Osteogenic potential of human dental pulp-derived mesenchymal stem cells in bone regeneration of rabbit

Eman E.A. Mohammed[a,b], Mohamed El-Zawahry[c], Abdel Razik H. Farrag[d], Nahla N. Abdel Aziz[a], Nourhan Abou-Shahba[a,b], Marwa Mahmoud[a,b], Wael A. El-Mohandes[e], Mahmoud A. El-Farmawy[f], Alice K. Abdel Aleem[a,b,g]

[a]Department of Medical Molecular Genetics, [b]Stem Cell Research Group, Medical Research Centre of Excellence, National Research Centre, [c]Department of Fixed and Removable Prosthodontics, National Research Centre, [d]Department of Pathology, National Research Centre, [e]Department of Oral and Maxillofacial Surgery, Faculty of Dental Medicine (Boys), Al-Azhar University, [f]Oral and Maxillofacial Surgery, El-Helal Hospital, Cairo, Egypt, [g]Neurology and Neuroscience Department, Weill Cornell Medical College, Doha, Qatar (from 2013 to 2019)

Correspondence to Alice K. Abdel Aleem, MB, BCh, MD, Department of Medical Molecular Genetics, Human Genetics and Genome Research Division, Stem Cell Research Group, Medical Research Centre of Excellence, National Research Centre, Al Bouhouth Street, Dokki, PO Box 12622, Cairo, Egypt.
Tel: +9744492 8450;
e-mail: aka2005@qatar-med.cornell.edu

Received: 24 August 2019
Revised: 24 October 2019
Accepted: 27 October 2019
Published: xx xx 2019

Journal of The Arab Society for Medical Research 2019, 14:102–112

Background/aim
Human dental pulp-derived mesenchymal stem cells (hDP-MSCs) offer a promising source of progenitor cells for regenerative medicine and bone tissue engineering. Cranial defects are common complications that can arise secondary to trauma, surgery, or infection. This study aimed to evaluate the osteogenic differentiation potential of hMSCs isolated from dental pulp of third molar teeth *in vitro* cultures and the bone regenerative capacity of hDP-MSCs transplanted into induced temporomandibular joint (TMJ) defect in rabbits.

Patients and methods
hDP-MSCs were isolated from third molar teeth and cultured. Alizarin staining was performed to assess the osteogenic differentiation at the 14th and 28th days. The therapeutic potential of hDP-MSCs in craniofacial bone defects was investigated in the left side of the rabbits' TMJ. The transplanted cells involved three groups: the osteogenic differentiated DP-MSCs (O), undifferentiated MSCs (M), and control group (cell-free matrix) (C). Cells were loaded on gel foam. Eighteen rabbits were used and sacrificed at subsequent three time points, 4, 6, and 9 weeks, after transplantation, with six rabbits/each time point and two rabbits/each cell group. Histopathological studies were applied to evaluate the healing potential of hDP-MSCs in the induced rabbit TMJ defect.

Results
hDP-MSCs showed a high proliferative potential and osteogenic differentiation *in vitro*. Histological results demonstrated a timely correlated mandibular defects' repair in all the experimental groups, including the control group, with more enhanced bone healing effect for the osteogenic differentiated DP-MSCs.

Conclusion
hDP-MSCs possess high proliferation capacity and osteogenic differentiation potential *in vitro*. Histological observations revealed the osteogenic differentiated DP-MSCs have higher bone healing potential than the undifferentiated DP-MSCs at 9 weeks after transplantation, and gel foam promotes bone formation in the control group. The bone regenerative potential of osteogenic differentiated DP-MSCs revealed a significant capacity when implanted in rabbit TMJ defect. Hence, hDP-MSCs could be a promising source for craniofacial bone regeneration.

Keywords:
human dental pulp-derived mesenchymal stem cells, human dental pulp, mesenchymal stem cells, temporomandibular joint, third molar teeth

J Arab Soc Med Res 14:102–112

1687-4293

Introduction

Human dental pulp-derived mesenchymal stem cells (hDP-MSCs) offer a very promising as well as easily accessible source of postnatal mesenchymal stem cells for clinical applications and regenerative medicine. This is owing to their inherit properties of high proliferative potential and expansion rate, capacity of self-renewal, and multilineage differentiation. hDP-MSCs exhibit a differentiation potential into different types of cells, including odontoblastic, adipogenic, and neural cells. This ranks DP-MSCs as highly promising and an attractive candidate in regenerative medicine and tissue engineering [1–3].

The hDP-MSCs have a pivotal role in repairing bone defects and bone tissue regeneration. The vertebral spine and craniofacial bone defects, which can be caused by injuries, congenital anomalies, trauma, and different diseases are examples [4–6]. hDP-MSCs have been used in bone tissue regeneration with the combined application of three-dimensional nanostructured scaffolds in induced critical size bone defects. Trials performed in calvarial defect's model rat revealed positive findings of bone formation assessed by immunohistochemistry and histopathology [7].

The temporomandibular joint (TMJ) is a site of intense morbidity for millions of people, especially teenagers and young premenopausal women. Several studies have suggested that sex hormones may influence TMJ dysfunction and cartilaginous breakdown. Central to TMJ illnesses are the cartilaginous tissues of the TMJ, especially those of the disc and condylar cartilage, which play crucial roles in the normal function of this joint. Compared with hyaline cartilage, the fibrocartilaginous TMJ disc has lower glycosaminoglycan content and contains primarily collagen type I, not collagen type II [8].

Cranial defects are a relatively common complication that can arise secondary to trauma, surgery, infection, neoplastic, or congenital malformation such as aplasia cutis; their reconstruction constitutes a challenge in craniofacial surgery [9].

The *in vivo* studies employing animal models have demonstrated the significance of MSCs when transplanted to increase the bone production and subsequently the stiffness of the regenerated structures. In various animal models, MSCs have been used to repair segmental bone defects of critical size. The most commonly used bone defect model is the radial defect model, in addition, to other rabbits models of skull and middle femur defects. As the modeling process is simple, these models are widely developed [10].

The implantation of scaffolds or carriers presents one of several potential strategies to facilitate the regeneration of damaged bone tissue. A number of scaffolds have been tested, such as synthetic polymers, demineralized bone matrix, hydrogel, titanic fibers, natural coral, and synthetic bioceramics. These scaffolds are used to carry the cells, and then are fitted ectopically on the bone defect site [11]. Gelatin sponge (gel foam) has the characteristics of flexibility, biocompatibility, and biodegradability as well as the potential to be used as an efficient carrier matrix (scaffold) to support osteoblasts and to promote bone regeneration in defective areas [12–14].

In present study, we assessed the proliferation and osteogenic differentiation capacity of hDP-MSCs *in vitro*. In addition, it tested its bone regenerative capacity *in vivo* using both undifferentiated hDP-MSCs and osteogenic differentiated DP-MSCs carried on gel foam scaffold in a rabbit mandible defect.

Patients and methods

Patients

Dental pulp of third molar teeth was collected from healthy participants (donors), who had neither infected nor carious teeth, and was used in the current study. The teeth were partially impacted with simple extraction without any traumatic injury to the tooth structures, leading to pulp exposure, and without being contaminated; this simple extraction preserves the sterile pulp. Another resource to get sterile dental pulp was extraction of premolar tooth for orthodontic treatment to obtain more space in dental arch for the future movements of all teeth in the right way. The extraction of pulp was done at the dental clinic of the National Research Centre, Egypt. Third molars were collected from four donors who were in the age range of 16–50 years old.

