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Original Article

Enhancement of bone healing through the direct injection of osteogenic and maintenance media concurrent with hydroxyapatite in a rat model of radial bone defects

Jannesar, A. M.[1]; Bigham Sadegh, A.[2*]; Oryan, A.[3]; Tabatabaie Naeini, A.[2] and Khanbazi, M. H.[1]

[1]Ph.D. Student in Veterinary Surgery, Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [2]Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [3]Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

[*]***Correspondence:*** *A. Bigham Sadegh, Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran. E-mail: dr.bigham@gmail.com*

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Abstract

Background: Healing critical bone defects remains a major challenge for orthopedic surgeons. Numerous studies have explored ways to enhance bone regeneration. **Aims:** This study aimed to evaluate the healing potential of osteogenic and maintenance cell culture media when applied directly to the defect site, either alone or in combination with hydroxyapatite (HA) granules. **Methods:** Seventy male Wistar rats were divided into seven groups: empty defect (n=10, negative control), autograft (n=10, positive control), HA (n=10), osteogenic medium (OM) (n=10), maintenance medium (MM) (n=10), MM+HA (n=10), and OM+HA (n=10). A 5 mm radial diaphyseal gap was created as a critical-sized defect. Culture media were injected transcutaneously into the gap on the fourth day post-surgery. Radiological evaluations were conducted on postoperative days 14, 28, 42, and 56. Histopathological assessments were performed on days 28 and 56. **Results:** OM and MM, both alone and combined with HA, demonstrated significant bone healing capacity. Radiologically, all treatment groups showed superior healing compared with the negative control. Bone regeneration in the OM group was comparable to the autograft group on days 42 and 56. Histopathologically, all groups exhibited advanced healing on days 28 and 56, except the HA group. Notably, bone healing in HA+OM and HA+MM on day 28, and in OM and MM on both days 28 and 56, was similar to that observed with autografts. **Conclusion:** Osteogenic and maintenance media exhibit promising bone regenerative properties and may serve as effective treatments for bone defects, provided that suitable delivery and support systems are developed.

Key words: Bone healing, Critical bone defect, Hydroxyapatite, Osteogenic medium, Rat model

Introduction

Despite the massive intrinsic regenerative capacity of bones, healing of the large bone defects is still a serious challenge for orthopedic surgeons (Verrier *et al*., 2016). Situations such as bone tumors or infections and intensive traumas cause abundant bone loss along with massive damage to the surrounding vascular supply and soft tissues, which in fact jeopardizes the chances of complete healing, leading to delayed unions and nonunions (Verrier *et al*., 2016). Although bone grafting remains the first-line treatment for such conditions, it is associated with several limitations, including limited availability, risk of infection, donor site morbidity, potential disease transmission, immune reactions, and the fact that the procedure can be time-consuming and costly (Shafiei *et al*., 2009; Marsell and Einhorn, 2011; Miller and Chiodo, 2016; Attia *et al*., 2022).

Classically, the bone healing process is either primary or secondary, although unlike soft tissues, no scar formation is expected (Marsell and Einhorn, 2011). Fractures treated with open reduction and rigid internal fixation, where the gap is completely obliterated, are expected to undergo direct bone healing or primary union (Marsell and Einhorn, 2011; Cottrell *et al*., 2016). Secondary healing, on the other hand, is dependent on callus formation and is the most common form of healing, since it occurs in nonoperative fractures or those treated with external or not fully rigid internal fixations, in which micro-motions remain and enhance endochondral ossification (Marsell and Einhorn, 2011; Cottrell *et al*., 2016). In the first few hours following the

trauma, hematoma forms and acute inflammation occurs. The blood clot acts as a matrix for inflammatory cells, fibroblasts and endothelial cells. This acute inflammatory phase lasts about 4 days. The inflammatory cells, release a wide range of growth factors and cytokines which ultimately begin the healing process. As a result, the pluripotent mesenchymal stem cells migrate into the fracture site leading to the next phase of healing known as fibroplasia or proliferative phase. During this phase, in the presence of glycosaminoglycans such as hyaluronic acid, dermatan sulfate, chondroitin sulfate and others and collagen type III, a soft callus begins to form gradually. In mice, the above-mentioned soft callus peaks formation 7-9 days after initial trauma. Cartilage is then gradually replaced by the woven bone through endochondral ossification process and a hard callus is formed. Ultimately, it takes months or even years for the newly formed bone to be fully remodeled and be replaced by lamellar bone and finally take back its original shape (Marsell and Einhorn, 2011; Oryan *et al*., 2015; Cottrell *et al*., 2016).

To date, autografts still remain the gold standard in healing of bone defects (Oryan *et al*., 2015). However, due to the existing limitations, numerous studies have been conducted to enhance the regenerative capacity of bone tissue. To do so, the use of platelet rich plasma alone or combined with other materials such as hydroxyapatite (Oryan *et al*., 2012; Oryan *et al*., 2016; Jamal *et al*., 2022), tissue engineered scaffolds along with collagen/chitosan/hydroxyapatite (Roddy *et al*., 2018), osteogenic cells (Shibli *et al*., 2022) and even 3D printing techniques (Mayfield *et al*., 2022) have all been investigated. Baring no surprises, each option has its own advantages and disadvantages, since for a defective bone to be ideally reconstructed, any material or tissue-engineered construct used to enhance bone reconstruction should be able to mimic the body's natural healing capacity (Roddy *et al*., 2018).

