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Research Article

A Novel Histological Study Evaluating the Combined Effect of Bovine-bone Graft and Royal Jelly on Socket Bone Healing in a Rabbit Model

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Abstract:

Background: The alveolar process, also called the alveolar bone, is a thickened ridge that contains the tooth sockets (dental alveoli) on the jaw bones that hold the teeth. After tooth extraction, several changes can occur in the alveolar process that may prevent or delay prosthodontic or other dental treatments. Recently, the Bovine-bone graft has been proposed to be applied in fresh extraction sockets to minimize the reduction in ridge volume. In recent experimental studies, Royal jelly has been shown to have antibacterial, antioxidant, antitumor, and anti-inflammatory properties, resulting in vascular dilatation and increased cell proliferation and differentiation.

Objective: To evaluate the efficacy of Bovine-bone graft and Royal Jelly separately and in combination on socket bone healing.

Methods: Forty-eight rabbits were divided into four groups (Control, Royal Jelly (RJ), Bovine-Bone Graft (BG), and Combination). The results were studied histologically after two and four weeks, postoperatively. A histological examination was performed under a light microscope for the section stained with hematoxylin and eosin (H&E). The study was conducted between November 2023 and ended in September 2024 in the laboratory of Histology in the College of Dentistry / University of Baghdad.

Results: In the RJ group, the socket area showed the formation of new bone trabeculae, lined by osteoblasts and osteocytes, with higher mean values than in other groups. Histomorphometric analysis revealed that RJ-treated sockets exhibited significantly higher trabecular bone area ($p<0.001$) and osteoblast/ osteocyte counts compared to the BG and Control groups.

Conclusion: The use of Royal Jelly separately or in combination with Bovine-bone graft is more beneficial than the Bovine-bone graft alone, for socket bone healing.

Keywords: Bovine-bone graft; Bone healing; Extraction sockets; Rabbits; Royal Jelly.

Introduction:

The alveolar process, also called the alveolar bone, is a highly specialized complex in structure and function. It is a thickened ridge that comprises the tooth sockets (dental alveoli) that hold the teeth on the jaw bones (1). Several changes can occur in the alveolar process after tooth extraction that may prevent or delay prosthodontic or other dental treatments (2). Maintenance of adequate alveolar ridge volume is also important to achieve a long-term, aesthetically acceptable, intraoral prosthesis or implant (3). Many methods have been used during the past two decades to maintain the architecture of the residual alveolar ridge, for example, the use of bone substitutes and collagen plugs packed into the extraction sockets (4,5). Given the increasing demand for aesthetic and functional outcomes in dental procedures (6), understanding the role of adjunctive therapies, such as, RJ in conjunction with bone grafting materials, is crucial for improving patient outcomes. Even as various materials and techniques have been explored to optimise healing after tooth extraction, the integration of biological agents such as RJ with traditional grafting methods remains under-researched. This study seeks to fill that gap by investigating it first on a Rabbit model, because Rabbits could well serve as experimental animals for assessment of alveolar bone healing, following tooth extraction (7,8).

BG is a xenograft that is widely utilized for oral regeneration, particularly for bone regeneration, due to its osteoconductive properties. (9). Bovine-origin bone substitutes were the first xenografts applied to patients, as they were commercially available in a wide range of products and were considered to be among the most documented materials in this category. They are characterized by osteoconductive properties, being deproteinized and lyophilized, causing no immune response (10).

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RJ is a white gelatinous substance with a sharp smell that is produced by the glands in the hypopharynx of the worker bees and is the main nutrient source for the queen bee and larvae. RJ has a high nutritional value and has been traditionally used as a food supplement. It consists of water (50%–70%), proteins (9%–18%), carbohydrates (7%–18%), lipid and fatty acids (3%–8%), mineral salts (1.5%) and very small quantities of vitamins and polyphenols (11). RJ has antibacterial, antioxidant, anti-tumor, and anti-inflammatory effects (12). Moreover, it has been shown to induce an estrogen-like effect in the osteoblast culture medium (13). It has been demonstrated that pure apisin, which is one of the major RJ glycoproteins (MRJPs), enhances cell proliferation and collagen production of healthy neonatal dermal fibroblasts (NB1RGB). Furthermore, apisin improves the differentiation rate of MC3T3-E1 (a mouse osteoblastic cell line). According to these findings, the RJ possibly induces inductive effects (partially due to the presence of apisin on cell proliferation and differentiation (14).