Experimental animals

A total of 18 New Zealand white rabbits, 6 months old, with a weight range of 1.5–2 kg, were used in the present study. All animals were treated humanely under the ethics approval committee of the National Research Centre. Rabbits were obtained and housed in cages in a cool room temperature out of direct sunlight and were protected from draughts, loud noises, and direct access to radiators in animal house at National Research Centre. Rabbits were fed on a commercial pelleted rabbit diet.

Experimental design

Rabbits were sacrificed after transplantation at three time intervals, 4, 6, and 9 weeks, and classified into three groups (six rabbits per each group). Each group included three subgroups (two each), as follows:

(1) The first group was transplanted with DP-derived stem cells, differentiated osteogenic progenitors cells (experimental group, O group),
(2) The second one was transplanted with matched DP-derived MSCs in proliferation media (control group, M group),
(3) The third one had received cell-free gel foam (negative control group, C group).

Ethical consideration

All participants were informed about the practical steps of this study and signed approval consent. This study was approved by the Ethics Committee of the National Research Centre and followed the ethical approvals of animal protocol (Approval No. 16/263).

Methods

In vitro study

Isolation of dental pulp-derived mesenchymal stem cells

Dental pulp tissue was digested by collagenase/dispase enzymatic digestion method [15]. Dental pulps were extracted from healthy third molar teeth by mechanical fracturing and a dentinal excavator that acts to gently remove the dental pulp. The removed pulp was immersed for an hour at 37°C in a digestive solution of two enzymes, collagenase type I (3 mg/ml) and dispase (4 mg/ml), and then centrifuged at 1000 r/min for 10 min. Cell pellets were collected and cultured in alpha minimal essential medium (α-MEM; Gibco BRL, Life Technologies B.V., Breda, Netherlands), containing 10% fetal bovine serum (FBS; Gibco ERL), 100 U/ml penicillin (Gibco ERL), and 100 U/ml streptomycin (Gibco ERL), and incubated at 37°C under standard conditions with 5% CO_2. Dividing cells at the third passage were used for the osteogenic induction experiment.

Culture and expansion of dental pulp-derived mesenchymal stem cells

Culture and expansion of hDP-MSCs were maintained in regular proliferation media to reach about 80% confluence, and then cells were passaged and reseeded. Manual scraping technique using cell scraper (Corning Incorporated, Costar, Mexico) was performed, and cell collection was done, followed by centrifugation and resuspension in regular proliferation media and then reseeding of cells in culture plates [15].

Population doubling time

The expansion rate of the hDP-MSCs was estimate by applying the population doubling time (PDT), which is the time required by the cells to double their population number within the specific culture period 12 days (288 h). The cell cultures at passage 3 (P3) and passage 6 (P6) of DP-MSCs were plated at 5000 cells/well in basic proliferation media incubated till reached confluence, and then culture was terminated and subjected to hemocytometer count. PDT is calculated according to the formulae, PDT=CT/PDN, where CT is the standard culture time and PDN is the population doubling number. PDN was calculated by the formula, PDN=log (N1/N0)×3.3, where N1 is the harvested cell number at the end of culture period and N0 is the initial cell number at the start of culture period [16].

Differentiation of dental pulp-stem cells into bone-forming cells

Proliferating human MSCs at 70% confluence of the third passage were used for osteogenic differentiation by exchanging the culture media into DMEM containing 20% FBS, L-ascorbic acid 2-phosphate, β-glycerol phosphate, and dexamethasone as well as 100 μg/ml penicillin/streptomycin and 1% glutamax [17]. A comparable control culture (MSCs in proliferation media) was made for each sample. Two similar sets that differ in incubation time were simultaneously generated; one set (plates in differentiation media and control plates) was maintained in corresponding culture media for 14 days before passed into characterization protocols, and the second set, with same kind of plates, was retained in its culture media for 29 days before characterization.

Characterization protocol for osteogenic differentiated cells

Characterization of osteogenic differentiation was done using alizarin red staining for the detection of mineralized nodules developed in the differentiated cultures. In brief, plates were washed three times with PBS and fixed in 70% ethanol at room temperature for an hour, and then washed with dH_2O before adding 1.3% alizarin red (pH 4.2). The plates were incubated at 37°C for an hour with gentle shaking. Plates were washed with dH_2O until the dye's color disappears. dH_2O was carefully aspirated, the plates were washed with PBS, and enough dH_2O was added to cover the cellular monolayer to be ready for image capture by inverted microscope [18].

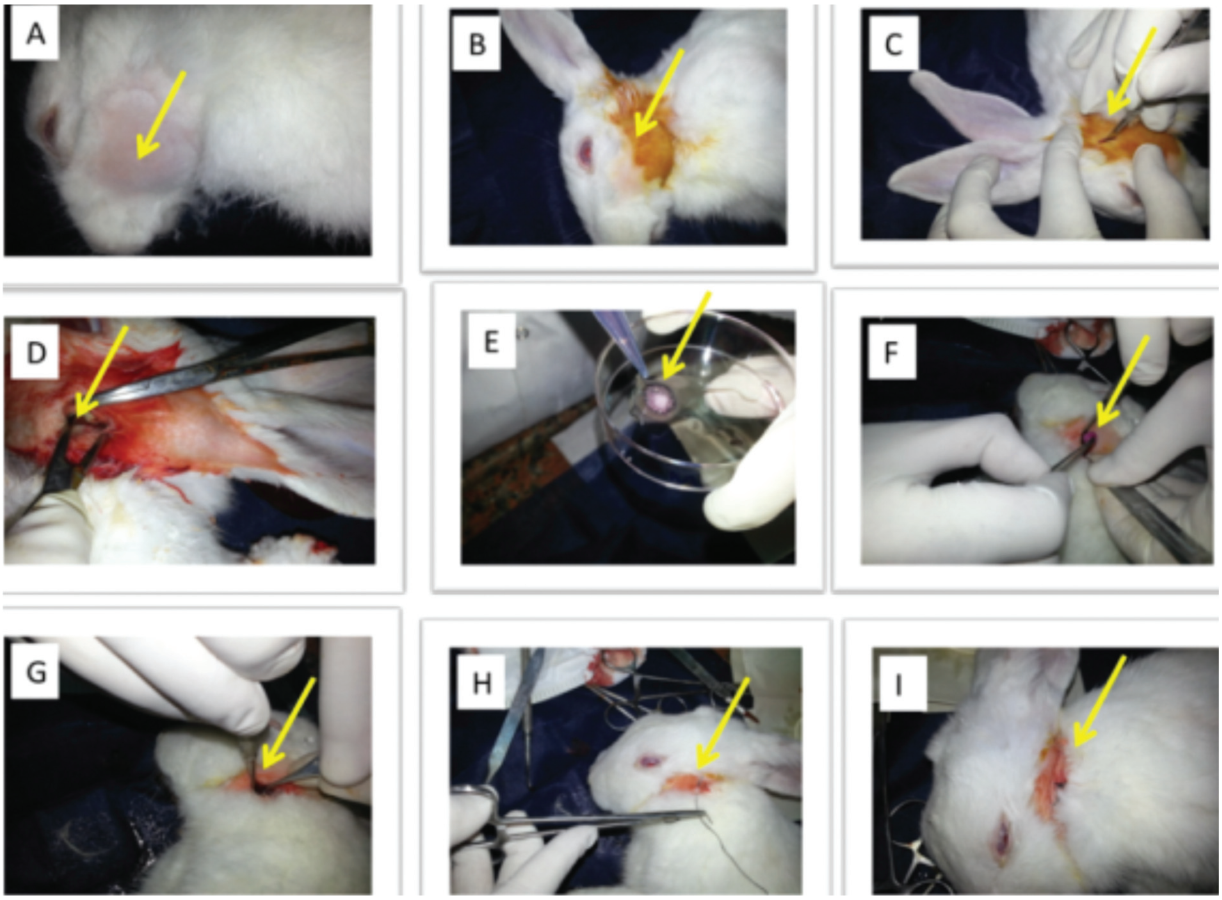
In vivo study

Induction of the rabbit's mandibular defect

Preoperatively, each rabbit was fasted for 12 h. General anesthesia was induced by intramuscular injection of ketamine hydrochloride (35 mg/kg body weight) and xylazine (5 mg/kg body weight) and maintained throughout the surgical procedure.

