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Bone Regeneration in Implant Dentistry: Role of Mesenchymal Stem Cells

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Abstract

This chapter focuses on a review of the activity of non-embryonic mesenchymal stem cells used to regenerate jaw bones in dentistry. Recent research of non-embryonic stem cells provides new possibilities for noninvasively obtaining new autologous bone from stem cells provided by various tissues from the same patient. Disaggregation of biologic tissue harvested from the patients during surgery permits extraction of stem cells from a small sample of connective tissue obtained from the patient's lingual mucosa or from the postextraction surgical site where the endosseous implant will be inserted.

Keywords: Bone regeneration, mesenchymal stem cells, scaffold, micrografts, socket preservation

1. Bone regeneration in implant dentistry

1.1. Bone components

Bone is formed by organic and inorganic components. Two-thirds of the volume comprises inorganic salts, including calcium, phosphate, carbonate, citrate, and hydroxyl ions (magnesium, sodium, and fluoride) in the form of crystals of hydroxyapatite [1]. The organic portion comprises 99% collagen type I and growth factors, such as osteocalcin, osteonectin, phosphoproteins, proteoglycans, and bone morphogenetic proteins [2].

Bone also includes cellular components, such as pre-osteoblasts, osteoblasts, osteocytes, and osteoclasts. Osteoblasts arise from mesenchymal pluripotent cells, which are cuboidal

mononuclear cells located along the bony margins, and are able to form new bone tissue. About 10–20% of osteoblasts are trapped within the matrix they produce by developing into osteocytes, which are considered mature osteoblasts. Osteocytes are smaller than osteoblasts, and have a higher nucleus-to-cytoplasm ratio and a larger number of extensions that allow for intercellular communication. Osteocytes are likely the cells responsible for bone regeneration [3]. Osteoclasts are large multinucleated cells that are polarized, have an average lifespan of 15–20 days, and are derived from bone marrow monocytes [4]. Osteoclasts facilitate bone resorption by reducing the surrounding pH.

1.1.1. Stem cells—mesenchymal stem cells

Stem cells are characterized by their ability to renew by cell division and to differentiate into a diverse range of specialized cell types. The two broad types of mammalian stem cells are embryonic stem cells, which are found in blastocysts, and adult stem cells, which are found in adult tissues such as the bone marrow. In adult organisms, stem cells give rise to progenitor cells that act as a repair system for the body by replenishing specialized cells and tissues. Because adult stem cells are obtained from a developed organism, their use in research and therapy is not as controversial as that of embryonic stem cells, which entail the destruction of an embryo [5].

Mesenchymal stem cells (MSCs) are multipotent adult stem cells with unique biologic properties that are typically associated with their mesodermal lineage (adipogenic, chondrogenic, osteogenic, or myogenic) [6–8]. MSCs were first discovered in 1968 by Friedenstein et al. [9], and are defined as adherent fibroblast-like cells that reside in the bone marrow and are capable of differentiating into bone. MSCs and an adequate blood supply are essential for the bone deposition process and healing. MSCs also contribute to the homeostasis of various tissues, including bone, in adults [10].

MSCs can be expanded in vitro for several passages, are easily accessible, and possess minimal immunogenic or tumorigenic risks, and are thus an excellent cell source of stem cells used in dental, craniofacial, and orthopedic regenerative surgery [11].

In 2006, the International Society for Cellular Therapy established the following definition of MSCs [12]:

1. Cells that are adherent to plastic under standard tissue-culture conditions;
2. Cells that are positive for surface markers CD105, CD73, and CD90, but negative for CD34, CD45, CD14, or CD11b, CD79a, or CD19, and human leukocyte antigen-D-related (HLA)-DR surface molecules;
3. Cells with the capacity to differentiate into osteoblasts, chondrocytes, and adipocytes;

MSCs, which represent ~10% of human stem cells, are rare and heterogeneous; they are part of the connective tissue and support hemopoiesis [13]. MSCs can be expanded in vitro and rapidly reach the desired cell counts for use in vivo [14].

