Research Article

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Regenerative Medicine

Association of mesenchymal stem cells and osteoblasts for bone repair

Aim: We tested the hypothesis that the association of bone marrow mesenchymal stem cells (MSCs) and osteoblasts (OBs) optimize bone repair. **Materials & Methods:** MSCs were cultured in growth or osteogenic medium and seeded into gelatin sponge prior to implantation. Defects were created into rat calvariae and implanted with gelatin sponge without cells, with MSCs, with OBs and with association of MSCs and OBs. Histological analysis and micro-CT-based histomorphometry were carried out after 4 weeks. **Results:** Increased bone formation was observed in defects treated with cells and bone volume was greater in defects treated with either OBs or MSCs/OBs. **Conclusion:** Association of MSCs and OBs did not increase the process of bone repair compared with cell-based therapy using either MSCs or OBs alone.

Keywords: bone marrow • bone repair • cell therapy • mesenchymal stem cell • osteoblast

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The repair of bone defects remains a major challenge in the field of dentistry and medicine. Large bone defects that are difficult to heal often occur as a result of trauma, cancer or metabolic diseases [1]. Therefore, treatment using different approaches such as bone grafts, scaffolds and cells has been employed in order to optimize the conditions under which the process of bone healing occurs [2].

Several types of grafts such as synthetic biomaterials, allografts, xenografts and autografts have been tested and used to repair bone defects [3]. Synthetic biomaterials have been suitable alternatives to avoid the risks associated with autografts and allografts. However, these biomaterials do not display osteogenic properties, acting only as scaffolds for bone formation [4]. Allograft bone is more readily available, but it has been associated with disadvantages such as risk of host reactions due to genetic differences, disease transmission, ethical and religious issues [3]. Autograft bone is referred as the 'gold standard' due to the promotion of osteoconduction, osteoinduction and osteogenesis as well as angiogenesis without the risk of disease transmission [5]. The disadvantages of autografts include the limited availability, morbidity at the harvest site and difficulty in shaping the graft to fit the bone defect [6]. Thus, considering the growing need for new therapies to the aforementioned treatments and that autograft success is largely related to the presence of cells and growth factors, the use of cells has been considered a promising alternative [7].

According to previous studies, bone marrow mesenchymal stem cells (MSCs) both undifferentiated and differentiated into osteoblasts (OBs) can induce bone repair [8–10]. Regarding the degree of OB differentiation, the use of OBs at early stage of differentiation resulted in greater bone formation [11]. Recently, by using tissue engineering strategies, we observed that MSCs and OBs differentiated for 7 days are equally able to stimulate bone formation while OBs differentiated for 14 days show a lower potential to induce bone repair in rat calvarial defects [12].

The mentioned results were obtained from either MSCs or OBs isolated and, to our knowledge, there are no studies evaluating the effect of MSCs combined with OBs on bone formation. Considering the evidences that both MSCs and OBs contribute to bone

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formation, we hypothesized that a cell therapy based on the association of MSCs and OBs could be more effective for bone repair than the use of these cells separately. To test this hypothesis, we evaluated the effect of the association of MSCs and OBs on bone repair of rat calvarial defects.

Materials & methods

Preparation of scaffolds

Gelfoam (Pharmacia & Upjohn Co., MI, USA), a sponge made of porcine gelatin was used as a scaffold. Prior to implantation in calvarial bone defects, they were manually cut with the aid of forceps and scalpel blade to obtain samples with 5 mm in diameter and 2 – 3 mm height. This preparation was carried out in a laminar flow cabinet to keep the scaffolds sterile.