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is the main component of hard bony tissue and is available in both synthetic and natural forms (Yang *et al*., 2018). It is highly biocompatible and has great osteoconductivity and osteoinductivity properties, making it an ideal option for tissue-engineering purposes (Lin *et al*., 2009). As it stands, hydroxyapatite (HA) can promote bone healing without local or systemic toxicity, inflammation or immune response (O'Hare *et al*., 2010; Sadat-Shojai *et al*., 2013; Liu *et al*., 2023). Apparently, hydroxyapatite has gene regulatory properties, such as ALP, collagen type I and osteocalcin genes; thus, it aids in differentiation of mesenchymal cells and proliferation of osteoblasts and osteoinduction (Lin *et al*., 2009; Borkowski *et al*., 2023). As of now, hydroxyapatite has had various biomedical applications including as an osseous defect substitute (Trombelli *et al*., 2010; Ji *et al*., 2020), a middle ear implant (Ye *et al*., 2001), in tissue-engineering systems (Lv *et al*., 2009; Seol *et al*., 2009), as a drug delivery system (Munir *et al*., 2021; Fan *et al*., 2022), in dental materials (Rajewska *et al*., 2023) and as a bioactive coating in bone implants (Blackwood and Seah, 2009). Several studies have shown the effect of HA usage in bone healing enhancement and/or mesenchymal stem cells (MSCs) differentiation (Shafiei-Sarvestani *et al*., 2012; Persson *et al*., 2018; Sattary *et al*., 2019; Bigham-Sadegh *et al*., 2020; De Luca *et al*., 2020).

Proper culture media is able to differentiate mesenchymal stem cells (MSCs) into osteoblasts, thus osteogenic medium (OM) which usually contains ascorbic acid, glycerol phosphate, sodium pyruvate, fetal bovine serum (FBS), dexamethasone along with glutamine and antibiotics is commonly used *in vitro* (Asti *et al*., 2010; Buttery *et al*., 2011; Ghali *et al*., 2015; Ciuffreda *et al*., 2016). In 2014, a study was conducted to evaluate the effect of direct application of OM into critical radial bone defects in rabbits, which in fact showed that OM enhanced the osteogenic capacity promisingly (Oryan *et al*., 2014). Formerly, the osteogenic medium and maintenance medium have been used several times to differentiate adipose-derived stem cells into osteoblasts to be used in enhancement of bone healing afterwards (Dudas *et al*., 2006; Yoon *et al*., 2007; Di Bella *et al*., 2008; Lin *et al*., 2013; Sandor *et al*., 2014; Lee *et al*., 2016; Li *et al*., 2022). Even co-application of the maintenance medium with omentum has been shown to have superior osteogenic properties compared with omentum alone (Bigham-Sadegh *et al*., 2012). Another study has shown that OM has been potentially more efficient in differentiation of human adipose stem cells into bone forming cells in a 3D culture than growth factors (Tirkkonen *et al*., 2013). In an *in vitro* study, Yang *et al*. (2018) have investigated the effect of hydroxyapatite nanoparticles in differentiation of human mesenchymal stem cells (hMSCs) towards osteoblasts. Thus, the mesenchymal cells were moved into an osteogenic medium after being cultured on the maintenance medium and were treated with HA nanoparticles first. Alkaline phosphatase (Ciuffreda *et al*., 2016) activity measurement, ALP staining, immunofluorescent staining for osteopontin, and real-time polymerase chain reaction (RT-PCR) examinations showed that the osteogenic differentiation of hMSCs was significantly improved (Yang *et al*., 2018). Therefore, the present study was designed to evaluate the effect of the concurrent direct application of osteogenic medium (OM) and hydroxyapatite (HA) on bone healing in experimentally induced critical-sized bone defects in rats. The underlying hypothesis was that the combined use of these materials could synergistically enhance bone regeneration by providing both osteoinductive and osteoconductive support.

Materials and Methods

Animals and surgical procedure

All animals received humane care in compliance with the Guide for Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1985). The study was approved by the local Ethics Committee of Shiraz

Table 1: Experimental grouping system

Treatment	Negative control	Positive control	HA	MM+HA (injection on day 4)	OM+HA (injection on day 4)	MM (injection on day 4)	OM (injection on day 4)
Autograft		•					
Hydroxyapatite			•	•	•		
Maintenance medium				•		•	
Osteogenic medium					•		•

University.

Seventy male Wistar rats, eight weeks old, weighing 200±20 g, were kept in separate cages and fed standard diet for one week. The animals were randomly divided into seven separate groups: the empty defect group (n=10, negative control), autograft group (n=10, positive control), HA group (n=10) [Industrial Hydroxyapatite granules (Reprobone®, England, UK); size of the granules: 4 mm], osteogenic medium (OM) group (n=10) [(OsteoPlus®, Tehran, Iran), it is maintenance medium combined with L-ascorbic acid 2-phosphate (50 μM), dexamethasone (100 nM), sodium pyruvate and β-glycerophosphate (10 mM)], maintenance medium (MM) group (n=10) [Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY), containing 10% FBS, 1% glutamine and 100 μg/ml streptomycin and 100 U/ml penicillin], MM+HA group (n=10) and OM+HA group (n=10) (Table 1). The animals were anesthetized with intramuscular injection of 10% ketamine (100 mg/kg) and 2% Xylazine hydrochloride (10% mg/kg). Hydroxyapatite (HA) granules were sterilized using the dry heat method. They were wrapped in surgical drapes and placed in a sterilization oven, where they were heated to 180°C for approximately 2 h. Presurgical preparations were done routinely on left forelimbs. A 1 cm incision was made in the craniomedial side of the radius and the soft tissues were dissected bluntly to expose the radial bone. A 5 mm diaphyseal defect was made osteoperiosteally, since the length of the defect should be at least twice the diameter of the bone to be considered a critical defect, clinically (Bolander and Balian, 1986). The defect was left empty and/or filled with autologous bone (harvested from the radial bone extracted for the critical gap) in the negative control and the positive control groups, respectively. The OM and MM groups were also left empty and the defects were filled directly with 0.5 ml of osteogenic medium and/or maintenance medium by transcutaneous injection, on the 4th day post-surgically, respectively. The other groups were filled by HA. The culture media were injected into the defect, in the OM+HA and MM+HA groups, as explained earlier. The incision was then closed routinely and the animals were housed in compliance with our institution's guiding principles "in the care and use of the animals".

