IJVR
ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol. 19 No. 4 Ser. No. 65 2018

IRANIAN JOURNAL OF VETERINARY RESEARCH

Effects of chitosan scaffold along with royal jelly or bee venom in regeneration of critical sized radial bone defect in rat

Meimandi-Parizi, A.[1*]; Oryan, A.[2]; Bigham-Sadegh, A.[3] and Sayahi, E.[4]

[1]Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [2]Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [3]Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran; [4]Resident of Veterinary Surgery, Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

[*]***Correspondence:*** *A. Meimandi-Parizi, Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran. E-mail: meimandi@shirazu.ac.ir*

(Received 27 Dec 2017; revised version 24 May 2018; accepted 12 Jun 2018)

Summary

The aim of this study was to compare the efficacy of honey bee venom (BV) and royal jelly (RJ) alongside chitosan scaffold (CS) in improving radius bone defect in rats. A total of 60 full thickness radial bone defects with a length of 5 mm were created in 60 male Wistar rats. Six healthy radial bones (3 rats) were also assigned as normal control for biomechanical studies. The defects were left empty (untreated group) or were filled by the autograft (autograft group), CS (CS group), CS alongside the BV solution (CS-BV group), and CS alongside the RJ solution (CS-RJ group). Healing of the bone defects were evaluated clinically and radiologically on days 0, 28, 42 and 56 after operation while the biomechanical testing and histopathological examination were performed on the 56th day after surgery. The autograft was more radiopaque than the untreated and CS groups at the 28th, 42nd and 56th postoperative days ($P<0.05$). The CS-BV and CS-RJ groups showed significantly higher radiographic outcomes than the untreated and CS groups at the 56th post-operative day ($P<0.05$). The density of osseous tissue (DOT) and the osteocytes and osteoblasts count of the CS-RJ and CS-BV groups were significantly higher than the CS and autograft groups ($P<0.05$). The biomechanical results of the CS-RJ group were significantly superior to the autograft, while the biomechanical properties of CS-BV group were not significantly different with the autograft group ($P>0.05$). The scaffolds in CS group were observable in the surgical site after 56 days. There was no significant difference in radiographs, DOT, cartilage tissue and fibrous tissue, and also biomechanical performances of the CS-BV and CS-RJ groups at the 42nd and 56th day after surgery. The untreated and CS groups showed weakest biomechanical results among all groups. It could be concluded that both treatment strategies in the CS-BV and CS-RJ groups were appropriate and useful in treating critical bone defects.

Key words: Bee venom, Bone healing, Chitosan, Royal jelly

Introduction

Bone autograft is gold standard in treatment of bone fractures, especially those fractures with lost parts or those suffered from delayed-union or non-union for some reasons (Buck and Murtha, 2017). However, bone grafting is associated with some well-known side effect that limit their applications (Oryan *et al*., 2014a). Tissue engineering is an excellent choice in treatment of bone fractures and includes scaffolds, enhancers of bone formation and stem cells (Oryan *et al*., 2016c). In fact, bone scaffold is the most important part of bone tissue engineering and can be divided into natural and artificial groups (Oryan *et al*., 2014b). Natural scaffolds are considerably more biocompatible, more biodegradable and have better regeneration properties than their synthetic counterparts (Zhang *et al*., 2008). In addition to the specific structure of these scaffolds, they can also transfer cells, medications or molecules to the fracture site.

Chitosan scaffold (CS) is a natural polysaccharide that has attracted much attention in medicine and pharmacology because of its unique biocompatibility, biodegradability and low toxicity (Sforcin *et al*., 2017). Chitosan is produced by partial deacetylation of chitin (Komi *et al*., 2017). It has a lot of biological properties, including beneficial effectiveness on wound healing and antimicrobial and antiviral activity (Komi *et al*., 2017). The CS molecules retain their structure in neutral environments, but they break down and degrade in acidic environments such as in inflammatory organs. Therefore, it is possible to use CS as a mean for delivering the drugs in an acidic environment (Rady *et al*., 2017).

The honey bee venom (BV) has been used in traditional medicine, since ancient times, as a curative agent for various diseases such as arthritis, rheumatism, back pain, tumors and skin diseases. Honey BV may be used as apitherapy, apipuncture, or local injection. It contains different peptides, enzymes and non-peptide components. Bee venom regulates function of the immune system and increases the production of cortisol (Rady *et al*., 2017). Combination of CS and BV as biofilm and hydrogel has been shown to have positive effects on wound healing in the healthy and diabetic rats (Amin *et al*., 2008; Amin and Abdel-Raheem, 2014). On the other hand, royal jelly (RJ) is produced by worker bees and is the permanent food of the queens and the first few days' larvae. Royal jelly contains water, proteins, carbohydrates, fat, fatty acids, mineral salts, vitamins and polyphenols (Sabatini *et al*., 2009). It has been

demonstrated that RJ has antibacterial, antioxidant, antitumor, and anti-inflammatory properties (Viuda-Martos *et al*., 2008; Ramadan and Al-Ghamdi, 2012). Experimental studies have shown that RJ results in vascular dilatation and increases cell proliferation and differentiation (Hattori *et al*., 2007; Zamami *et al*., 2008; Münstedt *et al*., 2009). It has been shown that apisin, one of the major glycoproteins in RJ, increases the proliferation of neonatal skin fibroblasts, and results in enhanced secretion of collagen, glycosaminoglycans and other extracellular matrices (Tsuruma *et al*., 2011). In addition, apisin promotes differentiation of the MC3T3-E1 cells into osteoblasts (Bogdanov, 2017). Combination of such beneficial effects of BV or RJ, along with CS scaffold may promote bone healing. Therefore, the aim of this study was to evaluate the clinical, radiological, macroscopical, histopathological and biomechanical effects of the CS-BV and CS-RJ scaffold composites on repair of radial bone defect in the rat model. The advantage of this model is that there is no need to use a stabilizing instrument that would interfere with the healing process (Okamoto *et al*., 2003). The hypothesis of this study was the possibility of BV and RJ positive effects along with the CS scaffold in improving the radial bone defect in rats.

Materials and Methods

Chitosan scaffold

An average molecular weight CS powder (Sigma-Aldrich) was slowly dissolved in acetic acid (0.5 M) (Merk, Germany) at 50°C. The resulted acidic solution was neutralized by adding NaOH solution (1 M) (Venkatesan and Kim, 2010). In order to create porosity, the CS (2% w/v) was freeze-dried (-80°C for 72 h) and cross-linked with 0.5% glutaraldehyde solution. After removing the glutaraldehyde residues (Campos *et al*., 2013), the scaffolds were fixed and dried in 96% ethanol and placed in sterile containers at 4°C before use.

Royal jelly and bee venom

The pure BV and fresh RJ were purchased from approved local bee fields. The natural products have different composition and quality between species and also in different seasons. The *Apis mellifera* RJ was collected in spring and was then characterized. The fresh RJ was freeze-dried immediately after purchase, to result in a white and soft powder that was kept away from light and humidity until use. The *Apis mellifera* BV was collected in spring and its HPLC test revealed melittin consisted 49% of its components.