Despite having some common features, MSCs have different characteristics depending on the tissue of origin. MSCs can be isolated from several different tissues, including bone marrow [15], placenta, cord blood [16], adipose tissue [17], muscle [18], periosteum [19], synovium [20], deciduous teeth [21], and brain, kidney, heart, epidermis, and periodontal ligaments [22–24]. Among these, bone marrow and adipose tissue are the most commonly used sources of MSCs because of their relative ease of harvesting. MSCs can differentiate into osteoblasts, adipocytes, chondrocytes, myoblasts, cardiomyocytes, hepatocytes, neurons, astrocytes, endothelial cells, fibroblasts, and stromal cells [25].

2. Bone regeneration

Since Horwitz et al. [26] first demonstrated that MSCs can improve osteogenesis in children with osteogenesis imperfecta, the role of MSCs in bone formation and regeneration has been intensively studied. Studies performed in several animal models revealed that the transplantation of MSCs improves bone regeneration and healing of bony defects [27, 28]. The therapeutic options clinically available for bone reconstruction and regeneration, however, are often unsatisfactory due to morbidity at the donor site or the complexity of allograft procedures.

Bone regeneration in maxillofacial reconstruction is one of the most important applications of MSCs [29]. The repair of craniofacial bone defects remains a challenge, however, and the results depend on the size of the defect, the quality of the soft tissues that cover the defect, and the reconstructive techniques used. In Europe, ~1.5 million patients undergo craniofacial reconstructions annually; ~20% of them continue to experience functional deficiency despite the intervention, and 30,000 patients per year develop donor-site morbidity following oral and maxillofacial reconstruction [30].

Traditional bone regeneration techniques involve autologous, homologous, heterologous, or allogeneic grafts. Autologous bone grafts are considered the best option for damaged tissue repair because of the low risk of immunogenicity or disease transmission compared with allografts (genetically different donors from the same species) or xenografts (donors from another species). Autologous bone grafts are limited due to the scarcity of available autologous tissue for repairing larger bone defects, donor-site morbidity, and potential wound-based infections, as well as the prolonged operative times. In addition, autologous bone grafts require additional surgical procedures, which increase the risk of both donor-site morbidity and significant resorption [31]. Alternative therapies continue to be explored [32], and researchers are attempting to identify the best material for bone regeneration.

Bone regeneration following the use of stem cells occurs through two mechanisms: a direct mechanism, which comprises the integration and differentiation into tissue-specific cells, and begins when transplanted cells take root in the target tissue [33]; and an indirect mechanism, which involves paracrine effects [34].

Differentiation of MSCs into osteoblasts was demonstrated *in vitro* by cultivating the cells in the presence of ascorbic acid, inorganic phosphate (beta-glycerophosphate), and dexamethasone [35].

methasone. In vivo studies suggest that transplanted adult stem cells can integrate into tissues that are different from those of the donor and, in some cases, contribute to their regeneration [35]. Demonstrating the in vivo differentiation of implanted cells is challenging, and researchers often assume that differentiation is the result of interactions between grafted cells and host-site cells, but the capacity of MSCs to release a number of trophic factors could also explain their therapeutic benefit.

Some recent reports suggest that the therapeutic properties of paracrine factors are a common feature of stem cells [36]. The paracrine effect could contribute to bone regeneration via the secretion of trophic and angiogenic molecules such as angiopoietin (Ang)-1, Ang-2, Ang-like-1, Ang-like-2, Ang-like-3, Ang-like-4, vascular endothelial growth factor (VEGF), and fibroblast growth factor-2. These molecules can activate local MSCs, promote tissue regeneration and angiogenesis [37], and inhibit fibrosis, apoptosis, and inflammation [38, 39]. They also have neurogenic, neuroprotective, and synaptogenic effects [40, 41]. Because the survival and differentiation of MSCs at the site of the lesion is limited, paracrine signaling is considered to be the primary mechanism of their therapeutic effects [42]. This hypothesis is supported by in vitro and in vivo studies showing that many cell types respond to paracrine signaling from MSCs, which leads to the modulation of a large number of cellular responses, such as survival, proliferation, migration, and gene expression [39].