External incision was made opposite to TMJ extending to the lower border of the mandible (angle of the mandible), and skin incisions (~1.5 cm) were initiated at the angle of the ramus of the mandible in the middle under the head of the condyle and ascending ramous at left sides under aseptic conditions, and a subperiosteally flap was raised to expose the angle of the mandible. Then, the incision was extended to the muscle layers till reaching the bone, and then a unicortical cavity (a defect hole created in the mandible left side) was performed in the body of the mandible 2.5×2.5 mm in diameter and in depth. The cavities were prepared using standard round bur in a contra-angle

Figure 1



The induction of rabbit mandible defect and hDPSCs transplantation. (A) Fur Shaving at the left side of mandible, (B) Sterilized with betadine, (C) Skin incisions at the angle of the ramus of the mandible, (D) Extension of incisions to muscle layers deep to bone level and cavity in the body of mandible 2.5 mm in diameter and in depth made by round bur, (E) DP-MSCs loaded on gel foam in plate, F & G) Transplantation of DP-MSCs into the rabbit mandible defect, (H) Flaps were repositioned, carefully and sutured in layers, and (I) transplantation operation was completed.

handpiece running at ~10 000 rpm and abundantly irrigated with saline solution. Finally, the flaps were repositioned carefully and sutured in layers (Fig. 1). Postoperative pain was managed by injection of 50 μg buprenorphine/kg each 2 h for the first day. This experiment was conducted following the national and European guidelines for animal experiments.

Approximately two million cells were loaded on the gel foam that was applied to and fitted in the induced defect area at the left rabbit's mandible, as shown in Fig. 1.

Histopathological investigation

The histological investigation was performed for qualitative evaluation of bone healing. Rabbits were sacrificed according to the recommendation of the ethical committee of the National Research Centre for Animal Experiment. The specimens of the left (defect) and the right (normal) sides of rabbit's mandibles were cut in blocks, 0.5–1 cm around (included) the defect, decalcified in 4% formic acid at room temperature, and embedded in paraffin. Latero-medial 5-μm-thick sections were obtained, stained with hematoxylin and eosin, and examined under the light microscope [19].

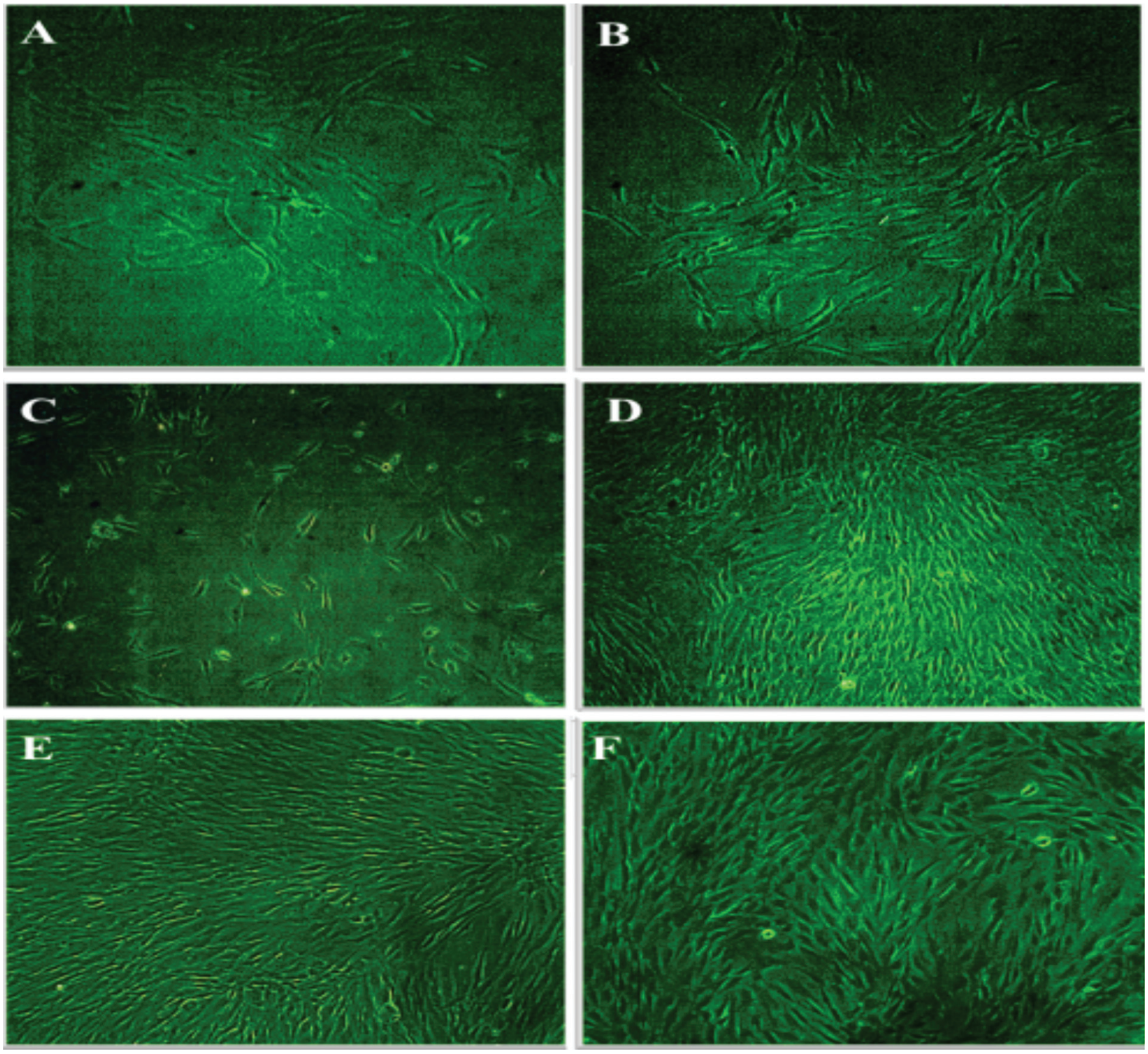
Results

In vitro study

Proliferative potential and morphological criteria of human dental pulp-derived mesenchymal stem cells

