

Assessment of the osteogenic potential of alendronate on isolated adipose-derived stem cells: an *ex-vivo* and *in-vivo* study

Mohamed S. Ayoub^{a,b}, Effat A. Abbas^b, Mohamed A. Abd El Hamid^d, Houry M. Baghdadi^a, Dina S. Abd El Fattah^e, Marwa M. Ellithy^c

^aDepartment of Oral Pathology, Faculty of Dentistry, Ain Shams University, ^bDepartment of Basic Sciences, ^cDepartment of Basic Dental Sciences, National Research Centre, ^dDepartment of Surgery, Faculty of Veterinary Medicine, ^eDepartment of Biochemistry, Faculty of Medicine, Cairo University, Cairo, Egypt

Correspondence to Marwa M. Ellithy, M.Sc., 7th Street Mohamed Khalaf, Gisir El Suez, Misr El Gedida, Cairo, Egypt
Tel: +20 2 2635 2130; fax: 002- 0224836312
e-mail: moonlight20_7@yahoo.com

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Background/aim

Tissue engineering relies on the principle that mesenchymal stem cells are capable of differentiating to optimize almost all craniofacial structures. Temporary biomimetic scaffolds are necessary for accommodating cell growth and tissue genesis. The aim of this study was to evaluate the effect of alendronate on adipose-derived stem cells (ADSCs) from dogs and to compare bone regeneration in critical-sized calvarial bone defect in dogs using ADSCs in the presence and the absence of locally delivered alendronate.

Materials and methods

Seven dogs were used for the study. After isolating the adipose tissue from the inguinal pad of fat, stem cells were harvested and expanded in culture. The effect of alendronate 1 mg/ml on stem cells' osteogenic differentiation was tested for 7 days. Three critical-sized calvarial defects were created in each dog. One defect was filled with stem cells seeded on a chitosan scaffold and soaked in an osteogenic media, the second was filled with stem cells seeded on a chitosan scaffold and soaked with osteogenic medium, and the third one was filled with stem cells seeded on a chitosan scaffold. Bone formation was tested histologically after 8 weeks in each defect.

Results

Alendronate is capable of inducing osteogenic differentiation of ADSCs after 7 days of in-vitro culture. Bones such as trabeculae were deposited in alendronate and osteogenic medium defects, whereas the control group showed only fibrous tissue formation. There was no statistically significant difference in the surface area of the deposited bone trabeculae between the alendronate group and the osteogenic medium group. The surface area of individual bone trabeculae in this group was 147.99 ± 14.803 compared with the osteogenic group.

Conclusion

Alendronate may be used locally at a concentration of 10 mg/ml to induce osteogenic differentiation of ADSCs both *in vitro* and *in vivo*. The combination of a local, short-term alendronate treatment with ADSCs and biodegradable chitosan scaffold enhances the bone repair of a critical-sized calvarial defect *in vivo*.

Keywords:

adipose-derived stem cells, alendronate, critical-sized calvarial defect

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Introduction

Skeletal defects represent a huge socioeconomic and biomedical burden. Bone defects can result from trauma, resection of tumors, or surgical correction of congenital defects [1]. These defects are currently treated by complex reconstructive surgical procedures. Autogenous, allogenic, and prosthetic materials are the currently available options for the reconstruction of osseous defects. Although autogenous grafts (bone taken from another part of the patient's body) arguably remain the best option for reconstruction because of the provision of osteogenic cells and a nonimmunogenic matrix, there are also a number of disadvantages [2].

Allogenic grafts are not without their inherent risks, including infection, disease transmission,

immunologic rejection, and graft-versus-host disease. As a result of these drawbacks, numerous materials have been developed and used with varying success, such as demineralized bone, hydroxyapatite, methyl methacrylate, silicone, and ceramics [3].

The ideal strategy for bone formation would be to combine a biomaterial scaffold with competent cellular elements and molecular and environmental cues. Current efforts are directed towards investigating each of these components [3].

Adipose-derived adult stromal cells (ADASs) have attracted researchers to investigate their ability to generate new bone in skeletal defects. They are currently focusing on the molecular events that occur

during osteogenic differentiation of ADAS and the optimization of this process [4].

