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Enhanced *In vivo* Bone Formation by Bone Marrow Differentiated Mesenchymal Stem Cells Grown in Chitosan Scaffold

Laeticia Nassif[1,2,3], Abdo Jurjus[3], Jihad Nassar[2], Joseph Ghafari[1] and Marwan El Sabban[3]*

[1]*Division of Orthodontics, American University of Beirut, Faculty of Medecine, Beirut, Lebanon*
[2]*Department of Otolaryngology Head and Neck surgery, American University of Beirut, Faculty of Medecine, Beirut, Lebanon*
[3]*Department of Human Morphology, American University of Beirut, Faculty of Medecine, Beirut, Lebanon*

Abstract

Objective: The aim of this study is to evaluate whether the differentiation of BMMSC to osteoblasts seeded in a biocompatible scaffold *in vitro*, would enhance bone formation *in vivo* over existing bone.

Background: The use of bone marrow derived mesenchymal stem cells (BMMSC) with appropriate scaffolds in critical size defects and ectopic sites has been proven to be a valuable bone tissue engineering (BTE) technique that could potentially overcome the drawbacks of the current bone graft modalities, but the use of such techniques to grow bone over existing cortical bone has not been investigated yet.

Materials and Methods: Autologous rabbit BMMSC grown in Chitosan scaffolds and pretreated with dexamethasone (Dx) were transplanted subperiosteally and bilaterally over the calvarial bone of syngeneic rabbits. Biopsies were analysed using radiographical, histological and histochemical methods for the assessment of bone formation within the scaffolds and its integration to the host calvarial bone.

Results: With Dx stimulation, alkaline phosphatase protein level and activity of BMMSC cultured *in vitro* increased by three folds and the proliferation rate decreased up 90%. *In vivo*, all cell seeded implants resulted in the formation of an outer collar of bone integrated with the host cortical, regardless of pre-differentiation.

Conclusion: BMMSC had osteogenic features *in vitro* and *in vivo* in conjunction with Chitosan scaffold which can have diverse clinical applications in maxilofacial bone regeneration.

Keywords: Stem cells; Chitosan; Scaffold; Bone; Tissue engineering; Maxillo-facial

Introduction

Bone autografts remain the gold standard in reconstructive surgery for enhancing bone repair. Lack of immunogenicity and the delivery of bone-forming cells have been considered [1]. Moreover, autologous bone grafts are thought to recruit mesenchymal stem cells and induce their differentiation into osteogenic cells through exposure to osteoinductive growth factors [2]. However, their limited availability, donor site morbidity and the need for a second operation site, emphasize the need for alternative approaches such as allografts [3]. Nevertheless, allografts have a poor degree of cellularity, reduced vascular supply, and a higher resorption rate compared to autologous grafts [4], resulting in a slower rate of new bone tissue formation [5]. Alloplasts, mostly ceramics (hydroxyapatite), have been used in surface contouring of the face and in alveolar ridge augmentation; however, they are associated with infection and resorption of the underlying bone [2]. More recently mesenchymal stem cells have been used in association with scaffolds and bone differentiation inducing factors to engineer bone. Chitosan has been frequently used as a matrix in association with bone marrow stem cells to generate bone in ectopic sites of the body. In our study use this engineered model to generate bone over existing cortical bone instead. This idea of bone regeneration beyond the skeletal envelope was already achieved using a titanium mesh as a barrier over the existing bone, without the use of any grafting material [6] however, in our model, we are using the chitosan as a barrier keeping the periosteum away in addition to the stem cells and the osteogenic inducing factors (dexamethsasone and collos E) to further enhance bone regeneration. This bone regeneration technique could potentially be applied in the field of oral-maxillofacial surgery, mainly in the restoration of alveolar ridge height and width, filling periodontal defects and alveolar clefts in addition to surface contouring of the face like bone augmentation of malar bones, chin and mandibular angle.