Post-operative evaluations

Radiological assessment

To evaluate the bone formation, union and remodeling, radiographs were taken on the day of the surgery and on the 14th, 28th, 42nd and 56th days postoperatively. Subsequently, the results were scored in a blinded manner, using modified Lane and Sandhu (1987) scoring system (Table 2).

Table 2: Radiographical findings for healing of the bone defect

Radiological finding	Score
Bone formation	
No evidence of bone formation	0
Bone formation occupying 25% of the defect	1
Bone formation occupying 50% of the defect	2
Bone formation occupying 75% of the defect	3
Bone formation occupying 100% of the defect	4
Union (proximal and distal ends evaluated separately)	
No union	0
Possible union	1
Radiographical union	2
Remodeling	
No evidence of remodeling	0
Remodeling of the medullary canal	1
Full remodeling of the cortex	2
Total possible points per category	
Bone formation	4
Proximal union	2
Distal union	2
Remodeling	2
Maximum score	10

Table 3: Emery's histopathological scoring system

Histopathological finding	Score
Empty gap	0
Gap filled only with fibrous connective tissue	1
Gap filled with fibrous connective tissue more than fibrocartilage	2
Gap filled with fibrocartilage more than fibrous connective tissue	3
Gap filled only with fibrocartilage	4
More fibrocartilage than bone	5
More bone than fibrocartilage	6
Gap only filled with bone	7

Histopathological assessment

Half of the animals in each group were euthanized and radial specimens were prepared for histopathological evaluation, on the 28th and 56th post-surgical days. The specimens were obtained and fixed in 10% neutral buffered formalin. Routine histopathological preparation was done. Longitudinal sections were obtained and stained with Hematoxylin & Eosin, so as to have the defect along with both distal and proximal fracture pieces in one plane. The sections were then blindly assessed and scored by two pathologists according to Emery's scoring system (Emery *et al*., 1994) (Table 3).

Statistical analysis

The radiological and histopathological data were compared with Kruskal-Wallis, non-parametric ANOVA, having P-values less than 0.05, then the Mann-Whitney U-test was performed for pairwise group

Table 4: Radiographical findings for healing of the bone defect (sum of the scores) in various intervals

Post-operative days	Med (min-max)							P[a]
	Untreated (n=10)	Autograft (n=10)	HA (n=10)	MM (n=10)	OM (n=10)	HA+MM (n=10)	HA+OM (n=10)	
14	0 (0-2)[b]	6 (3-7)	2 (0-3)	3 (0-5)	2 (0-5)	3 (2-4)	3 (0-7)	0.00
28	1 (0-2)[b]	6 (5-8)	3 (2-6)	5 (1-7)	3 (2-6)	3 (1-5)	3 (0-7)	0.00
42	1 (1-3)[b]	8 (8-10)[c]	3 (3-6)	3 (2-6)	5 (4-9)	4 (3-6)	5 (2-6)	0.001
56	1 (1-3)[b]	9 (9-10)[c]	5 (5-8)	6 (5-8)	8 (5-10)	4 (3-6)	6 (4-8)	0.001

Significant P-values are in bold face. [a] Kruskal-Wallis non-parametric ANOVA, [b] Compared with autograft, HA, MM, OM, HA+MM and HA+OM by Mann-Whitney U-test. There was a significant difference (P<0.05) between the untreated group and all the other groups in 14th, 28th, 42nd and 56th days post-operation, except for the HA group in 42nd post-operative day (P>0.05), and [c] Compared with OM group by Mann-Whitney U-test. There was not a significant difference in 42nd and 56th days post-operation (P>0.05)

Table 5: Histopathological findings for healing of the bone defect (sum of the scores) in different intervals

Post-operative days	Med (min-max)							P[a]
	Untreated (n=5)	Autograft (n=5)	HA (n=5)	MM (n=5)	OM (n=5)	HA+MM (n=5)	HA+OM (n=5)	
28	3 (3-4)[b]	5 (4-6)	4 (3-5)	6 (5-6)[d, e]	5 (4-5)[d]	5 (4-5)[f]	5 (5-6)[f]	0.05
56	4 (3-5)[b]	6 (6-7)	5 (4-5)[c]	6 (5-7)[d]	6 (5-6)[d]	5 (5-6)[g]	6 (5-6)[g]	0.01

Significant P-values are in bold face. [a] Kruskal-Wallis non-parametric ANOVA, [b] Compared with autograft, HA, MM, OM, HA+MM and HA+OM by Mann-Whitney U-test. There was a significant difference (P<0.05) between the untreated group and all the other groups in 28th and 56th days post-operation, except for the HA group, [c] Compared with autograft MM, OM and HA+OM by Mann-Whitney U-test. Bone healing was significantly inferior in HA group (P<0.05), [d] Compared with autograft group by Mann-Whitney U-test. There was no significant difference in 28th and 56th post-operative days (P>0.05), [e] Compared with HA and OM groups by Mann-Whitney U-test. MM group showed significantly superior healing in 28th day post-operation (P<0.05), [f] Compared with autograft group by Mann-Whitney U-test. There was no significant difference in 28th day post-operation (P>0.05), and [g] Compared with autograft group by Mann-Whitney U-test. Autograft group showed superior bone healing capacity in 56th post-operative day (P<0.05)