The secretion of bioactive factors is thought to play a critical role in the paracrine activity of MSCs. These factors and cytokines can be collected in a conditioned medium (CM), which, when transplanted into animal models of different diseases, has effects that are similar to those exerted by MSCs and can increase the tissue-repair process in acute myocardial infarction [43], wound healing [44, 45], and neuroprotection [46]. Encouraging results have also been obtained following the graft of MSCs obtained from the bone marrow cleft at the level of the maxillary sinus and alveolar schisis [47, 48].

Preliminary studies of bone regeneration used MSC populations that were not expanded from bone marrow due to the reduced number of MSCs in the bone marrow (0.01% of the bone marrow cell population). The use of unexpanded MSCs, however, produced unpredictable results [49], and later advances made it possible to cultivate and characterize MSCs. The osteogenic potential of expanded and purified MSCs has been studied extensively, but with mixed results [50, 51]. Factors that may affect the results relate to the donor site, blood supply, and inadequate osteoblastic differentiation of the implanted cells.

In summary, stem cells are effective for tissue regeneration and future research is warranted despite the low number of clinical studies compared to those in preclinical animal models. The use of MSCs is still limited because of their low accessibility, difficult collection, and poor long-term stability. Stem cells are used mainly in combination with scaffolds or biomaterials to improve their efficacy and stability. Scaffold material is often used to provide mechanical support and as a substrate for cell attachment, proliferation, and differentiation. Regardless of the scaffold used for bone reconstruction, however, bone healing depends mainly on two pivotal factors: the capacity to recruit progenitor cells to the injury site and the presence of healthy vasculature near the injury site. Researchers have identified several different tissue types that can be considered valid MSC donors.

2.1. Dental pulp stem cells

Dental pulp is a source of neural crest-derived stem cells that is easily accessible and characterized by low morbidity after collection [52, 53]. Dental pulp comprises both ectodermic and mesenchymal components, and is divided into four layers (outer to inner). The external layer is made up of odontoblast-producing dentin. The second layer, called the “cell-free zone,” is poor in cells and rich in extracellular matrix. The third layer, called the “cell-rich zone,” contains progenitor cells that exhibit plasticity and pluripotent capabilities [52]. Finally, the inner layer comprises the vascular area and nerve plexus.

In the context of the oral and maxillofacial area, dental pulp stem cells (DPSCs) and periosteal stem cells may be optimal alternatives to MSCs and display high potential for differentiating into a variety of cell types, including osteocytes, suggesting their effective use in bone regeneration, although clinical studies are limited. In addition to DPSCs and periosteal stem cells, adipose tissue also serves as a source of MSCs [17]. In fact, adipose-derived stromal cells can differentiate into chondrocytes, osteocytes, or myocytes, as indicated by several studies in animal models [54–57], although clear and conclusive data about their osteogenic potential are limited.

In 2005, Laino et al. [58] successfully isolated and selected a distinctive and highly enriched population of stem cells derived from dental pulp in adult humans. This stem cell population was self-expanding and differentiated into pre-osteoblasts able to self-maintain and renew. These stem cells differentiated into osteoblasts and produced living autologous fibrous bone tissue in vitro after 50 days of culture. Transplantation of this tissue in vivo led to the formation of lamellar bone with osteocytes without the need for scaffolding. The differentiated cells and living autologous fibrous bone could be frozen at -80°C and stored for extended periods of time with no clear effect on their bone-forming ability. The same research group subsequently demonstrated that DPSCs differentiate into osteoblasts that secrete abundant extracellular matrix [59].

