Original Article

Immunohistochemical Localization for Osteopontin on Healing Process of Intra-Bony Defect Treated by Royal Jelly and Mangosteen in Rats

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Abstract

Background: Osteopontin (OPN) is a noncollagenous glycoprotein that is, extensively phosphorylated and glycosylated and is found in mineralized tissues. It is produced and expressed by a variety of cell types, including osteoblasts and osteocytes. **Objectives:** The goal is to examine the immunohistochemical presence of osteopontin in defect areas of new bone formation and remodeling to investigate how local applications of royal jelly and mangosteen, separately and together, affect bone healing. **Materials and Methods:** Thirty-two albino male rats were used in this study. Intra-bony defects were created in both the right and left femur bones of each rat, and the bone defect was left to heal normally without treatment in the control group. At the same time, the experimental groups were treated with local applications of royal jelly and mangosteen peel extract powder, both separately and in combination. An immunohistochemical study was performed in all groups. **Results:** The immunohistochemical examination of osteopontin by stromal and bone cells shows a significant difference between the experimental and control groups. **Conclusion:** The results of this study show that osteopontin is capable of bone formation and remodeling.

Keywords: Bone, mangosteen, osteopontin, royal jelly

Introduction

Bone healing is a complicated, reformative process. In general, bone tissue heals on its own, but in complicated instances, such as massive bone defects, it does not. There are three stages to bone healing: the inflammatory, reparative, and remodeling phases.[1,2]

Bone remodeling includes both bone resorption (carried out by osteoclasts) and bone synthesis (carried out by osteoblasts) of the extracellular matrix of bone tissue. Hydroxyapatite and collagen fibers are the main components of the extracellular matrix. Noncollagenous sialylated glycoproteins and proteoglycans are present in the extracellular matrix as well as on the surfaces of bone cells.[3] Noncollagenous proteins such as bone sialoprotein, osteopontin, and osteocalcin have recently been proven to improve bone fracture resistance. Diffuse damage occurs in rat and human bones, allowing the bones to diffuse energy without causing overt fractures.[4]

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In osseous tissue, osteopontin is released by osteoblasts and osteoclasts involved in bone formation. However, OPN expressed by osteoclasts has been shown to inhibit hydroxyapatite, implying that OPN is related to bone destruction. Numerous studies have found that OPN promotes macrophages and T cells during inflammation.[5]

Previous studies indicate that osteopontin is synthesized and secreted during the differentiation and mineralization of osteoblasts. It is an early and effective marker of bone formation.[6] The strong positive reaction of OPN, particularly at the demarcation line, is attributable to an

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increase in the number of osteoblasts required for the synthesis of reformative bone tissue.[7] In other studies, osteopontin has been demonstrated to be a marker of bone remodeling encountered during the reparatory phase in patients with femoral head osteonecrosis.[8]

During the process of bone resorption, OPN binds to osteoclasts and enhances their adherence to the mineral. Enzymes, particularly phosphatases alkaline phosphatase and tartrate-resistant acid phosphatase, in addition to matrix metalloproteases and other signaling factors, are a small but potentially interesting amount of noncollagenous proteins in the mineralized matrix of bone.[9] Since osteopontin's primary function is adhesive, its main role is in the early phases of bone repair.[10] Furthermore, Icer and Gezmen-Karadag suggest that osteopontin may minimize the incidence of bone fractures by modifying osseous cell adhesion and matrix mineralization.[5]

Osteopontin expression increases during osteoclastogenesis in monocytes; exogenous osteopontin might inhibit osteoclastogenesis. Osteopontin, or an alternative protein that includes sialic residues that have been predeposited on bones, may inhibit osteoclastogenesis and serve as a mark that signals subsequent osteoclasts to avoid resorbing the same region.[11]

The immunohistochemical expression of osteopontin is associated with bone remodeling and maturation changes. Furthermore, the hypothesis that osteoclasts are the source of OPN in bone cement lines during remodeling is confirmed. Because exogenously supplied OPN has no access to these areas, the detection of OPN expression within lacunae is a clear confirmation of OPN secretion by osteoclasts.[12]

The aim of this study was to determine the effects of royal jelly and mangosteen peel extract on osteopontin expression in bone defect areas, separately and in combination.

