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 made great advancements in stem cell biology, with the aim of regenerating tissues injured by disease. 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 [(120)].

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 unspecialied, capable of sustaining self-renewal 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 [(51)].

Stem cells may remain quiescent for long periods of time until they are activated by disease, by tissue injury or by a physiological need for more cells to maintain tissues. Thus, the primary role of adult stem cells is to maintain and repair the tissue in which they are found [(20)].

Different adult tissue sources for obtaining mesenchymal stem cells (MSCs) have been discovered. However, bone marrow aspirates still remain the principal source of MSCs for most preclinical and clinical studies, owing to the fact that bone marrow aspirates represent the most accessible and minimally invasive procedure to obtain MSCs [(110)].

Adipose tissue is an abundant source of MSCs and has been extensively studied in the field of regenerative medicine as a stem cell source. Adipose-derived MSCs (ASCs) can be readily harvested via lipectomy or by lipoaspiration from areas such as the chin, upper arms, abdomen, hips, buttocks and thighs in large numbers with low donor-site morbidity. ASCs application is much less expensive than bone marrow derived stem cells (BMSCs) use and the stem cell numbers that can be extracted from isolated adipose tissue is higher than bone marrow [(132)].

Oral mucosa derived stem cells (OMSCs) have several advantages over BMSCs. OMSCs are easy to isolate and proliferate faster than BMSCs without any growth factor. They show a stable morphology and do not lose MSC characteristics even at higher passages. Thus, it was found that oral mucosa is a better source of MSCs than the bone marrow[(177)].

In the future, stem cells will be used to completely restore the hard and soft tissue in the patient's mouth cavity, thus by-passing problems with histocompatibility. There is a possibility for stem cells to be placed in biocompatible molds in the shape of the anatomical structures that are to be repaired. So far, tissue engineering on animal models has been conducted for the purpose of repairing oral, dental and craniofacial structures - soft tissue grafts, dental tissues, temporomandibular joint and facial bones [(188)].

The dental pulp responds to irritants (deep carious lesions) that cause odontoblastic cell death and this is repaired by forming new odontoblasts resulting in the formation of reparative dentinal matrix. The exact mechanism for the production of odontoblast-like cells, which thus

produce reparative dentin is, however, not very clear. In light of tissue regeneration, creating odontoblast-like cells and subsequently reparative dentine in vitro would be an ideal restorative material for lost tooth structure, compared to the materials currently in use [39].

Articular cartilage has poor regenerative capacity following injury and degradation due to its avascular nature. For some patients, autologous chondrocyte implantation (ACI) is a viable cartilage repair strategy. However, this procedure requires the isolation of chondrocytes via a preliminary surgery, which itself may result in further cartilage degeneration. Adult MSCs may be an alternative autologous source for such applications due to their multi-potency and relative ease of isolation and expansion [123].

Review of Literature

Regenerative Dentistry and Tissue Engineering

Tissue engineering is an approach that utilizes specific biodegradable synthetic or natural scaffolds as well as advanced molecular techniques in order to replace tissue function. There has been a clear and distinct hypothetical shift in regenerative medicine from using medical devices and whole tissue grafts, to a more explicit approach that utilizes specific bioactive, biodegradable synthetic or natural scaffolds combined with cells and/or biological molecules, to create a functional replacement tissue in a diseased or damaged site [(141)].

Tissue engineering provides a new era for therapeutic medicine; it is progressing very rapidly and extends to involve all tissues in our body. Three decades ago, tissue engineering was an idea and today it has become a potential therapy for several conditions. For a more regenerative breakthrough to develop and lead to off-the-shelf bioproducts to replace a variety of lost tissues and organs, a thorough understanding of embryonic development and stem cell biology are required [(31)].

Regenerating oral tissues, in particular, is very challenging and requires recapitulation of the biological development of several tissues and interfaces [(155)]. The progress in this field is taking several routes including cell biology, the development of novel scaffolds/fabrication methods/ characterization techniques.

Recent advances in tissue engineering suggest that significant changes in the more traditional areas of clinical dentistry are beginning to

occur. Thus, there has been a recent surge in guided tissue engineering methods to establish new therapies to manage periodontal diseases beyond the traditional approaches based solely upon infection control [(29)].