Chitosan scaffold is a polymer characterized by its sterilizablity osteocompatiblity, osteoconductivity and biodegradability [7]; thereby it can be used as a bone grafting matrice. It is made up of β (1-4)-linked N-acetyl-D-glucosamine and D-glucosamine subunits and is produced industrially by alkaline hydrolysis of chitin [8]. Chitin can be found widely in the exoskeletons of arthropods, shells of crustaceans, and the cuticles of insects. N-Acetyl-glucosamine is present in glycosaminoglycans of the extracellular matrix, and of cell surface, usually linked to core proteins as proteoglycans. Chitosan degree of acetylation (DA) of 4% can thus provide a non-protein matrix structurally similar to extracellular proteoglycans for 3D cell viability, spreading and growth [9].

On the other hand, BMMSC are multipotent cells that can differentiate into diverse cell lineages, such as bone, cartilage and muscle

***Corresponding author:** Marwan El Sabban, American University of Beirut P.O.Box 11-0236 / (Human Morphology) Riad El-Solh / Beirut 1107 2020 Lebanon, Tel: 961-3-658575; E-mail: me00@aub.edu.lb

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cells among others [10]. The local microenvironment, in- vivo, is critical to support the desired differentiation of stem cells and to sustain the phenotype of the stem cell-derived *in vitro* differentiated cells. This local microenvironment comprises a physical support supplied by the organ matrix as well as tissue-specific factors. Consequently, when BMMSC are used to enrich orthopedic grafting matrices, it almost invariably produced faster and more consistent defect healing compared with the carrier matrix alone [11].

Previous research has proven that dexamethasone and growth factors like BMP-2, and 1,25-dihydroxyvitamin D are osteogenic inducers of BMMSC [4] and enhance the *in vivo* osteogenic potential. An inexpensive alternative to recombinant growth factors, the medical device Colloss E®, a lyophilizate of bone proteins extracted from the extracellular matrix of equine cortical diaphyseal bone, has demonstrated the ability to stimulate new bone formation out of human BMMSC both, *in vivo* [12] and *in vitro* [13].

In this report, we demonstrated that BMMSC seeded onto a Chitosan scaffold *ex vivo*, would enhance bone formation *in vivo*. The importance of osteoblastic pre-differentiation of BMMSC in the scaffold using Dx may be elucidated by increasing sampling tissue over time.

Materials and Methods

Animal model

The model used in this study is based on a study by Yamada et al. [14] where titanium caps were sealed bilaterally over the calvarial bone of rabbits and bone generation underneath the caps was assessed. In our study, seven three month- old syngeneic New Zealand rabbits were used for bone marrow aspiration and isolation of BMMSC. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC). All surgeries were performed under strict aseptic conditions. Rabbits were later subjected to surgical implantation of an engineered material over the parietal bone, bilaterally. Three other rabbits of the same age were used as controls. The animals did not present any medical complication during the whole experiment and were not subjected to any systemic drug administration that would interfere with the results.

Isolation of BMMSC and chitosan preparation

Bone marrow aspirates were collected from the superior medial surface of the tibia, inferior to the medial condyle and medial to the tibial tuberosity, then subjected to density gradient centrifugation (d=1.077g/ml) (Ficoll-Paque™ Plus, Amersham Bioscience, UK). The mononuclear buffy coat cells were washed and resuspended in culture medium composed of Dulbecco's modified Eagles medium low glucose (L-glutamine, $NaHCO_3$ and pyridoxine HCl), with 1% penicillin/streptomycin (Sigma-Aldrich Co Ltd, Irvine UK), 20% fetal bovine serum(FBS) (GIBCO-BRL) and incubated at 37°C in a 95% air and 5% carbon dioxide and 100% humidity. After reaching ~90% confluency at around day 14, cells were passaged by trypsinization and gentle scraping. Cells at passage 3 were used in all assays.

Cells were released from the culture vessel then counted using trypan blue. $2x10^4$ BMMSC were seeded into 6- well plates (falcon). After 24 hours, adherent cells were treated either with standard medium (control group), medium containing 10^{-8} mol/l of dexamethasone (Decadron 4mg/ml) (experimental group), or medium containing Colloss E at a concentration of 32µg/ml (positive control group) [13]. Cultures were replenished with fresh media (with or without treatment) every 2 days.