Animals and surgical operation

Sixty-three male Wistar rats, aging eight weeks and weighing 200 to 250 g, were purchased from an approved local animal house. Three rats (n=6; number of radial bones) were randomly selected and considered as the normal bone group to compare the biomechanical performance of the intact radius and ulna bone complexes with the injured treated or untreated bones of other groups. Two weeks before the study started, the animals were moved to new cages to become accustomed with the new environment and diet. Water and food were freely accessible throughout the experiment. During the experiment, animal care was performed for all rats in accordance with the Animal and Health Guidelines, published by the National Institutes of Health (NIH Publication No. 85-23). Animal studies were approved by our Veterinary School's Ethics Committee.

After induction of anesthesia, using ketamine (10%, 75 mg/kg) and xylazine (2%, 10 mg/kg BW) (both from Alfasan, Woerden, Netherlands), a 5 mm bone piece from the middle of the radius bone was removed to produce a non-union model (Bigham-Sadegh and Oryan, 2015). Based on the implanted material, the radial bone defects were randomly divided into 5 groups (n=12/group). The first two groups were the untreated and autograft groups. The bone defects in the untreated group remained empty and in the autograft group, the defects were filled with the extracted bone pieces from the contralateral radius. Groups 3-5 were the CS, CS-BV, and CS-RJ groups, in which the bone defects were implanted with the CS scaffolds, with similar size to the harvested radial bone segments (dimensions = 2 × 2 × 5 mm^3). After implanting the scaffolds and closing the approach, 0.1 ml of the diluted BV solution (1 mg/ml), or RJ solution (50 mg/ml) was injected percutaneously into the defect site, in the CS-BV and CS-RJ groups, respectively. Injection of the BV, and RJ was similarly repeated in the third postoperative day. Flunixin meglumine (2.5 mg/kg) was used for pain relief, and Enrofloxacin (Enrofan 5%) was administered intramuscularly for 5 days, for antibiotic therapy. The animals were euthanized 56 days after the operation.

Clinical examinations

Clinical behavior, physical activities, and weight bearing on the injured limb were blindly evaluated throughout the experiment, in terms of pain expression at digital touch and presence of inflammatory responses such as hyperemia, swelling and edema.

Evaluation of the diagnostic imaging

Lateral radiography was performed from the injured area of forelimbs in the anesthetized animals [ketamine (10%, 75 mg/kg) and xylazine (2%, 10 mg/kg BW) (both from Alfasan, Woerden, Netherlands)] at the 0th, 28th, 42nd and 56th days after injury. Blind evaluation of the radiographs was blindly done by two orthopedic surgeons and bone formation, bone union and bone remodeling were scored based on the Lane and Sindhu system (Lane and Sandhu, 1987) which has been described in Table 1.

Histopathological evaluations

The animals were euthanized at the 56th post-surgical day by rapid intracardiac injection of potassium chloride (10%) in the deep stage of anesthesia [ketamine (10%, 75 mg/kg) and xylazine (2%, 10 mg/kg BW) (both from

Alfasan, Woerden, Netherlands)] (Tasker, 2008). The radius and ulna complex were extracted and the healing area of the radius was evaluated macroscopically. The radius and ulna bone complexes (n=6/group) were fixed in 10% formalin buffer solution. They were decalcified with 0.5% nitric acid, dehydrated with graded ethanol, cleared by xylol, and finally embedded in paraffin blocks. The paraffin blocks were cut in 5 µm thick slices and then stained with hematoxylin and eosin. Blind assessments of tissue sections were blindly performed by two experienced pathologists. Three different microscopic fields of the healing area in each tissue section (×400) were photographed, using a digital camera (Olympus, Tokyo, Japan) connected to a typical optical microscope (Olympus, Tokyo, Japan). Fibroblasts /fibrocytes, chondroblast/chondrocytes, osteoblasts/ osteocytes, osteoclasts, polymorphonuclear and mononuclear inflammatory cells as well as blood vessels (BV) in each field were counted. The density of fibrous tissue (DFT), density of cartilaginous tissue (DCT) and DOT were also assessed in the ×400 magnification.

Biomechanical evaluations

The samples considered for biomechanical evaluations (n=6 in each group) were wrapped in sterile gas soaked with phosphate buffer solution (PBS) and were kept at -20°C until biomechanical test [three-point bending test using a universal tensile testing machine (Instron, London, UK)]. After measuring the diameter of each bone sample, they were horizontally placed on two supporting bars at a distance of 20 mm. A third rod was lowered at 10 mm/min in the middle of the healing area until fracture occurred. The load-deformation curve was drawn and the ultimate load, maximum stress, yield load, bending stiffness, ultimate strain, and yield strain were measured and calculated. All the extracted data from the load-deformation curve were presented as mean± standard deviation (SD), as previously described (Oryan *et al*., 2016b).

Table 1: Modified Lane and Sandhu radiological scoring system

Radiological healing time pattern	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 evaluated separately)	
No union	0
Possible union	1
Radiographic union	2
Remodeling	
No evidence of remodeling	0
Remodeling of medullary canal	1
Full remodeling of cortex	2
Total point possible per category	
Bone formation	4
Proximal union	2
Distal union	2
Remodeling	2
Maximum score	10

Statistical analysis

The quantitative data such as the biomechanical and histomorphometric results were presented as mean±SD and were analyzed statistically by one-way ANOVA followed by Tukey post-hoc test. The qualitative or scored data were presented as mean (min-max) and subjected to Kruskal-Wallis, non-parametric ANOVA, and subsequent Mann-Whitney U test. P-values lower than 0.05 were considered statistically significant. The whole statistical analysis was done by SPSS software, version 19.0 (SPSS, Inc., Chicago, USA).

Results

The diagnostic imaging outcomes

The radiographic results are shown in Table 2. The untreated, CS, and CS-RJ groups showed significantly inferior results to the autograft group at day 28 (P=0.000, P=0.000, and P=0.007 respectively). Although the radiological score of the CS-BV group were superior to the CS and the untreated groups, the differences were not significant.