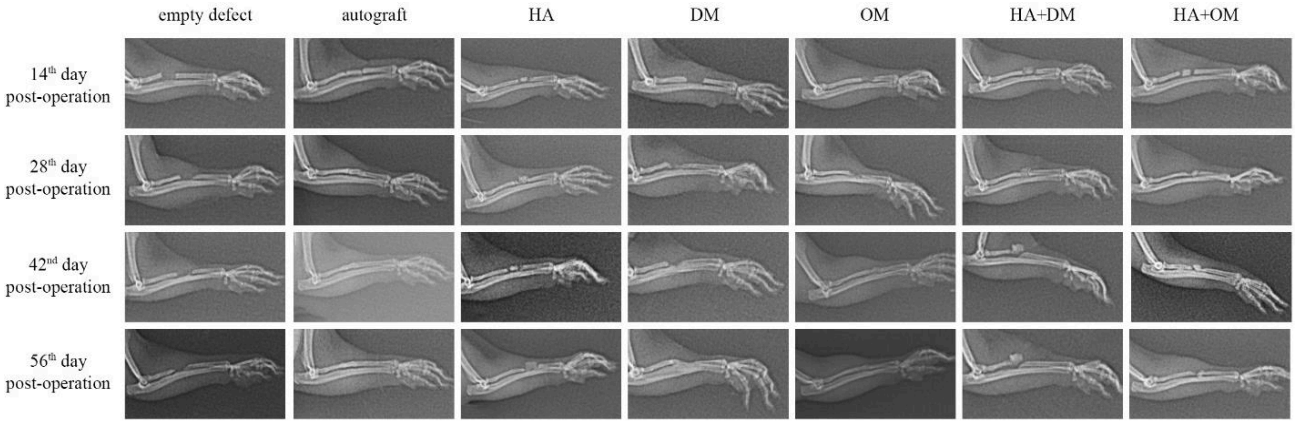

Fig. 1: Lateral radiographs of radial defects of animals of the different groups in 14th, 28th, 42nd and 56th post-operative day. The bone healing was significantly superior in all the groups compared with the untreated group (P<0.05), except for the HA group in 42nd post-operative day (P>0.05). There were no significant differences between OM group and autograft in 42nd and 56th days post-operation (P>0.05)

comparisons.

Results

Radiological findings

In terms of evaluation, there was a significant difference in the radiologic scores between the untreated group and all other experimental groups in all weeks except for the HA group on the 42nd post-operative day (P<0.05). Bone healing was also significantly superior in the autograft group in all weeks compared with other groups (P<0.05), except for the OM group on the 42nd and 56th days post-operation (P>0.05). No significant difference was found between the HA, MM, OM, MM+HA and OM+HA groups (P>0.05), although bone formation was superior in the OM & MM groups compared with the other groups in the 42nd and 56th days post-operation (Table 4 and Fig. 1).

Histopathological findings

There was a significant difference in the histopathological scores between the untreated group and

all the other groups in the 28th and 56th post-operative days (P<0.05), except for the HA group which did not show a significant difference compared with the untreated group. Statistical evaluations of the autograft group indicated that no significant difference was seen between the autograft group and the two experimental groups of HA+OM and HA+MM (P>0.05), in the 28th post-operative day, while bone healing was significantly superior in autograft group (P<0.05), in the 56th post-surgical day. On the other hand, bone healing was not significantly superior in the autograft group compared with the OM and MM groups in either 28th or 56th days post-operation (P>0.05). Significant differences were observed between the HA and MM groups, and MM and OM groups (P<0.05) in the 28th post-surgical day, with the MM group being superior in both comparisons. Bone healing was significantly superior in the MM, OM and HA+OM groups compared with the HA group (P<0.05), in the 56th day post-operation (Table 5, Figs. 2A-G and 3A-G).