Alendronate (Aln) is one of the most commonly used bisphosphonates for osteoporosis treatment. Aln suppresses osteoclast activity by inhibiting the activity of farnesyl pyrophosphate synthetase [5]. It is suggested that Aln enhances the osteogenesis of bone marrow mesenchymal stem cells (BMMSCs). However, the use of Aln as an osteoinductive factor to stimulate the osteogenesis of hADAS has not been reported previously [6].

This study aimed to evaluate the effect of Aln on the osteogenic differentiation of adipose-derived stem cells (ADSCs) isolated from dogs and to compare the bone regeneration potential in critical-sized bone defects in dogs using ADSCs in the presence and the absence of locally delivered Aln.

Materials and methods

Adipose-derived stem cell isolation, culture, and propagation

ADSCs used in this study were isolated from fat tissue excised from the inguinal pad of fat of dogs. The cells were resuspended at a density of 10 000 cells/cm² in complete culture media containing α -MEM with L-glutamine, supplemented with 10% fetal bovine serum, antibiotics (penicillin G, 100 U/ml; streptomycin, 100 μ g/ml), and antimycotic agent (Fungizone, Gibco, Invitrogen Life technologies, USA 0.25 μ g/ml) in sterile 25-cm² polystyrene filter cap cell-culture plates labeled by cell type and date and incubated in a CO₂ incubator at 37°C in a humidified atmosphere of 5% CO₂ (Sigma-Aldrich, Taufkirchen, Germany). Once the cells became 70–80% confluent, they were passaged. Cell cultures from the third passage were used for cell differentiation experiments.

In-vitro study of the effect of alendronate on the osteogenic differentiation of adipose-derived stem cells

Testing the mineralization capability of cultured cells using alizarin red stain

On day 7 of culture, the monolayers were stained using alizarin red stain to identify mineralization. Bone-forming cells containing calcium deposits were stained orange-red by the alizarin red solution (R&D Systems, Boston, Massachusetts, USA).

The alkaline phosphatase enzyme assay

Alkaline phosphatase (ALP) in a sample is determined calorimetrically in accordance with the German Clinical Chemistry Department (DGKC), using kits from BD Biosciences Co. (Boston, Massachusetts, USA).

Polymerase chain reaction for the detection of different osteoblast-specific genes

The total RNA was extracted from the cultured cells, and RT-PCR was performed to analyze the mRNA level of the osteoblastic differentiation marker gene, osteonectin (Lonza Co., Gampel, Swiss, USA).

In-vivo study of the repair of critical-sized calvarial defects in different experimental groups of dogs

The surgical procedure

Seven healthy adult mongrel dogs aged from 12 to 24 months at an average weight of 20 kg were included in this study. The dogs were chosen carefully to have a small-sized head, average 10–15 cm in the greatest diameter.

The dogs were anesthetized using a mixture of xylazine HCL (1 mg/kg weight) and ketamine HCL 5 mg/kg body weight through a 23-G intravenous cannula through the cephalic vein. Anesthesia was maintained through the operative time by venous drip of a mixture of 0.5 g thiopental sodium and 500 ml dextrose 5% with a drip rate ranging from 28 to 40 drops/min. A curvilinear sagittal incision (2.5 cm) was made in the cutaneous and the subcutaneous tissues in the parietal, the nasal, and the frontal bones. The underlying periosteum was incised similarly, and the flaps were elevated to expose the calvarial bones. A standard critical-sized surgically created defect was performed using a trephine bur 1 cm in diameter. A small unibevell osteotome and a small mallet facilitated the removal of the cortical plate. Three full-thickness bone defects with a size of 1 cm in diameter were created at the parietal, the nasal, and the temporal bones of each dog, and the periosteum of 1 cm surrounding the defect was removed. The size of the bone defects was determined on the basis of the fact that a cranial defect with a size of 1/10th of the skull cannot heal spontaneously during the lifetime according to a previous report. Parietal defects were repaired with the autologous ADAS/chitosan+Aln scaffolds, whereas nasal defects (control) were treated with the ASC scaffold alone. Temporal defects were repaired with the autologous ASCs/chitosan+osteogenic media. The wounds were then closed routinely in layers using vicryl material size 0 (Figs. 1 and 2).

Histological examination

Tissues from the defect areas of all groups were harvested at 8 weeks after implantation. The samples were fixed in 10% formalin in PBS for 24 h and demineralized in a solution of 8% hydrochloric acid, 5% formic acid, and 7% aluminum chloride for another 48 h. The tissues were then embedded in paraffin, sectioned in 5 μ m thickness, processed for hematoxylin and eosin (H&E) staining, and examined by light microscopy [6].