There was also no significant difference between the autograft group and CS-BV and CS-RJ groups at the 42nd day after surgery. There was no significant difference between the CS group and the untreated group at this stage. The CS-BV group was superior to the CS and untreated groups and was closer to the autograft group at this stage, but it still did not show a significant difference with any of them at the 42nd day post-injury. There was also no significant difference between the CS and CS-RJ despite the superiority of the CS-RJ to CS group (P=0.051). There was a significantly higher radiological score in the autograft over the CS and untreated groups (P<0.05). There were no significant

Table 2: The sum of radiographically scored bone healing at determined postoperative intervals

Postoperative days	Groups					p[a]
	Autograft (1) (n=12)	Untreated (2) (n=12)	CS (3) (n=12)	CS-BV (4) (n=12)	CS-RJ (5) (n=12)	
28	5.27 (3-8)[b]	1.58 (1-2)	1.2 (0-4)	3.5 (0-8)	2.54 (0-6)	0.000
42	5.90 (4-10)[c]	2.91 (1-4)	2.5 (1-4)	3.83 (0-10)	4.18 (1-8)	0.031
56	6.36 (4-10)[d]	3.00 (1-5)[e]	3.7 (2-5)[f]	5.91 (0-10)	6.72 (4-9)	0.001

CS: Chitosan scaffold, CS-BV: Chitosan scaffold-Bee venom, and CS-RJ: Chitosan scaffold-Royal jelly. [a] Kruskal-Wallis non-parametric ANOVA, [b] P<0.05 (1 vs. 2, 3, 5), [c] P<0.05 (1 vs. 2, 3), [d] P<0.05 (1 vs. 2, 3), [e] P<0.05 (2 vs. 4, 5), and [f] P=0.003(3 vs. 5)

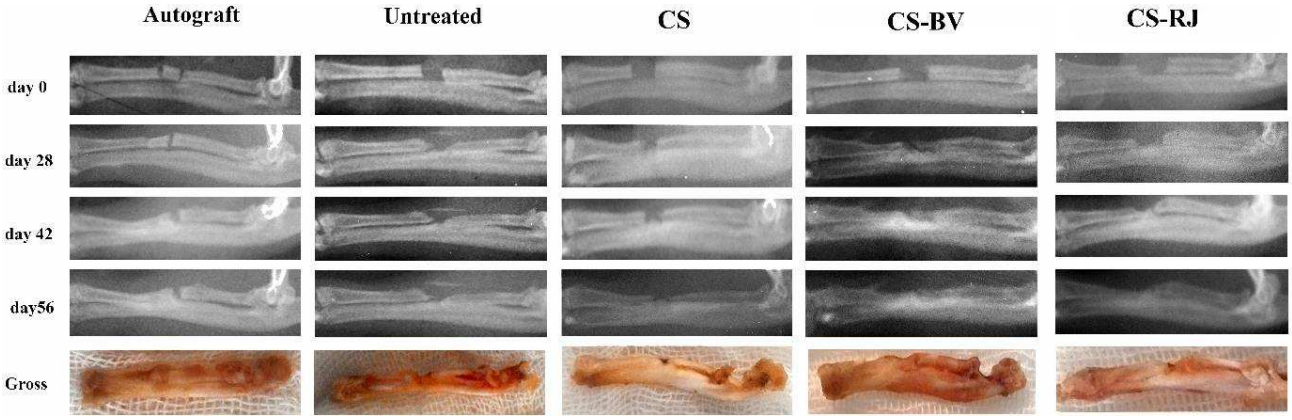

Fig. 1: Macroscopic and radiographic findings of the radial defects at 56 days after injury. In the gross evaluation, the defects in the autograft group were filled by hard connective tissue including cartilage and bone which connected to cut edges of the radius and the ulnar periosteum. In the CS-RJ and CS-BV groups the healing areas were filled with bone and cartilage tissues. While in the untreated defect group the defect area was mostly filled by loose soft connective tissues. The autograft group demonstrated significant superiority over the untreated defect group at the 56th post-operative day ($P<0.05$). The untreated defect and CS groups demonstrated significantly inferior bone formation in comparison to the autograft, CS-RJ and CS-BV groups at the 56th post-operative day ($P<0.05$). CS: Chitosan scaffold, CS-BV: Chitosan scaffold-Bee venom, and CS-RJ: Chitosan scaffold-Royal jelly

differences between other groups at this stage.

The CS-BV and CS-RJ groups had no significant difference with the autograft group at the 56th day after injury ($P>0.05$). There were significant differences in radiographic scores between the CS-BV and CS-RJ with the untreated group ($P=0.045$, $P=0.000$, respectively) at this stage. Radiographic evaluations demonstrated significant superiority of the CS-RJ over CS group ($P=0.000$) at this time point. Despite the superiority of CS-BV scores over the CS group, there was no significant difference between them ($P=0.107$), at the 56th postoperative day. There were no significant differences between other groups at this stage (Fig. 1).

Gross and histopathological findings

During the 56 days of the experiment, no death occurred, and normal activities, normal weighing on the limbs and normal weight gain in animals were observed in all groups. A fascia-shaped soft tissue was formed and the bone union did not occur in the bone defect of the untreated group. The scaffold in the CS treated group, as well as the autograft group, was not completely degraded and their remnants were visible at the defect site after 56 days. A combination of soft tissue and hard tissue, including cartilage, was found in the CS group, while the defect area in the autograft, CS-BV, and CS-RJ groups were replaced with a bone-like hard tissue.

The implanted scaffolds in the CS-RJ and CS-BV groups were degraded and the defect areas were replaced by a combination of woven bone (WB), cartilage, fibrocartilage, and dense connective tissue (DCT) (Fig. 2). New bone formation was dominant at the edge of the bone and in the middle of the defect area, in these groups. The defects treated with CS-RJ were filled with WB and hyaline cartilage (HC). Addition of the BV and RJ probably led to a faster degradation of these scaffolds in the defect site. A combination of WB and HC was attached to the edges of the bone, in the autograft group, while a DCT was visible on the surface of the defect area. A loose areolar connective tissue (LACT) composed of collagen fibers, fibroblasts, BV and a small amount of cartilage and bone was formed near the radius edges, in the untreated group.

There was a significantly higher number of osteoblasts + osteocytes and DOT in the CS-RJ group than the CS group ($P=0.000$). The number of primary osteons (PO) in the CS, CS-BV, and CS-RJ groups was significantly more than the untreated group ($P<0.05$). On the other side, there was a significant superiority in the number of osteoblasts + osteocytes and DOT in the CS-BV over the CS group ($P<0.05$). The CS-BV and CS-RJ treated groups were significantly superior to the autograft group in the DOT ($P=0.000$, and $P=0.000$, respectively). There was no significant difference between the CS-BV and autograft groups in the number of osteoblasts + osteocytes. The chondrocytes + chondroblasts count and density of cartilage tissue (DCT) were significantly higher in the autograft group than the untreated group ($P=0.027$, $P=0.000$, respectively). The least healing was observed in the untreated group so that the main regenerated tissue in the defect sites was FCT (fibroblasts + fibrocytes, collagen fibers and BV), and the highest DFT belonged to this group ($P<0.05$). There was a significant superiority in the number of osteocytes + osteoblasts and DOT in the autograft group, over the CS group ($P=0.007$ and $P=0.001$, respectively) (Fig. 3).

The histomorphometric results indicated that the untreated defects had significantly higher number of fibroblasts + fibrocytes and greater DFT and significantly fewer osteoblasts/osteocytes, chondroblasts/chondrocytes and PO in comparison to the autograft, CS-BV, and CS-RJ treated groups (Table 3). Inflammatory cell infiltration was higher in the untreated and CS-BV groups than other groups. The highest percentage of bone tissue density was seen in the CS-RJ group.