In 2007, d’Aquino et al. [60] provided direct evidence that osteogenesis and angiogenesis mediated by human DPSCs are regulated by distinct mechanisms that lead to the organization of adult bone tissue after stem cell transplantation. In this study, stromal stem cells from human dental pulp were extracted, cultivated, and characterized in vitro. After 30 days of culture, the cells began to differentiate, lost their stem cell markers, and expressed differentiation markers. After 40 days, the cells differentiated into two cytotypes from a common progenitor: osteogenic progenitor cells (70% of total cells) and endothelial progenitor cells (EPCs, 30%), demonstrating synergic differentiation into osteoblasts and endotheliocytes. After 50 days, woven bone was obtained in vitro and its transplantation into immunocompromised rats resulted in a tissue structure with an integral blood supply similar to that of human adult bone. These findings suggest that osteogenesis and vasculogenesis are interdependent, and that this process is essential to obtain adult bone tissue suitable for transplantation and surgical or clinical applications in tissue repair.

DPSCs grafted into immunosuppressed rats generated complete and well-vascularized lamellar bone [61]. DPSCs are easily managed because they have a long lifespan, can be safely

cryopreserved, and are able to interact with biomaterials [62]. Finally, *in vitro* and *in vivo* experiments revealed that both the quality and quantity of bone regenerated by DPSCs blended from stem cells and biomaterials [58, 60, 61, 63].

DPSCs can be applied for oral and maxillofacial bone repair in the maxillofacial area and, on appropriate resorbable scaffolds, promote the formation of an efficient biocomplex in patients with a mandibular defect, as reported by d'Aquino et al. [60]. In that study, a biocomplex constructed from dental pulp stem/progenitor cells and a collagen-sponge scaffold was used for oral and maxillofacial bone tissue repair. Stem/progenitor cells obtained from the upper third of molars previously extracted were gently placed with a syringe onto a collagen-sponge scaffold and used to fill the space left by the lower third of the molar extraction procedure. Thirty days after surgery, X-ray controls exhibited a high rate of mineralization; 3 months after the surgery, samples collected from the regeneration site showed well-organized and well-vascularized bone with a lamellar architecture surrounding the Haversian canals. Bone from control sites was immature and showed fibrous bone entrapped among new lamellae, incomplete and large Haversian canals, and evidence of bone resorption. Moreover, immunofluorescence analyses showed high levels of bone morphogenetic protein-2 and VEGF in regeneration samples. This clinical study demonstrated that dental pulp stem/progenitor cells can be used for oral and maxillofacial bone repair and that collagen sponges can be considered an optimal support for stem/progenitor cells in cell-guided regeneration.

The same group published a 3-year follow-up [64]. Histology and in-line holotomography revealed that regenerated bone was uniformly vascularized and qualitatively compact rather than the physiologic type of bone found in that region—cancellous (i.e., spongy). The authors speculated that the regeneration of compact bone probably occurs because grafted DPSCs do not follow the local signals of the surrounding spongy bone. Although the bone that regenerated at the graft site was not the proper type found in the mandible, it seemed to have a positive clinical outcome because it created steadier mandibles, increased implant stability, and may have improved resistance to mechanical, physical, chemical, and pharmacologic agents.

Although the use of DPSCs is valid for tissue regeneration in the maxillofacial area, the identification of an accessible site from which to collect these cells can be challenging, and the amount of cells that can be obtained is very limited. DPSCs can be cultured by two methods. In the enzyme-digestion method, pulp tissue is collected under sterile conditions and digested with the appropriate enzymes, and the resulting cell suspensions are seeded in culture dishes [65]. In the explant outgrowth method, the extracted pulp tissues are cut, anchored via microcarriers onto a suitable substrate, and directly incubated in culture dishes [66]. From a clinical point of view, these methods are not appropriate for therapeutic applications because of the manipulation of dental pulp. A new, efficient, and safe method for isolating dental pulp was reported by Brunelli in 2013 [67], in which a new instrument called a Rigenera® (Torino, Italy) machine was used to create micrografts of disaggregated dental pulp that was subsequently poured onto a collagen sponge. This micrograft was injected into the sinus cavity, and 4 months after the intervention newly formed bone was observed with twice the mineral density of native bone [67].