Human mesenchymal stem cells were isolated from dental pulp and cultured in regular proliferation media. The morphological criteria during the expansion/proliferation phase of DP-MSCs from a 28-year-old donor are shown in Fig. 2A-F. The original cell count for DP-MSCs was 100×10^3 cells. The primary culture (P0) of hDP-MSCs appears after 7–10 days forming colonies at the periphery of the plates (Fig. 2A and B). After 3–5 weeks, these

Figure 2



The proliferation and the morphological characteristics of hDP-MSCs derived from 28-year-old donor (AKF) During the expansion phase: hDP-MSCs were cultured at different passages. (A & B) Primary culture (P0) at the 9^{th} &11^{th} days respectively, (C & D) First Passage (P1) at the 3^{rd} & 6^{th} days respectively, (E) Second Passage (P2) at the 4^{th} day, (F) Third Passage (P3) at the 5^{th} day. ×100 magnifications.

colonies formed a confluent monolayer of typical elongated fibroblast-like cells that grow at passage 1 (P1) (Fig. 2C and D). The dominant typical spindle mesenchymal stem cell morphology was shown in the subsequent passages demonstrating the high proliferative potential of hDP-MSCs. The cells adhered to each other, fusing together, and forming a web structure at passage 2 (P2) (Fig. 2E). Then the cells were detected microscopically as yellowish white dense spots that deposited on the culture surface at passage 3 (P3) (Fig. 2F).

Population doubling time

PDT was used to evaluate the ability of the cells to duplicate its number, a direct marker of the proliferative ability of the cells. In this study, we analyzed the PDT of DP-MSCs cultured in proliferation growth medium. PDT was calculated for DP-MSCs at P3 and P6 (Table 1). The proliferation potential and expansion rate were observed in DP-MSCs, which tended to double their number in 38 h at P3 and 40 h at P6 in the culture period of 12 days (288 h). The DP-MSCs derived from a 28-year-old donor showed rapid

Table 1 Population doubling time for dental pulp-derived mesenchymal stem cells through proliferation and expansion phase

Passage no.	N0	Ni	PDN	CT	PDT
P3	$5×10^3$	$970×10^3$	7.57	288	38
P6	$5×10^3$	$720×10^3$	7.1	288	40

CT, the standard culture time; N0, the initial cell number at the start of culture period; Ni, the harvested cell number at the end of culture period; PDN, the population doubling number; PDT, the population doubling time.

proliferation and a higher expansion rate at P3 than that at P6.

Osteogenic differentiation potential of dental pulp-derived mesenchymal stem cells

Alizarin red staining assay was performed for detection of the calcium deposits, resulting from the osteogenic differentiation induction of hDP-MSCs. The calcium deposits (mineral deposits) chelate with alizarin forming an alizarin red calcium complex detected as orange-red staining spots (Fig. 3). There were no staining spots in the control sets incubated in regular proliferation media at the two time points: 14^{th} and 28^{th} days (Fig. 3A and D, respectively). Alizarin red staining achieved after 14 days of the osteogenic differentiation shows moderate staining in the central of the osteogenic field and intense staining in the peripheral of the field (Fig. 3B and C, respectively). Alizarin red staining performed after 28 days of osteogenic induction revealed enhancement of the production of mineralized matrix by the differentiated cells. Several stained spots were obviously detected on the day 28^{th} of the osteogenic differentiation, centrally and peripherally throughout the osteogenic field (Fig. 3E and F, respectively).

In vivo study

Evaluation of rabbit mandibular defect's healing after transplantation

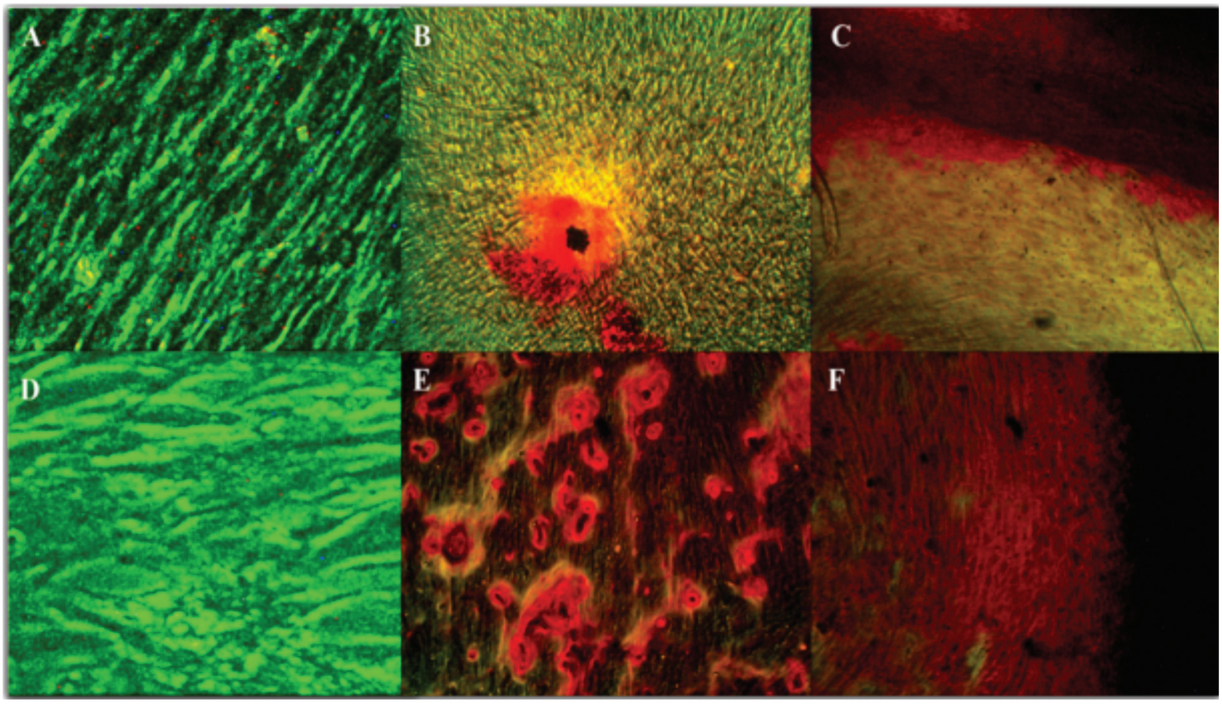
Rabbits were sacrificed at subsequent three time points, 4, 6, and 9 weeks, after implantation and followed up by qualitative histopathological analysis, which revealed the following results.

Histopathological results

Histopathological photomicrographs of different derived grafts, including the cell-free/gel foam graft (C), undifferentiated DP-MSCs (M), and osteogenic differentiated DP-MSCs (O), are shown in Figs 4–6.