Biomechanical performance

There was no significant difference between the CS-

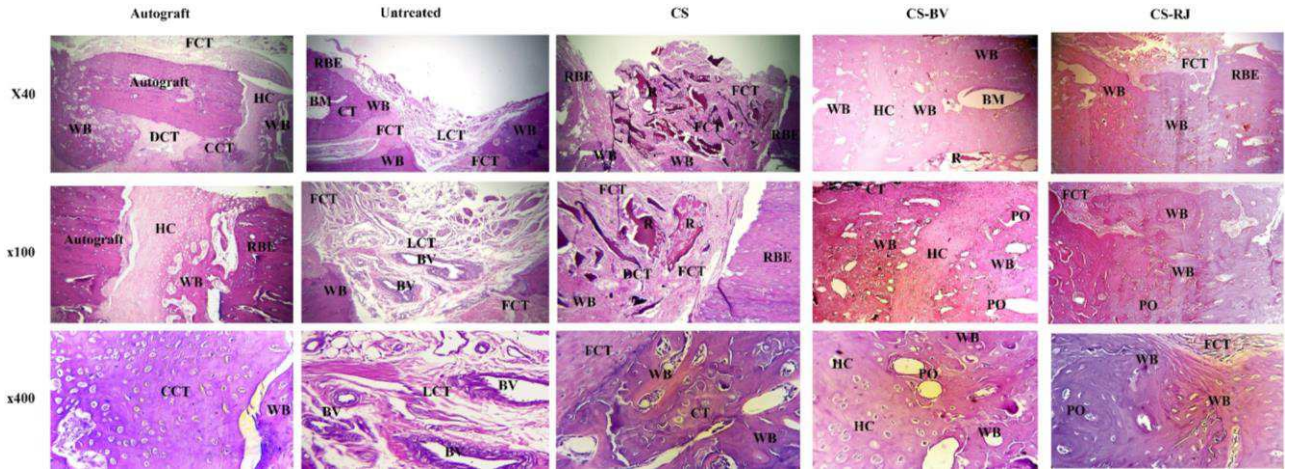

Fig. 2: Tissue sections from the radial bone defects at the 56th post-operative day. In the CS-RJ and CS-BV groups a remarkable superiority in bone formation and a significant decrease in cartilage and fibrous tissue is evident compared to the untreated, and CS groups. The scaffolds in the CS-RJ and CS-BV groups participated more effectively in healing than in the CS group. The scaffold was replaced by woven bone and hyaline cartilage in the CS-BV and CS-RJ groups, at the 56th post-operative day. Primary osteons were present very often in the newly formed osseous tissue which indicates early stages of remodeling in the CS-BV and CS-RJ groups. Cartilage and woven bone filled the distance between the radial bone edges in autograft group in which the graft is still visible in the defect area. The healing area, in the untreated group, is filled by loose areolar connective tissue, and minimum amounts of bone and cartilage tissue which are observed in the vicinity of the radial bone edges. Residual of the scaffolds still remain in the healing area of the CS group after 56 day of implantation while surrounded by fibrous connective tissue. Major part of the regenerated tissues consists of cartilage and osseous tissue, in the CS group (H&E staining). CS: Chitosan scaffold, CS-BV: Chitosan scaffold-Bee venom, and CS-RJ: Chitosan scaffold-Royal jelly, CT: Cartilage tissue, LCT: Loos connective tissue, FCT: Fibrous connective tissue, RBE: Radial bone edge, WB: Woven bone, BV: Blood vessel, CCT: Calcified cartilaginous tissue, HC: Hyaline cartilage, DCT: Dense connective tissue, BM: Bone marrow, R: Remnants of the scaffold, and PO: Primary osteon

Table 3: Histomorphometric findings in the lesions of different experimental groups after 56 days of injury

Parameter	Autograft (1)	Untreated (2)	CS (3)	CS-BV (4)	CS-RJ (5)	p[a]
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	
Inflammatory cells (n)	7.0 ± 2.05	10.86 ± 4.48[b]	9.71 ± 3.35[c]	10.0 ± 2.73[d]	6.0 ± 1.8	0.000
Fibroblast + fibrocyte (n)	18.6 ± 7.35[e]	65.8 ± 15.57[f]	54.14 ± 5.49[g]	13.1 ± 4.30	11.2 ± 2.27	0.000
Chondroblast + chondrocyte (n)	52.4 ± 10.54[h]	26.14 ± 6.98[i]	41.14 ± 10.12[j]	17.6 ± 5.26[k]	24.7 ± 4.38	0.032
Osteoblast + osteocyte (n)	46.9 ± 7.78[l]	7.86 ± 2.26[m]	34.57 ± 7.85[n]	45.2 ± 4.55[o]	4.5 ± 7.92	0.001
Osteoclast (n)	4.2 ± 1.54[p]	0[q]	1.29 ± 0.75[r]	1.60 ± 0.89[s]	3.56 ± 1.13	0.531
Blood vessels (n)	3.9 ± 1.72[t]	10.0 ± 2.30[u]	2.57 ± 1.27[v]	0.8 ± 1.3[w]	5.33 ± 1.22	0.000
Osteons (n)	2.1 ± 1.37[x]	0.86 ± 0.69[y]	2.43 ± 1.07[z]	4.4 ± 1.94	5.89 ± 1.69	0.260
Density of fibrous tissue (%)	13.50 ± 4.76[aa]	54.14 ± 9.77[ab]	37.71 ± 4.23[ac]	14.6 ± 4.82	10.78 ± 1.85	0.000
Density of cartilage tissue (%)	39.5 ± 6.60[ad]	22.0 ± 6.75	28.71 ± 6.52[ae]	20.0 ± 5.29	23.56 ± 3.39	0.000
Density of osseous tissue (%)	35.6 ± 6.96[af]	6.57 ± 2.14[ag]	24.14 ± 4.56[ah]	51.4 ± 2.51	51.67 ± 3.87	0.000

CS: Chitosan scaffold, CS-BV: Chitosan scaffold-Bee venom, and CS-RJ: Chitosan scaffold-Royal jelly. [a] One-way ANOVA followed by Tukey post-hoc test, [b] P<0.05 (2 vs. 5), [c] P<0.05 (3 vs. 5), [d] P<0.05 (5 vs. 5), [e] P<0.05 (1 vs. 2, 3, 5), [f] P<0.05 (2 vs. 4, 5), [g] P<0.05 (3 vs. 4, 5), [h] P<0.05 (1 vs. 2, 3, 4, 5), [i] P<0.05 (2 vs. 3, 4), [j] P<0.05 (3 vs. 4, 5), [k] P<0.05 (4 vs 5), [l] P<0.05 (1 vs. 2, 3, 5), [m] P<0.05 (2 vs. 3, 4, 5), [n] P<0.05 (3 vs. 4, 5), [o] P<0.05 (4 vs. 5), [p] P<0.05 (1 vs. 2, 3, 4), [q] P<0.05 (2 vs. 3, 4, 5), [r] P<0.05 (3 vs 5), [s] P=0.005 (4 vs. 5), [t] P<0.05 (1 vs. 2, 4), [u] P<0.05 (2 vs. 3, 4, 5), [v] P<0.05 (3 vs. 4, 5), [w] P<0.05 (4 vs 5), [x] P<0.05 (1 vs. 2, 5), [y] P<0.05 (2 vs. 3, 4, 5), [z] P=0.000 (3 vs. 5), [aa] P<0.05 (1 vs. 2, 3), [ab] P<0.05 (2 vs. 3, 4, 5), [ac] P<0.05 (3 vs 5), [ad] P<0.05 (1 vs 2, 3, 4, 5), [ae] P=0.000 (3 vs. 4), [af] P<0.05 (1 vs. 2, 3, 4, 5), [ag] P<0.05 (2 vs. 3, 4, 5), and [ah] P<0.05 (3 vs. 4, 5)