2.2. Periosteal stem cells

In addition to dental pulp, the periosteum is a surprising source of stem cells. After bone fracture in animal models, periosteal progenitor cells undergo an impressive expansion, followed by differentiation into osteoblasts and chondrocytes [68]. This remarkable property of the periosteum has prompted extensive research into the use of periosteum-derived cells for regenerative approaches, and preclinical studies have demonstrated the potential of these cells. The success of periosteal cells in preclinical animal models has also given rise to several exploratory clinical studies using ex vivo expanded periosteal cells for bone regeneration.

In 1992, chick tibial periosteal cells were cultured, combined with porous calcium phosphate ceramics, and subcutaneously implanted into athymic mice [69]. These cells eventually gave rise to bone tissue via two different mechanisms. Intramembranous bone formation occurred early in the peripheral pores of the ceramics, and endochondral bone formation occurred later in the central pores. These results raised the possibility that composite grafts of cultured periosteal-derived cells and porous ceramics could be clinically used as bone-graft substitutes for bone augmentation or regeneration.

In 2001, Vacanti et al. [70] first used culture-expanded periosteal cells derived from the radius in combination with a porous hydroxyapatite scaffold to replace the distal phalanx of the thumb. In this study, coral alone seeded with cells derived from the periosteum and placed in the subcutaneous tissue that was not adjacent to native bone formed new bone.

The use of periosteum-derived bony matrix for augmentation in the posterior maxilla before implantation results in bone formation 4 months after transplantation with trabecular bone containing viable osteocytes [71, 72]. The graft provides a reliable basis for the simultaneous or secondary insertion of dental implants.

Springer et al. [73] compared mandibular periosteum cells that were cultured and seeded onto a collagen matrix and maxillary bone cells that were cultured and seeded onto natural bone minerals. They concluded that the first method produced a significantly higher amount of new living bone.

Taken together, these reports demonstrate the clinical potential of periosteal-derived cells for bone regeneration therapies. The last three studies described, however, did not use stem cells but rather only cultures of differentiated periosteal cells.

2.3. Bone marrow-derived MSCs

Bone marrow-derived mesenchymal stem cells (BMSCs) are a readily available and abundant source of cells for tissue-engineering applications. BMSCs may be useful tools for regenerating bone, but the method of bone marrow aspiration from patients is associated with significant morbidity at the donor site [74].

BMSCs can differentiate into osteoblasts in vitro [75] and have osteogenic ability in vivo [76]. The addition of BMSCs to a biomaterial improves the quality of regenerated lamellar bone [77]. In 2008, BMSCs were successfully used in association with biphasic hydroxyapatite/ β -tricalcium phosphate in a sinus-augmentation procedure [78].

In a recent study [79], tissue repair cells isolated from bone marrow were successfully used to repair bone defects in a human model. In this study, bone marrow cells were collected, cultivated, and characterized. Flow cytometry demonstrated the presence of mesenchymal and vascular phenotypes. The cellular suspension, carried by a gelatin sponge, was implanted in a postextraction site and covered by a resorbable collagen membrane. Six weeks after the implantation, biopsy revealed the presence of highly vascularized and mineralized bone tissue. McAllister et al. [5] used an MSC-heterologous bone graft harvested from cadavers for sinus-augmentation procedures. The authors demonstrated the presence of MSCs in the commercial bone preparation derived from cadavers and harvested within 24 h of death and stored at -80°C . Moreover, they rapidly formed bone from a commercially available cellular bone matrix that contained heterologous MSCs.

In one study [80], researchers seeded Geistlich Bio-Oss (GeistlichPharma North America, Princeton, NJ, USA) with stem cells and found that this construct was superior to Bio-Oss mixed with autogenous bone in terms of bone formation 3–4 months after surgery. This study, however, presented some issues regarding data reporting and statistical analysis.

In a well-documented preliminary report, Behnia et al. [81] used BMSCs in association with platelet-rich plasma. They used biphasic hydroxyapatite/tricalcium phosphate as a scaffold and implanted the graft in an alveolar cleft, achieving cleft closure and a mean postoperative defect filling of 51.3% at 3 months after surgery. The same research group used demineralized bone mineral and calcium sulfate in association with BMSCs to treat alveolar clefts, but did not achieve similar positive results [47]. They concluded that the latter material was not a suitable scaffold for MSC-induced bone regeneration.