Results

Adipose-derived stem cell isolation, culture, and propagation

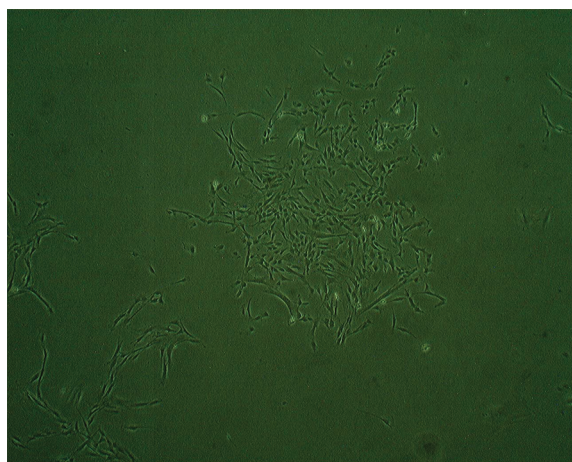
After the isolation and culturing procedures, ADSCs were kept for 1 week before they began to attach to the bottom of culture dishes. The cells were either spindle-like or stellate-shaped, reflecting the diversity of the morphology of the isolated cells (Fig. 3). Cells continued to proliferate and propagate until 70–80% of the dish area became covered with cells by day 16 (confluence 70–80%), indicating a high proliferative cell population in the fat tissue (Fig. 4). After confluence, the cells were passed successfully up to the third passage. ADSCs maintained their stellate and spindle-like appearance, indicating an undifferentiated state in the conventional culture media.

Figure 1



A photograph showing a curvilinear sagittal incision made in the cutaneous and the subcutaneous tissues in bones.

Figure 3



A photomicrograph showing isolated adipose-derived stem cells on the 10th day of culture showing spindle-shaped stem cells (mesenchymal-like appearance) (×4).

In-vitro study of the effect of alendronate as a culture medium on osteogenic differentiation of adipose-derived stem cells

The capability of cultured cells to differentiate into active osteoblasts was tested on the seventh day after culture in 10 mg/ml alendronate sodium trihydrate as mentioned in the Materials and Methods section.

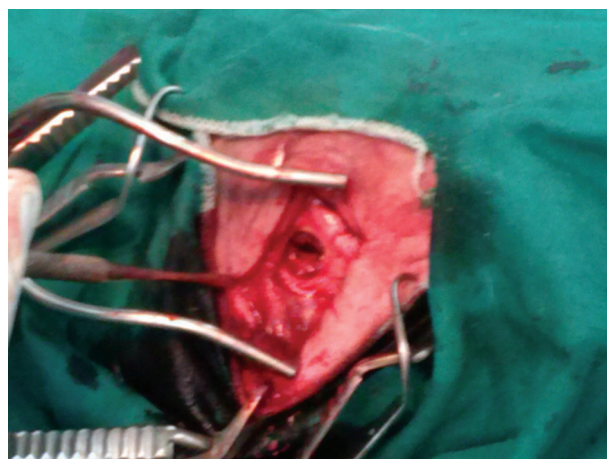
Alizarin red stain

Plates showed colorimetric changes, indicating calcium crystal deposition and successful osteogenic differentiation of ADSCs (Fig. 5).

Alkaline phosphatase result

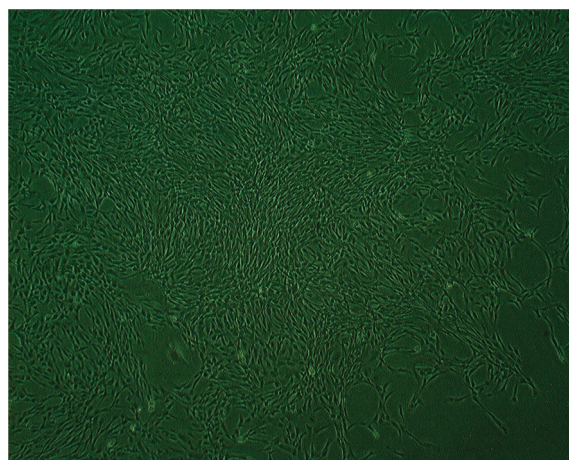
Readings given by the spectrophotometer indicated the formation of ALP enzyme by the cells cultured in alendronate sodium trihydrate, reflecting

Figure 2



A photograph showing full-thickness bone defects with a size of 1 cm in diameter.