This study aimed to evaluate the efficacy of the BG and Royal Jelly separately and in combination on socket bone healing. As far as we know there was no previous study about this combination's effects on socket healing .

Material and Methods:

The study was conducted between November 2023 and September 2024, in the Histology laboratory in the College of Dentistry / University of Baghdad.

Animal Model and Grouping

The upper two central incisor teeth (average length of each tooth ranged about 25 mm) (15) for each of 48 rabbits were extracted after administration of general anesthesia. These rabbits were divided randomly into two periods (two and four weeks). The consensus in the literature indicates that the cycle of bone repair in rabbits is completed in approximately 42 days. Therefore, the evaluation periods of 2 and 4 weeks allowed us to analyze both the initial and final stages of bone repair effectively. After extraction, the 96 sockets of 48 rabbits were divided as described in Figure 1.

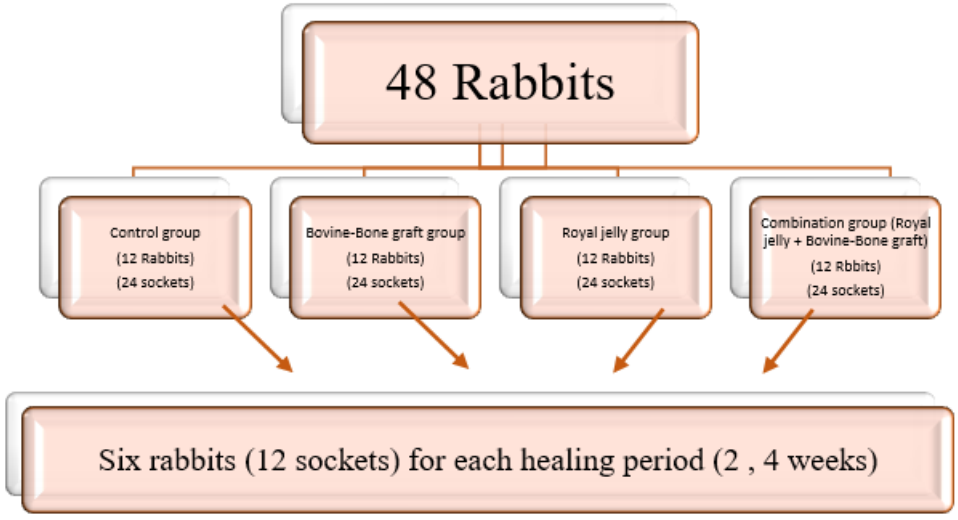

Figure (1): Experimental design.

Materials:

1 - Pure RJ lyophilised powder containing 10-hydroxy-2-decenoic acid (10-HAD) 6%, from (Amazon) (China).

2 - Natural Bovine-bone grafting material for filling bone defects composed of Calcium phosphate (100% pure hydroxyapatite, mineral phase) from Botiss (Germany).

Ethical Approval: All experimental procedures were carried out in accordance with the ethical principles of animal experimentation of the College of Dentistry, University of Baghdad (**Ref. number: 880, Date: 3-12-2023**).

Inclusion and exclusion criteria: Forty-eight male New Zealand white rabbits, weighing between 1 and 1.5 kg and aged between six and eight months, were enlisted as experimental subjects. Female rabbits were specifically excluded due to cyclic hormonal variations and the potential for pregnancy throughout the experimental timeline.

Surgical procedure: The rabbits were kept in a private animal house, in separate cages, and were fed with the standard diet (Barseam, green vegetables, and pellets) and had free access to tap water following the Animal Research: Reporting of *In-Vivo* Experiment guidelines (ARRIVE). All the instruments were autoclaved at 134ºC / 210 kPa for 15 minutes for sterilization. General anesthesia was induced by intramuscular injection of ketamine hydrochloride 50 mg/ml (1 ml/kg of body weight) plus xylazine 2% (0.2 ml/kg of body weight) (16). Following the application of the check retractor for each rabbit, the upper two central incisors were extracted, first by separation with a dental probe. Mobilization of the tooth was done by using straight elevators, and then extraction of the upper central incisors was done by using a pediatric forceps (Figure