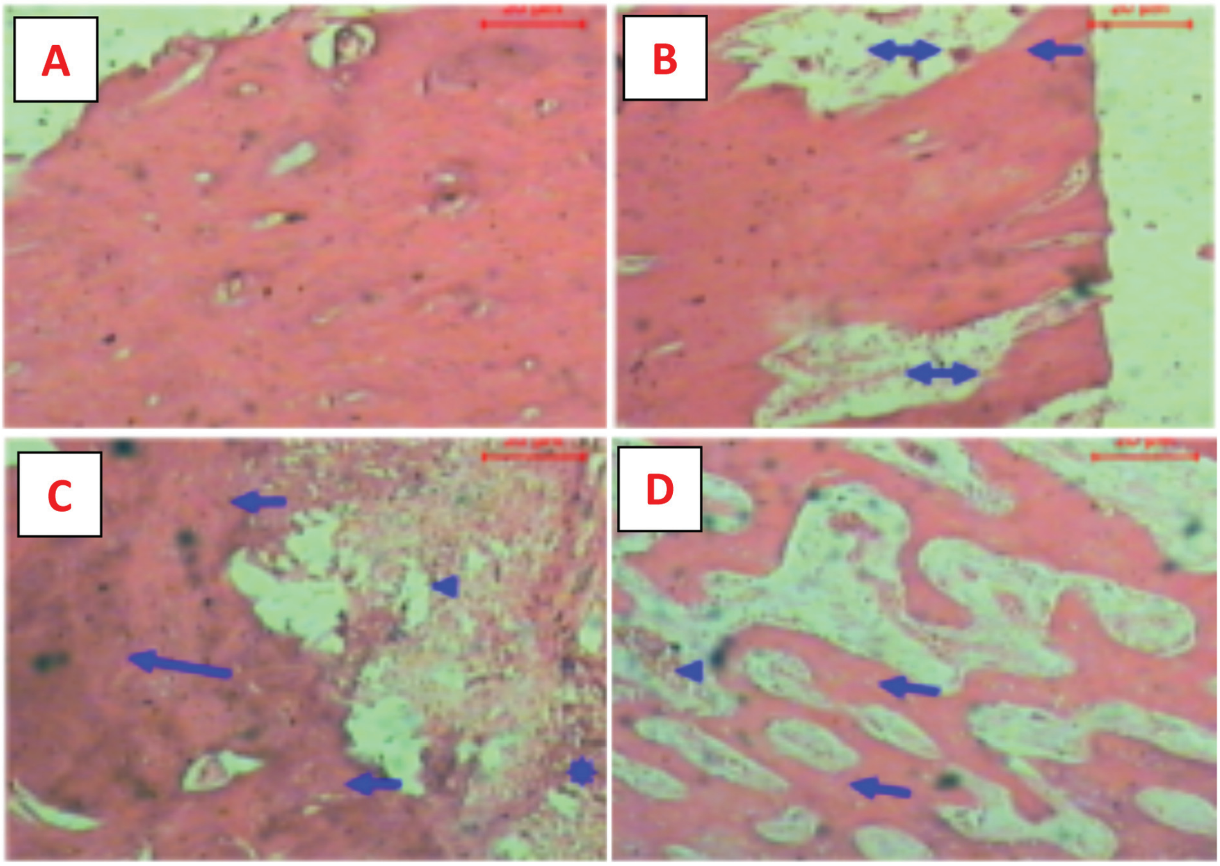
The histological changes presented in Fig. 4A–D were the outcomes of 4 weeks after transplantation. A normal bone structure of the rabbit mandibular right side was shown in Fig. 4A. The examined specimen for the cell-free/gel foam (C) graft-treated defect showed small area of new bone formed in the depth of the defect, and the remaining defect areas were empty except for a little cellular debris observed (Fig. 4B). At the same time point, the M graft (undifferentiated DP-MSCs loaded on gel foam)-treated defect showed a large osteoid tissue (numerous osteocytes with round

Figure 3



Osteogenic differentiation of DP-MSCs cultured in the osteogenic differentiated media by Alizarin Red staining on the 14 & 28 days. (A & D) unstained control DP-MSCs on 14^{th} & 28^{th} days respectively, (B & C) Represented photos for staining in the osteogenic field on the 14th day (B) Moderate Alizarin staining in between the induced cells (C) Strong staining pattern in peripheries (E) Strong Alizarin staining on the 28th day, in the osteogenic set in between the induced cells; (F) Strong intense Alizarin staining in the periphery of the osteogenic field. Magnification power ×100.

Figure 4



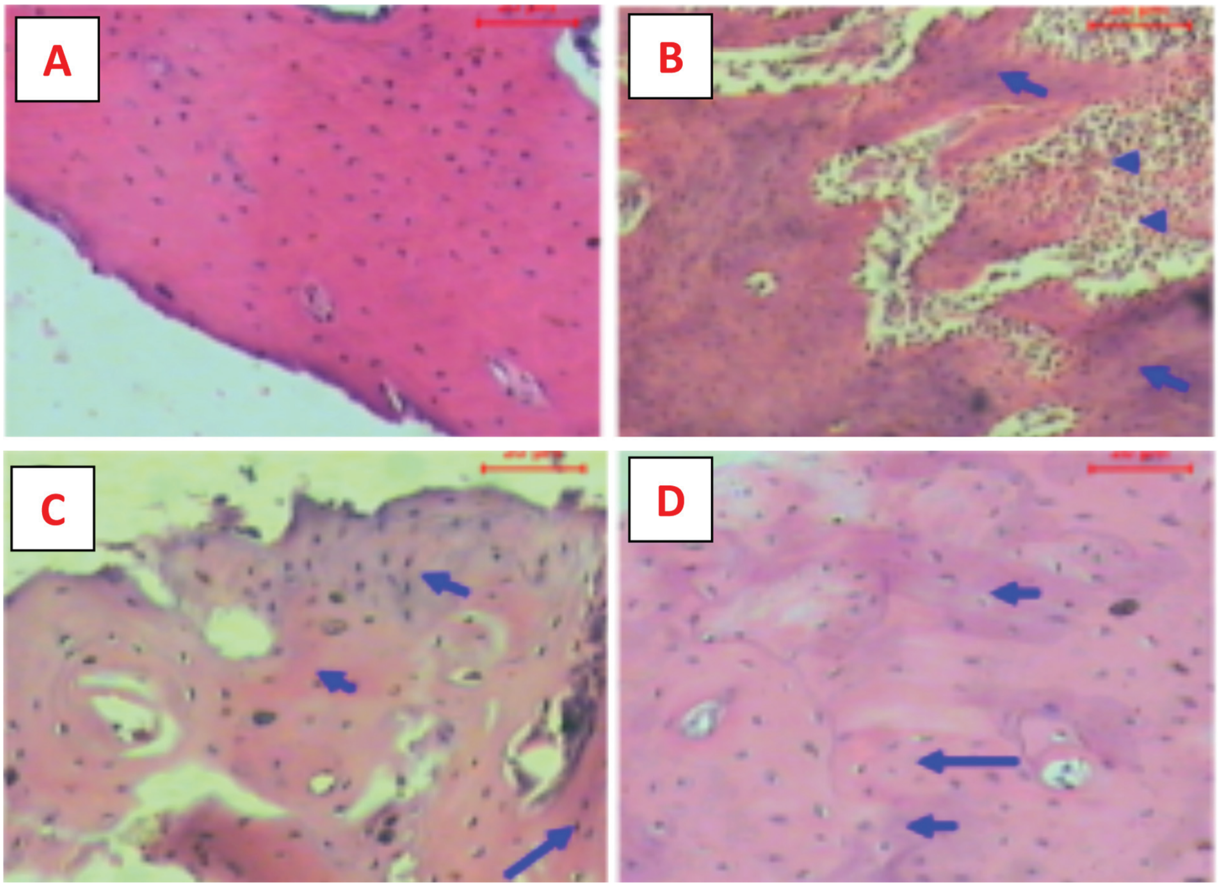
Sections from rabbits mandible defect at 4 weeks of (A) shows right normal mandible side, (B) control that received media loaded gel foam shows a small area of newly formed bone in the depth of the defect with large empty defect area except for a little cellular debris, (C) rabbit mandible defect transplanted with undifferentiated DP-MSCs shows newly formed bone in contact to the old bone, large osteoid tissue, and cartilage area; (D) experimental defect shows the implant contained a newly formed bone with a thick trabecular pattern and minimal osteoid tissue (H & E; Scale Bar: 20 μm). Short arrow: newly formed bone, long arrow: old bone, arrowhead: osteoid, asterisk: cartilage.

cell bodies inside a homogenous matrix without lamellation). This tissue extended from the defect's margin to the nearby host bone with small areas of new bone formation that was visible immediately adjacent to the host bone (Fig. 4C). New formed bone with thick trabecular pattern and less osteoid tissue was observed in the experimental rabbits transplanted with osteogenic differentiated DP-MSCs, the O graft (Fig. 4D).