Chitosan scaffold consisted of ready to use porous discs of 15 mm diameter and 5mm height. The discs were donated by Dr. Christina Teixeira from the craniofacial biology department at New York University. They were prepared from 2% Chitosan solution in 0.2 M acetic acid. The polymer was allowed to dissolve for 24 h, centrifuged for degassing, and poured in 12- well polystyrene plates. The Chitosan samples were then incubated in the -78°C freezer and lyophilized for 24 h, resulting in spongy discs. Chitosan scaffolds presented average pores values ranging from 60 to 90µm, with few pores outside the 47µm to 102 µm range [9]. Prior to cell seeding, the lyophilized scaffolds were cut into pieces of about $5mm^3$ and immersed in absolute ethanol for sterilization. Hydration was accomplished by sequential immersion in serially diluted ethanol solutions of 70, 50, and 25%, during intervals of 30 min each. Scaffolds were finally equilibrated in phosphate-buffered saline (PBS) followed by standard culture medium (30 min; 3 times), and then placed in 12-well tissue culture plates ready to be seeded with the proper volume of cell suspension [9].

At passage 3, BMMSC derived from one rabbit were trypsinized, counted using trypan blue and a total of $10x10^4$ viable BMMSC were seeded into each Chitosan cube after a brief suction of the media contained in the scaffolds. Scaffolds were incubated at 37°C for 4 h, after which 1 ml of culture medium was added.

Characterization of cells *in vitro* 2D and 3D culture

Cells from each group were trypsinized and counted using trypan blue at days 5, 15 and 20. Counting was repeated 3 times and the mean value considered. Morphological changes were documented by photo-microscopy on a daily basis. Hematoxylin and Eosin (H and E) stain was used to assess the morphology of the cells at days 5, 10 and 20 after treatment. Alkaline phosphatase protein levels in total cell lysates were evaluated by western blot using specific antibodies (Abcam, UK). Alkaline phosphatase enzyme activity was assessed in medium-free cell lysates using 5 mM of p-nitrophenyl phosphate (Amresco, USA) in 2-amino-2-methyl-1 propanol (Sigma-Aldrich Co Ltd, Irvine UK) alkaline buffer solution [15].

Alkaline Phosphatase activity in BMMSC seeded Chitosan was assessed in medium-free cell lysates as described by Oreffo et al. [10]. After 5 days of culture, scaffolds were stained with the nuclear stain Hoechst and resulting fluorescence was detected by fluorescence microscopy.

BMMSC/Chitosan *in vivo* transplantation

Seven cubes seeded with BMMSC were treated with the standard medium, 7 others were treated with medium containing 10^{-8} mol/l of dexamethasone (Decadron 4g/l ampules) and 6 cubes were left empty and incubated with standard control medium. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C and replenished with fresh media every 2 days.

Lateral longitudinal skin incisions were made on both sides over the parietal bones, the periosteum elevated and a subperiosteal pocket was created. Prior to implantation, the underlying cortical bone was perforated using a bur mounted on a rotary hand piece to induce blood supply. On one side, we implanted BMMSC/Chitosan scaffolds treated with media supplemented with dexamethasone whereas the other side received the BMMSC/Chitosan scaffolds treated with standard media. The remaining exogenous FBS was eliminated prior to implantation of the scaffolds by rinsing the implants with incomplete media. The site of implantation on each side was about one centimeter caudal to the posterior orbital ridge and 5 mm lateral from the midline. Incisions were

closed with 4.0 vicryl resorbable sutures. The 3 control animals received the cell free scaffolds bilaterally. All animals recovered from anesthesia and had never had any post-operative complications. Incision sites were treated with topical application of penicillin/streptomycin gel once a day for 5 days. No anti-inflammatory drugs were administered.