2). In the control group, the socket was left to heal spontaneously without any treatment. In the RJ group, 0.10 - 0.15 mg of RJ was applied to treat the sockets using a cumine, a dental instrument with a spoon-like end designed for material delivery. An amount of 0.065 mg of the Bovine Bone Graft was used to treat the sockets in the Bovine Bone Graft group, and was also applied with the cumine. For the combination group, the sockets were treated with 0.165 mg of both RJ and Bovine Bone Graft (0.10 mg RJ + 0.065 mg BG), delivered using the same cumine instrument.

Figure 2 :A- Extraction of two central incisors ,B: Material application , C- Socket area on day of sacrifice.

Histological and histomorphometric analysis: The animals were sacrificed by inducing an overdose of general anesthesia after a healing period lasting either two or four weeks after teeth extraction. The premaxillae that hold the sockets of the upper two central incisors of each rabbit were dissected by cutting the disk and engine handpiece. After fixation in 10% neutral buffered formalin, the specimens underwent a washing process followed by decalcification in 10% formic acid. The samples were dehydrated by placing them in increasing alcohol concentrations, followed by clarification in xylene and finally embedding them in paraffin wax. Sections of paraffin blocks, 4 µm in thickness, were sliced and then stained with Hematoxylin and Eosin for microscopical examination. Histomorphometric analysis was performed by double blind examination (Inter and intra-examination) of bone cells (Osteoblasts, Osteocytes and Osteoclasts) and microarchitectural parameters (Bone Trabecular (BT) area and Bone Marrow (BM) Area) was done by using a light microscopic photomicrograph, equipped with ImageJ software (version 1.53e) (17),(18).

Statistical Analysis: SPSS 26.0 software was used for statistical analysis and graphs. The normality test for the distribution of the variables (BT Area, BM Area, Number of Osteoblasts, Osteocytes, and Osteoclasts) was assessed using the Shapiro-Wilk test. ANOVA, and the Kruskall-Wallis test; the Post-hoc and Bonferroni tests were used for identifying the group difference in the area of the extracted socket, at each healing period (two and four weeks), for duration difference (two and four weeks) in each area of the extracted socket and each group with a *p*-value of less than or equal to 0.05.

Results:

Clinical Observations: All the rabbits endured the procedure well and recovered after extraction without significant clinical complications. Throughout the healing period and on the day of sacrifice, there was no sign of infection or any negative clinical observation noticed at the extraction sites in any of the rabbits.

Histological Findings: The histological assessment of the socket areas of all groups for both healing periods (2 and 4 weeks) was examined in three parts (coronal, middle, and apical)

Two-week period: The histological assessment of the socket area of the Control (C) group showed formation of immature bone trabeculae in the apical part of the socket area that contained numerous arranged osteocytes, lined by active osteoblasts. The central part of the socket was filled with bone marrow and fibrous connective tissue, which contained scattered blood vessels. Reversal lines were seen between the socket wall and the new bone. In the RJ group, more bone formation was seen all over the socket area. The trabeculae were thicker than those of the control group, especially in the apical half of the socket. The bone trabeculae contained more well-organized osteocytes, lined by a large number of osteoblasts, which indicates the presence of active bone formation, and numerous reversal lines were also detected. The bone marrow tissues were well-vascularized. In the BG group, the socket area exhibited the formation of several new bone trabeculae that appeared to be attached to the remnants of the bone graft, surrounded by a highly vascular bone marrow area. Meanwhile, in the Combination (Comb.) group, newly formed bone trabeculae of moderate thickness could be seen, along with osteocytes entrapped inside the new bone. Osteoblasts made a continuous layer on the trabecular border, accompanied by numerous large osteoclasts. The bone marrow area showed fewer remnants of bone graft particles, with an abundance of blood vessels present within the marrow spaces, as seen in Figure 3.