Periodontal diseases are some of the most common oral diseases worldwide, after caries, and have been found to have a role in more general systemic diseases such as diabetes and cardiovascular disease. The need for more reproducible oral tissue replacement therapies is therefore considerable [(91)]. To date, the regeneration of small or medium-sized periodontal defects using in vitro engineered cell-scaffold constructs is technically feasible. However, the predictable reconstruction of the innate organization and function of whole teeth as well as their periodontal structures remains challenging. Future possibilities depend on an improved fundamental understanding of cellular and molecular mechanisms involved in the regeneration of all periodontal tissues, the differentiation potential of stem cells, the biocompatibale stem cells and materials with host tissues [(30, 187)].

Major bone reconstructions because of trauma, cancer or augmentation for dental implants are current examples of how tissue engineering can be also be used for craniofacial applications [(186)].

The most recent advances in restorative dentistry involve the development of techniques and materials to regenerate the whole tooth complex in a biological manner. Tissue engineering-based approaches certainly have the potential to achieve this and the future research drive seems to be diverting from a metal-based implant to a biological, cell-based one. Thus, the absolute minimum requirement for tooth regeneration of this type is the successful formation of a heterogenous

and dynamic array of tissues including roots, the periodontal ligament, nerve and vascular tissues, as well as the essential dentine-pulp complex. Perhaps the least important anatomical structures are the mineralized tissues of the crown as current synthetic tooth crowns function more than adequately, as well as being matched for size, shape, color and occlusion[(180)].

Despite some limited progress and minor successes, there remain distinct and important challenges in the development of reproducible and clinically safe approaches for oral tissue repair and regeneration. Clearly, there is a convincing body of evidence which confirms the need for this type of treatment, and public health data worldwide indicates a more than adequate patient resource. The future of these therapies involving more biological approaches and the use of dental tissue stem cells is promising and advancing. As more and more information is collated and knowledge acquired with respect to dental stem cells and tissues, there may well be a significant interest of their application and wider potential to treat disorders beyond the craniofacial region of the body [(50)].

In the future, stem cells will be used to completely restore the hard and soft tissue in the patient's mouth cavity, thus by-passing problems with histocompatibility. Craniofacial skeletal defects are most often results of operative procedures to treat neoplasms, infections, congenital malformations and progressive diseases that lead to deformation of the craniofacial system. Transplantation of bone marrow derived stem cells (BMSCs) cell populations containing mesenchymal stem cells can provide in the future an alternative approach to the reconstruction of craniofacial defects, thus avoiding the dangers of auto and allotransplants[(63, 193)].

Stem Cells

Stem cells are immature, unspecialized cells that have the potential to develop into many different cell lineages via differentiation. By the conventional definition, these cells can renew themselves indefinitely through self-renewal [(159)], and they vary in terms of their location in the body and the type of cells that they can produce [(76)].

There are two primary sources of stem cells: adult stem cells and embryonic stem (ES) cells. In addition to these stem cells, which are naturally present in the human body, induced pluripotent stem (IPS) cells have been recently generated artificially via genetic manipulation of somatic cells [(168)]. ES cells and IPS cells are collectively referred to as pluripotent stem cells because they can develop into all types of cells from all three germinal layers. In contrast, most adult stem cells are multipotent, as they can only differentiate into a limited number of cell types [(169)].

In 2006, a ground breaking approach allowed researchers to induce pluripotency in somatic cells by introducing four transcription factors (OCT4 and SOX2 with the combination of either KLF4 and MYC or NANOG and LIN28) into dermal fibroblasts. This discovery has shed light on the possibility of obtaining autologous pluripotent embryonic-like stem cells without the need of dealing with nuclear transfer and embryos, the so-called induced pluripotent stem cells (IPS) [(168)].

Adult stem cells are also called somatic stem cells or postnatal stem cells, and they are found in many tissues and organs. Although very few of these cells are present in adult tissues, they undergo self-renewal and differentiation to maintain healthy tissues and repair injured tissues [(80)].