Cell culture

Bone marrow MSCs were obtained from femora of male Wistar rats weighting 140–150 g, under approval of the Committee of Ethics in Animal Research of the University of São Paulo, as previously described [13]. MSCs were selected by adherence from the whole bone marrow and cultured for 3 days in 75 cm^2 culture flasks (Corning Incorporated, MA, USA) containing growth medium constituted by α-MEM (Gibco-Invitrogen, NY, USA) supplemented with 15% fetal calf serum (Gibco-Invitrogen), 50 μg/ml gentamycin (Gibco-Invitrogen), 50 μg/ml vancomycin (Acros Organics, Geel, Belgium) and 0.3 μg/ml fungizone (Gibco-Invitrogen). After that, to eliminate cell passage, they were kept in the same flasks and cultured for 7 days either in growth medium to keep MSC features or in osteogenic medium to differentiate into OBs. Osteogenic medium was growth one plus 5 μg/ml ascorbic acid (Gibco-Invitrogen), 7 mM β-glycerophosphate (Sigma-Aldrich, MO, USA) and 10^{-7} M dexamethasone (Sigma). During the culture period, cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed every 48 h, and 24 h before seeding, cells were deprived of fetal calf serum to minimize the risk of foreign body reaction.

Cell seeding

Centrifugal cell immobilization (CCI) technique was used to seed cells into gelatin sponge [14]. Scaffolds and 5 ml of medium containing 5×10^5 cells/mg scaffold were centrifuged at 300 × *g* for 1 min four-times. Between each cycle, the medium was stirred to resuspend nonattached cells into pore structure and viable and nonviable cells in the medium were detected by Trypan blue and counted using an automatic cell counter (Countness Cell Counter, Invitrogen, NY, USA). The seeding efficiency was defined by the number of cells seeded into scaffolds as determined from the difference between the initial and the final number of cells in the medium. In order to evaluate the ability of CCI to force cells within the internal walls of scaffolds, cells were stained with MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) before seeding. After that, cells and scaffolds were fixed at room temperature with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 and sectioned. Cell penetration was observed and recorded using a digital camera (Axiocam ICc3; Carl Zeiss, Oberkochen, Germany) attached to a stereomicroscope MZ6 (Leica Microsystems, Wetzlar, Germany).

Implantation procedure

Twenty male Wistar rats weighting 250–300 g were anesthetized by a combination of ketamine (7 mg/100 g body weight; Agener União, Embu-Guaçu, SP, Brazil) and xylazine (0.6 mg/100 g body weight) (Calier, Juatuba, MG, Brazil). The cranium of each rat was shaved and disinfected with povidone-iodine alcoholic solution (Rioquímica, São José do Rio Preto, SP, Brazil). An incision was made along the sagittal suture to expose the parietal bones and unilateral calvarial bone defects with 5 mm in diameter were created with a trephine under saline irrigation. Defects were randomly implanted (five for each implant) with gelatin sponge scaffolds without cells (control) or seeded with either MSCs, OBs or the association of MSCs/OBs in a 1:1 proportion followed by skin suture. Then, one dose of antibiotics, benzathine benzylpenicillin (156,000 IU/100 g body weight), benzylpenicillin procaine (78,000 IU/100 g body weight), benzylpenicillin potassium (78,000 UI/ 100 g body weight), dihydrostreptomycin base (65 mg/100 g body weight) and streptomycin base (65 mg/100 g body weight; Fort Dodge, Campinas, SP, Brazil) and Flunixin meglumine analgesic (10 mg/100 g body weight; Schering-Plough, Vila Olímpia, SP, Brazil) were administered. Four weeks postimplantation, animals were euthanized and samples collected and processed for histological and histomorphometric analyses.

Histological analysis

Samples of implantation areas were fixed in 10% buffered formalin for 36 h. Then, they were decalcified in buffered EDTA (Merck, Darmstadt, HE, Germany) for 10 days and embedded in paraffin. Six-micrometer-thickness sections from central region of samples were stained with hematoxylin and eosin and images were obtained using light microscopy (Axioskop 40, Carl Zeiss) attached to a digital camera (Axiocam ICc3, Carl Zeiss).