Four-week period: The histological examination of the socket area in the Control group showed thick new trabecular bone, with numerous new blood vessels in between, and osteoblasts lined the surface of the new bone. In the RJ group, more mature bone trabeculae were seen all over the socket area. The trabeculae were thicker compared to those in the control group,

especially in the apical third of the socket. Small osteons were also seen. The bone marrow area was well-vascularized. In the BG group, the mature bone trabeculae appeared attached to the remnants of the bone graft with a high number of blood vessels in the marrow area. Meanwhile, in the Combination group, mature trabecular bone formation was seen, especially in the apical third of the socket, surrounding remnants of the bone graft, which contained regularly arranged Haversian canals (osteons), osteocytes, and large osteoclasts, as seen in Figure 3.

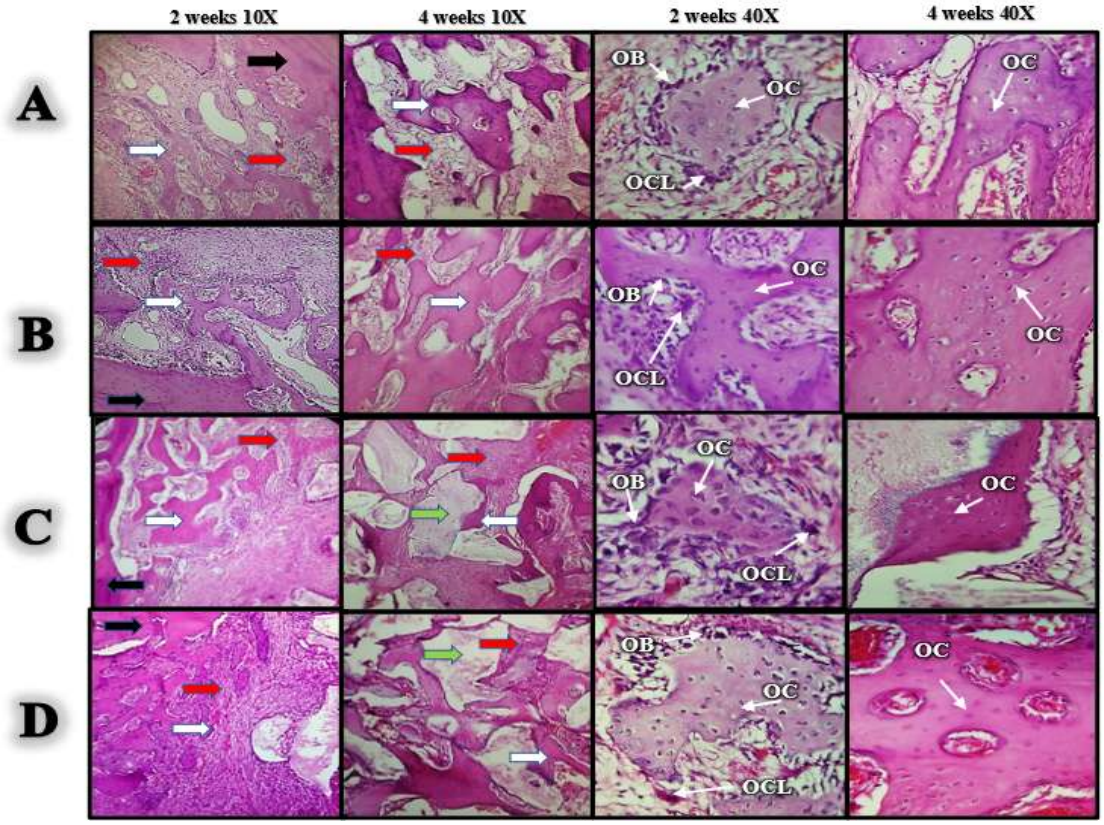

Figure (3): Photomicrograph of the histological sections in the socket area in H&E stain, black arrows represent native socket wall bone, white arrows represent new bone trabeculae, red arrows represent the bone marrow area and green arrows represent remnants of Bovine-bone graft, OB: Osteoblast, OC: Osteocyte, OCL: Osteoclast.
A: Control group, B: Royal Jelly group, C: Bovine-bone graft group, D: Combination group

Statistical Results:

1- **Bone Trabecular (BT) area parameter:** Following the two-week and four-week healing periods, the BT values demonstrated higher mean values in the sockets treated with the RJ and Combination groups, respectively. Concurrently, the trabecular area values in the socket region exhibited a progressive increase over time across all studied groups. Notably, there was a highly significant difference in the trabecular area values between the studied groups, as illustrated in Table 1 & 2.