Specimen collection

Rabbits were sacrificed 2 months after the surgery and implants with surrounding tissues harvested (host cortical bone, transplant and overlying periosteum and scalp skin). A rectangular skin incision surrounding the implant area was performed and a rectangular groove penetrating the skull was prepared in the bone around this area and in the midline, separating the two transplants on each side of the midline. Samples were fixed in 10% phosphate buffer formalin. All fixed tissues were radiographed in a dental X-ray machine set at 25 kVp and 2.5 mA for 3 s, using a dental film, then were demineralized for 1 day in 3% EDTA solution, dehydrated in alcohol series, embedded in paraffin, and 5µm-thick sections mounted on glass slides and stained with H & E, Masson-Trichrome that stains collagen in green, and Alizarin red that stains calcium deposits in orange. A rating system going from zero to five, regarding integration, cellularity and collagen formation was adopted on 4 sections selected randomly from each of the harvested 20 specimen. Expression of Stro-1 and osteopontin was evaluated by fluorescence immunocytochemistry using specific antibodies (R&D systems, USA). After deparaffinization, sections were blocked in 3% normal goat serum (NGS) after which primary antibody, mouse anti-Stro-1 antibody, or goat anti-osteopontin (Chemicon, RandD systems, USA) were applied at a concentration of 5µg/ml and the secondary antibody, anti mouse IgM FITC-conjugated or anti-goat IgG FITC-conjugated (Molecular Probes USA) at a concentration of 1:1000. A rating system going from zero to three, regarding the degree of fluorescence of the concerned antigen was adopted on duplicates from each of the harvested specimen.

0	No integration
1	1 contact
2	2 contacts
3	3 contacts
4	4 contacts
5	More than 4 contacts

Table 1: Integration rating (contact and merging between the host cortical bone and the implant).

0	No cells
1	Few cells
2	25% confluent
3	50% confluent
4	75% confluent
5	100% confluent

Table 2: Cellularity rating (degree of confluency of osteoblastic cells).

0	Absence of collagen fibers
1	Sparse fibers
2	Thicker sparse fibers
3	More abundant fibers
4	Spaced bundles of collagen
5	Homogenous collagen bundles

Table 3: Collagen formation rating

	Osteopontin	**Stro-1**
0	No signal	No signal
1	Slight signal	Slight signal
2	More intense signal	More intense signal
3	+ control fluorescence	+ control fluorescence

Table 4: Osteopontin and Stro-1 fluorescence rating relative to a negative control (rate 0) and a positive control (rate 3).

Statistics

Statistical analysis was determined by analysis of variance (ANOVA). Significant differences in the final *in vivo* outcome were assessed by the Mann–Whitney U test. The p-value referred to a comparison of a measured parameter in the experimental group with that of the control; Significance was set at $p<0.05$.

Results

Mononuclear cells suspended in culture medium and incubated, started adhering after 4 to 5 days in culture and formed colonies. Around day 14, cells were ~90% confluent and ready to be passaged (Figure 1A).

