be the main cause of the negative result.[58]

In this context, a key aspect is the three-dimensional macrostructure that mimics the physiological functions of extracellular matrix (ECM). Porosity is important for good bone growth; pore size must be between 100 and 400 μm[59], and they should be open and interlinked to enable diffusion, penetration, adherence, and proliferation of cells.[60] The presence of surface microcavities leads to a greater osteogenic response of stem cell. In fact, N-methyl-pyrrolidone treatment of PLGA scaffolds was demonstrated to improve cell adhesion and polarity, mainly by increasing the presence of microcavities on the scaffold.[61] Furthermore, the cells in this microenvironment released larger amounts of BMP-2 and VEGF in the culture medium and increased alkaline phosphatase activity.[61]

An important factor in the construction of the scaffold is the material used to produce it. A suitable material in bone tissue engineering should attract osteogenic elements (*i.e.*, be osteoinductive) and support tissue regeneration (*i.e.*, be osteoconductive). In recent years, numerous types of scaffold materials and structures have been compared.[62] Scaffolds can be of a natural or a synthetic origin. Those of a natural origin have the advantage of being biocompatible and bioactive, whereas for synthetic ones it is possible to control the rate of degradation, the microstructure, the porosity, and the mechanical strength.[63,64] An example of a natural polymer is coral. The natural structure of coral has excellent mechanical properties: in fact, it has an interconnected porous structure that allows it to withstand compressive breaking stress, and has good reabsorbability and biocompatibility. Its three-dimensional structure, with the presence of interconnected pores of around 260 μm in diameter, is similar to that of spongy bone, encouraging the spread of the new bone ingrowth. Recently, it has been shown that stem cells rapidly move inside Biocoral cavities and later begin to differentiate into osteoblasts.[65] Examples of synthetic polymers are PLGA and titanium, which possess high biocompatibility without rejection and inflammatory reactions.[62–65] Researchers have also demonstrated[66] that there is complete osteointegration on a titanium surface. They have tested laser-sintered titanium (LST) and acid-etched titanium surfaces with both human osteoblasts and DPSCs. The LST surfaces induced better and quicker differentiation of DPSCs into osteoblasts.[66]

Although an *in vitro* culture system offers a simplified model to investigate differentiation ability, the potential of this system for dental pulp tissue engineering needs to be verified *in vivo*. In fact, potential development and formation of an appropriate histological structure often cannot be fully realized *in vitro*. Thus, studies on DPSC behaviour *in vivo* have been conducted in animal models, implanting constructs composed of cells seeded onto a wide variety of biomaterials, including calcium phosphates, collagen hydroxyapatite, poly (D,L) lactide-based materials, bioactive glasses, and methacrylate-based biomaterials.[56,66–70] These systems can generate highly ordered collagenous matrix deposition and give rise to bone containing vessels, fundamental for complete integration with the host.

It is important also to consider various animal models with bone defects used for tissue engineering in bone regeneration. Dog, mice or rats, pig, sheep, and rabbit are those more used in this field. Unfortunately, there isn't an ideal animal model. Each model has advantages and/or disadvantages. Certainly,

dog, pig, and sheep are more similar to humans than the others[71]; mice and rats are the worst models due to their dissimilarities in terms of size and bone structure compared to humans.[71] Despite this, many studies report that DPSCs form bone in animal models in which cranial and mandibular bone defects have been generated. Only one study has reported a complete failure to form bone by DPSCs loaded on a porous HA/TCP ceramic scaffold.[72] However, to overcome the limitations of *ex vivo* grown stem cell transplants, recent reports have explored the recruitment of endogenous progenitor cells as a novel strategy for *in situ* tissue regeneration.[73] It has been demonstrated that the interaction between the chemokine SCF and its receptor c-Kit is a potent aid in the regeneration of dental pulp and other mesenchymal tissues, and is capable of inducing cell homing, angiogenesis, and tissue remodelling.[73] In summary, DPSCs are an excellent model to study bone formation on substrates appropriate for clinical bone-remodelling applications.[61,74]