The histological changes 6 weeks after transplantation are presented in Fig. 5A–D, where normal bone structure has been observed in the rabbit's mandible right side (Fig. 5A). New bone with trabecular pattern and large areas of osteoid tissue were observed in the sections of the rabbit defect transplanted with cell-free/gel foam (Fig. 5B). Bone with a trabecular pattern that appears separated from the top of the host bone with area of fibrous tissue observed in-between the newly developed and the host bones in the undifferentiated DP-MSCs cells loaded on gel foam (M) graft-treated defect, is shown in Fig. 5C. Bone union that is the newly developed bone is in contact with the host bone was observed in the examined specimen of the O graft (osteogenic differentiated DP-MSCs cells loaded on gel foam)-treated defect (Fig. 5D).

The histological observations derived in nine weeks after transplantation are presented in Fig. 6A–D. Normal bone structure was noticed in the untreated mandible right side (Fig. 6A). New bone in the depth of the defect that appeared separated from the top of the peripheral host bone, small marrow space, and thin fibrous layer were observed in the sections of the rabbits defect transplanted with cell-free gel foam (Fig. 6B). A fibrous connective tissue extending at the margin of the defect preceded a large area of newly developed bone, with small marrow spaces, adjacent to the host bone in the section of the rabbits defect transplanted with the undifferentiated DP-MSCs cells loaded on gel foam (Fig. 6C). More new developed bones in the depth of the surgically created defect in contact and on top of the peripheral host bone have been observed in the section of the experimental rabbit transplanted with the

Figure 5



Sections from rabbits mandible defect at 6 weeks of (A) shows right normal mandible side, (B) rabbit mandible defect that not received DP-MSCs at 6 weeks shows a newly formed bone with a wide trabecular pattern and large areas of osteoid tissue; (C) implanted with undifferentiated DP-MSCs at 6 weeks showed a newly formed bone with the trabecular pattern not in contact and on top of the old bone, fibrous tissue area observed in between the newly developed and old bone; (D) the experimental defect shows the implant contained a newly formed bone in contact and on top of the old bone (H & E; Scale Bar: 20 μm). Arrow: newly formed bone, arrowhead: osteoid.

osteogenic differentiated DP-MSCs progenitor cells loaded on gel foam (Fig. 6D).

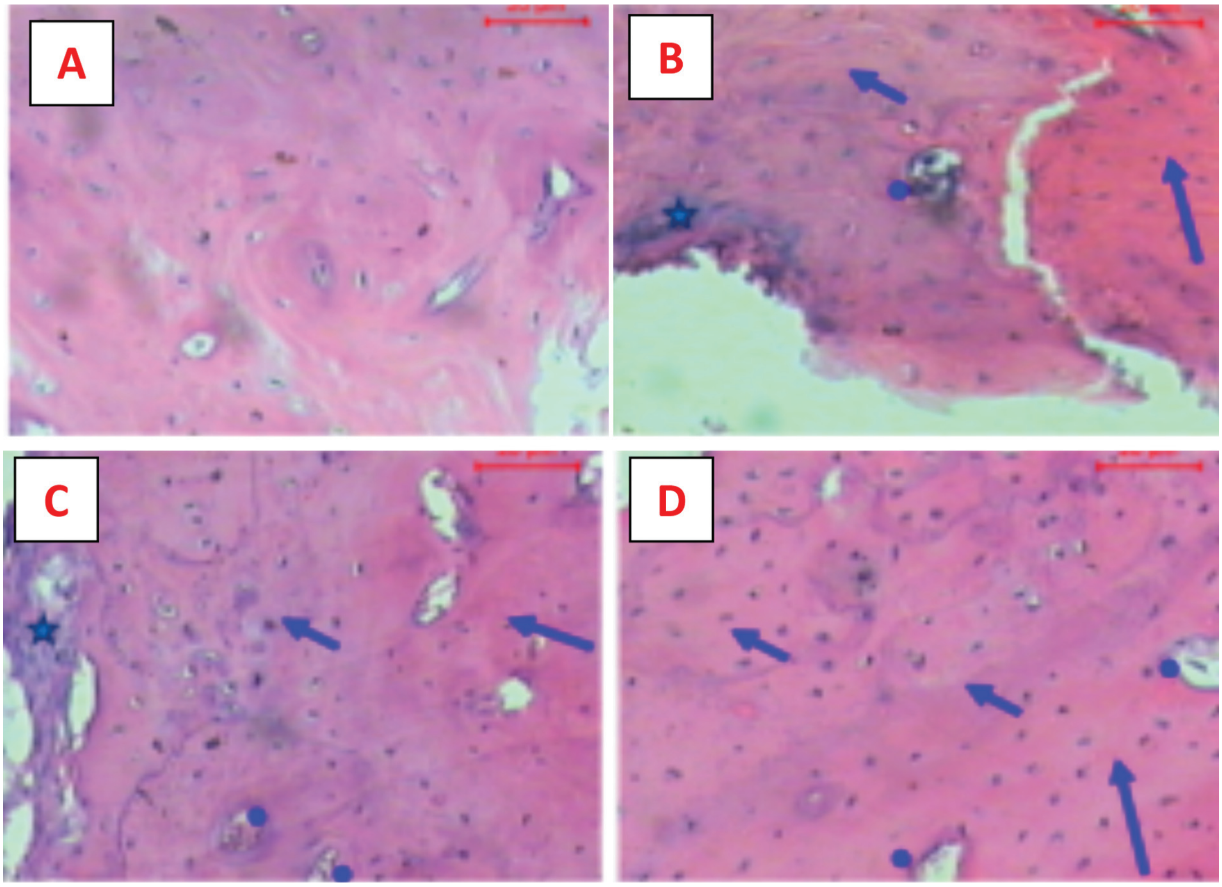
Discussion

MSCs are suggested to be the source of osteoblastic cells during normal bone growth and remodeling *in vivo*. Their relative ease of isolation from different sources, *in vitro* expansion potential, and the well-recognized osteogenic differentiation capability makes them potentially promising for several applications including bone repair and regeneration.

Reconstruction of complex craniofacial deformities is a clinical challenge in situations of injury, congenital defects, or acquired diseases. The use of cell-based therapies represented one of the most advanced methods for enhancing the regenerative response of craniofacial wound healing process in both skin and the oral cavity [20–22]. Mesenchymal stem cells have been adopted in the treatment of complex osseous defects, for their particular characteristics of proliferation, osteogenic differentiation capacity, and immunomodulatory properties [23]. Based on this background, the isolation of high-quality human postnatal mesenchymal stem cells from accessible resources and evaluating their osteogenic potential and *in vivo* constitute important goals for stem cell research and its application in regenerative medicine.