Table 4: Results of three-points bending biomechanical test on the radius-ulna complex 56 days after injury

Parameter	Untreated (1)	Autograft (2)	Normal (3)	CS (4)	CS-BV (5)	CS-RJ (6)	p[a]
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	
Maximum load (N)	29.9 ± 2.8[b]	39.0 ± 3.98[c]	47.1 ± 7.1[d]	35.8 ± 6.3[e]	42.9 ± 7.71	49.23 ± 10.0	0.001
Maximum stress (N/mm^2)	4.3 ± 0.78[f]	8.1 ± 1.36[g]	10.1 ± 1.07[h]	5.5 ± 2.1	6.87 ± 0.5	7.12 ± 1.8	0.000
Yield load (N)	24.4 ± 2.2[i]	33.2 ± 2.3[j]	39.7 ± 5.7[k]	29.3 ± 5.9[l]	34.3 ± 7.47	44.1 ± 9.01	0.000
Bending stiffness (N/mm)	19.42 ± 3[m]	32.56 ± 5.2[n]	45.83 ± 7.13[o]	22.73 ± 6.09[p]	43.76 ± 9.87	51.87 ± 6.69	0.000
Ultimate strain (%)	8.5 ± 2.1[q]	5.7 ± 1.0[r]	3.79 ± 1.01[s]	7.54 ± 1.9[t]	6.01 ± 1.44	4.49 ± 1.8	0.000
Yield strain (%)	6.68 ± 1.4[u]	5.04 ± 0.44[v]	2.53 ± 1.3[w]	6.1 ± 1.5[x]	3.98 ± 1.27	3.4 ± 1.6	0.000

Normal: Intact radius-ulna bone complex of rat, CS: Chitosan scaffold, CS-BV: Chitosan scaffold-Bee venom, and CS-RJ: Chitosan scaffold-Royal jelly. [a] One-way ANOVA followed by Tukey post-hoc test, [b] P<0.05 (1 vs. 2, 3, 4, 5, 6), [c] P=0.043 (2 vs. 3), [d] P<0.05 (3 vs. 4), [e] P=0.026 (4 vs. 6), [f] P<0.05 (1 vs. 2, 3, 6), [g] P<0.05 (2 vs. 3, 4), [h] P<0.05 (3 vs. 4, 5, 6), [i] P<0.05 (1 vs. 2, 3, 4, 5, 6), [j] P<0.05 (2 vs. 3, 6), [k] P<0.05 (3 vs. 4), [l] P<0.05 (4 vs. 6), [m] P<0.05 (1 vs. 2, 3, 5, 6), [n] P<0.05 (2 vs. 3, 4, 5, 6), [o] P<0.05 (3 vs. 4), [p] P<0.05 (4 vs. 5, 6), [q] P<0.05 (1 vs. 2, 3, 6), [r] P=0.010 (2 vs. 3), [s] P<0.05 (3 vs. 4), [t] P<0.05 (4 vs. 6), [u] P<0.05 (1 vs. 3, 5, 6), [v] P<0.05 (2 vs. 3), [w] P<0.05 (3 vs. 4), and [x] P<0.05 (4 vs. 5, 6)

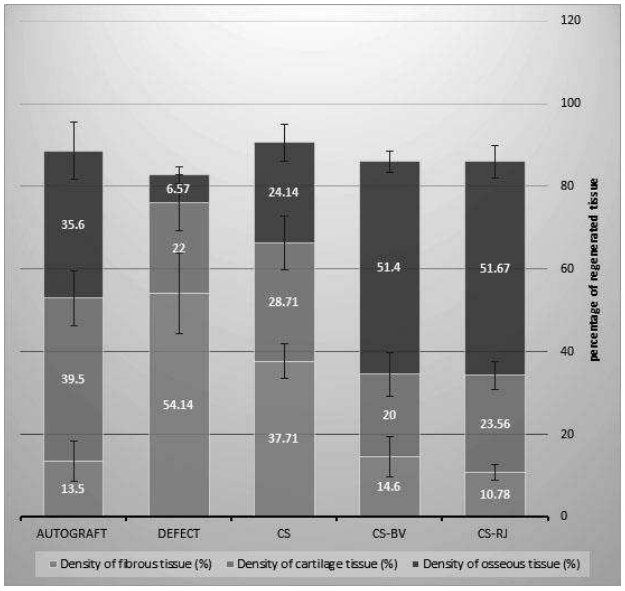

Fig. 3: Density of fibrous, cartilage and osseous tissues in the regenerated tissue of different groups at 56 days post-injury. The autograft had the highest DCT and the defect demonstrated the highest DFT. The CS-RJ and CS-BV have the highest DOT (P<0.05). The least amount of DOT belonged to the defect group while the lowest DFT contributed to the CS-RJ group (P<0.05). CS: Chitosan, CS-BV: Chitosan-Bee venom, CS-RJ: Chitosan-Royal jelly, DCT: Density of cartilage tissue, DFT: Density of fibrous tissue, and DOT: Density of osseous tissue

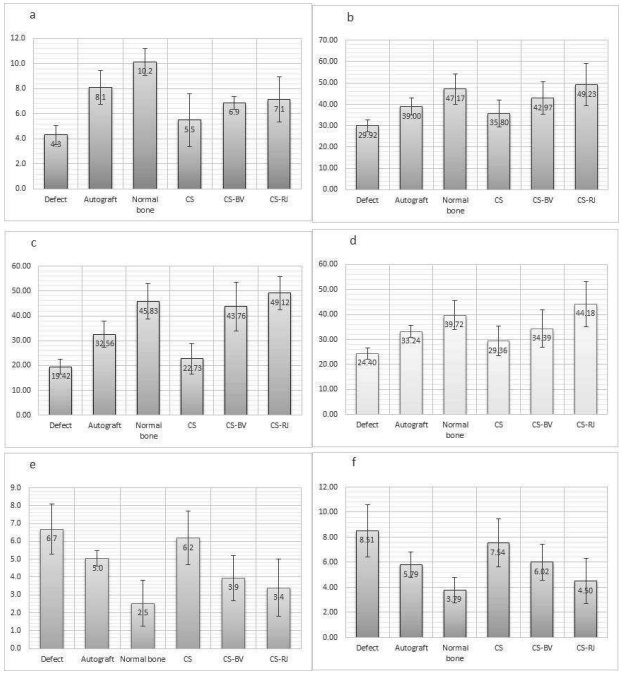

Fig. 4: Biomechanical properties of the radius and ulnar bone complex, 56 days after injury. Maximum stress (**a**), Maximum load (**b**), bending stiffness (**c**), yield load (**d**), yield strain (**e**), and ultimate strain (**f**) included for all the untreated and treatment groups. CS: Chitosan, CS-BV: Chitosan-Bee venom, and CS-RJ: Chitosan-Royal jelly