Bone marrow aspiration, however, is severely painful for donors, often requires general anesthesia, and may be associated with adverse events [74, 82].

2.4. Blood-derived stem cells

Peripheral blood is a source of MSCs that can be isolated with minimal invasiveness compared to extraction from bone marrow [83, 84]. According to some authors [85], blood-derived stem cells have characteristics and bone-regeneration abilities that are similar to those of BMSCs both *in vitro* and *in vivo* and are a promising source for bone regeneration for clinical use; by contrast, other authors [83] report that blood-derived stem cells have less multipotency than bone BMSCs.

2.5. Adipose-derived stem cells

Adipose tissue is an alternative source of MSCs that can differentiate into chondrocytes, osteocytes, or myocytes [17, 54, 86, 87]. An *in vivo* study demonstrated that adipose-derived stem cells are capable of bone regeneration and are useful for reconstructing critical-size defects in rats [55].

2.6. Secretomes

MSCs enhance wound healing, but the mechanisms are unclear. The use of MSCs for tissue repair was initially based on the hypothesis that these cells migrate to and differentiate within injured tissues, becoming specialized cells. It now appears that only a small proportion of transplanted MSCs actually integrate into and survive in the host tissue. Thus, the predominant mechanism by which MSCs participate in tissue repair seems to be related to their paracrine activity. Indeed, MSCs provide a suitable microenvironment that includes a multitude of trophic and survival signals, including growth factors and cytokines. Factors secreted from stem cells into a medium are called *secretomes* and have attracted much attention [45] because of their ability to support regenerative processes in the damaged tissue, induce angiogenesis, protect cells from apoptosis, modulate the immune system, and recruit endogenous stem cells to the grafted site. Compared to stem cells from other sources, BMSCs secrete distinctively different cytokines and chemokines, including greater amounts of VEGF- α , insulin-like growth factor 1, epidermal growth factor, keratinocyte growth factor, Ang-1, stromal-derived factor 1, macrophage inflammatory protein-1 α and -1 β , and erythropoietin [45], which are important for normal wound healing.

In vitro, the CM from the culture of BMSCs (MSC-CM) enhances the migration, proliferation, and expression of osteogenic marker genes such as alkaline phosphatase, osteocalcin, and Runt-related transcription factor 2 of MSCs, and contains cytokines such as insulin-like growth factor 1, VEGF, transforming growth factor- β 1, and hepatocyte growth factor. The concentrations of cytokines contained in MSC-CM are relatively low, and the use of MSC-CM does not induce the severe histologic inflammatory responses observed with the clinical use of recombinant human bone morphogenetic protein 2 [88]. Implantation of MSC-CM in association with a collagen sponge or agarose produced early bone regeneration in rat calvaria, suggesting that MSC-CM has potential for cell-free bone regeneration [88, 89].

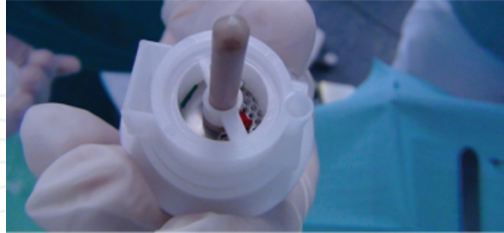
MSC-CM recruits endogenous stem cells to the graft site and promotes early bone and periodontal regeneration in rat calvarial bone defects and periodontal tissue [88, 90]. Some authors [88] noted a stronger effect on bone regeneration and autogenous MSC migration when MSC-CM, rather than MSCs alone, was used in the graft, demonstrating that MSC-CM induces bone regeneration via mobilization of endogenous stem cells. The recent use of MSC-CM in various oral and maxillofacial bone regeneration procedures demonstrated osteogenic potential [91].

The use of MSC-CM for bone regeneration is a unique concept in which the paracrine factors of stem cells are used without cell transplantation.