4. Human grafts

The majority of studies evaluating *in vivo* applications of DPSCs are performed on animal models. There have been few clinical trials. In one clinical study,[75] bone formation subsequent to DPSC transplantation was evidenced radiographically. Researchers extracted cells from dental pulp of drawn third molars and seeded them onto collagen scaffolds. The two mandibular third molars of each patient were extracted simultaneously; one side received a biomaterial construct loaded with cells, and the other side, used as a negative control, was filled with biomaterial alone. Three months after injury, radiographical and histological analysis showed a noticeable difference in bone regeneration between the two sites (Fig. 3): the control side had a significantly lower amount of regenerated bone.[75] That study gave clear evidence of the possibility of employing DPSCs to repair bone defect in humans. After three years of follow-up,[76] both stability and the characteristics of the regenerated bone were examined by means of the novel synchrotron radiation-based holotomography (HT) technique. The use of histological methods and synchrotron radiation-based HT showed that 3 years after surgery, the regenerated bone was uniformly vascularized and qualitatively compact. This result demonstrated that DPSCs successfully repair bone. In this context, compact bone regenerated after DPSC engraftments in the mandible could be considered of fundamental importance to limit pathologic fracture and to guarantee better quality of life in oral cancer patients.[76] More clinical trials on

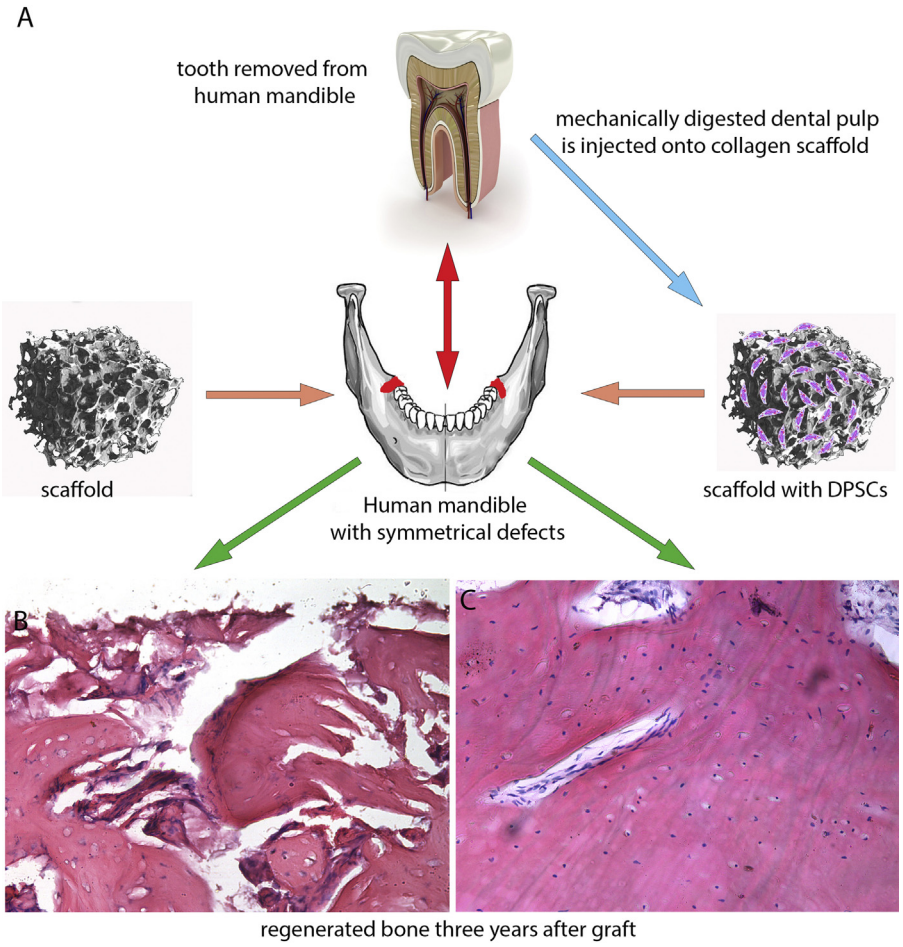

Fig. 3 – (A) Representation of human mandible defect repair using DPSCs. (B) Histological evaluation (3 years after grafting) showing a reabsorbed spongy bone in the control site (original magnification 200×). (C) Histological evaluation (3 years after grafting) at the test site, showing a compact bone (original magnification 200×).

bone loss should be conducted to confirm the potential of DPSCs for a regenerative therapeutic approach.

5. Realistic perspectives

New strategies are needed to fully develop the clinical application potential of these studies. This should involve both researchers/clinicians and improvements in good manufacturing practice (GMP) procedures/rules. We must think how to do more research that is focused towards clinical application rather than the publication of articles without an effective therapeutic prospect.

The GMP procedures that must be followed by researchers/clinicians, even for the engraftment of autologous cells, are not really orientated for research on humans. They are much too difficult, too long, and extremely expensive, so much so that often researchers and clinicians are discouraged to start with a clinical trial application in the first place. The long bureaucratic procedures, the continuous requests for the tiniest of details within protocols, and the demand of fixed or over-fixed steps, are not compatible with the clinical development of stem-cell-based therapy. This type of procedure is devoted to patients with differing needs, whose