Among all possible stem cell sources, adult stem cells have a number of advantages over ES for regeneration of many dental, oral and craniofacial structures. Adult stem cells are chronologically closer to the target dental, oral and craniofacial structures than ES. They are not subjected to the ethical controversy associated with ES. They can be autologous and isolated from the patient, whereas ES cannot be autologous. It is also impossible for ES to be used as autologous cells until these cells are banked. The risk of immune rejection is present for non-autologous cells, whereas autologous stem cells are free from immune rejection [(90)].

Mesenchymal stem cells are among the most promising adult stem cells for clinical applications; they were originally found in the bone marrow, but similar subsets of MSCs have also been isolated from many other adult tissues, including skin [(42)], adipose tissue [(56)], fat [(192)], muscles [(118)], dermis [(26)] and various dental tissues [(47)]. They are localized to specific niches where the regulation of stem cell proliferation, survival, migration, fate and aging occur. Whether cells undergo either prolonged self-renewal or differentiation depends on intrinsic signals modulated by extrinsic factors in the stem cell niche [(33)].

Mesenchymal stem cells, with a capacity of self renewal and multi-lineage differentiation, have attracted worldwide attention during the past few years as attractive progenitor cell sources for tissue engineering and regeneration. In the field of dentistry, several potential sources of MSCs have been identified as candidates for dental tissue engineering. These include MSCs from bone marrow [(84,112,192)], adipose tissues [(95, 185)], umbilical cord blood [(121)] and dental tissues [(26, 53, 86, 111)].

Several sources of dental stem cells (DSCs) have been identified in different dental tissues, including the bone marrow from orofacial bone (BMSCs), the periosteum (PSCs), the dental pulp of permanent teeth (DPSCs), the naturally exfoliated deciduous teeth (SHED), the apical papilla (SCAP), the periodontal ligament (PDLSCs), the dental follicle (DFSCs), the tooth germ (TGPCs), the salivary gland (SGSCs), the oral epithelium (OESCs) and the gingiva (GMSCs) (Fig.1). In comparison to BMSCs, the most extensively studied MSCs these DSCs derived from oral and maxillofacial areas, have also been demonstrated to possess potent self-renewal/colony forming and multipotent differentiation capabilities [(167)].

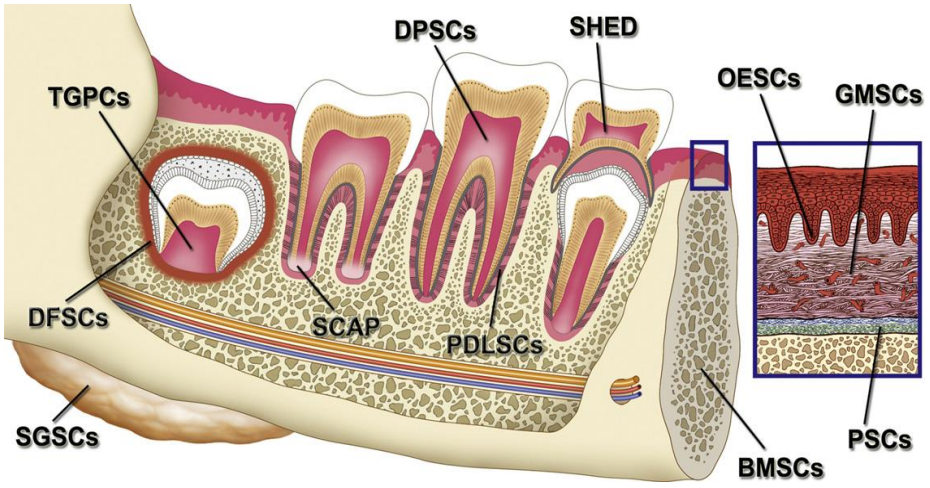

Fig. 1: Sources of adult stem cells in the oral and maxillofacial region.