3. Cell isolation

The isolation of cells is often difficult, and the methods of extraction, such as enzymatic digestion or mechanical disaggregation, require several minutes to a few hours, which can reduce cell viability. A recent study [92] demonstrated the efficacy of a new medical device

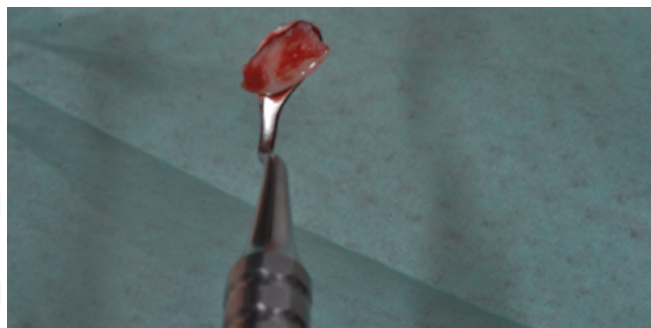
called Rigeneracons® (CE certified Class I; Human Brain Wave, Turin, Italy) (**Figures 1 and 2**) to provide autologous periosteal micrografts (**Figure 3**) for clinical practice that are enriched with progenitor cells and are able to regenerate and differentiate.



Figures 1. Rigeneracons® medical device.



Figures 2. Detail of the blades system which disaggregate the periosteal tissue.



Figures 3. 1-2 mm² of periosteal tissue harvested after flap elevation can be disaggregate to get progenitor cells that will be seeded on a scaffold to be grafted in the bone defect.

The protocol is very simple. A 1–2-mm periosteal tissue harvested from the flap elevated at extraction or other surgical site is disaggregated mechanically (2 min at 15 Ncm and 75 round/min) after adding 1 ml sterile saline. The Rigeneracons® has 100 holes each provided with six microblades. A filter allows only the cells smaller than 50 μ (eight progenitor cells) to drop into a tank. The solution is then seeded on a polymeric scaffold (polylactic-co-glycolic acid-hydroxyapatite (PLGA-HA)) and grafted in the bone site (socket preservation, sinus lifting,

periodontal defects, etc.) Although in vitro data about the Rigenera protocol are limited, a recent study demonstrated the efficacy of the Rigenera machine for obtaining stem cells from dental pulp [67]. These cells were positive for mesenchymal cell-line markers and negative for hematopoietic and macrophage markers. The percentage of viable cells derived from periosteum samples was high, however, suggesting that the device provides effective extraction.

4. Scaffold

MSCs grafted from a cell suspension require scaffolds to provide support, cohesion, and stability. Several types of materials are used as scaffolds. Advances in cell therapy have been accompanied by advances in novel scaffold fabrication techniques, yielding greater control over the surface topography, internal microstructure, and pore interconnectivity. Porous scaffolds have been widely explored for cell attachment because of the importance of allowing adequate room for tissue ingrowth and vascularization (i.e., pore size of 150–500 nm) [93]. Although natural materials retain their bioactivity, synthetic scaffolds present several advantages, including added flexibility in manufacturing, reproducibility, sterilization, storage times, and nonimmunogenicity. Solid free-form fabrication, a rapid three-dimensional printing technology for prototyping, was recently adapted for use in bone regeneration. Other researchers have developed hydrogels to encapsulate stem cells for tissue engineering, some with tunable degradation rates, but hydrogels may not provide the strength necessary for bone repair in load-bearing locations [94–96].

Not all researchers agree about the efficacy of combinations of stem cells and scaffolds, and a recent study reported that tissue-engineered complexes did not significantly improve bone-induced regeneration processes. Further studies are needed to elucidate the role of stem cells and scaffolds in tissue regeneration [97].

5. Epigenetic regulation

Epigenetic factors play a fundamental role in regulating the regenerative processes of MSCs [98, 99]. In stem cell differentiation processes, some genes may be upregulated and others repressed. Epigenetic modifications result in significant functional genomic alterations without changes in the nucleotide sequence [100].