BV and CS-RJ in the evaluated biomechanical factors. There was no significant difference in biomechanical parameters between the CS-RJ and CS-BV groups and the normal group except for maximum stress (P=0.009, and P=0.001 respectively) and ultimate strain for CS-BV (P=0.008) which indicated superiority of the normal group. The normal group showed significantly higher maximum load, maximum stress, yield load, and bending stiffness and significantly lower ultimate strain and yield strain (P<0.05) compared to the autograft, untreated, and CS groups (P<0.05). There were no significant differences in the assessed biomechanical factors between the CS-BV and the autograft group. The CS-RJ group was significantly superior to the autograft group in the yield load (P=0.030) and bending stiffness (P=0.001). There was no significant difference in bending stiffness between the normal and autograft groups. The biomechanical properties of CS-BV were significantly higher than the CS in bending stiffness (P=0.006) and lower in yield strain (P=0.034). The biomechanical properties of CS-RJ group were statistically higher than the CS group in the maximum load (P=0.026), yield load (P=0.010), and bending stiffness (P=0.000). The ultimate strain and yield strain were significantly lower in the CS-RJ group than the CS group (P=0.027 and P=0.025, respectively). The biomechanical findings are shown in Table 4 and Fig. 4.

Discussion

Since ancient times, bee products have been used in many ways to treat various problems. The ability of honey as the main product of the hive in wound healing has led to significant results (Oryan and Zaker, 1998; Oryan *et al*., 2016a). The efficacy of the CS-RJ and CS-BV treated groups in improving bone formation in the defects area, in the present study, was superior to other groups after 56 days of surgery. The CS-RJ group was better than CS-BV group in most assessed criteria, but its superiority was not statistically significant. The CS scaffolds used in this study did not show infection and necrosis in tissue samples and clinical examination, which suggests its proper biocompatibility. However, the presence of the CS scaffold remnants in the defect area and lack of replacement of bone tissue in the lesions of the animals of this group, after 56 days of surgery, indicated its poor biodegradability and improper osteoconductivity. In addition to the CS-RJ group, the CS-BV group also showed proper biocompatibility, biodegradability and osteoconductivity. The CS scaffolds in the CS-BV and CS-RJ treated groups were completely decomposed and replaced with bone and cartilage tissues.

Chitosan scaffold has many promising characteristics, including non-toxicity, biocompatibility, anti-microbial, and antioxidant properties. Most reports regarding the antioxidant activity of CS are based on the ability of amine and hydroxyl groups to trap free radicals to form stable macromolecule radicals (Tamer *et al*., 2016). Alidadi *et al*. (2017) measured the potential of CS and demineralized bone matrix scaffolds in improving the repair of critical radial bone defects and reported the low biodegradability of CS scaffold that agrees with the

results of our study.

Bee venom could change bone metabolism by four main mechanisms. Melittin, as one of the main proteins of the BV, is responsible for the first mechanism which is suppression of the osteoclast formation by obstructing the RANK-RANKL system and also inhibiting the effect of interleukin (IL)-1β osteoclastogenesis (Badr *et al*., 2016). The second mechanism is based on the immune responses of hyaluronidase enzymes and phospholipase A in the BV that result in release of histamine and heparin from the mast cell granules, resulting in production and release of IL-1α and IL-6 (Yang *et al*., 2014; Al Subaie *et al*., 2016; Pak, 2017). Pharmacological studies have shown that IL-1, TNF-α1, and other proinflammatory signals have major roles in bone regeneration (Oryan *et al*., 2014; Rutkowski, 2014; Schmidt-Bleek *et al*., 2014). The third mechanism of BV in improving bone repair is probably based upon its antioxidant activity. Badr *et al*. (2016) reported that the antioxidant activity of BV led to a reduction in reactive oxygen species, nitric oxide and anti-inflammatory cytokines (Badr *et al*., 2016). Finally, BV can increase growth factors such as transforming growth factor-β and vascular endothelial growth factor in the healing area, leading to cellular proliferation and differentiation (Jaasma *et al*., 2007; Dang *et al*., 2011; Wu *et al*., 2012; Freudenberg *et al*., 2015; Schliephake *et al*., 2015; Zhao *et al*., 2015; Badr *et al*., 2016). In the present study, the CS-BV treated group showed increased biodegradability and osteoconductivity. Unfortunately, in the present study immunological assays did not perform and the exact mechanism responsible for bone healing improvement by BV could not be determined. However, decrease of osteoclasts in CS-BV group in comparison to other groups in histomorphometrically assessments probably emphasize on the role of melittin, which contains 49% of the applied BV, in suppression of osteoclast. The CS scaffold in the CS-BV group was biologically degraded and more integrated with fresh bone and cartilage tissues than the other groups. Probably destruction of the scaffold in this group was the result of severe inflammation, enzymatic activity and enormous phagocytosis in the inflammatory and early proliferative stages of wound healing.

Royal jelly is composed of water, proteins, amino acids, lipids, vitamins, sugar and steroids (Mateescu, 1999). Various properties have been attributed to this natural compound, including anticoagulant, anti-inflammatory, anti-bacterial, anti-carcinogenic, as well as diminishing blood pressure and enhancing wound healing (Okamoto *et al*., 2003; Kashima *et al*., 2014; Siavash *et al*., 2015). It has been suggested that major RJ proteins (MRJPs) play a significant role in RJ biological activities, especially in wound healing (Siavash *et al*., 2015). It has also been reported that the effects of RJ on estrogen receptors increase the proliferation of MC3T3-E1 cells, the precursor cell of osteoblast (Kaku *et al*., 2014). There is evidence that RJ has high mitogenic properties and also stimulates expression of extracellular bone matrix (Narita *et al*., 2006; Musa *et al*., 2014; Chen *et al*., 2016). Antioxidant activity of RJ also has an important role in bone healing process as Ozan *et al*. (2015) stated in regeneration of maxillary bone in rats. Another potential mechanism of RJ in enhancing bone healing is associated with increase in TGF-β production (Koya-Miyata *et al*., 2002; Satomi *et al*., 2004). The CS-BV and CS-RJ scaffolds showed superior biomechanical and histological results than the other treatment strategies including the autograft group, in the present study. Improvement in biomechanical performance in the CS-RJ group may be due to modulation of the inflammatory phase in the early stages of recovery and also enhanced proliferation and differentiation of the mesenchymal cells in the wound site in the proliferative and maturation phases of the healing process, which results in the earlier use of the damaged limbs. This weight-bearing behavior may increase the regeneration process and ultimately strengthen the biomechanical function of the injured limb.