Characterization of cells *in vitro* and when grown in Chitosan

Adherent cells cultured at passage three exhibited a characteristic

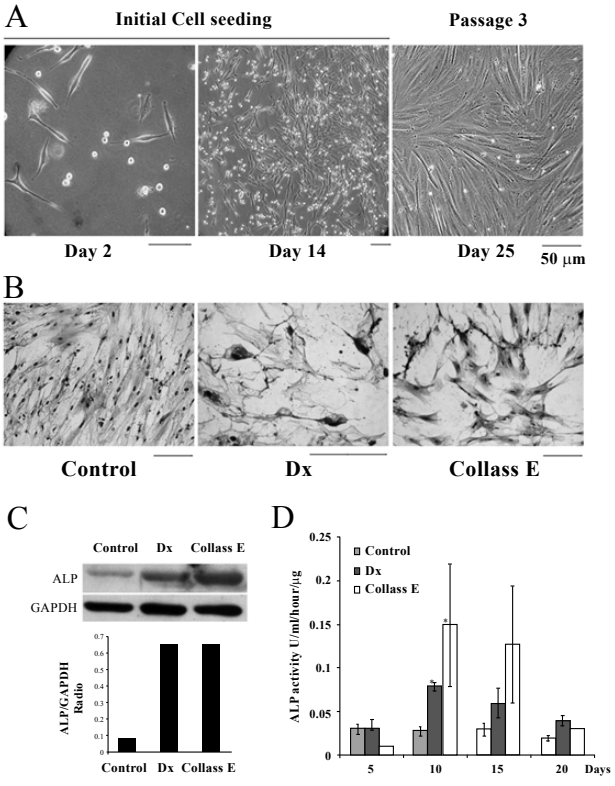

Figure 1: Establishment and characterization of BMMSC in culture
A: Adherence and proliferation of BMMSC.
B: Morphological changes during BMMSC osteocytic differentiation at day 10 following treatment with dexamethasone or Colloss E on Hematoxylin and Eosin stained cover slips.
C: ALP protein expression in treated versus untreated BMMSCs at day 8 post treatment. GAPDH was used to ensure equal loading of cell lysates.
D: Expression of alkaline phosphatase activity in U/ml/hour/µg of protein. The peak was detected at day 10 post-treatment with Dexamethasone and Colloss E.
A statistically significant increase in ALP activity * was detected in treated versus non treated cells.

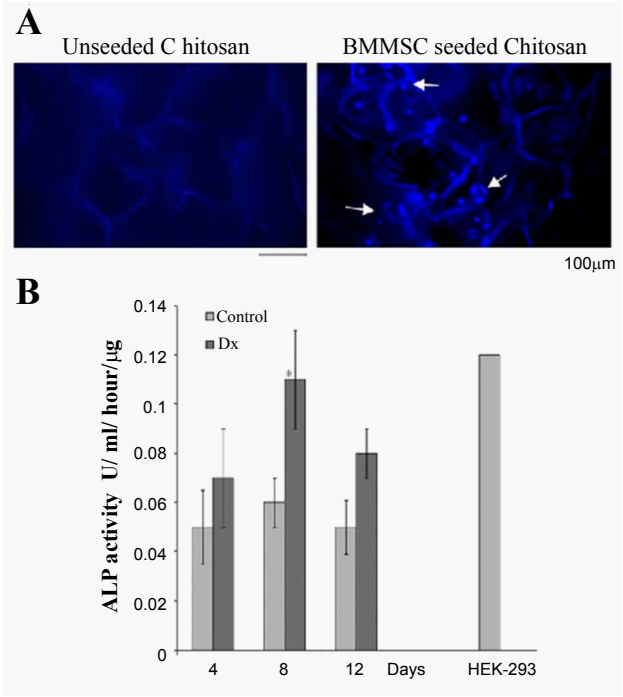

Figure 2: Characterization of BMMSC grown in Chitosan scaffold in-vitro
A: Successful growth and adherence of seeded cells (6.10^4 /5mm^3) to Chitosan was assessed by nuclear stain of the sponge at day 4. Arrows indicate the nucleii of adherent BMMSC.
B: Increased ALP activity of dexamethasone treated Chitosan seeded with BMMSC expressed as U/ml/hour/µg of proteins. Hek 293 cells were used as positive control. There was a statistically significant increase ALP activity * of treated versus non treated sponges seeded with BMMSC.