In the present study, the dental pulp tissues derived from permanent third molars teeth that were commonly surgically removed owing to dental related issues were used as the source of postnatal DP-MSCs. The isolated DP-MSCs were known for their high proliferative clonogenic potentials as well as their differentiation into osteoblast-like cells, when grown in an osteogenic inducing media producing mineralized extracellular matrix. The results of this study revealed the ability of transplanted osteogenic differentiated DP-MSCs to produce new osteoid tissue helps in repairing an induced rabbit mandibular defect in a time and differentiation phase-dependent manner.

Figure 6



Sections from rabbits mandible defect at 9 weeks of (A) shows right normal mandible side, (B) control that received media loaded gel foam shows a newly formed bone in the depth of the defect that appears separated from the top of the peripheral old bone, Small marrow space, and a thin fibrous layer; (C) the implanted defect with undifferentiated DP-MSCs shows newly formed bone that associated with narrow bone marrow spaces and fibrous tissue area in contact and on top of the peripheral old bone; (D) experimental rabbit shows newly developed bone in the depth of the surgically created defect in contact to the old bone with wide marrow spaces (H & E, scale bar=20 μm). Short arrow: newly formed bone, long arrow: old bone, circle: bone marrow.

In our previous study, evaluation of proliferation potential and expansion rate of DP-MSCs cells of two different age donors, 50 and 16 years old, were performed by applying growth curve and PDT at P3 and revealed different proliferation and expansion rate. The DP-MSCs derived from young donor took 37 h for PDT and reached the plateau stage faster than the cells derived from the old age donor that took 39 h [24].

In this study, PDT was applied for hDP-MSCs derived from 28-year-old donor at two different culture passages, early and intermediate passages (P3 and P6), and showed higher proliferation and expansion rate at early passage (PDT=38 h) than that at the intermediate one (PDT=40 h).

The osteogenic differentiation potency of DP-MSCs was reported by other investigators [25–27]. The osteoblastic phenotype was examined for calcium deposition by alizarin red staining, which chelates the mineral nodules, forming orange-red spots. Our mineralization findings showed a strong positive staining in the osteogenic cultures, indicating osteogenic differentiation under the applied protocol. Absence of staining in the control cultures highlights the lack of spontaneous osteogenic differentiation. Weak staining patterns were observed at the early time point (the 14^{th} day of osteogenic incubation), which indicates less amount of calcium deposit produced by the osteogenic differentiated cells. The variably intense staining patterns shown at different parts of the osteogenic field (centrally and peripherally) at the later time point (the 28^{th} day of induction) could be attributed to individuals' variability.

Extensive research in the era of bone tissue engineering was to explore the potential role of combined use of osteogenic-differentiated DP-MSCs and various scaffold materials in bone tissue regeneration. In a previous study, there was formation of a bone-like hard tissue following the implantation of differentiated rat-derived DP-MSCs seeded on a three-dimensional porous calcium phosphate ceramic scaffold as a cell carrier into subcutaneous sites of an immunocompromised mouse [25].

A previous clinical study revealed clinical and radiographic evidence of repairing alveolar bone defects of human mandible, using autologous implantation of DP-MSCs extracted from their maxillary third molars and expanded *ex vivo* in combination with collagen sponge scaffold. Additionally, the histopathology observation of this study showed complete healing of human mandible defect, indicating the healing potential of the biocomplex DP-MSCs/collagen sponge for repair and bone tissue regeneration [28].

In the present study, we have explored the feasibility of using DP-MSCs in mandibular bone regeneration in an attempt to treat carinorofacial bone defects. We have surgically created a critical size defect in the left side of rabbit mandible and used this as a preclinical animal model. The regeneration efficiency of the gel foam/DP-MSCs implants was evaluated after implantation at three different time intervals, 4, 6, and 9 weeks, by qualitative histological analysis, which revealed the formation of new bone at the induced defect of rabbit's mandible. Histological results displayed a timely correlated osteoid tissue formation in all tested groups including the control ones with the enhanced bone healing effect observed in the group transplanted with osteogenic differentiated DP-MSCs. The bone healing in the control group transplanted with cell-free gel foam may be a function of the defects being smaller than critical size, enabling self-repair with endogenous progenitor cells and differentiation factors or/and the osteoinductive effect of the implanted material, gel foam.

In our previous study, the comparison of human amniotic stem cells (AF-MSCs) and human bone marrow stem cells (BM-MSCs) *in vitro* and *in vivo* revealed hAF-MSCs have a better performance *in vivo* bone healing, bone regeneration, and spine fusion surgeries than that of hBM-MSCs. Moreover, the usage of gel foam as a scaffold proved as an efficient cell carrier that showed biocompatibility with cells, biodegradability, and osteoinductivity *in vivo* [29].

In this study, the *in vivo* bone regenerative capacity of undifferentiated versus osteogenic differentiated DP-MSCs carried on gel foam scaffolds revealed different healing potential; however, the osteogenic differentiated DP-MSCs cells showed the highest healing efficiency of almost complete healing of the defect site at 9 months.

In the present study, the histological observations revealed that the osteogenic differentiated DP-MSCs progenitor cells have a higher bone healing potential than the undifferentiated DP-MSCs at 9 weeks after transplantation. Histological results in the control group of the scattered newly formed bone at the induced rabbit TMJ defect compared with the osteogenic group may be secondary to the gel foam that could promote bone formation *in vivo*. Moreover, the histological findings suggest the 9-week post-transplantation group had a higher healing potential than either 6-week group or 4 week post-transplantation group. Further time period after transplantation is required to achieve the complete bone healing and regeneration.

The histological observations of Song *et al.* [30] revealed the full osteogenic differentiation induction of DP-MSCs was achieved when it was incubated in osteogenic differentiation media for 3 weeks at least *in vitro*, which enhanced bone regenerative capacity when seeded on ceramic cubes and implanted subcutaneously on both sides of the dorsal surface of severe combined immunodeficiency mice. There are many studies that have discussed the utility of pre-implantation osteoinduction of DP-MSCs in treatment of bone defects [26,30,31]. Their results were similar to our present observations; however, in the present study, the comparison of osteogenic differentiated cells (DP-MSCs) versus un-differentiated mesenchymal cells (DP-MSCs) was performed under the same setting of the same *in vivo* experiment and revealed that the transplanted osteogenic differentiated DP-MSCs cells loaded on gel foam have a higher bone healing potential in rabbit mandible bone defect than that of the undifferentiated transplanted one.The results of Gronthos *et al.* [2] and d'Aquino *et al.* [28] have shown the osteogenic differentiation potential of hDP-MSCs *in vitro*, and calcium deposit was detected by alizarin red staining technique. Development of a more complex animal model of larger animals, such as pigs, dogs, sheep, and horses, applied to assess the ability to reconstitute different defect sites with natural bone graft with the advantage of closely mimicking the human bone physiology, will be more helpful than do the rat models of rat calvarial defects and rat femoral defects [9].

Conclusion

In conclusion, hDP-MSCs have a high proliferation capacity and osteogenic differentiation potential *in vitro*. The *in vitro* osteogenic differentiation of MSCs derived from human dental pulp before its *in* *vivo* implantation promotes its osteogenic/bone-forming capacity that enhanced their healing effect. Our findings revealed that hDP-MSCs have a potential healing effect on the craniofacial bone injury and could be a promising source for bone regeneration applications.