Materials and Methods

Experimental design and surgical procedure

Thirty-two albino rats (4–6 months old, weighing 250–350 g) utilized in this study were evaluated by veterinarian staff in the private animal house to assess their general health and exclude ill animals. The surgery was carried out in a sterilized environment. Intramuscular injections of ketamine hydrochloride 50 mg/mL (1 mL/kg body weight) and xylazine 2% (0.2 mL/kg body weight) were used to induce general anesthesia. To prevent dehydration, eye ointments have been applied to the rats' eyes. Both rat femurs had been prepared for surgery. Skin and underlying tissue were incised, then muscles were dissected using surgical scissors, and bone was exposed by reflecting both skin and muscle with a surgical reflector. Intra-bony defects were performed by micro-engine using intermitted drilling and continuous cooling with normal saline; the access opening was made with a round bur and enlarged with a fissure bur at a rotating speed of 1500 rpm until bone defects (about 2 mm in diameter and 3 mm in depth) were created in both the right and left femurs on the distal side.[13] The bone defects were created and left to heal without treatment in the control group, while the bone defects in the three experimental groups were filled with either 1 mg of royal jelly, 1 mg of mangosteen peel extract powder, or a combination of 1 mg of royal jelly and mangosteen peel extract powder. The rats were euthanized 2 and 4 weeks after surgery [each group had 8 rats with 16 bone defects].

Histological and immunohistochemical preparation

All bone specimens were fixed in 10% freshly made formalin for 24 h, then decalcified in 10% formic acid for 8–10 days before being embedded in paraffin wax.[14,15]

Deparaffinization, rehydration, antigen retrieval, endogenous peroxidase blocking, and the addition of primary and secondary antibodies to the slides were all immunohistochemical steps. The primary antibody utilized in this study was a polyclonal osteopontin antibody (OPN) from Abcam, UK (ab63856) at a 1:5 dilution. A two-step poly HRP anti-rabbit and mouse IgG detection kit (E-IR-R213) from Elabsience was used.

The scoring system

Using a light microscope at 40× magnification and then images captured using an iPhone 7 camera with a 1× zoom; two histologists independently examined five random fields from each section to determine IHC expression. The intensity of staining and the percentage of positive bone cells were used to score OPN. As a result, protein expression was assessed semi-quantitatively.[10,16] Scales for staining intensity ranged from 0 (no staining) to 3+ (intense staining), and the mean positive bone cell percentage was classified as follows: negative to 0 of (0%–5% positive cells), 1 for (5%–20% positive cells), 2 for (20%–50% positive cells), and 3 for (50%–100% positive cells).[17]

Statistical analytical

The Shapiro–Wilk test was employed to determine whether the distribution of the variables was normal, and it was found to be so. Statistics were presented in many varieties, including mean standard deviation, minimum, maximum, *F*-test, and probability value. The 26 version of SPSS (Statistical Package for the Social Sciences) was used to analyze the data. Statistical significance is assumed at the 0.05 level.[18,19]

Ethical approval

All experimental protocols and animal handling were carried out in line with animal experimentation ethical rules provided by the College of Dentistry, University of Baghdad/Iraq (Ref. number: 296 on April 1, 2021).

Results

Depending on the positive and negative controls, the presence of a brown cytoplasmic stain indicated a positive reading, while the absence of immunological reactions indicated a negative reading.[20]

At 2 weeks, significant differences in the number of immunohistochemically stained cells were seen in all groups, revealing moderate to strong positive osteopontin antibody expression in osteoblast (OB), osteocyte (OC), osteoclast (OCL), and bone marrow stromal cells (BMSCs) in all groups [Figures 1–4]. At 4 weeks, there was a significant decrease in the number of immunostained bone cells seen in all groups; mild to moderate positive expression was recognized in osteoblasts, osteocytes, and osteoclasts. The osteopontin expression in the new bone is positive [Figures 5–8]. The higher mean values of immunoreactivity scoring of BMSCs to osteopontin antibody were seen in the royal jelly group among both healing durations (7.5 ± 2.2) and (3.8 ± 1.1), respectively, for osteoblasts in the combination group were (8.6 ± 1) in 2 weeks and (4.3 ± 1.9) for the royal jelly group in 4 weeks, osteocytes were (7.8 1.5) and (3.3 ± 0.9) in both (2 and 4 weeks) were seen, respectively, in the combination group, and for osteoclasts in both healing durations (3.6 ± 1.5) and (2.3 ± 0.7), respectively, in the control group [Tables 1–4].