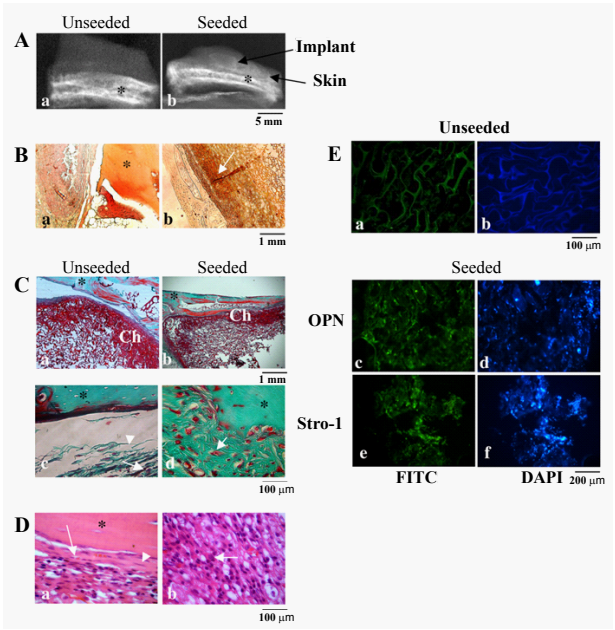

Figure 3: Evidence of bone formation within BMMSC loaded Chitosan sponges
A: Unseeded control Chitosan showing no radioopacity within the implant (a). Radiographic evidence of bone formation around the BMMSC seeded implants (arrow) compared with the underlying host cortical bone (*) (b).
B: Alizarin red positive staining of the host cortical bone (*) used as positive control (a) and the newly generated tissue that forms a collar (arrow) around the implants seeded with BMMSC (b).
C: Masson-trichrome stained frontal sections of the harvested Chitosan implants (Ch) with the surrounding underlying host cortical bone (*) and overlying periosteum. The control unseeded implants show fibroblastic cells embedded among sparse collagen fibers (arrowhead) stained in green (a, c), whereas several osteoblastic cells (arrow) (d) are embedded within the newly formed collagenous matrix which is integrated with the host cortical bone (*) (b, d).
D: Hematoxylin and Eosin stained frontal sections of the newly generated tissue showing new bone spicules (arrowhead) with embedded osteocytes (arrow) (a). Evidence of basophilia within the newly forming bone matrix with its numerous osteoblastic cells (arrow) (b).

elongated morphology of a mesenchymal stem cell. Treatment with dexamethasone or Colloss E, but not with medium alone, induced characteristic morphological changes, starting at day 5 and becoming more obvious at day 10 and 15. In general, cells became flattened and exhibited cytoplasmic projections suggestive of osteoblastic differentiation (Figure 1B). The morphological changes were accompanied by a marked decrease in total cell proliferation along with a significant increase in alkaline phosphatase protein expression (Figure 1C) and enzyme activity (Figure 1D) in medium-free cell extracts around day 10.

On 2D projections of 3D stack images of BMMSC seeded scaffolds, 4 and 8 days after seeding, an almost uniform distribution of viable cells was observed. Chitosan scaffolds supported cell anchorage and growth of BMMSC (Figure 2A). The ALP activity of dexamethasone treated scaffolds seeded with BMMSC was significantly higher than the ALP activity of non treated scaffolds with a peak being situated around day 8 post-treatment (Figure 2B).

These results strongly suggest that both dexamethasone and Colloss-E induce osteoblastic differentiation of *in vitro* expanded rabbit BMMSC.

Bone formation induction by Chitosan seeded with BMMSC

Radiographic observations showed a callus spanning the contour of the transplant with the same degree of radiopacity as the cortical calvarial bone underneath (Figure 3A) and with evidence of calcium deposition (Figure 3B). Implants unseeded with BMMSC did not show any evidence of radiopacity or calcium deposition. There was an obvious continuation of the margin of the BMMSC seeded implants and not the unseeded implants to the host cortical bone.

Furthermore, histological examination of the limits of the BMMSC seeded implants revealed the presence of a continuous collar of newly generated tissue consistently demonstrating integration to the host cortical bone whereas, the control unseeded implants showed an obvious discontinuity between the newly formed tissue and the host cortical bone. Higher magnification revealed that the periphery of the seeded implants contained a collagenous matrix stained in green by the Masson-Trichrome stain and in which, multiple high cuboidal cells were embedded. In the control unseeded implants, the newly formed tissue at the cortical bone-implant interface consisted of fibroblastic cells embedded among sparse collagen fibers. The collagenous matrix gets replaced with a non collagenous matrix towards the center of the implants, where we only find the Chitosan matrix depleted of cells (Figure 3C). Upon H & E staining, the newly generated tissue in the BMMSC seeded implants showed a basophilic component indicating activity in contrast with the eosinophilic component of the Chitosan matrix in addition to bone spicules (Figure 3D). Union at the host bone-implant interface appeared to be primarily due to the presence of BMMSC with a centripetal osteoconduction pattern, emanating from

the cortex of the transplant towards the center. On the other hand, and on the sub-periosteal side of the implant, BMMSC seeded implants showed a collar of new matrix formation with cuboidal osteoblastic cells whereas the control unseeded implants did not show any new tissue formation at the periosteum-Chitosan interface. There were no significant differences related to the pretreatment with dexamethasone regarding the integration of the implant to the host cortical bone, cellularity within the outer collar of the implant and collagen secretion, whereas differences were significant with the control cell free implants.