In 2006, the International Society for Cellular Therapy (ISCT) proposed minimal criteria to define human multipotent MSCs; notably, the ISCT termed MSCs as mesenchymal stromal cells, regardless of the tissue from which they are isolated [(82)]. According to the ISCT criteria, MSCs must be adherent to tissue-culture-treated plastic when maintained

in standard culture conditions. Additionally, MSCs must express CD105, CD73 and CD90 and lack the expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Finally, MSCs must be able to differentiate to osteoblasts, adipocytes and chondroblasts in vitro [43]. Recently, other unique cell surface markers for human MSCs, such as CD271 [25] and MSC antigen-1[18], have been reported. In addition to the use of surface marker analysis, the selection of MSCs using stable mRNA markers specifically expressed in the MSCs has been proposed [87, 104].

More recently, various cell-surface markers have been used in attempts to identify putative MSCs. Markers that are more consistently reported are STRO-1, CD73, CD90, CD105, CD146, Oct4, Nanog, beta 2 integrin positive, and CD14, CD34, CD45, and HLA-DR negative expression [17, 43, 70, 71].

CD90 (Thy-1) is a 25–37 kDa glycosyl phosphatidylinositol (GPI)-anchored glycoprotein expressed mainly in leukocytes and is involved in cell–cell and cell–matrix interactions. CD90 is expressed in BMSCs. Various researchers have reported the expression of CD90 in most adult stem cells [143].

CD105, also known as endoglin, is a homodimeric membrane glycoprotein primarily associated with the human vascular endothelium. Endoglin is a component of the transforming growth factor-beta (TGF -β) receptor complex and binds TGF β-1, and is reported to be expressed in most adult stem cells [143].

CD45 is a protein tyrosine phosphatase located in hematopoietic cells except erythrocytes and platelets. CD45 is also called the common

leukocyte antigen. CD45 is uniformly distributed in plasma membrane and enrich regions of T cell and B cell contact. CD45 is a protein that has several isoforms and the hematopoietic cells express one or more of the isoforms and it is commonly used to identify a population of hematopoietic stem cells. It is reported to be negatively expressed in most MSCs [(143)].

Flow Cytometry

Flow cytometry (FC) is defined as a method for the qualitative and quantitative measurement of biological and physical properties of cells and other particles suspended within a high-velocity fluid stream and passing through a laser beam in a single file. A flow cytometer can provide information on the intrinsic and extrinsic characteristics of the analyzed cells including size, shape, density, DNA, RNA, protein content, internal or external receptors, membrane structure, apoptosis, necrosis, calcium flux, intracellular pH, viability, cell cycle, cell physiology and immunophenotype [(5)].

Flow cytometry is a well-established powerful analytical tool that has led to many revolutionary discoveries in cell biology and molecular disease diagnosis. In conventional flow cytometry, cells are introduced into a high speed laminar artificial flow. After focusing the cells into a single file, laser-induced fluorescence, and/or forward and sideways scattered lights emitted from the cells are detected using photodetector arrays with spectral filters. This highly accurate technology provides fast (a few million cells in a minute), multiparameter quantification of the biological properties of individual cells at subcellular and molecular

levels, including their functional states, morphology, composition, proliferation and protein expression [48].

Any suspended particle or cell from 0.2–150 micrometers in size is suitable for analysis. Cells from solid tissue must be disaggregated before analysis. The characteristics of the cells are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence [123].

Much effort is underway to determine cell surface marker signatures of MSCs that will provide a quality control for their successful isolation and for their differentiation potential. Flow cytometry is the method of choice for immunephenotyping MSCs [105, 140].

Isolation of Stem Cells

Previous studies have shown that the isolation method and the culture conditions can give rise to different populations or lineages of cells under in vitro passage [86]. It was also observed in another publication that seeding with a higher density and a confluence superior to 80% causes changes in cell morphology and phenotype. It is recommended that the density of cells being seeded should be very low (from 80-100 cells per cm^2), and the cultures should be 60 to 70% confluent before passaging [3].

In a protocol of isolation, the tissue should be cut into as small pieces as possible (~1 mm). Tissue pieces should be washed twice with sterile PBS and placed in a solution of 2 mg/mL collagenase and dispase for 30-45 min at 37 °C with gentle agitation on an orbital shaker [145]. As for the isolation procedure, controversies still remain in obtaining reproducible

results by the published methods especially where the differentiation protocols are concerned [190]. Meanwhile, different isolation methods have striking impacts on the differentiation potential of adult stem cells [14]. The lack of consistency between the established protocols of different laboratories adds to the challenge of interpreting previously reported data [190].