Well-known epigenetic mechanisms include DNA methylation and histone modifications. Cytosine methylation downregulates gene expression, and the absence of methylation is essential for gene expression. In bone regeneration, however, methylation is essential. During MSC differentiation into osteoblasts in vitro, methylation of the osteocalcin promoter is significantly decreased, leading to the upregulation of osteocalcin [98]. Also, during osteoblast differentiation, increased methylation of the promoter of LIN28, a gene responsible for the maintenance of stem cell characteristics, reduces the expression of this gene, which facilitates osteogenesis [101].

Gene transcription is also regulated by histone modifications [100]. Histones are positively charged proteins that strongly bind the light-chain bearing structure of the double strand of phosphate-deoxyribose DNA. The binding of histone DNA determines the accessibility of transcription factors [102]. The most studied modifications are acetylation and methylation. Acetylation reduces DNA binding, allowing for greater gene expression. Conversely, deacetylation leads to a more compact chromatin structure, thus decreasing gene expression [103]. During differentiation, osteoblastic regions of the osteocalcin and osteocalcin promoters exhibit high levels of acetylation, which allows for greater accessibility of transcription factors. In addition, the downregulation of histone deacetylase-1 is an important process during osteogenesis [104]. These examples highlight the complexity of the effects of epigenetic regulation during bone regeneration.

6. Issues

Despite the initial success regarding the use of MSCs, some challenges remain as follows:

1. MSC removal requires invasive procedures that are associated with morbidity.
2. MSC proliferation and osteogenic differentiation potential decrease with age.
3. Inadequate vascular grafts of MSC carriers lead to cell death.
4. Difficulty accessing the repair site may limit the application of MSCs.

Recent studies revealed that implanted cells do not survive long [105]. One study showed a significant loss of cells within 24 h, and low numbers of transplanted cells survived at 12 weeks. Cells that did survive, however, underwent differentiation [106].

A crucial issue for autografts is cell viability; after collection, viability decreases to less than 50%, thereby reducing the regenerative capacities of the autografts. Cell death results from vessel interruption and subsequently reduced nutrition. Inadequate graft dimensions and tissue-size reductions to facilitate feeding can also lead to cell death. A promising approach to address this problem is the use of an instrument that preserves graft viability, such as by selecting small cells that are less susceptible to cell lysis.

Graft vascularization is a determining factor in cell survival, engraftment, and bone regeneration. In 1997, circulating EPCs were identified [107, 108]. EPCs participate in neovascularization processes [109], angiogenesis, vascular repair, restoration of blood flow after ischemia, distraction osteogenesis [110, 111], healing of fractures [111], and bone regeneration [112], and have an osteogenic potential. They are located mainly in the bone marrow and are mobilized as a result of biologic signals. The *in vitro* cultivation of mononuclear cell fractions under favorable conditions produces two EPC subtypes: early- and late-outgrowth endothelial cells [113]. Early-outgrowth endothelial cells survive less than 7 days *in vitro*, are characterized by a low rate of duplication, and induce transient angiogenesis principally for paracrine effects; late-outgrowth cells can expand to 100 cell population doublings, take root at the site of

engraftment, and can differentiate into osteoblasts [114]. A 2009 study demonstrated the successful application of blood-derived EPCs for healing bone defects [115].

7. Safety of transplanted MSCs

Clinical trials to evaluate the safety of MSCs for the treatment of graft-versus-host disease, ischemic heart disease, spinal cord injury, and systemic lupus erythematosus have not revealed any significant adverse effects [116–119]. While pluripotent cells have been obtained from adult somatic tissues by reprogramming methods [120], these cells can differentiate into different tissues and have wrongly been considered a source of MSCs for tissue regeneration. Indeed, they are known to cause teratoma formation and significant efforts to address the safety concerns are required before their application in patients [120]. By contrast, MSCs obtained without genetic reprogramming have a high capability to differentiate into many tissues without developing into tumor cells.

Studies of the role of MSCs in tumorigenesis have identified the ability of MSCs to interact with tumor cells and to support angiogenesis by providing a matrix to support cancer cells [121, 122]. MSCs may thus facilitate the growth of existing tumors [123, 124]. Transdifferentiation of MSCs has been observed in vitro, but this phenomenon could be due to contamination by tumor cells [125, 126].

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