The presence of Stro-1 cell surface marker was used to indicate the presence of a multipotential bone marrow stem cell population with an osteogenic potential whereas osteopontin secretion was evaluated to comfirm the presence of differentiated osteocytes. The sections showed positive staining for Stro-1 antigen in all seeded implants, regardless the pretreatment with dexamethasone and negative staining in control unseeded implants. The signal was present only within the cellular collar of the implants. Hoechst staining confirmed the presence of cell nuclei where fluorescence was evident (Figure 3E).

Discussion

Despite their osteoinductive properties, autografts present a variable resorption rate and limited availability. However, allografts compensate for this problem but still lack cellulalrity and osteoinductive potential resulting in a slower rate of new bone tissue formation. Our study showed an alternative approach using an engineered material (Chitosan/BMMSC) created *in vitro* to generate bone when transplanted *in vivo*. This approach has important applications in orthopedics including the field of oral and maxillofacial surgery, where restoration of alveolar ridge height and width, filling periodontal defects, alveolar clefts and surface contouring of the face, are of utmost concern.

All possible alternatives were considered regarding the choice of the proper combination scaffold/cells to generate the best osteoinductive construct. Currently, osteoblast transplantation using polymer scaffolds is a promising strategy on several criteria [12]. First, the material should allow for uniform loading and retention of cells. Second, the carrier should support rapid vascular growth. Third, the matrix should be composed of materials that are resorbed and replaced by newly formed bone. Fourth, the material should allow or enhance osteoconductive bridging of host bone by the new bone. Finally, the cell-matrix combination should be easy for the physician to handle in a clinical setting. To date, the most consistent bone formation by human BMMSC has been demonstrated with the use of synthetic hydroxyapatite/ tricalcium phosphate (HA/TCP) ceramics [7]. However, these materials are not well-resorbed, but instead persist for long periods after transplantation. More recent studies used engineered cartilaginous scaffolds to engineer bone *in vivo* [6]. We attempted to prepare a hybrid material with osteoinductive capacity by the association of autologous BMMSC with a porous Chitosan scaffold. This carrier was selected because its characteristics match with the current concepts of bone regenerative medicine [16]. The deacetylated Chitosan (4%) has the advantage of slowing down the degradation rate [17] and favoring cell adhesion [9]. The porous microarchitecture improves scaffold colonization by those cells and cell yield [5]. In addition, Chitosan scaffolds can maintain space and inhibit fibrous tissue invasion, an important consideration in preventing new bone tissue generation [14].