Acknowledgments

All authors acknowledge the National Research Centre for funding this project. We thank the participants for providing their samples.

Financial support and sponsorship

This research was funded by the National Research Centre.

Conflicts of interest

There are no conflicts of interest.

References

1 Graziano A, d'Aquino R, Laino G, Papaccio G. Dental pulp stem cells: a promising tool for bone regeneration. Stem Cell Rev 2008; 4:21–26.

2 Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, *et al.* Stem cell properties of human dental pulp stem cells. J Dent Res 2002; 81:531–535.

3 Akiyama K, Chen C, Gronthos S, Shi S. Lineage differentiation of mesenchymal stem cells from dental pulp, apical papilla, and periodontal ligament. Methods Mol Biol 2012; 887:111–121.

4 Undale AH, Westendorf JJ, Yaszemski MJ, Khosla S. Mesenchymal stem cells for bone repair and metabolic bone diseases. Mayo Clin Proc 2009; 84:893–902.

5 Kern S, Eichler H, Stoeve J, Kluter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 2006; 24:1294–1301.

6 Leyendecker Junior A, Gomes Pinheiro CC, Lazzaretti Fernandes T, Franco Bueno D. The use of human dental pulp stem cells for in vivo bone tissue engineering: a systematic review. J Tissue Eng 2018; 9:2041731417752766.

7 Jaquiery C, Schaeren S, Farhadi J, Mainil-Varlet P, Kunz C, Zeilhofer HF, *et al.* In vitro osteogenic differentiation and in vivo bone-forming capacity of human isogenic jaw periosteal cells and bone marrow stromal cells. Ann Surg 2005; 242:859–867. discussion 867–858.

8 Athanasiou KA, Almarza AA, Detamore MS, Kalpakci KN. Tissue engineering of temporomandibular joint cartilage. Tissue engineering of temporomandibular joint cartilage. Williston, VT: Morgan & Claypool; 2009.

9 de Mendonca Costa A, Bueno DF, Martins MT, Kerkis I, Kerkis A, Fanganiello RD, *et al.* Reconstruction of large cranial defects in nonimmunosuppressed experimental design with human dental pulp stem cells. J Craniofac Surg 2008; 19:204–210.

10 Liu M, Lv Y. Reconstructing bone with natural bone graft: a review of in vivo studies in bone defect animal model. Nanomaterials (Basel) 2018; 8:999.

11 Arthur A, Rychkov G, Shi S, Koblar SA, Gronthos S. Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. Stem Cells 2008; 26:1787–1795.

12 Kaigler D, Pagni G, Park CH, Tarle SA, Bartel RL, Giannobile WV. Angiogenic and osteogenic potential of bone repair cells for craniofacial regeneration. Tissue Eng Part A 2010; 16:2809–2820.

13 Dudas JR, Marra KG, Cooper GM, Penascino VM, Mooney MP, Jiang S, *et al.* The osteogenic potential of adipose-derived stem cells for the repair of rabbit calvarial defects. Ann Plast Surg 2006; 56:543–548.

14 Rohanizadeh R, Swain MV, Mason RS. Gelatin sponges (Gelfoam) as a scaffold for osteoblasts. J Mater Sci Mater Med 2008; 19:1173–1182.

15 Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A 2003; 100:5807–5812.

16 Sessarego N, Parodi A, Podesta M, Benvenuto F, Mogni M, Raviolo V, *et al.* Multipotent mesenchymal stromal cells from amniotic fluid: solid perspectives for clinical application. Haematologica 2008; 93:339–346.

17 Zhang Q, Wang X, Chen Z, Liu G. Semi-quantitative RT-PCR analysis of LIM mineralization protein 1 and its associated molecules in cultured human dental pulp cells. Arch Oral Biol 2007; 52:720–726.

18 Tsukamoto Y, Fukutani S, Shin-Ike T, Kubota T, Sato S, Suzuki Y, Mori M. Mineralized nodule formation by cultures of human dental pulp-derived fibroblasts. Arch Oral Biol 1992; 37:1045–1055.

19 Carleton HM, Drury RAB, Wallington EA. Carleton's histological technique. Wallington EA, editor. USA: Oxford University Press; 1980.

20 desJardins-Park HE, Mascharak S, Chinta MS, Wan DC, Longaker MT. The spectrum of scarring in craniofacial wound repair. Front Physiol 2019; 10:322.

21 Pagni G, Kaigler D, Rasperini G, Avila-Ortiz G, Bartel R, Giannobile WV. Bone repair cells for craniofacial regeneration. Adv Drug Deliv Rev 2012; 64:1310–1319.

22 Maruyama T, Jeong J, Sheu TJ, Hsu W. Stem cells of the suture mesenchyme in craniofacial bone development, repair and regeneration. Nat Commun 2016; 7:10526.

23 d'Aquino R, De Rosa A, Laino G, Caruso F, Guida L, Rullo R, *et al.* Human dental pulp stem cells: from biology to clinical applications. J Exp Zool B Mol Dev Evol 2009; 312B:408–415.

24 Mohammed Eman EA, Mohamed E-Z, Abdel Aziz Nahla N, Nourhan A-S, Marwa M, Abdek Aleem KA. The role of donor's age on the proliferation and osteogenic differentiation of human dental pulp stem cells. Curr Sci Int 2018; 7:680–689.

25 Karbanova J, Soukup T, Suchanek J, Mokry J. Osteogenic differentiation of human dental pulp-derived stem cells under various ex-vivo culture conditions. Acta Med (Hradec Kralove) 2010; 53:79–84.

26 Laino G, d'Aquino R, Graziano A, Lanza V, Carinci F, Naro F, *et al.* A new population of human adult dental pulp stem cells: a useful source of living autologous fibrous bone tissue (LAB). J Bone Miner Res 2005; 20:1394–1402.

27 Bakopoulou A, Leyhausen G, Volk J, Tsiftsoglou A, Garefis P, Koidis P, Geurtsen W. Comparative analysis of in vitro osteo/odontogenic differentiation potential of human dental pulp stem cells (DPSCs) and stem cells from the apical papilla (SCAP). Arch Oral Biol 2011; 56:709–721.

28 d'Aquino R, De Rosa A, Lanza V, Tirino V, Laino L, Graziano A, *et al.* Human mandible bone defect repair by the grafting of dental pulp stem/progenitor cells and collagen sponge biocomplexes. Eur Cell Mater 2009; 18:75–83.

29 Mohammed EEA, El-Zawahry M, Farrag ARH, Aziz NNA, Sharaf-ElDin W, Abu-Shahba N, *et al.* Osteogenic differentiation potential of human bone marrow and amniotic fluid-derived mesenchymal stem cells in vitro & in vivo. Open Access Maced J Med Sci 2019; 7:507–515.

30 Song IH, Caplan AI, Dennis JE. In vitro dexamethasone pretreatment enhances bone formation of human mesenchymal stem cells in vivo. J Orthop Res 2009; 27:916–921.

31 El-Backly RM, Massoud AG, El-Badry AM, Sherif RA, Marei MK. Regeneration of dentine/pulp-like tissue using a dental pulp stem cell/poly(lactic-co-glycolic) acid scaffold construct in New Zealand white rabbits. Aust Endod J 2008; 34:52–67.