The mean number of immunoreactivity scoring (IRS) of positively expressed bone marrow stromal cells, osteoblasts, osteocytes, and osteoclasts for OPN antibody at each healing duration (two and four weeks) was compared across groups using an ANOVA test, as shown in [Tables 1-4]. IRS of positively expressed BMSCs, OB, OC, and OCL varied significantly at two week periods of

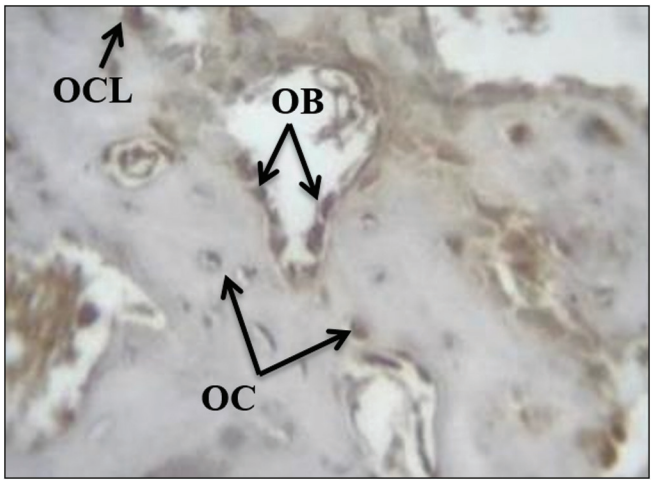

Figure 1: An immunohistochemical view of the control group after 2 weeks showed strong positive expression of osteopontin in OC, OB, and OCL. DAB with hematoxylin counterstain 40×

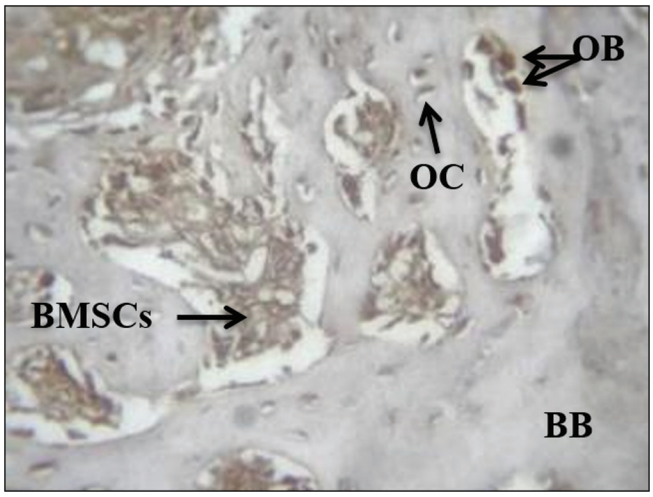

Figure 3: An immunohistochemical view of the mangosteen group after 2 weeks showed strong positive expression of osteopontin in OC, OB, and BMSC. Basal bon BB and DAB with hematoxylin counterstain 40×

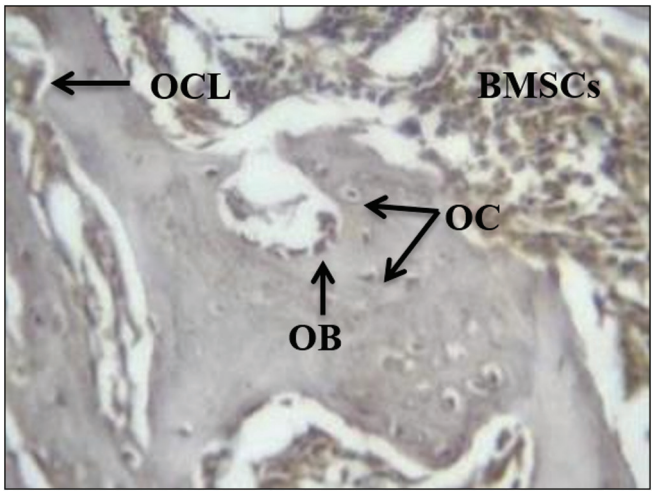

Figure 2: An immunohistochemical view of the royal jelly group after 2 weeks showed strong positive expression of osteopontin in OC, OB, OCL, and BMSCs. DAB with hematoxylin counterstain 40×