Histomorphometric analysis

Morphometric analysis was carried out before histological processing by micro-computed tomography (micro-CT), using the SkyScan 1172 system (SkyScan, Belgium). Images were obtained at 60 kVp and 200 mA and reconstructed (NRecon software, Bruker-Skyscan, Belgium) with smoothing 1, ring artifact correction 5 and beam hardening correction 20%. Bone volume, percentage of bone volume, bone surface, bone surface/volume ratio, trabecular number, trabecular thickness and trabecular separation were measured using the 3D Ctan software (BruKer-Skyscan) [15,16].

Statistical analysis

The results were expressed as mean ±standard deviation (n = 5) and compared by Kruskal–Wallis test followed by Mann–Whitney test if appropriated. The level of significance was set at $p \leq 0.05$.

Results

Cell seeding

The CCI method was carried out immediately before the implantation procedure and it was highly efficient to seed cells into the scaffolds. It was noticed that the number of seeded cells gradually increased along the centrifugation cycles (Figure 1A). The efficiency of CCI method was around 72% and irrespective of the cell type and centrifugation cycles, cell viability remained close to 100% (Figure 1B). Also, CCI method was very effective in carrying cells into scaffold internal walls (Figure 1C).

Effect of cells on bone repair

Three-dimensional micro-CT reconstructions showed that none of the treatments was effective in fully regenerating bone defects (Figure 2A–D). However, some bone formation was observed in cell-treated defects without remarkable differences related to which cell had been used. Defects treated only with gelatin sponges showed connective tissue with no signs of bone formation. The histological observations confirmed these findings, with no evidence of bone formation in defects treated with gelatin sponge and a heterogeneous bone formation in defects treated with cells. Newly formed vessels and bone and connective tissue were observed in all defects, without relevant histological differences among the treatments irrespective of the use of cells (Figure 2E–H). At the defect edges, in all groups there was new bone displaying osteocyte lacunae with some areas presenting a cement line in the interface between this new bone and lamellar bone (Figure 2I). In animals treated with cells, we found large amounts of newly formed blood vessels and osteoid deposition lined by osteoblasts (Figure 2K &L) and bone islets inside the dense connective tissue that filled the defect (Figure 2L). Furthermore, only in defects treated

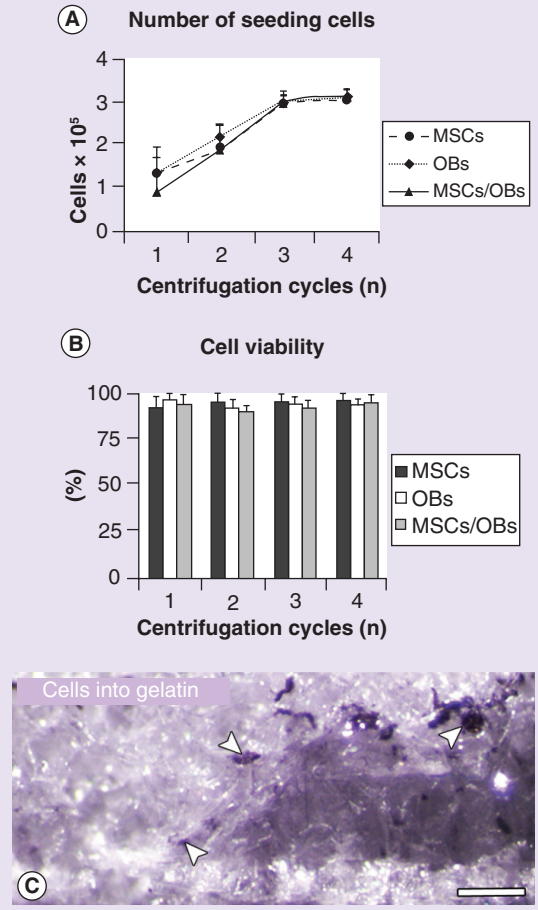

Figure 1. Cell seeding into gelatin sponge. The number of seeded cells gradually increased with the progression of centrifugation cycles. **(A)** Data are expressed as number of cells and reported as mean ±standard deviation (n = 5). Cell viability related to the number of cycles of centrifugation and resuspension. **(B)** Data are expressed as percentage of viable cells and reported as mean ±standard deviation (n = 5). **(C)** Cell aggregates stained with MTT (blue) were observed in the internal walls of the gelatin sponge pores (arrowheads, **C**). Scale bar: 250 μm.
MSC: Mesenchymal stem cell; OB: Osteoblast; MSC/OB: Association of mesenchymal stem cell and osteoblast.
For colour images see online at: www.futuremedicine.com/doi/full/10.2217/RME.14.75