The findings generated in the first part of the study support previous evidence that dexamethasone [11] and Colloss E [13] would stimulate the differentiation of BMMSC *in vitro*, along the osteogenic lineage. It was interesting to assess whether it enhances osteoblastic differentiation *in vivo*. Colloss was used as a positive control based on the study of El Sabban et al. [13] and dexamethasone was selected to enhance the differentiation into the oesteoblastic lineage *in vivo*. The decrease in the proliferation rate, the morphological changes observed in the cultures along with the high levels of both alkaline phosphatase activity and alkaline phosphatase protein expression indicated osteoblastic differentiation. These data mainly verified the identity and the behavior of our extracted cell population even when seeded onto Chitosan. In the second part of the study, Chitosan proved to be an appropriate scaffold for (BTE) techniques. The 3D Chitosan scaffold could support BMMSC adhesion and viability *in vitro* as shown upon Hoechst staining as well as differentiation characterized by the increase in ALP activity. Osteoinduction *in vivo* was mainly related to the presence of BMMSC regardless of their predifferentiation *in vitro* with dexamethasone, denying our previous assumption that it might enhance the osteoblastic differentiation but many factors may be involved in these findings. Chitosan's cationic nature allows for pH-dependent electrostatic interactions with anionic glycosaminoglycans, proteoglycans and other negatively charged molecules, accumulating these molecules within the scaffold after implantation. Since a large family of growth factors and cytokines are known to be bound and modulated by GAGs, (in particular heparin and heparan sulfate), a scaffold incorporating a Chitosan-GAG complex may provide a means of retaining and concentrating desirable factors secreted by colonizing cells. Such a system may even allow the recruitment of desirable growth factors from surrounding tissue and enhance differentiation of progenitor cells [18] which explain the presence of osteoblastic cells, osteopontin and calcium deposits in the scaffolds seeded with undifferentiated BMMSC. The presence of a positive signal to stro-1 in the scaffolds seeded with predifferentiated BMMSC confirms the presence of osteoprogenitor cells in those implants [2] that might have been recruited from the surrounding tissues by the seeded cells. Integration of the seeded implant to the host cortical bone proves that the binding of the newly generated tissue to the cortical bone is mainly due to the presence of cells and the subsequent formation of an extracellular matrix in contact with the matrix of the host bone. The collar formation of new bone in a circumferential manner around the implant is concomitant with the work of Gauthier et al. [4] whereby histological analyses revealed the formation of a cortical layer of bone on the surface of the experimental calcium-phosphate scaffold at two months post-implantation with the presence of osteocytic cells in that layer. No collar formed around any of the implants that had not been seeded with cells. Radiographic interpretations corresponded favorably with the histological observations.

One should consider potential reasons for the similar findings related to the presence of osteoprogenitor cells and differentiated osteocytes in the pretreated and non pretreated implants regarding the higher *in vivo* osteogenic potential of dexamethasone-treated cultured rabbit MSCs seeded onto a hydroxyapatite scaffold [19]. Timing could be a major reason for those findings. We extended our study for 2 months of observation which is the minimal duration that characterizes most (BTE) approaches to study the potential of generated bone to grow [20] but if the implants were collected earlier we probably would not have found positive indicators of maturation in the non treated samples or osteoprogenitor cells in the treated ones. The absence of cells and tissue formation towards the middle of the implant is most likely related to the lack of blood perfusion towards the middle of the implant, shortly after *in vivo* implantation. This assumption is further supported by the vascular invasion of the cellular layer only at the periphery of the implant. These results would suggest the use of a thinner

Chitosan scaffold and its placement in a better vascularized region or the engineering of a cartilaginous scaffold and subsequent *in vivo* intramembranous bone formation [6]. The ratio of Chitosan volume to cell number is still a critical factor that requires further investigation. The number of cells seeded was based upon the in- vitro studies of Amaral et al. [9] using chondrocytic cells seeded onto $5mm^3$ scaffolds, but the number was mainly based on our microscopical evaluation of the density of cells following Hoechst staining of the hybrid material. The proliferation rate in- vitro, using quantitative techniques, needs to be evaluated in a more precise way.

Conclusion

This study provides evidence that the combination of Chitosan with BMMSC contained signals necessary to induce new orthotopic bone formation in- vivo, while an empty scaffold failed to do so. Pretreatment of the BMMSC seeded Chitosan implants with dexamethasone did not affect their osteogenic potential at 2 months post-implantation but results may be associated with sampling frequency. It is possible that pretreatment of BMMSC initiates the bone formation process at an earlier time point than considered in this study. Colloss E which efficiently differentiated BMMSC into osteoblasts could be used for pretreatment of the scaffold seeded with BMMSC. This procedure may provide a microenvironment that better resembles bone milieu. An optimal cell to carrier ratio may exist for optimal *in vivo* results but further research is needed to determine such ratio. Other techniques such as *in vitro* engineering of cartilaginous scaffolds might also be envisaged for better *in vivo* intramembranous bone regeneration.

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