Figure 4



A photomicrograph showing adipose-derived stem cells reaching 70–80% confluence (×4).

successful osteogenic differentiation of cultured ADSCs (Table 1).

Real time PCR (RT-PCR)

The Glyceraldehyde 3 phosphate dehydrogenase (GADPH) gene was used as a housekeeping gene to ensure the reliability of the isolated RNA. The osteonectin gene was detected in plates on the seventh day of culture in alendronate sodium trihydrate, indicating successful osteoblastic differentiation of the isolated cells (Fig. 6).

In-vivo study of the repair of critical-sized calvarial defects in the different experimental groups of defects

Alendronate group medium

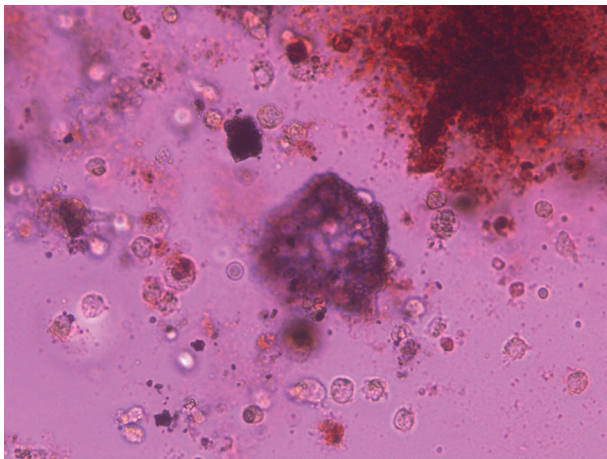
Defects showed proper healing with newly formed bone-like tissue rich in cellular activity. The newly

formed tissue consisted of islands of bone-like tissue and large marrow spaces with fat cells and hematopoietic cells. Osteocyte-like cells were found in their lacunae. The defect size decreased significantly more than in the ADSC-free group. Osteoblast-like cells were numerous, plump, and rimming the bone-like trabeculae. The surface area of individual bone trabeculae in this group was 147.99 ± 14.803 (Figs. 7 and 10).

Osteogenic medium groups

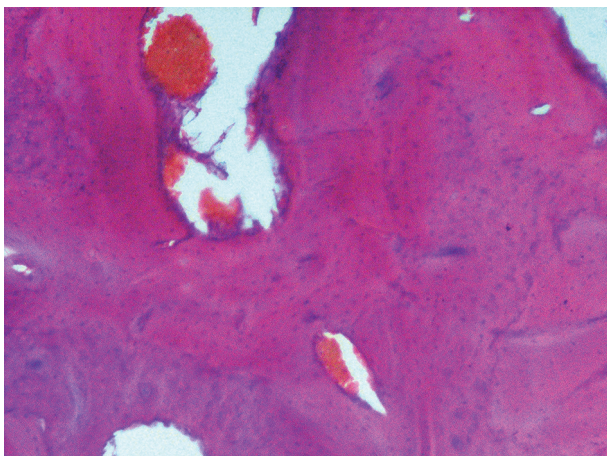
Defects showed proper healing with newly formed bone-like tissues rich in cellular activity. The newly formed tissue was in varying sizes, consisting of islands of bone-like tissue and large marrow spaces with fat cells and hematopoietic cells. Osteocyte-like cells were found in their lacunae. Defects showed more bone-like trabeculae with increased individual trabeculae surface area. Osteoblast-like cells were numerous, plump and

Figure 5



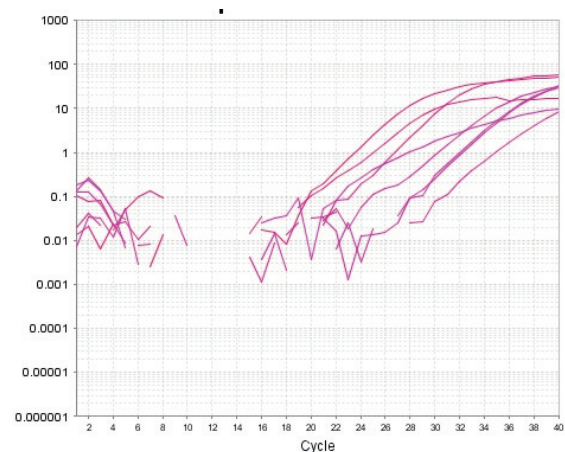
A photomicrograph showing orange to red alizarin red stain, indicating calcification in plates after 7 days of culture in alendronate medium ($\times 20$).