with cells we observed the presence of residual gelatin sponge (three out of five for each treatment) suggesting an unpredictable degradation behavior of gelatin sponge (Figure 2J). None of the groups showed foreign body reaction, chronic inflammation or infection. Among the parameters evaluated by micro-CT analysis, only the volume of bone tissue showed a statistically significant difference between groups ($p = 0.045$) (Figure 3A–G). The volume of bone tissue was higher in defects treated with either OBs or MSCs/OBs, without statistically

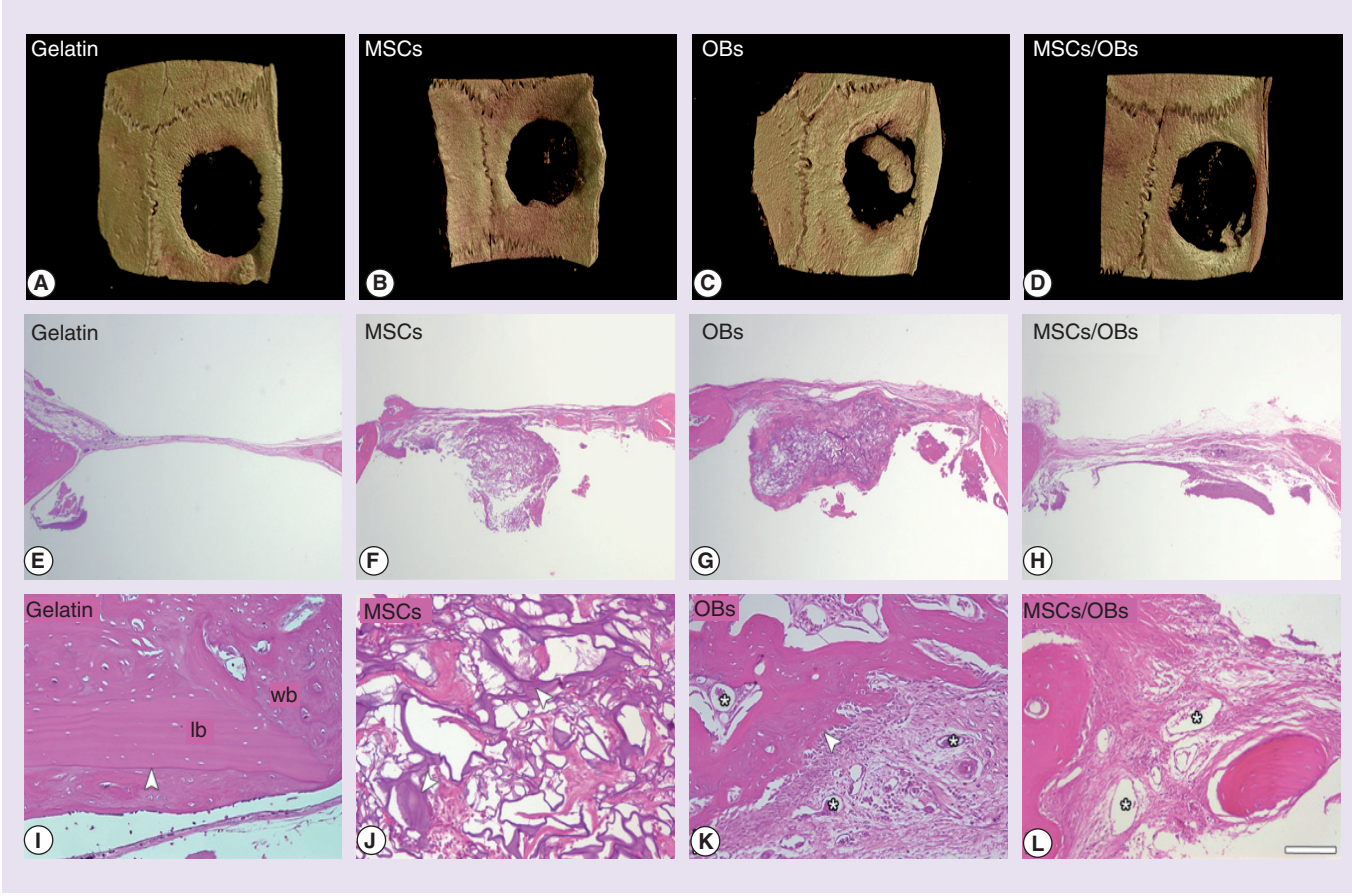

Figure 2. 3D reconstructed micro-CT images and light microscopy of rat calvarial bone defects. Treated with gelatin sponge either **(A, E & I)** without, **(B, F & J)** with MSCs, **(C, G & K)** OBs or **(D, H & L)** MSCs/OBs at 4 weeks. **(E)** Bone defects treated with gelatin sponges without cells were filled with connective tissue and a slight bone formation occurred in the defects edges. **(F–H)** Greater bone formation was observed in bone defects treated with gelatin sponges associated with cells. **(I)** The arrowheads indicate cement line between lamellar bone and woven bone, **(J)** the remaining gelatin sponge and **(K)** osteoblast layer covering the osteoid. **(K&L)** Asterisks indicate blood vessels. Histological details described were present in all bone defects regardless of the type of treatment (MSCs, OBs or MSCs/OBs). Hematoxylin-eosin stain. Scale bar: A–D = 2.5 mm; E–H = 800 μm; I–L = 100 μm.
Gelatin: Gelatin sponge; MSC: Mesenchymal stem cell; OB: Osteoblast; MSC/OB: Gelatin sponge seeded with association of mesenchymal stem cell and osteoblast; lb: Lamellar bone; Wb: Woven bone.