Table 1: Descriptive statistics of Bone Trabecular area and ANOVA test

Group Statistics and ANOVA test after 2 weeks							
BT Area						F	*p*-value
	Period	N	Mean	Std. Deviation	Std. Error		
Control Group	2 weeks	12	2.89075	.427467	.123399	14.431	<.001*
Royal Jelly Group	2 weeks	12	5.83067	1.545014	.446007		
Bone graft Group	2 weeks	12	3.43433	.827910	.238997		
Combination Group	2 weeks	12	4.35750	1.499401	.432840		
Group Statistics and ANOVA test after 4 weeks							
	Period	N	Mean		Std. Error		
BT Area				Std. Deviation		F	*p*-value
Control Group	4 weeks	12	5.22692	1.112721	.321215	13.505	<0.001*
Royal Jelly Group	4 weeks	12	8.05958	1.640190	.473482		
Bone graft Group	4 weeks	12	4.65617	1.625031	.469106		
Combination Group	4 weeks	12	5.99992	1.141646	.329565		

* $0.05 \geq p > 0.01$ = significant

Table 2: Post-hoc analyses of T.A. values after 2 and 4 weeks

Post-hoc analyses of T.A		Two-week Period		Four-week Period	
Groups		Mean Difference	p-value	Mean Difference	p-value
Control	Royal jelly	-2.939917*	<0.001*	-2.059667*	0.005*
	Bone graft	-.543583	>0.99	1.343750	0.141
	Combination	-1.466750*	0.022*	.773000	>0.99
Royal jelly	Bone graft	2.396333*	<0.001*	3.403417*	<0.001*
	Combination	1.473167*	0.022*	2.832667*	<0.001*
Bone graft	Combination	-.923167	0.362	-.570750	>0.99
The p-values were adjusted according to the Bonferroni correction for multiple analyses.					

2-Bone Marrow (BM) area parameter: Concerning the Bone Marrow area values, there was a noticeable decrease in the marrow area over time. After the four-week period, the lowest mean values were recorded in the RJ and Combination groups, while after 4 weeks, Although the ANOVA test showed a significant difference in the mean B.M area after 4 weeks, adjusting the p-values of the post hoc tests according to the Bonferroni correction showed no significant differences between any two groups after 4 weeks. Meanwhile, during the two-week period, there was no significant difference in Bone Marrow values among the studied groups, as presented in Tables 3&4.

Table 3: Descriptive statistics of Bone Marrow area and ANOVA test

Group Statistics and ANOVA test after two weeks							
BM Area	Period	N	Mean	Std. Deviation	Std. Error	F	*p*-value
Control Group	2 weeks	12	5.56275	1.045338	.301763	2.518	0.070
Royal Jelly Group	2 weeks	12	6.14658	1.822180	.526018		
Bone graft Group	2 weeks	12	4.69717	1.830004	.528277		
Combination Group	2 weeks	12	6.19133	1.218173	.351656		
Group Statistics and ANOVA test after 4 weeks							
BM Area	Period	N	Mean	Std. Deviation	Std. Error	F	*p*-value
Control Group	4 weeks	12	3.53683	1.344275	.388059	3.093	0.037*
Royal Jelly Group	4 weeks	12	3.32758	.550685	.158969		
Bone graft Group	4 weeks	12	4.40483	1.065186	.307493		
Combination Group	4 weeks	12	3.34725	.883594	.255072		

* $0.05 \geq p > 0.01$ = significant

Table 4: Post hoc tests for B.M area values after 4 weeks

Multiple Comparisons				
Dependent Variable: B.M Area				
Bonferroni				
Groups		Mean Difference	Std. Error	*p*-value
Control	Royal jelly	.209250	.409557	<0.99
	Bone graft	-.868000	.409557	0.238
	Combination	.189583	.409557	<0.99
Royal jelly	Bone graft	-1.077250	.409557	0.070
	Combination	-.019667	.409557	<0.99
Bone graft	Combination	1.057583	.409557	0.079
The p-values were adjusted according to the Bonferroni correction.				