Figure 7



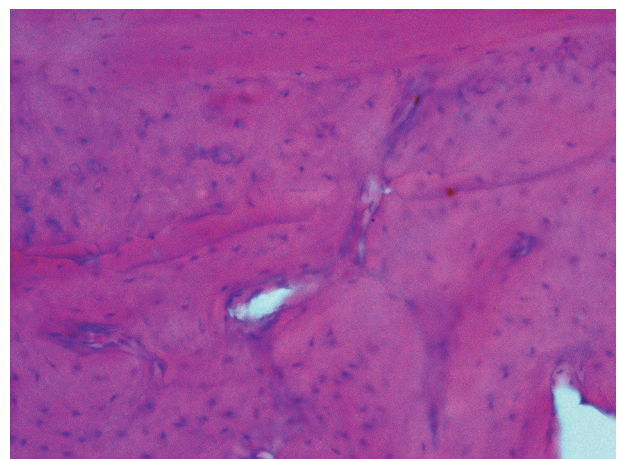
A photomicrograph showing bone-like trabeculae deposited in the bony defect filled with alendronate and adipose-derived stem cells. Note the osteoblastic rimming of newly formed bone-like trabeculae (hematoxylin and eosin, $\times 10$).

Figure 6



A real-time amplification plot of the osteonectin gene.

Figure 8



A photomicrograph showing bone-like trabeculae deposited in the defect filled with osteogenic medium and adipose-derived stem cells. Note the osteoblastic rimming (hematoxylin and eosin, $\times 10$).

were rimming the bone-like trabeculae. The surface area of individual bone trabeculae in this group was 172.97 ± 19.021 ($P = 0.0179$) (Figs. 7, 8 and 10).

The control group

Defects showed healing with minimal regeneration, and no evidence of newly formed bone-like tissue was noted. Dense bundles of residual collagen were observed. Fibrotic tissue was observed within the defects. At all levels, defects were filled with fibrovascular tissue and fat cells. The outer levels showed very few bone-like spicules (Fig 9).

Discussion

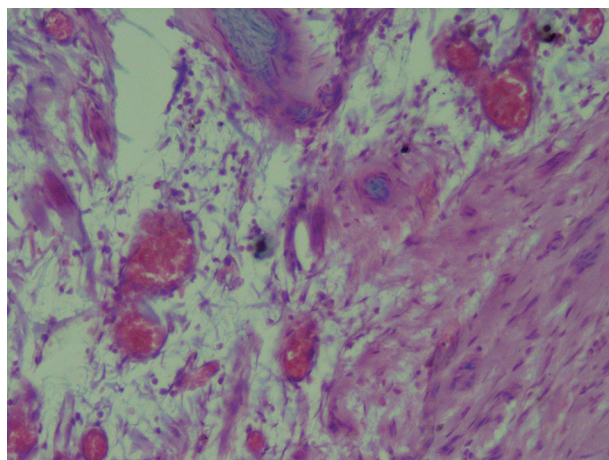
Bone marrow-derived stem cells have been shown to differentiate into various cell types. However, for various reasons, such as surgical trauma caused by bone marrow isolation procedures or bone marrow-related diseases, much of the stem cell research has focused on finding alternatives with no or minimally invasive collection procedures [7].

In this study, ADSCs were used to fill calvarial defects combined either with alendronate sodium trihydrate or with an osteogenic medium consisting of dexamethasone, ascorbic acid, and b-glycerophosphate.

Table 1 *In-vitro* alkaline phosphatase readings after 7 days of culture in alendronate 10 mg/ml

Time	First plate	Second plate	Third plate
60 s	1.7	1.21	1.37
120 s	1.65	1.33	1.38
180 s	1.61	1.36	1.33
Mean (U/l)	471	151	390

Figure 9



A photomicrograph showing fibrovascular tissue filling the bony defect of the control group (hematoxylin and eosin, $\times 10$).