significant difference between them (p = 0.310). Bone volume was higher in defects treated OBs compared with those treated with gelatin sponge (p = 0.032) and MSCs (p = 0.05) and no differences were noticed when defects treated with MSCs/OBs were compared with those treated with gelatin sponge (p = 0.101) and MSCs (p = 0.114)

Discussion

The present study was designed to evaluate whether a combination of MSCs and OBs would be able to stimulate bone formation in rat calvarial defects. MSCs, OBs and MSCs/OBs seeded into gelatin sponges were implanted into rat calvarial defects and after 4 weeks, bone formation was evaluated by micro-CT and histological analyses. It is important to mention that this period of evaluation was selected because we previously compared three time-points, 2, 4 and 8 weeks and noticed that at 4 weeks relevant differences in tissue formation could be detected [17]. Also, the concentration of cells and the 1:1 proportion of MSCs and OBs were based on *in vitro* experiments carried out prior to this *in vivo* study.

Different methods have been described to seed cells into scaffolds, dynamic, static, perfusion bioreactor, centrifugation, injection of the cell suspension directly on the surface and cells cultured with the scaffold [18,19]. For seeding cells into gelatin sponge, the CCI method was chosen since it allows an efficient seeding and homogeneous distribution of the cells without affecting cell viability [14]. Indeed, in this study, it was confirmed that around 72% of the cells, regardless of cell type, were seeded and consequently around 10^6 cells were delivery to each calvarial defect as the average weight of the gelatin sponge was 3 mg, which is in accordance with the literature [8,9]. Moreover, cells maintained via-

bility and were uniformly distributed throughout the gelatin sponge that kept their 3D structure even after four centrifugation cycles.