Stem cells are usually cultured in Eagle‟s basal medium (EBM), Eagle‟s minimum essential medium (MEM), α -MEM or Dulbecco‟s modified Eagle medium (DMEM). **Govindasamy et al.** [69] cultured dental pulp cells in four different media: DMEM, DMEM low glucose (DMEM-LG), DMEM, and minimum essential medium-alpha (α -MEM), all supplemented with 10 % FBS. They observed that α -MEM and DMEM are the most optimal culture conditions for MSC in terms of their proliferation, morphology, cell surface marker analysis and population doubling time.

Kerkis and Caplan [101] stressed on the importance of the selection of the method of isolation and cell culture conditions in cell populations isolated and cultured in vitro, to achieve the balance in quantity of stem cell and progenitors at different stages. And so, quality and quantity of fetal bovine serum (FBS) added in basal culture medium, use of enzymatic digestion, addition of growth factors and other changes introduced into the protocol for stem cell isolation can contribute to selection or propagation of different populations of stem cells and/or progenitors.

Fetal bovin serum provides all of the growth factors, vitamins, co-factors, hormones, attachment factors (fibronectin, laminin), transport

factors (albumin, globulin, transferrin), nutrients (nucleosides, amino acids, fatty acids, lipids), trace elements and other factors which limit free radicals, toxins and heavy metals. Isolation and maintenance of cells require special culture conditions. Most of the studies are still conducted using FBS as a supplement to culture media since cells are unable to proliferate without essential growth factors, hormones and nutrients that are present in the serum. Serum-free cell culture would represent an adequate alternative, but it requires careful adaptation of sensitive cell lines and often results in lower proliferation rates [(97)].

Serum is a very complex product and only a small percentage of the components have been fully identified. For this reason, and in the absence of a valid alternative, it remains the most effective growth product for cell culture available today. Most sera used in cell culture are from animal, mainly bovine origin. This brings some disadvantages such as antibodies which may impair or damage cell growth, the possibility of presence of adventitious animal viruses and the possible contamination with endotoxins which can damage fragile cell lines [(139)].

High FBS concentration has been shown to induce osteogenesis, wherein a subpopulation of DPSCs was capable of forming woven bone in vitro [(108)]. However, considering the clinical application of tissue-engineered bone grafts, the use of animal-derived components are not recommended, because they can elicit an immune response in patients upon implantation. This is because human cells are able to take up animal proteins and present them on their membranes, thereby initiating an immune response leading to failure of the tissue-engineered graft. Thus, alternative serum supplementation is needed [(102)].

With respect to the pathogenic risks due to addition of FBS in culture media, recent researches are directed towards studying autologous Human Serum (HS) as a safer alternative excluding the transfer of animal derived infections and related immunogenic reactions. Therefore, to safely produce DPSCs for clinical applications, **Ferro et al.** [57] formulated and tested an isolation/proliferation media, reducing as much as possible serum percentage presence and substituting it by adding specific cytokines and growth factors in order to obtain a well-defined composition. They concluded that DPSCs cultured in presence of isolation/proliferation medium added with low HS percentage were obtained without immune-selection methods and showed high uniformity in the expression of stem cell markers, proliferated at a higher rate and demonstrated comparable osteoblastic potential with respect to DPSCs cultured in 10% FBS. In their study, it was demonstrated that a chemically defined medium added with low HS percentage, derived from autologous and heterologous sources, could be a valid substitute to FBS-containing media and should be helpful for adult stem cells clinical application.

The ability and efficiency of various techniques to purify adult stem cells from a heterogeneous cell population is an important factor in the successful characterization and application of stem cells. Existing cell separation methods can be classified into two main groups. The first is based on physical criteria like size, shape and density differences and includes filtration and centrifugation techniques. These methods are commonly used for debulking heterogeneous samples. The second group comprises affinity methods such as capture on affinity solid matrix (beads, plates, fibers), fluorescence-activated cell sorting (FACS) and