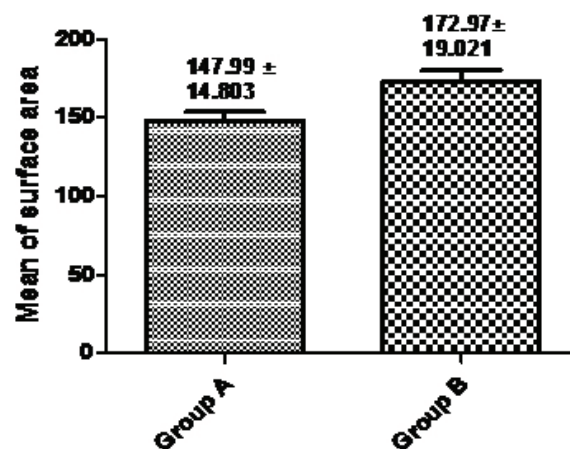
Okumura *et al.* [8] and Yoshikawa *et al.* [9] cultured bone marrow-derived rat mesenchymal stem cells with dexamethasone on porous coralline hydroxyapatite to permit the attachment of the cells to the matrix and promote lineage progression, while still *in vitro*.

ADSC cells have several advantages over BMMSCs from the perspective of clinical application. Thus, the yield of cells is much higher from fat than from bone marrow. Furthermore, the proliferation rate of ADSCs was substantially higher than that of BMMSCs during subsequent *in-vitro* expansion. Because ADSCs proliferate rapidly in culture, populations can readily reach the enormous cell numbers needed for clinical application. The ease of harvest, the large number of cells, and the rapid *in-vitro* expansion are attractive advantages of ADSCs over BMMSCs when contemplating clinical strategies [10].

ALP staining, von Kossa staining, and measurement of calcium in the extracellular matrix all revealed no significant difference between the ability of juvenile and adult ADSCs for bone formation *in vitro*. This observation is particularly encouraging for skeletal regeneration in an aging population, suggesting that ADSCs with the proper cues can still maintain their osteogenic potential and bone formation despite increased age [3].

ADSCs were isolated from the inguinal pad of fat of an adult 20 kg mongrel female dog. The inguinal pad of fat allowed the excision of a large amount of fat tissue, thus promoting a greater number of stem cells in contrast to the subcutaneous region that permits the excision of only a small quantity of fat tissue. This was in agreement with Schäffler and Büchler [11], who showed that increasing the amount of bone marrow

Figure 10



A histogram showing the mean surface area measurement in different groups.

does not coincide with a high number of stem cells, whereas increasing the amount of adipose tissue does. In contrast, Cui *et al.* [12] demonstrated autologous ADSCs isolated from subcutaneous adipose tissue to be effective in treating bone defects.

Zheng *et al.* [13] isolated ADSCs from the visceral fat of the abdominal cavity of mouse. They showed, in accordance to the present work, the proliferative capacity of isolated cells and their mesenchyme-like appearance in culture. Osteogenic inductive medium was used to examine the osteogenic differentiation capability of isolated cells. In this study, in addition to the ordinary osteogenic medium, 10 µg/ml of alendronate sodium trihydrate was also used to allow osteogenesis.

García-Moreno *et al.* [14] revealed, in accordance to this study, that Aln *in vitro* did not affect the viability, the proliferation, and the mineral deposit capacity of human osteoblasts at the concentration at which it inhibited the resorptive capacity of osteoclasts by 50%.

To test mineralized nodule formation in culture plates, alizarin red stain was used as a colorimetric measure for calcium deposition inside and outside cultured cells. By the seventh day of ADSC culture in 1 mg/ml sodium alendronate, osteogenic differentiation was evident by calcified nodule deposition either inside or outside cells. In accordance to the present study, Saugspier *et al.* [15] used alizarin red stain for the quantitative measurement of hard nodule formation.

ALP activity and osteonectin gene expressions are considered as markers of osteoblast differentiation [16] as early progenitor cells (ADSCs) do not express these osteoblast markers and only cells that have differentiated into a mature osteoblast phenotype express them. In the present work, it was found that ALP activity and expression of the osteonectin gene were achieved by the seventh day when adding alendronate sodium trihydrate to the culture medium, indicating that the drug used is capable of inducing osteogenesis of cultured ADSCs. Using the same technique, Cui *et al.* [12] studied the effect of routine osteogenic medium on the expression of ALP by ADSCs isolated from the subcutaneous fat tissue. The activity was increased from day 1 of culture and reached its peak on day 7 of culture.