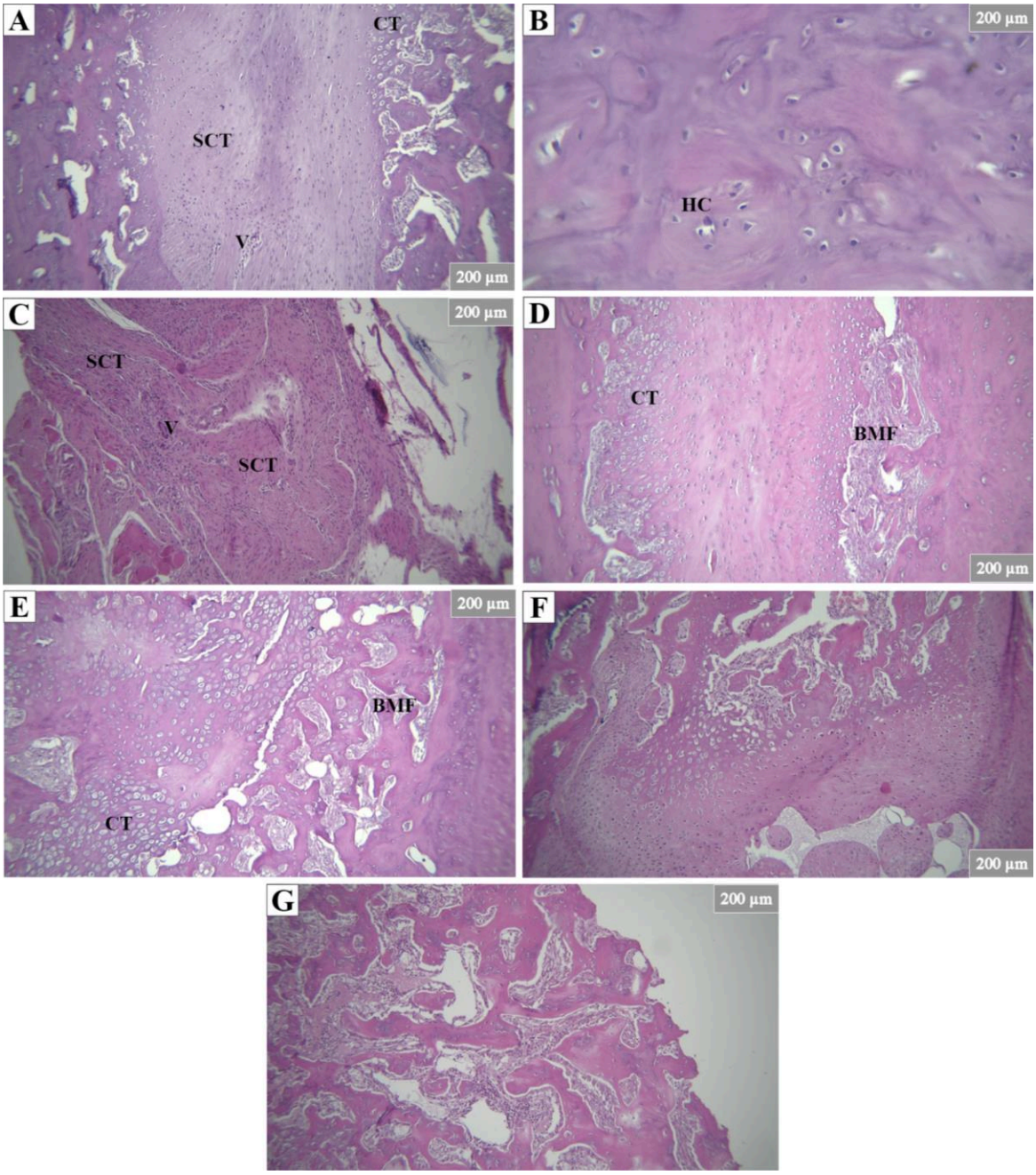

Fig. 2: Histopathological sections of the injury site in animals of different groups in the 28th post-operative day. (**A**) Untreated group: Soft connective tissue is the main core and blood vessels can be seen, (**B**) Autograft: The haversian canals are forming and bone is the main tissue in the defect, (**C**) HA: The defect is mostly filled with SCT, (**D**) DM: Cellularity is diminishing and bone marrow formation can be seen, (**E**) OM: The defect is mostly filled with bone and cartilage and SCT is not spotted, (**F**) HA+DM: Cartilaginous tissue is seen along with bone marrow formation, and (**G**) HA+OM: Bone marrow is forming and the gap is mainly filled with bone. SCT: Soft connective tissue, CT: Cartilaginous tissue, V: Blood vessel, HC: Haversian canal, and BMF: Bone marrow formation (H&E; scale bars A-G: 200 µm)