3-Bone Cells Count: The osteoblast (OB) and osteocyte (OC) numbers recorded higher mean values in the RJ group compared to the other groups after the two-week and four-week periods. Meanwhile, the Osteoclast number (OC) recorded higher mean values in the Combination group after a two-week period. On the other hand, after four weeks**,** the number of Osteoclasts (OCL) recorded higher mean values in the Control group, as seen in Figure 4.

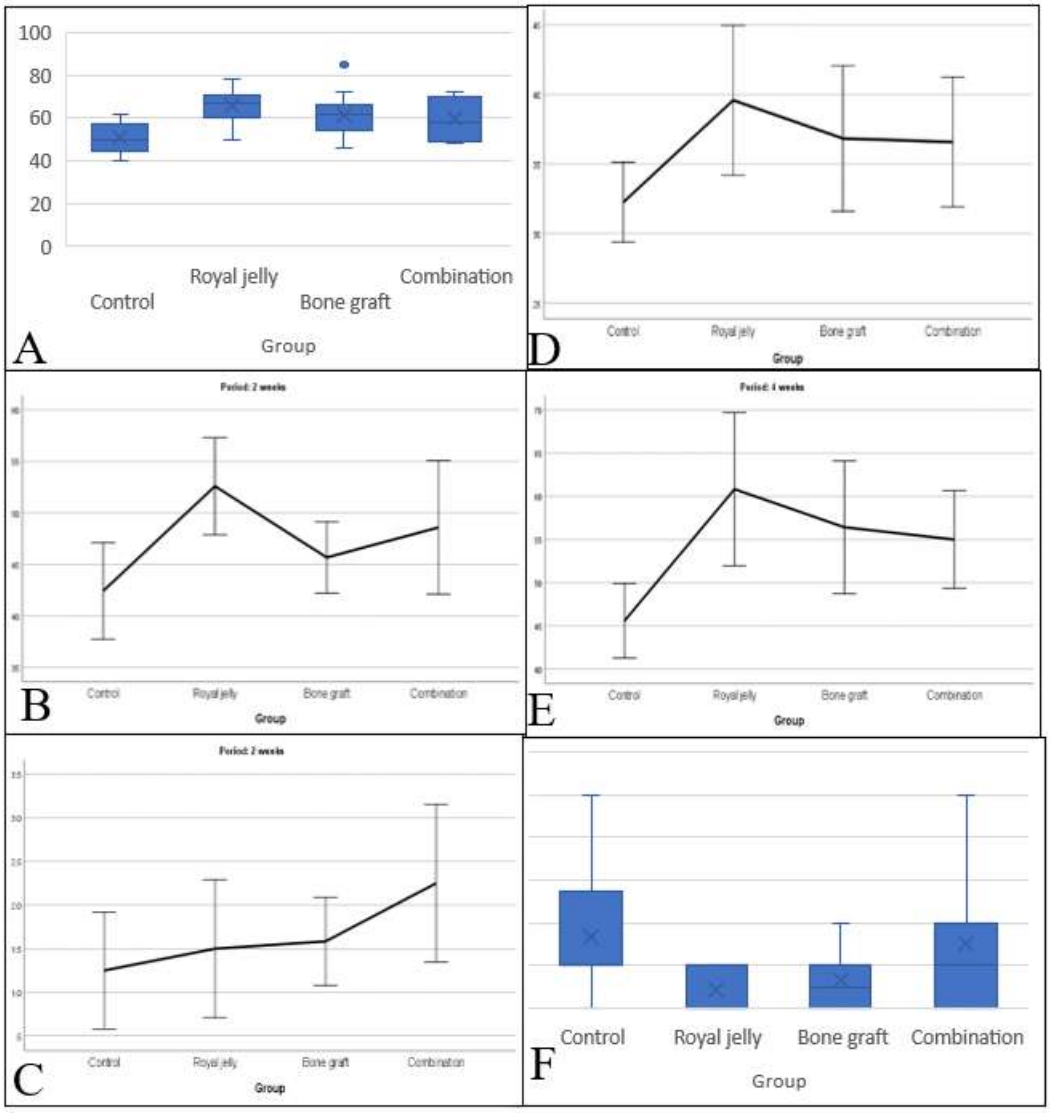

Figure 4: Mean numbers after two weeks: A-Osteoblasts, B- Osteocytes, and C-Osteoclasts
Mean numbers after four weeks: D-Osteoblasts, E-Osteocytes, and F- Osteoclasts