A critical sized defect (CSD) may be defined as the smallest size intraosseous wound in a particular bone and species of animal that will not heal of its own volition during the lifetime of the animal. Attempted repair of a CSD results in the formation of fibrous connective tissue rather than bone [17]. A calvarial model has many similarities to the maxillofacial

region morphologically and embryologically as the calvaria develops from a membrane precursor and thus resembles the membranous bones of the face [17]. Anatomically, the calvaria consists of two cortical plates with regions of intervening cancellous bone similar to the mandible. Physiologically, the avascular nature of the cortical bone in the calvaria resembles an atrophic mandible [18].

Animal models have the limitations of recreating only limited cranial defects, but are the best option currently available. The primary benefit is the greater control and precision that the larger size allows intraoperatively. Large animals such as rabbits, guinea pigs, dogs, sheep, and monkeys provide the opportunity for more control in the surgical area. In contrast, these animals are more expensive to purchase and maintain, and they also take up a lot of space [19].

The current study used canine (dog) calvarial defects as the calvarium allows for a reproducible defect that can be generated quickly and does not require fixation for stabilization of the skeleton, as is generally required with femoral defects. Dog critical-sized 10-mm circular defects in a time interval of 2 months with three calvarial defects per dog were used in this study. Liu *et al.* [20] demonstrated, in a similar way, that osteoinduced ADSCs successfully repaired the defect when seeded on coral scaffolds when CSDs were made in canines. Cui *et al.* [12] confirmed that the reossification accomplished by implanted ADSCs remains present at 6 months after implantation. In accordance, Jeon *et al.* [21] proved that osteoinduced ADSCs repaired CSD in rabbits when seeded on polylactic acid scaffolds.

In this study, chitosan, which is considered as one of the natural polymers, was used as a scaffold due to its biocompatibility, biodegradability, high cell adhesiveness, and porosity (stable in three-dimensional microstructure) [22]. Chitosan proved to support ADSCs well during surgery. It does not interfere with healing and regeneration progress postoperatively. By the end of the experimental period, it showed complete degradation, which was evident by its absence in the H&E sections.

In agreement, Biazar *et al.* [23] used a chitosan/hydroxyapatite nanoparticle scaffold to reconstruct rat calvarial defects. Results of the computed tomography (CT) analysis showed bone regeneration on the scaffolds with or without stem cells, although presence of stem cell showed an increased bone regeneration. Hicok *et al.* [24] found that ADSCs produced more osteoid when being seeded on HA-TCP than cells that were cultured in a collagen/HA-TCP composite,

indicating that the osteogenic capacity of ADSCs could be heavily influenced by the scaffold in which cells were seeded. Ng *et al.* [25] combined silk and chitosan to produce a blended scaffold using the lyophilization technique.

In the present study, ADSCs cultured in Aln (10 µg/ml) and loaded on a chitosan scaffold were able to heal a dog critical size 10 mm circular defect successfully in a time interval of 2 months with three calvarial defects per dog, similar to the manner in which ADSCs were cultured in osteogenic medium and loaded on a chitosan scaffold. Dexamethasone, which is the main component of osteogenic medium used to enhance healing in bony defects, has contrasting dual functions in bone metabolism; it is a prerequisite supplement for the differentiation of stem cells into osteoblasts, and at the same time, it is a catabolic factor inducing loss of pre-existing bone or osteoporosis with prolonged administration in a human system [26]. In addition, dexamethasone is a corticosteroid with all the drawbacks of steroids, most importantly, delaying healing of surgical sites when applied clinically. The present study focused on proving the possibility of substituting the use of an ordinary osteogenic medium containing a steroid by a bisphosphonate (alendronate sodium trihydrate) having a comparative osteogenic capability.

In this study, the positive effects of local bisphosphonate treatment at a 10 mg/ml dosage with regard to new bone formation have been proved.

Treating bones locally with a bisphosphonate gives protection against resorption, without affecting the entire skeleton. Higher local concentrations of bisphosphonate can be achieved than with systemic treatment [6]. An earlier study showed that local pretreatment of an allograft with a bisphosphonate solution (1 mg/ml) can protect the graft from resorption.