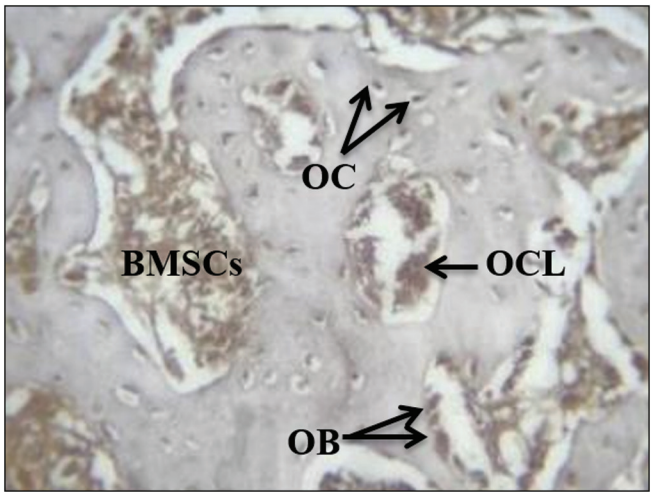

Figure 4: Immunohistochemical view of the combination group after 2 weeks showed strong positive expression of osteopontin in OC, OB, OCL, and BMSCs. DAB with hematoxylin counterstain 40×

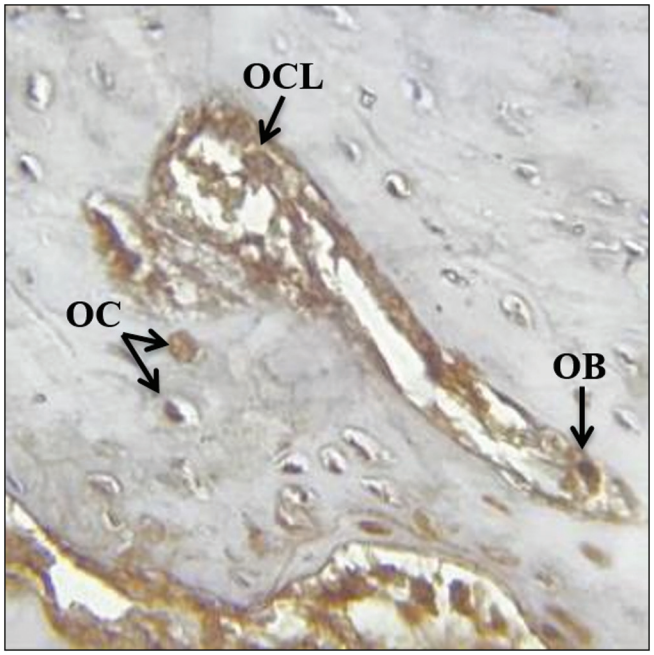

Figure 5: View after 4 weeks of the control group with moderately positive expression of osteopontin in OC, OB, and OCL. DAB with hematoxylin counterstain 100×

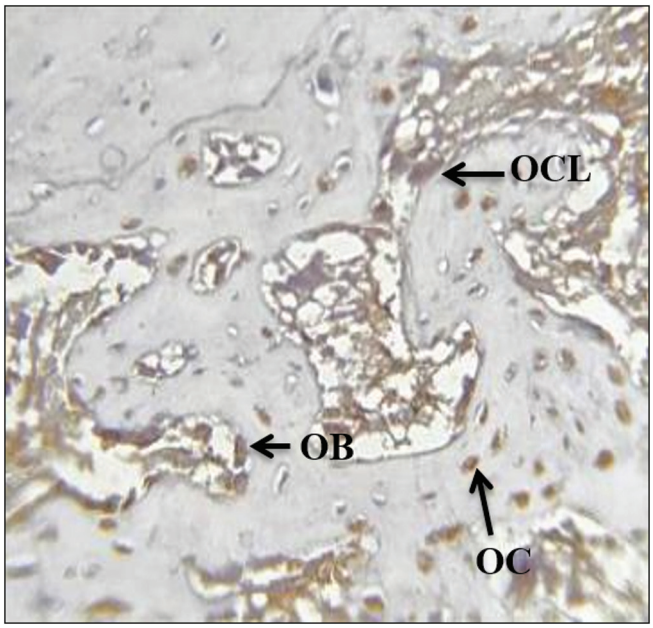

Figure 7: View after 4 weeks of mangosteen group with moderate positive expression of osteopontin in OC, OB, and OCL. DAB with hematoxylin counterstain 40×