The lack of more extensive bone repair of calvarial defects observed here could be attributed to the use of gelatin sponge as scaffolds. Its degradation behavior is unpredictable and seems to be influenced by the presence of cells as gelatin sponges without cells had completely disappeared after 4 weeks while those carrying cells were still present in three out five defects of each treatment. Such behavior has been reported for bone, periodontal and cartilage regeneration [18–23]. Regarding

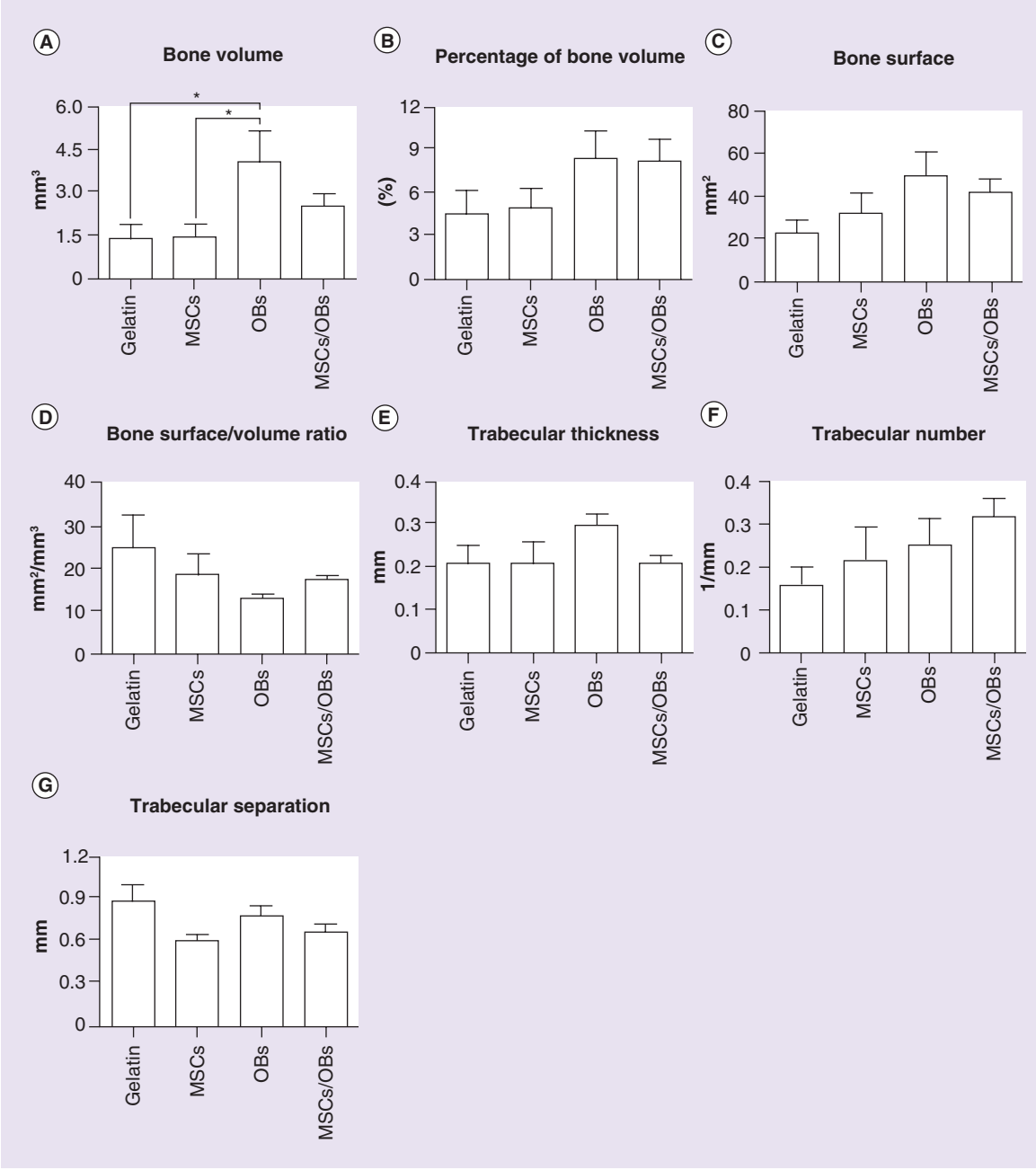

Figure 3. Morphometric parameters of 3D reconstructed images obtained from micro-CT of new bone formation in rat calvarial defects treated with gelatin sponge either without or with cells at 4 weeks. (A) Bone volume, **(B)** percentage of bone volume, **(C)** bone surface, **(D)** bone surface/volume ratio, **(E)** trabecular thickness, **(F)** trabecular number and **(G)** trabecular separation. Asterisk indicates statistically significant difference ($p \leq 0.05$).

Gelatin: Gelatin sponge; MSC: Mesenchymal stem cell; MSC/OB: Gelatin sponge seeded with association of mesenchymal stem cell and osteoblast; OB: Osteoblast.

this observation, it is possible to speculate that, in cases where the gelatin sponge remained for a longer time the cells acted as a protection from degradation mechanisms since they covered a large surface area of the scaffold.

Bone repair has been observed in rat and rabbit calvarial defects treated with either MSCs or OBs isolated, without remarkable differences between both cell types [8,9,12]. Here, we observed that all cell treatments, MSCs, OBs or MSCs/OBs, stimulated bone formation. Such finding is mainly based on qualitative differences as revealed by 3D reconstructions and histological observations. Among all histomorphometric parameters analyzed, only bone volume was greater in defects treated with OBs compared with MSCs and gelatin alone. Additionally, bone volume was not statistically significantly different in defects treated with OBs and MSCs/OBs or with MSCs and MSCs/OBs. Despite not being statistically significant, the percentage of bone volume and bone surface tended to be higher in defects treated with OBs and MSCs/OBs while those parameters measuring bone trabecular characteristics failed to show any difference among the treatments.