The chief advantage of topical application is the possibility to administer a single dose to stimulate new bone formation. The present study showed that topically applied alendronate sodium trihydrate enhanced local bone conditions significantly. In the Aln and the osteogenic groups, a notable increase in individual trabeculae surface area was observed ($P < 0.05$).

In agreement, Srisubut *et al.* [27] reported that a single dose of local delivery of Aln improved bone formation. It can be hypothesized that the topical application of Aln will modify the local osteoclastic activity and thereby slow down the bone resorption during initial remodeling, leading to better bone formation in the defect area.

Unlike Srisubut *et al.* [27], Bodde *et al.* [28] showed no increase in bone formation around Aln-loaded bioactive bone cements in femoral defects. Jakobsen *et al.* [29] found decreased biomechanical fixation of all the implants soaked in Aln.

Yun *et al.* [30] studied the capacity of Aln/polycaprolactone (PCL) nanofibrous scaffolds to regenerate new bone in a rat calvarial defect model. New bone formation *in vivo* was evaluated by radiography, micro-computed tomography, and histological analysis. Eight weeks after implantation, Aln/PCL scaffolds had a positive effect on bone regeneration and matrix formation.

Nobre *et al.* [31] stated that local application of Aln did not contribute to bone repair, but it might be responsible for extracortical bone formation in spontaneously hypertensive rats. Altundal and Güvener *et al.* [5] demonstrated that osteoblastic activity was less in the Aln-treated group than in the saline-treated group after tooth extraction. These different results may be due to differences in compounds, durations of treatment, dosages, methods of administration, and research models. In the present study, in contrast to Srisubut *et al.* [27], who dissolved Fosamax to place it into the bone defect, pure alendronate sodium trihydrate was dissolved in saline and mixed with ADSCs and loaded on a chitosan scaffold before application in the intrabony defect area of the dog. Srisubut *et al.* [27] reported that the ingredients of this drug, other than Aln may have contributed to the stimulation of bone growth.

Results of light microscopic evaluation in this work showed that healing progression was from outside towards the central part of the defect in all sections. This was obvious in both groups of defects filled with ADSC-seeded scaffolds. In accordance to the results obtained by the presented work, Wang *et al.* [32] demonstrated that local delivery of Aln, a potent osteoinductive factor, enhances hADSC osteogenesis and bone regeneration. He studied the in-vivo effect of locally administered Aln on bone repair in a rat critical-sized (7-mm) calvarial defect that was implanted with a hADSC-seeded poly(lactic-co-glycolic acid) (PLGA) scaffold. Aln (5 mmol/l/100 ml/day) was injected locally into the defect site for 1 week.

Accordingly, Cowan *et al.* [33] proved that implanted, apatite-coated, PLGA scaffolds seeded with ADSCs can heal CSD mouse calvarial defects.

In the present work, cell-loaded groups (osteogenic and Aln groups) demonstrated partial new bone formation, observed as islands within the defects. The newly formed bone-like tissue did not completely bridge

the defects. However, the level of bone maturation regarding bone lamellae appearance was not different between these two groups.

Wang *et al.* [32] demonstrated that PLGA-ADSC-Aln-implanted defects showed more new bone formation than the PLGA-ADSC group after 8 weeks of healing. At 12 weeks after implantation, the cell-unloaded defects (control and PLGA-Aln groups) demonstrated partial new bone formation, observed as islands within the defects. In contrast, the cell-loaded defects (PLGA-ADSC and PLGA-ADSC-Aln) were markedly filled with mature bone.

Cowan *et al.* [33] proved, by H&E stained sections, that bone deposition increased eventually, forming a thickness comparable to that of the uninjured left parietal bone. In the present study, the newly formed bone-like tissue in the operation area had immature characteristics with many marrow spaces and disordered osteocytes that were numerous, plump, and rimming the bone-like trabeculae. The surface area of individual bone trabeculae in the osteogenic group was significantly greater than the surface area of individual bone trabeculae in the Aln group. Although it seems evident that the bone regeneration in the osteogenic group was accelerated (increased individual trabeculae surface area) compared with the Aln group, the differences seem negligible if we consider the drawbacks of the steroid-containing osteogenic medium.

Defects of the control group showed healing with minimal regeneration, and no evidence of newly formed bone-like tissue was noted. These results proved that autogenous ADSCs can heal CSDs in the calvarium successfully with evidence of new bone formation compared with unfilled calvarial bony defects.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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