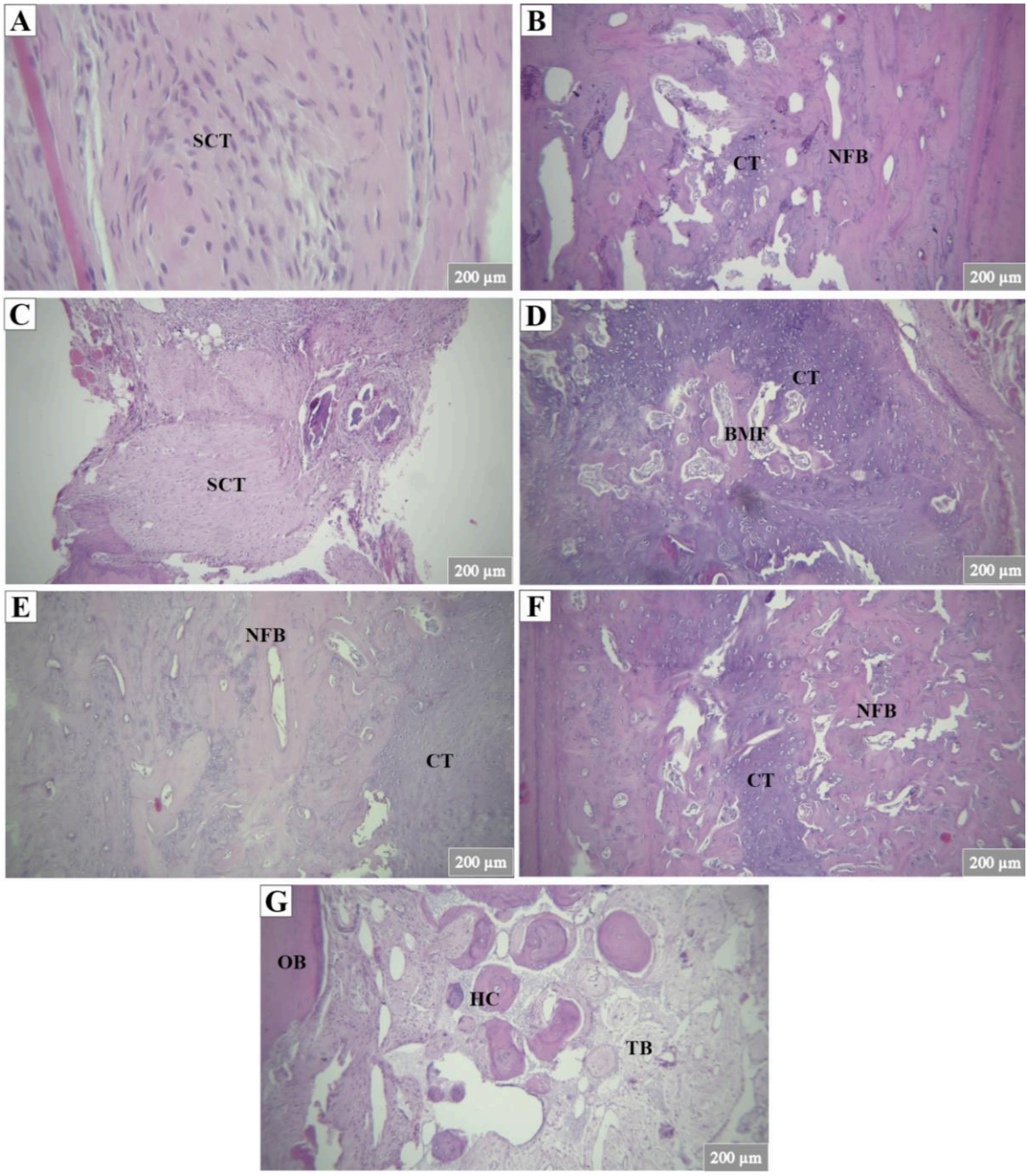

Fig. 3: Histopathological sections of the injury site in animals of different groups in the 56th post-operative day. (**A**) Untreated group: The defect is filled with SCT and fibrocytes and fibroblasts can be seen, (**B**) Autograft: Bone formation and cartilaginous tissue can be seen with no SCT, (**C**) HA: The defect is mostly filled with SCT and to lesser extent with bone, (**D**) DM: BMF and cartilage can be seen, (**E**) OM: The newly formed bone can be seen along with cartilaginous tissue, (**F**) HA+DM: The defect is mostly filled with bone and cartilage and cellularity is considerabily diminished, and (**G**) HA+OM: No SCT is seen and the defect is filled with bone and the haversian canals and trabecular bone are detectable. Abbreviations: SCT: Soft connective tissue, CT: Cartilaginous tissue, NFB: Newly formed bone, HC: Haversian canal, BMF: bone marrow formation, OB: Old bone, and TB: Trabecular bone (H&E; scale bars A-G: 200 µm)

The gap was mostly filled with soft connective tissues and less with cartilage, in the untreated group, in the 28th day post-operation. No bone formation was seen in the lesions of the animals of the untreated group, at this stage and the main cells were fibroblasts, fibrocytes and few chondrocytes. Vascular buds could also be seen, at this stage. Bony tissue was the main core in the lesions of the autograft group, at this stage. Cartilaginous tissue was on the verge of transforming to bone and haversian canals were being formed in the lesions of the animals of this group. The main cells in the autograft lesions were osteoblasts, chondroblasts and few fibrocytes at this stage. Soft connective tissue was seen more than cartilaginous tissues, in the defects of the HA treated group. The main cells were fibrocytes and fibroblasts, however, inflammatory cells, including macrophages and

few neutrophils, could still be seen in the lesions of the animals of this group, at this stage. Bony tissue was on the verge of formation, in some specimens of this group. The gaps were mainly filled with cartilaginous and soft connective tissues. Also, bone formation was started and bone trabeculae could be seen in the lesions of the animals of the HA+MM and HA+OM treated groups. The gaps were primarily filled with bone and cartilage, and the soft connective tissue density was diminishing, in the lesions of the MM group. Bone and cartilage were the main core and soft connective tissue was spotted sparsely, in the defects of the OM group. Also, bone marrow was formed in some tissue sections of the animals of this group (Table 5, Figs. 2A-G and 3A-G).

The gaps were mostly visible and filled with soft connective tissue, in the untreated group, on the 56th day post-operation. Cartilage and non-compact bone were formed in the extremities of the gap. Vascularization could also occasionally be seen in the lesions of the animals of this group. The gaps were mostly filled with non-compact bone and some degrees of cartilage in the lesions of the autograft group. Soft connective tissue was rarely seen. Bone marrow and haversian canals were formed. The gaps were mostly filled with soft connective tissue and cartilage, and rarely with bone, in the HA treated group. Bone formation was mostly seen in the defect extremities of the animals of this group. Cartilage, bone and soft connective tissue were all seen in the gaps of the HA+MM treated group. Cancellous bone was also formed in the lesions of the animals of this group. Bone formation could mostly be seen, with bone marrow being formed, in the HA+OM treated group. Soft connective tissue was rarely spotted in the defective area of the animals of this group. The gaps in the MM group were completely filled with mostly bony tissue followed by cartilaginous tissue. Soft connective tissue was rarely seen, in the lesions of the animals of this group. The gaps were mostly filled with bone and cartilage, while soft connective tissue was rarely spotted, in the lesions of the animals of the OM group. Bone marrow was also formed in the defects of the animals of this group. In some animals of this group, soft connective tissue was being directly transformed into the bone (Table 5, Figs. 2A-G and 3A-G).

Discussion

The aim of this study was to evaluate and compare the effectiveness of two types of cell culture mediums, namely osteogenic and maintenance, when applied directly into a bone defect gap. The evaluation was conducted individually as well as in combination with Hydroxyapatite granules, to assess their ability to promote healing. Formerly, the osteogenic and maintenance medium had been applied in radial critical bone defects directly and showed promising osteogenic capacity (Oryan *et al*., 2014). In this study, we hypothesized that the addition of HA to the osteogenic and maintenance media could potentially act as an osteoconductive agent and result in better bone healing capabilities. We chose to create a radial bone defect in rats as it does not require any external or internal fixation due to the inherent splintage provided by the ulna (Kim and Kim, 2013). After eight weeks, no unions were seen in the untreated group, indicating that the gap was completely critical.

The inflammatory stage of the healing process lasts approximately four days. Following this, the fibroplasia stage begins, during which the hematoma and fibrin clot serve as a natural scaffold for cellular infiltration and mediator activity (Marsell and Einhorn, 2011). Therefore, the media were injected into the hematoma on the fourth postoperative day. Both radiological and histopathological evaluations demonstrated that the OM and MM groups exhibited superior bone healing characteristics. Radiographically, no significant difference was observed between the autograft and OM groups on postoperative days 42 and 56, highlighting the strong regenerative potential of OM. Furthermore, histopathological analysis revealed that all four groups treated with OM and MM showed healing capacities comparable to the autograft group, which served as the optimal standard in this study. Specifically, the defects in the OM and MM groups were predominantly filled with bone, followed by cartilage, with minimal presence of soft connective tissue and a markedly reduced inflammatory cell count. In contrast, the untreated group showed no evidence of bone formation, and union was absent in both radiological and histopathological assessments.