In the present study, we used MSCs, OBs and the association of these cells (MSCs/OBs). OBs in early stage of differentiation were chosen due to previous evidences that these cells increase bone repair compared with more mature OBs [12,24]. The lack of remarkable differences between the use of OBs alone and MSCs/OBs might be attributed to several aspects such as: the higher ability of OBs to induce bone repair compared with MSCs. Thus, the proportion of bone repair would be positively related to the number of OBs, which was twice in defects treated with only OBs compared with those treated with MSCs/OB; the reduced amount of local factors capable of inducing differentiation of MSCs into OBs; a crosstalk between MSCs and OBs with inhibitory effect on OB activity; the fact that OBs stay longer than MSCs in the bone defects [25].

Conclusion

Taken together, our results suggest that cell-based therapy using MSCs associated with OBs promoted similar bone repair than OBs alone in a rat calvarial bone defect.

Future perspective

Cell therapy has arisen as a promising and powerful tool in the field of regenerative medicine in the past few years. Regarding bone repair, before the implementation of this therapy in the clinical routine, several key points that are now under investigation should be better clarified, such as: the most suitable cell source, number of cells in relation to the defect size, delivery way either locally or systemically, number of doses and the time-point of delivery during the bone repair progression. Additionally, despite our study suggests that OBs may elicit a better bone response compared with MSCs, the cell differentiation stage and the combination of different cell types should be deeply investigated. We believe that the multiple efforts of researchers from both basic and translational sides will shed lights on these issues and open new perspectives of using cell-based therapies as a feasible alternative to repair bone tissue in a plethora of clinical situations in the near future.

Financial & competing interests disclosure

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

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.

Executive summary

Cell seeding

- Centrifugal cell immobilization method is simple, feasible and was highly efficient to seed cells into the gelatin sponges without affecting cell viability.

Scaffold

- Gelatin sponge elicited an unpredictable degradation behavior.

Effect of cells on bone repair

- Defects treated with only gelatin sponges showed connective tissue with no signs of bone formation.
- Bone repair was enhanced by the presence of cells compared with the gelatin sponge.
- The association of mesenchymal stem cells and osteoblasts elicited similar bone repair compared with osteoblasts alone.

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