stem cells are often unique in many features. This does not mean that a safe protocol cannot be drawn up and used, but it must be a flexible one. We believe and strongly suggest that only general rules should be standardized, not everything. Importantly, this would not have an effect on the safety of patients, but may very well affect the results, which cannot be identical for all patients due to physiological diversity in the biology of each patient. We hope that the above will stimulate in a short time the opening up of the possibility for advanced therapies that enhance the well-being of patients in need.

Stem cell research has expanded at an exponential rate, but its therapeutic applications have progressed much more slowly. Despite this, stem cell therapy represents a fascinating new approach for the management of disease. Worldwide scientific advances using stem cells in therapeutic, reconstructive, orthopaedic, and cosmetic applications are the future of personalized and regenerative medicine. Stem cell therapy allows the repair of defective tissues or functions through the transplantation of live cells. However, immune rejection is a major risk for cell transplantation, so biosecurity is a crucial point for cell therapy, requiring control of cell transformation and a protocol for cellular biobanking.

In the next few years, the targets of cell therapy will be to identify and understand cell differentiation mechanisms, so that optimal cell populations and culture conditions can be used in a more efficient treatment of disease. A thorough knowledge of stem cell physiology may enhance development of novel and more competent therapeutic approaches and fulfil the huge impact that stem cell therapy will have for future healthcare. In parallel, it is important to evaluate the role of specific and new molecules that recruit endogenous stem cells for tissue regeneration. This strategy is hopefully an easier way towards a clinically acceptable procedure, side-stepping difficulties caused by bureaucracy and policy. Therefore, we expect that increased application of stem cells will strengthen the translatability to supporting clinical trials.

6. Conclusions

DPSCs are mesenchymal stem cells expressing mesenchymal, haematopoietic and stemness markers. These cells:

i. differentiate into different tissues of mesenchymal origin, but also into functional melanocytes and neurons;
ii. maintain their characteristics after cryopreservation for years if stored as selected stem cells and not as part of whole pulp;
iii. differentiate into bone-like tissues in animal models when loaded on scaffolds;
iv. regenerate bone in human grafts;
v. are an excellent model for the study of bone formation on substrates appropriate for clinical bone remodelling applications.

Unfortunately, clinical trials using DPSCs have not been widely reported. However, the basic and clinical data reported in this review should encourage the development of DPSC-based procedures with practical applications for humans.

Disclosure

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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