Discussion:

The present study has aimed to evaluate the efficacy of RJ alone or in combination with BG for enhancing the healing process following tooth extraction, a common procedure that often results in significant bone loss and compromised soft tissue integrity. Our findings indicate that the application of RJ significantly reduces ridge volume loss and promotes better healing compared to the standard extraction protocols. Nearly all the animals included in the current study demonstrated effective healing of the socket areas, particularly in the group treated with RJ, throughout all healing periods, with no signs or symptoms of inflammation observed. This result may be due to the effectiveness of local application of RJ for bone regeneration, which served as a graft material in the socket area and promoted osteoblast differentiation. The bone healing process was thereby accelerated by the application of RJ, which agrees with the findings of Hattori *et al.* (19) and Bigham-Sadegh *et al.* (20).

There highly significant increase in the number of bone cells (osteoblasts and osteocytes), new bone formation, resorption and remodeling two weeks following extraction, in sockets treated with RJ, compared to controls, bone graft and combination groups, agrees with the findings of Ozan *et al.* (21), who confirmed the osteogenic properties of RJ after using it to treat radial bone defects.

Microscopic examination of sockets treated with RJ revealed significant new bone formation, characterized by an abundance of bone trabeculae and a higher count of osteoblasts compared to the control group. This increase in osteoblastic activity, led to an expansion of the trabecular bone area in the sockets treated with RJ. It was likely that the activity of RJ contributed to its antioxidant properties by reducing nitric oxide (NO) and reactive oxygen species (ROS) levels in the cells. Additionally, RJ exhibited an inhibitory effect on the production of inflammatory mediators through the JNK, p38, and NF-kB pathways and promoted bone stimulation, which might account for these results as suggested by You

et al. (22). RJ appeared to not only enhance bone regeneration in the socket area, but also facilitated healing. The observed increase in the proliferation of bone-forming cells, such as osteoblasts and osteocytes, indicated heightened activity in bone formation. This study supported the findings of Ozan *et al..*, (21), demonstrating that RJ promoted the development of new bone tissue in rats, which aligned with the results of the current investigation.

Two weeks postoperatively, histological and histomorphometric analysis indicated new bone formation in the socket area treated with BG. This was evidenced by the presence of numerous bone trabeculae and a slight increase in the number of osteoblasts and osteocytes, as compared to the control group. The new bone formation was attributed to the osteoconductive properties of the graft, which was primarily composed of hydroxyapatite (HA). One of its key characteristics was its chemical composition, which closely resembled that of human HA, with a calcium/ phosphate ratio of 1.67 that was identical to that found in human bone by de Freitas, *et al.* (23).

In the case of the Combination group, the use of combined materials with xenografts is emerging as an interesting trend, not only in research (24), but also in industrial and clinical applications (25). Some studies suggest that this combination — representing a new generation of bone grafts — offers improved clinical outcomes (25, 26). The findings of this study demonstrate that the use of RJ alone or as a delivery material for BGs will influence the bone healing process. These results indicate that the bone graft particles placed in the socket area facilitate reconstruction, serve as a scaffold within the bone space, promote cell differentiation into osteoblasts, and enhance the scaffold attachment to the bone. Therefore, the combined application of bone graft and RJ improves bone healing.

At four weeks post extraction, the microscopic examination revealed that the RJ group exhibited an increased trabecular area and a decreased bone marrow area compared to the other groups, which agrees with the findings of Ibrahim *et al.* (27). The bone trabeculae had become denser and were populated by more osteocytes than observed during the two-week healing period. These results aligned with those reported by Oryan *et al..*,(28), who found that injecting RJ into a significant radial bone defect in rats, 56 days post-injury, led to an increase in osteocyte numbers and bone tissue density compared to the control group. This observation was also supported by the findings of Ozan *et al..*, (21) and Bigham-Sadegh *et al..*, (20).