The radiographic, histopathologic and biomechanical findings indicated a marked improvement in quality and quantity of the newly regenerated bone in the CS-BV and CS-RJ treated groups compared to the untreated and CS groups. Unfortunately, our study did not include *in vitro* study for determining possible mechanisms or compounds responsible in enhancing bone healing. The CS scaffolds along with BV and RJ are possible choices in healing of the critical radial bone defect, but certain mechanisms in enhancing bone healing by these natural substances needs to be widely investigated. Although further studies and evidence are still needed for the safe and uncomplicated use of these compounds, the results of this study highlight the capability of CS-BV and CS-RJ as potential alternatives and enhancers of bone graft substitute materials.

Acknowledgements

The authors would like to thank the authorities of Veterinary School, Shiraz University for providing facilities and supports. They are also thankful to Iran National Science Foundation (INSF) for financial support (grant No. 96006039).

Conflict of interest

There is no conflict of interest.

References

Alidadi, S; Oryan, A; Bigham-Sadegh, A and Moshiri, A (2017). Comparative study on the healing potential of chitosan, polymethylmethacrylate, and demineralized bone matrix in radial bone defects of rat. Carbohydr. Polym., 166: 236-248.

Al Subaie, A; Emami, E; Tamimi, I; Laurenti, M; Eimar, H; Abdallah, MN and Tamimi, F (2016). Systemic administration of omeprazole interferes with bone healing and implant osseointegration: an *in vivo* study on rat tibiae. J. Clin. Periodontol., 43: 193-203.

Amin, MA and Abdel-Raheem, IT (2014). Accelerated wound healing and anti-inflammatory effects of physically cross linked polyvinyl alcohol-chitosan hydrogel containing honey bee venom in diabetic rats. Arch. Pharm. Res., 37: 1016-1031.

Amin, M; Abdel-Raheem, I and Madkor, H (2008). Wound healing and anti-inflammatory activities of bee venom-chitosan blend films. J. Drug Deliv. Sci. Technol., 18: 424-430.

Badr, G; Hozzein, WN; Badr, BM; Al Ghamdi, A; Saad Eldien, HM and Garraud, O (2016). Bee venom accelerates wound healing in diabetic mice by suppressing activating transcription factor-3 (ATF-3) and inducible nitric oxide synthase (iNOS)-mediated oxidative stress and recruiting bone marrow-derived endothelial progenitor cells. J. Cell Physiol., 231: 2159-2171.

Bigham-Sadegh, A and Oryan, A (2015). Selection of animal models for pre-clinical strategies in evaluating the fracture healing, bone graft substitutes and bone tissue regeneration and engineering. Connect. Tissue Res., 56: 175-194.

Bogdanov, S (2017). Royal jelly, bee brood: composition, health, medicine: a review. Lipids. 3: 8-19.

Buck, B and Murtha, YM (2017). The management of bone defects in periarticular knee injuries: a review article. J. Knee Surg., 30: 194-199.

Campos, E; Coimbra, P and Gil, M (2013). An improved method for preparing glutaraldehyde cross-linked chitosan–poly (vinyl alcohol) microparticles. Polym. Bull (Berl)., 70: 549-561.

Chen, D; Xin, XX; Qian, HC; Yu, ZY and Shen, LR (2016). Evaluation of the major royal jelly proteins as an alternative to fetal bovine serum in culturing human cell lines. J. Zhejiang Univ. Sci. B., 17: 476.

Dang, Y; Liu, B; Liu, L; Ye, X; Bi, X; Zhang, Y and Gu, J (2011). The 800-nm diode laser irradiation induces skin collagen synthesis by stimulating TGF-β/Smad signaling pathway. Lasers Med. Sci., 26: 837.

Freudenberg, U; Zieris, A; Chwalek, K; Tsurkan, MV; Maitz, MF; Atallah, P; Levental, KR; Eming, SA and Werner, C (2015). Heparin desulfation modulates VEGF release and angiogenesis in diabetic wounds. J. Control Release. 220: 79-88.

Hattori, N; Nomoto, H; Fukumitsu, H; Mishima, S and Furukawa, S (2007). Royal jelly and its unique fatty acid, 10-hydroxy-trans-2-decenoic acid, promote neurogenesis by neural stem/progenitor cells *in vitro*. Biomed. Res., 28: 261-266.

Jaasma, MJ; Jackson, WM; Tang, RY and Keaveny, TM (2007). Adaptation of cellular mechanical behavior to mechanical loading for osteoblastic cells. J. Biomech., 40: 1938-1945.

Kaku, M; Rocabado, JMR; Kitami, M; Ida, T and Uoshima, K (2014). Royal jelly affects collagen crosslinking in bone of ovariectomized rats. J. Funct. Foods., 7: 398-406.

Kashima, Y; Kanematsu, S; Asai, S; Kusada, M; Watanabe, S; Kawashima, T; Nakamura, T; Shimada, M; Goto, T and Nagaoka, S (2014). Identification of a novel hypocholesterolemic protein, major royal jelly protein 1, derived from royal jelly. PloS one., 9: e105073.

Komi, DEA; Shafaghat, F and Zwiener, RD (2017). Immunology of bee venom. Clin. Rev. Allergy Immunol., 54: 386-396.

Koya-Miyata, S; Takei, Y; Ushio, S; Iwaki, K; Ikeda, M and Kurimoto, M (2002). Royal jelly and ascorbic acid 2-O-alpha-glucoside (AA-2G) increase collagen synthesis in normal hamster skin fibroblast cultures. Nat. Med., 56: 191-194.

Lane, JM and Sandhu, H (1987). Current approaches to experimental bone grafting. Orthop. Clin. North Am., 18: 213-225.

Mateescu, C (1999). Enhanced nutritive, functional and therapeutic action of combined bee products in complex food supplements. Rom. Biotechnol. Lett., 4: 163-172.

Münstedt, K; Henschel, M; Hauenschild, A and von Georgi, R (2009). Royal jelly increases high density lipoprotein levels but in older patients only. J. Altern. Complem. Med., 15: 329-330.

Musa, M; Nasir, NFM and Thirumulu, KP (2014). Evaluation of royal jelly as an alternative to fetal bovine serum in cell culture using cell proliferation assays and live cell imaging. Afr. J. Tradit. Complement Altern. Med., 11: 148-155.

Narita, Y; Nomura, J; Ohta, S; Inoh, Y; Suzuki, KM; Araki, Y; Okada, S; Matsumoto, I; Isohama, Y and Abe, K (2006). Royal jelly stimulates bone formation: physiologic and nutrigenomic studies with mice and cell lines. Biosci. Biotechnol. Biochem., 70: 2508-2514.