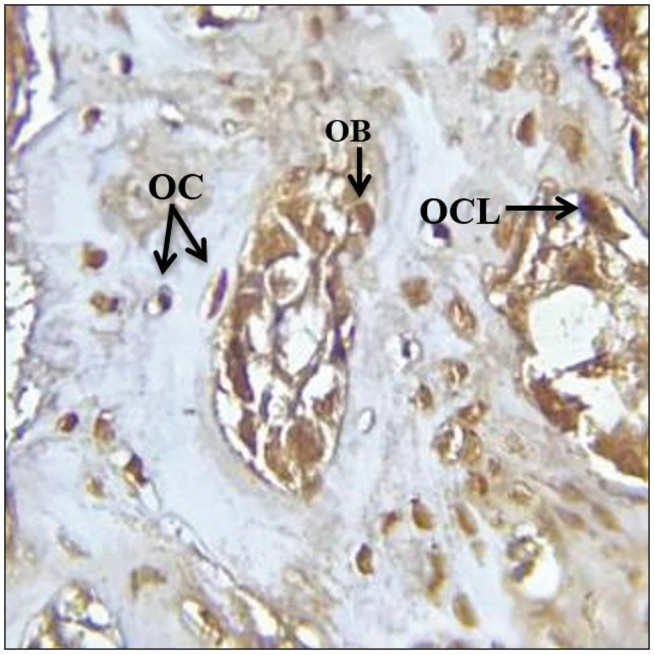

Figure 6: View after 4 weeks of royal jelly group with moderate positive expression of osteopontin in OC, OB, and OCL. DAB with hematoxylin counterstain 100×

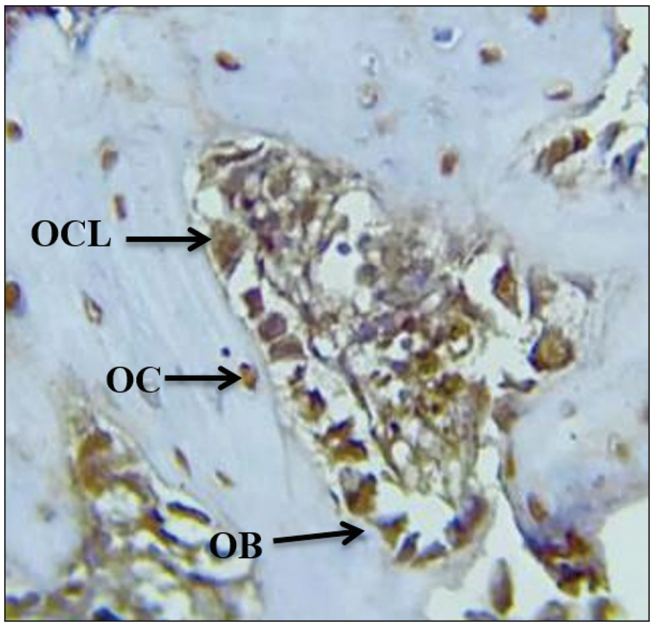

Figure 8: Moderately positive expression of osteopontin was detected in OC, OB, and OCL after 4 weeks in the combination group. DAB with hematoxylin counterstain 100×

all groups, also the results revealed significant difference at 4 week period in osteoclasts.

Discussion

Osteopontin has a crucial role in regulating bone formation, mineralization, and turnover.[21] Bone release of osteopontin increases and reaches a peak at day 14 of the fracture.[22]

Immunostaining was performed to evaluate the activity of osteopontin (OPN) which plays an important role in osteogenic activity in the defect region, evaluating the positive labeling of OPN in the area of defect bone treated with royal jelly and mangosteen, from

Table 1: Group comparison difference by ANOVA test for the mean immunoreactivity scoring of bone cells for osteopontin for stromal cells

Duration	Study group	Bone stromal cells		*F* value	*P* value
		Mean ± SD	Range		
2 weeks	Control	4.3 ± 1.7	2–6	5.18	0.006*
	Royal jelly	7.5 ± 2.2	3–9		
	Mangosteen	7.1 ± 2.2	3–9		
	Combination	7.8 ± 1.5	6–9		
4 weeks	Control	2.5 ± 1.4	2–6	1.1	0.35
	Royal jelly	3.8 ± 1.1	2–6		
	Mangosteen	3.5 ± 1.7	2–6		
	Combination	3.3 ± 1.7	2–6		

*$P < 0.05$

Table 2: Group comparison difference by ANOVA test for osteoblast

Duration	Study group	Osteoblast		*F* value	*P* value
		Mean ± SD	Range		
2 weeks	Control	5.8 ± 1.8	2–9	4.15	0.015*
	Royal jelly	8.2 ± 2.1	3–9		
	Mangosteen	7.8 ± 1.5	6–9		
	Combination	8.6 ± 1	6–9		
4 weeks	Control	4 ± 1.5	2–6	0.18	0.906
	Royal jelly	4.3 ± 1.9	1–6		
	Mangosteen	3.8 ± 1.5	2–6		
	Combination	3.7 ± 1.9	2–6		