Limitations:

While this study provides valuable insights into the effects of Royal Jelly and Bovine-bone graft on socket bone healing, several limitations should be acknowledged:

Exclusive Use of Incisors: The study exclusively focused on incisors, omitting canines, premolars, and molars in both jaws, which might respond differently to treatment. This limitation may affect the applicability of the results to a broader range of dental applications.

Short Follow-Up Period: The histological evaluations were conducted only at two and four weeks postoperatively. Longer follow-up periods could provide more comprehensive insights into the long-term effects of Royal Jelly and Bovine-bone graft on bone healing and integration.

Exclusion of Female Subjects: The study exclusively used male rabbits, which may limit the applicability of the findings to female subjects and could introduce gender-related variability in the healing process.

Conclusion:

Based on the findings of the current study, the local application of the Royal Jelly alone or with Bovine-bone graft could serve as an effective therapeutic option for future clinical applications aimed at accelerating bone healing after extraction.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to the current study, have been given permission for re-publication attached to the manuscript. Authors sign on ethical consideration's Approval-Ethical Clearance: The project was approved by the local ethical committee in in accordance with the ethical principles of animal experimentation of the College of Dentistry, University of Baghdad, according to the code number (Ref. number: 880, Date: 3-12-2023).

Conflict of Insert: None

Funding: None.

Authors' contributions:

Study conception & design: (Shatha Mahir Alhamrany, Nada M.H Al-Ghaban and Mahdi Mutahar). Literature search: (Shatha Mahir Alhamrany and Nada M.H Al-Ghaban). Data acquisition: (Shatha Mahir Alhamrany). Data analysis & interpretation: (Shatha Mahir Alhamrany). Manuscript preparation: (Mahdi Mutahar). Manuscript editing & review: (Nada M.H Al-Ghaban and Mahdi Mutahar).

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دراسة نسيجية جديدة لتقييم التأثير المشترك لطعم عظام البقر وغذاء الملكات على التئام عظمة التجويف في نموذج الأرانب

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الخلاصة:
الخلفية: تم إقتراح إستخدام طعوم عظام الأبقار في تجاويف الإستخراج الطازجة لتقليل الانخفاض في حجم التلال. وقد أظهرت الدراسات التجريبية الحديثة أن غذاء ملكات النحل له خصائص مضادة للبكتيريا ومضادة للأكسدة ومضادة للأورام ومضادة للالتهابات مما يؤدي إلى توسع الأوعية الدموية وزيادة تكاثر الخلايا وتمايزها.
الغرض: تقييم فعالية طعوم عظام الأبقار وغذاء ملكات النحل بشكل منفصل وبالاشتراك مع بعضها البعض في التئام عظم التجويف.
المنهجية: تم تقسيم ثمانية وأربعين أرنبا ذكرا (نيوزيلنديا) بالتساوي إلى 4 مجموعات بعد استخراج القواطع المركزية العلوية تحت التخدير العام: المجموعة الأولى (المجموعة الضابطة)؛ المجموعة الثانية (غذاء ملكات النحل)، المجموعة الثالثة (طعوم عظام الأبقار)، المجموعة الرابعة (المجموعة المركبة). تمت دراسة النتائج نسيجيا بعد أسبوعين وأربعة أسابيع من الجراحة. تم إجراء فحص نسيجي تحت المجهر الضوئي للجزء الملطخ بالهيماتوكسيلين والإيوسين.
النتائج: في مجموعة غذاء ملكات النحل، أظهرت منطقة التجويف تكوين عوارض عظمية جديدة مبطنة بالخلايا العظمية والخلايا العظمية بقيم متوسطة أعلى من المجموعات الأخرى. كشف التحليل النسيجي لمعلمة مساحة العظم الإسفنجي أن العوارض المعالجة بغذاء ملكات النحل والمزيج (طعم عظمي تم تسليمه بغذاء ملكات النحل) سجلت قيما متوسطة أعلى من طعم عظام البقر وحده ومجموعات التحكم.
الاستنتاج: أظهرت النتائج أن استخدام غذاء ملكات النحل بشكل منفصل أو بالاشتراك مع طعم عظام البقر يمكن أن يكون أكثر فائدة من طعم عظام البقر لالتئام عظم التجويف.
الكلمات المفتاحية: غذاء ملكات النحل، طعم عظام البقر، عوارض الاستخراج، التئام العظام، الأرانب.