Okamoto, I; Taniguchi, Y; Kunikata, T; Kohno, K; Iwaki, K; Ikeda, M and Kurimoto, M (2003). Major royal jelly protein 3 modulates immune responses *in vitro* and *in vivo*. Life Sci., 73: 2029-2045.

Oryan, A; Alemzadeh, E and Moshiri, A (2016a). Biological properties and therapeutic activities of honey in wound healing: a narrative review and meta-analysis. J. Tissue Viability., 25: 98-118.

Oryan, A; Alidadi, S; Bigham-Sadegh, A and Moshiri, A (2016b). Comparative study on the role of gelatin, chitosan and their combination as tissue engineered scaffolds on healing and regeneration of critical sized bone defects: an *in vivo* study. J. Mater. Sci. Mater. Med., 27: 155.

Oryan, A; Alidadi, S; Bigham-Sadegh, A; Moshiri, A and Kamali, A (2017). Effectiveness of tissue engineered chitosan-gelatin composite scaffold loaded with human platelet gel in regeneration of critical sized radial bone defect in rat. J. Control Release., 254: 65-74.

Oryan, A; Alidadi, S; Moshiri, A and Maffulli, N (2014a). Bone regenerative medicine: classic options, novel strategies, and future directions. J. Orthop. Surg. Res., 9: 18.

Oryan, A; Moshiri, A and Meimandi-Parizi, A (2014b). Implantation of a novel tissue-engineered graft in a large tendon defect initiated inflammation, accelerated fibroplasia and improved remodeling of the new Achilles tendon: a comprehensive detailed study with new insights. Cell Tissue Res., 355: 59-80.

Oryan, A; Papaioannou, N; Kamali, A and Stylianaki, I (2016c). Mesenchymal stem cells and immunomodulation: implications in bone tissue engineering. Int. Clin. Pathol. J., 2: 60.

Oryan, A and Zaker, S (1998). Effects of topical application of honey on cutaneous wound healing in rabbits. Transbound Emerg. Dis., 45: 181-188.

Özan, F; Çörekçi, B; Toptaş, O; Halicioğlu, K; Irgin, C; Yilmaz, F and Hezenci, Y (2015). Effect of royal jelly on new bone formation in rapid maxillary expansion in rats. Med. Oral Patol. Oral Cir. Bucal., 20: e651.

Pak, SC (2017). Chemical composition of bee venom. In: Alvarez-Suarez, J (Ed.), *Bee products - chemical and biological properties*. Springer, Cham. doi: 978-3-319-59689-1_13.

Rady, I; Siddiqui, IA; Rady, M and Mukhtar, H (2017). Melittin, a major peptide component of bee venom, and its conjugates in cancer therapy. Cancer Lett., 402: 16-31.

Ramadan, MF and Al-Ghamdi, A (2012). Bioactive compounds and health-promoting properties of royal jelly: a review. J. Funct. Foods., 4: 39-52.

Rutkowski, JL (2014). Controlled early inflammation and bone healing-potential new treatments. J. Oral Implantol., 40: 229-230.

Sabatini, AG; Marcazzan, GL; Caboni, MF; Bogdanov, S and Almeida-Muradian, L (2009). Quality and standardisation of royal jelly. J. ApiProd. ApiMed. Sci., 1: 1-6.

Satomi, KM; Okamoto, I; Ushio, S; Iwaki, K; Ikeda, M and Kurimoto, M (2004). Identification of a collagen production-promoting factor from an extract of royal jelly and its possible mechanism. Biosci. Biotechnol. Biochem., 68: 767-773.

Schliephake, H; Rublack, J; Förster, A; Schwenzer, B; Reichert, J and Scharnweber, D (2015). Functionalization of titanium implants using a modular system for binding and release of VEGF enhances bone-implant contact in a rodent model. J. Clin. Periodontol., 42: 302-310.

Schmidt-Bleek, K; Schell, H; Lienau, J; Schulz, N; Hoff, P; Pfaff, M; Schmidt, G; Martin, C; Perka, C and Buttgereit, F (2014). Initial immune reaction and angiogenesis in bone healing. J. Tissue Eng. Regen. Med., 8: 120-130.

Sforcin, JM; Bankova, V and Kuropatnicki, AK (2017). Medical benefits of honeybee products. Evid. Based. Complement Alternat. Med., doi: 10.1155/2017/2702106.

Siavash, M; Shokri, S; Haghighi, S; Shahtalebi, MA and Farajzadehgan, Z (2015). The efficacy of topical royal jelly on healing of diabetic foot ulcers: a double-blind placebo-controlled clinical trial. Int. Wound J., 12: 137-142.

Tamer, TM; Valachová, K; Mohyeldin, MS and Soltes, L (2016). Free radical scavenger activity of chitosan and its aminated derivative. J. Appl. Pharma. Sci., 6: 195-201.

Tasker, L (2008). Methods for the euthanasia of dogs and cats: comparison and recommendations. World Society for the Protection of Animals, London.

Tsuruma, Y; Maruyama, H and Araki, Y (2011). Effect of a glycoprotein (apisin) in royal jelly on proliferation and differentiation in skin fibroblast and osteoblastic cells. Nippon Shokuhin Kagaku Kogaku Kaishi., 58: 121-126.

Venkatesan, J and Kim, SK (2010). Chitosan composites for bone tissue engineering—an overview. Mar. Drugs. 8: 2252-2266.

Viuda-Martos, M; Ruiz-Navajas, Y; Fernández-López, J and Pérez-Álvarez, J (2008). Functional properties of honey, propolis, and royal jelly. J. Food Sci., 73: 117-124.

Wu, CS; Wu, PH; Fang, AH and Lan, CC (2012). FK506 inhibits the enhancing effects of transforming growth factor (TGF)-β1 on collagen expression and TGF-β/Smad signalling in keloid fibroblasts: implication for new therapeutic approach. Br. J. Dermatol., 167: 532-541.

Yang, L; Murota, H; Serada, S; Fujimoto, M; Kudo, A; Naka, T and Katayama, I (2014). Histamine contributes to tissue remodeling via periostin expression. J. Invest. Dermatol., 134: 2105-2113.

Zamami, Y; Takatori, S; Goda, M; Koyama, T; Iwatani, Y; Jin, X; Takai-Doi, S and Kawasaki, H (2008). Royal jelly ameliorates insulin resistance in fructose-drinking rats. Biol. Pharm. Bull., 31: 2103-2107.

Zhang, X; Awad, HA; O'Keefe, RJ; Guldberg, RE and Schwarz, EM (2008). A perspective: engineering periosteum for structural bone graft healing. Clin. Orthop. Relat. Res., 466: 1777-1787.

Zhao, D; Shi, Y; Dang, Y; Zhai, Y and Ye, X (2015). Daidzein stimulates collagen synthesis by activating the TGF-β/smad signal pathway. Australas J. Dermatol., 56: e7-14.

Supporting Online Material

Refer to web version on PubMed Central® (PMC) for Supplementary Material.