*$P < 0.05$

Table 3: ANOVA test for osteocyte group comparative difference

Duration	Study group	Osteocyte		*F* value	*P* value
		Mean ± SD	Range		
2 weeks	Control	4.2 ± 1.9	1–6	6.29	0.002*
	Royal jelly	7.1 ± 2.2	3–9		
	Mangosteen	7.5 ± 1.6	6–9		
	Combination	7.8 ± 1.5	6–9		
4 weeks	Control	2.8 ± 1.4	2–6	0.28	0.835
	Royal jelly	3 ± 1	2–4		
	Mangosteen	3.1 ± 0.9	2–4		
	Combination	3.3 ± 0.9	2–4		

*$P < 0.05$

Table 4: ANOVA test for osteoclast immunoreactivity scoring for osteopontin group comparison

Duration	Study group	Osteoclast		*F* value	*P* value
		Mean ± SD	Range		
2 weeks	Control	3.6 ± 1.5	2–6	4.443	0.011*
	Royal jelly	2.6 ± 1	0–3		
	Mangosteen	1.8 ± 1.5	0–3		
	Combination	1.1 ± 1.5	0–3		
4 weeks	Control	2.3 ± 0.7	0–3	4.84	0.008*
	Royal jelly	1.1 ± 1.2	0–3		
	Mangosteen	0.87 ± 1.2	0–3		
	Combination	0.37 ± 1	0–3		

*$P < 0.05$

the osteoblast differentiation stage to the synthesis of the organic matrix, mineralization, and finally bone remodeling. Information about osteopontin expression in the defect area is very important, especially when there is a biomaterial filling the defects; as osteopontin is the most important marker during the early stages of bone formation.[6,23]

In the present study, the strong positive expression cells of osteopontin antibody could be found in the BMSCs, osteoblasts, osteocytes, osteoclasts, and new bone matrix in all groups at 2 weeks of healing periods; and moderately stained cells at 4 weeks of healing period. The current result also illustrates the highest mean of strong positive expression of OPN in BMSCs, osteoblasts, and osteocytes in experimental groups when compared to the control group, and especially in the combination group (royal jelly/mangosteen) of 2 weeks, because the combination group showed a higher percentage of new bone formation with the highest number of cells than the other groups after a 2-week period of healing. The more intense expression suggests a more advanced stage in the bone formation and remodeling processes, as OPN is considered an early and effective marker of bone formation that is, released by osteoblasts. The present result is in agreement with.[5,7,22] This suggests the potential role of royal jelly (which contains bioactive substances like 10-hydroxy-2-decenoic acid) and mangosteen (which contains xanthones) in modulating healing responses and increasing new bone formation, bone cell differentiation, and proliferation due to their properties (anti-oxidant, anti-inflammatory, and wound healing) in the royal jelly and mangosteen groups, and this agrees with[24,25]; and this may suggest that the combined action of both materials in the combination group provides better healing.

Also, according to the results of the current study and the immunohistochemical analysis performed, in the experimental and control groups, there was a reduction in OPN expression with time in all groups in BMSCs and osteoclasts, as well as in osteoblasts and osteocytes localized within the new bone; due to increased bone deposition and decreased bone marrow area, osteopontin expression for cells decreased over time in the defect area, and these findings agree with.[12,26]

Although it decreased with time, the mean value of osteoclasts was higher in the control group at 2 and 4 weeks of healing, as the percentage was higher in the control group compared to the experimental groups. Since osteopontin is released by osteoclasts and its expression increases during osteoclastogenesis and bone remodeling, also this may indicate that the healing process is slower in the control group than in the experimental groups. The present results are in agreement with other studies.[6,11,12,21,26]

The study included numerous instances of limitations. First, getting the animals is challenging and takes more time and effort. Second, the provision of sophisticated research centers is also essential for studies involving animals, like the one under consideration. Finally, to prevent animal losses before the study's end, the animals' housing is also crucial. The application or interpretation of the study results may be affected or influenced by these limitations.

Conclusion

Mangosteen peel extract and royal jelly, separately and in combination, could increase osteopontin expression in bone, which is associated with bone remodeling and maturation.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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