Although treatment with HA showed positive efficacy in bone healing improvement, its effect was not as promising as OM and MM groups. Many other studies had previously declared HA osteoinductive and osteoconductive capacity (Lin *et al*., 2009; Bigham-Sadegh *et al*., 2020; Khotib *et al*., 2021). As previously mentioned, the primary objective of this study was to use hydroxyapatite (HA) primarily for its osteoconductive properties and to serve as a scaffold for the culture media. Although radiological assessments in the HA group indicated enhanced bone healing compared with the untreated group, these findings were not corroborated by histopathological evaluations. Moreover, the concurrent application of HA with culture media did not result in superior healing outcomes compared with the use of culture media alone. In the HA group, pronounced inflammatory responses persisted within the defect site, accompanied by increased vascularization and predominant filling of the gap with soft connective tissue. These observations suggest that HA alone is insufficient to effectively promote bone regeneration in critical-sized defects.

Moreover, in both HA+OM and HA+MM groups, histological healing quality was comparable to that of the autograft group only up to the fourth postoperative week. Beyond this point, healing in these groups declined significantly. This suggests that sustained the availability of the culture media within the defect site is a critical factor for optimal bone regeneration. In this study, HA

did not function effectively as a conductive scaffold capable of maintaining the presence of the media throughout the defect, thereby limiting their therapeutic potential. In contrast, histopathological evaluations of the OM and MM groups revealed significantly enhanced bone healing on both days 28 and 56 post-surgery compared with the untreated group, reinforcing the conclusion that HA alone could not facilitate the full effectiveness of the culture media. Importantly, no signs of intolerance, immune response, or inflammatory reaction related to the application of the culture media were observed in gross examinations or histopathological analyses.

The maintenance medium that was used in the present study, was the DMEM culture media which is normally used for cell culturing purposes *in vitro*, and contains 10% FBS, 1% glutamine along with antibiotics. The osteogenic medium used *in vitro* to promote bone regeneration from the mesenchymal stem cells, is a combination of DMEM with L-ascorbic acid 2-phosphate, dexamethasone, sodium pyruvate and β-glycerophosphate. The promising bone healing capacity of these mediums is thought to be related to their contents. It has been shown that the intravenous injection of glutamine resulted in quicker and more regular bone healing and enhanced cartilaginous tissue formation, in rats (Polat *et al*., 2007). These positive effects were thought and shown to be related to the role of glutamine in the attainment of positive nitrogen balance. Recently, a glycerol phosphate containing hydrogel was developed for tendon-bone healing purposes, resulting in early healing and increased total bone volume (Huang *et al*., 2020). Also, it was shown that beta-glycerophosphate accelerated calcification in the cultured bovine vascular smooth muscle cells (Shioi *et al*., 1995). According to another study, beta-glycerophosphate modifies key parameters of mitochondrial function and cellular bioenergetics in vascular smooth muscle cells through increasing basal respiration and mitochondrial ATP production (Alesutan *et al*., 2020). It is shown that beta-glycerophosphate provides an external phosphate source, thereby facilitating the mineralization of the extracellular matrix (Schack *et al*., 2013). It also upregulates the expression of late osteoblastic markers (Yevlashevskaya *et al*., 2023). Recently, the bone marrow mesenchymal stem cells (BMSCs) treated by L-ascorbic acid, a well-known antioxidant, were used for wound healing purposes. The results indicated that the wounds by ascorbic acid treated BMSCs resulted in a better angiogenesis and more collagen fiber deposition in the wound bed (Yi *et al*., 2022). Besides, it has been shown that the presence of L-ascorbic acid 2-phosphae significantly enhanced human skin fibroblasts (Hata and Senoo, 1989), emphasizing that cell proliferation and collagen synthesis were significantly influenced. Another study investigated the effect L-ascorbic acid on the growth and differentiation of human osteoblast-like cells, showing that collagen synthesis and alkaline phosphatase activity were positively influenced (Takamizawa *et al*., 2004). Hinoi *et al.* (2002) showed that pyruvate deficiency had led to cell death in proliferative cultured osteoblasts. The results of their study suggest that pyruvate has a critical role in cell growth in the proliferative stage of healing. Also, more studies have shown promising antioxidant effect of pyruvate and its role in the prevention of cell damage and necrosis (Moriguchi *et al*., 2006; Alvarez-Elizondo *et al*., 2019). Dexamethasone is also shown to promote osteoblastic differentiation of vascular smooth muscle cells *in vitro* (Mori *et al*., 1999). Carpenter *et al*. (2010) cultured equine bone marrow-derived mesenchymal stem cells in dexamethasone supplemented culture media and the results showed better osteoblastogenic differentiation. It has also been demonstrated that dexamethasone has positive effects on the cellular differentiation of bone potentially (Langenbach and Handschel, 2013; Gasson *et al*., 2021). Morsczeck and Reichert (2017) have also investigated the effect of dexamethasone in osteogenic differentiation of the dental follicle cells. Dexamethasone binds to specific receptors on cells related to bone formation, triggering signaling pathways that improve cell proliferation, differentiation, and migration as well as angiogenesis (Mai *et al*., 2023). One key pathway through which dexamethasone exerts its positive effects involves the activation of SMAD proteins which play an important role in osteogenic differentiation (Yuasa *et al*., 2015). FBS which is present in both culture mediums contains various growth factors and extracellular molecules which are of great benefit in osteogenic proliferation and differentiation (Martin *et al*., 2005; Sundin *et al*., 2007). Therefore, the great capacity of osteogenic media in bone healing is thought to be related to these biomaterials. However, there are a lot to investigate about the exact cellular mechanisms and pathways by which the above-mentioned biomaterials and stimulating factors exert their function and effects *in-vivo*.

Osteoblasts are formed through the differentiation of mesenchymal stem cells (MSCs) from a fibroblastic to a cuboidal shape, then they form the extracellular bone matrix (ECM). ECM is classically divided into two parts, the protein-like part and mineralized part. Osteoblasts themselves are in charge of the formation of these two parts and secreting them to the extracellular area. After the completion of bone matrix, some of the osteoblasts are entrapped and turned into osteocytes. Osteoblasts and young osteocytes have an important role in secreting the protein-like part of ECM (aka osteoid), which consists of different types of collagen fibers. Two of the most important proteins, which are of high importance and necessity in the rate of bone formation, are ALP and osteocalcin. The expression of both ALP and osteocalcin is strictly bounded to the osteoblasts activity. These organic materials finally serve as a template for the formation and mineralization of hydroxyapatite (Vater *et al*., 2011; Bellido *et al*., 2014; Camal Ruggieri *et al*., 2021). As mentioned earlier, ALP has an important role in the deposition of HA along the collagen fibers. As described above, L-ascorbic acid significantly increases ALP activity and collagen synthesis. There is a high rate

of osteoblastic apoptosis, indicating that only some of the mature osteoblasts will finally mature into osteocytes (Bellido *et al*., 2014). Thus, the role of pyruvate in preventing cellular damage might be related to higher osteocyte maturation rate following the application of OM, in the present study. The superior bone healing observed in the OM and MM groups suggests that these media may actively support key cellular and molecular mechanisms involved in bone regeneration. OM likely promotes osteoinduction by enhancing the differentiation of mesenchymal stem cells into osteoblasts, while MM may help maintain a favorable microenvironment for sustained cellular activity and matrix deposition. Histopathological findings—such as increased densities of chondroblasts, chondrocytes, osteoblasts, and osteocytes—indicate that both media facilitate progression through the endochondral ossification pathway. The reduced inflammatory cell count and minimal soft tissue infiltration further suggest that OM and MM may modulate the local immune response, potentially accelerating the transition from inflammation to repair. Although the exact molecular pathways remain to be elucidated, these results point to a synergistic interplay between cellular maturation, scaffold-independent signaling, and immunomodulation as key contributors to the observed regenerative outcomes.

Based on the radiological findings of this study, the primary efficacy of osteogenic medium (OM) application became evident during the later stages of the healing period, particularly at 56 days post-operation. The greatest similarity between the OM and autograft groups was observed on postoperative days 42 and 56, suggesting that OM plays a critical role in long-term bone regeneration. This distinction was especially notable when compared with the untreated and HA-only groups. Histopathological analysis further revealed that the density of chondroblasts, chondrocytes, osteoblasts, and osteocytes was higher in the OM and MM groups compared with those treated with HA in combination with culture media on day 56. Whether this enhanced healing capacity is due to more advanced osteoblastic maturation into osteocytes, the increased expression of specific proteins, or a combination of these and other mechanisms remains to be elucidated. However, the findings of this study indicate that co-application of HA with culture media did not significantly enhance these regenerative processes.

In the recent years, there have been massive research and development regarding bone tissue engineering. In order to enhance bone healing, several biomaterials and composite materials have been used and various scaffolds have been developed. Several structural and biological features must be considered for a scaffold to properly and efficiently enhance bone healing. An ideal and suitable scaffold for bone tissue engineering should maintain and enhance cell viability, cell proliferation, osteogenic differentiation and even load bearing if needed (Roseti *et al*., 2017). Also, other criteria such as biodegradability, bioactivity, porosity, non-immunogenicity and ability to maintain a sustained release properties are important characteristics in the selection of a scaffolds to be used as a bone implant (Roseti *et al*., 2017). In so doing, a proper scaffold can apply osteoinduction and/or osteoconduction for bone healing purposes (Marsell and Einhorn, 2011; Cottrell *et al*., 2016). In other words, the more a scaffold resembles the biology and biodynamics of the bone tissue, the more it could be integrated into bone healing properties (Roseti *et al*., 2017). The use of different forms of hydroxyapatite in scaffolds has been vastly investigated and various scaffolds have been developed to date (Oliveira *et al*., 2017). Although osteoinductivity and osteoconductivity of HA has previously been well demonstrated by others (Lin *et al*., 2009; Oliveira *et al*., 2017), the results of the present study did not particularly show significant positive findings regarding its usage, as discussed earlier. However, structural and topographical factors are imminent. Thus, it seems that the predevelopment of a HA-integrated scaffold, which is able to provide sustained release and maximal availability and effectiveness along with mechanical support, can be investigated to better and more efficiently exert osteogenic capacities of OM for bone healing purposes. We acknowledge that the lack of a carrier system such as a hydrogel may have limited the duration of exposure and spatial stability of the media, particularly in the HA co-application groups. This could partly explain the reduced efficacy observed when HA was combined with the media, as HA alone did not provide sufficient structural support to retain the liquid phase. Future studies should explore the use of biocompatible carriers—such as hydrogels or composite scaffolds—that can enhance retention, prolong release, and potentially improve the regenerative performance of culture media. Despite this limitation, the direct liquid application in our model still yielded promising results, especially in the OM and MM groups, which showed healing comparable to autografts.

Apart from the above-mentioned factors, one of the shortcomings of the present study was a lack of sufficient methodological evaluation systems due to financial limitations. Micro-CT analysis of bone volume and density could better delineate the efficacy of osteogenic medium. Also, investigating bone regeneration specific markers could potentially elucidate the specific pathways by which its great osteogenic properties are exerted.

Our study demonstrates that osteogenic and maintenance culture media possess strong osteogenic potential in the healing of critical long bone defects when applied directly in rats, either alone or in combination with hydroxyapatite (HA) granules. Radiological and histopathological findings revealed that both media produced healing outcomes nearly equivalent to those of autografts, which served as the gold standard in this study. These results suggest that osteogenic and maintenance media hold significant promise for the development of effective bone scaffolds, potentially offering a viable solution for developing applicable scaffolds and treating large bone defects in the future.

Conflict of interest

The author of this article/document hereby declares that they have no relevant financial ties, affiliations, or interests, personal or professional, that could be perceived as a potential conflict of interest in relation to the subject matter discussed. All data, statements, and opinions presented are solely those of the author and are not influenced by any third-party organizations or individuals.

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