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Ultrasonographic and Radiographic Evaluation of Zeolite/Collagen Nanocomposite Scaffolds Compared With Nanohydroxyapatite on Experimental Bone Defect Healing in Rabbit Femur

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Abstract

Objectives: The use of a biomaterial scaffold can improve the healing process of bone defects. Using radiologic and ultrasonographic methods, this study aimed to evaluate the effects of zeolite/collagen nanocomposite and nanohydroxyapatite (nHA) bone scaffolds on the healing process of bone defect in rabbit femur.

Materials and Methods: Twenty-eight mature male New Zealand white rabbits were classified into four equal groups (n=7 in each). In the first group, the defect was made, and the wound was closed with no treatment; in the second group, the nHA was implanted into the defect; in the third group, the nanocomposite of zeolite/collagen was implanted; and in the fourth group, the defect was filled using autograft. Radiologic (Sedecal Veterinary X-Ray System, Model No. A6544-2) and ultrasonographic (Mindray Z5 Veterinary Ultrasound Scanner) examinations were done on days 0, 15, 30, 45, and 60 postoperatively.

Results: There were no healing effects on days 0 and 7 in any of the studied groups in the radiologic examination. The highest and lowest healing effects were related to treatment with zeolite/collagen nanocomposite and control group on day 60 after operation, respectively. There was no angiogenesis on day 0 in any group in the ultrasonographic examination. The highest and lowest levels of angiogenesis were related to rabbits treated with zeolite/collagen nanocomposite and the control group on day 30 after operation, respectively. Also, bone filling and angiogenesis in rabbits treated with zeolite/collagen nanocomposite were higher than other groups.

Conclusions: Zeolite/collagen nanocomposite scaffolds bear a crucial capability in the reconstruction of bone defects and can be used in bone fractures.

Keywords: Bone scaffolds, Femur bone defect, Hydroxyapatite, Radiology, Ultrasonography, Zeolite/collagen nanocomposite

Introduction

Tissue engineering aims to restructure living tissues such as bones for substitution of injured or lost organs with healthy ones, hoping to preserve, restore, or increase part or whole organ function of living organisms. The designation of a biodegradable scaffold using biological and molecular cells is one of the major purposes of bone tissue engineering (1).

Clinical issues continue to arise in the repair of bone defects in orthopedic surgery. Loss of function, pain, the occurrence of local injuries in the harvesting procedure, limited supply of bone, the transmission of several diseases, host rejection, and lack of osteo-inductive properties are the most important limitation of conventional methods used for repair of bone defects (2). Using bone scaffolds prepared from natural biological and molecular materials is one of the best ways to increase the possibility of bone regeneration. A perfect scaffold material should be degradable, biocompatible, and have suitable mechanical properties. Additionally, the materials should have the same structure and composition of extracellular matrices of natural bone tissue (3).

In today's bioceramic market, nanohydroxyapatite (nHA) with the chemical formula of Ca10(OH)2(PO4)6 is one of the most versatile materials. Human bone, enamel, and dentine are primarily composed of this mineral. It has been applied for bone regeneration due to its bioactivity, biodegradability, and osteoconductive properties (4). So, biocomposite scaffolds containing nHA were fabricated in previous studies (4,5). There are no published data about inflammatory, toxic, or carcinogenic reactions due to the use of nHA as a bone scaffold (6). It also increases osteoblast proliferation, osseointegration and direct connection between implants and newly formed bone tissue (4,7,8).

Silica-based materials, such as zeolite, are commonly

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Key Messages

- This study proved that bone scaffolds are effective in bone healing.
- Bone scaffolds are easy-to-use and effective.

used as bone substitutes, implant coats, and drug delivery systems. One of the main benefits of silicate-based biomaterials is that they can form apatite when immersed in a simulated body fluid. Implants and human bones are bonded together by this action (9). The crystalline aluminosilicates classified as mesoporous materials like zeolite have many advantages over amorphous porous silica, such as large surface areas, rapid diffusion, and adjustable porosity (10, 11). In the biomedical field, zeolites are used for a variety of purposes, including bone grafts, implant coatings, antimicrobial agents, drug carriers, hemostatic agents, and diagnostic agents due to their non-cytotoxicity, biocompatibility, and mechanical strength. Studies have shown that zeolites play an important role in bone formation. This is probably due to the presence of silicon, which can stimulate bone growth (10-13) .

As another component of bone, collagen can enhance cell adhesion, proliferation, and differentiation. The collagen composites not only showed favorable biocompatibility but also enhanced adherent and differentiated bone marrow stromal cells. Furthermore, collagen scaffolds did not exhibit any aberrant events that hampered the regenerative process (14-16).

There is limited data about the application of zeolite/collagen nanocomposite and hydroxyapatite bone scaffolds. Hence, using radiologic and ultrasonographic methods, this study aimed to evaluate the effects of zeolite/collagen nanocomposite and nHA bone scaffolds on the healing process of bone defect in rabbit femur.

Materials and Methods

Using zeolite and collagen from Sigma-Aldrich (St. Louis, MO), the matrix was constructed, while glutaraldehyde solution (Sigma-Aldrich, St. Louis, MO) served as the cross-linking agent. We also used analytical grade reagents and solvents. Sodium hydroxide was dissolved in deionized water to obtain an alkali solution (pH 12).

Animals

In the present study, we purchased 28 healthy mature male New Zealand white rabbits (age range: 6–8 months; mean weight: 2.5–3 kg) from Pasteur Institute in Tehran, Iran. To reduce genetic variability, all rabbits were obtained from the same source. A standard pellet diet and tap water were provided to the rabbits (one rabbit per cage). Animal houses were in standard environmental conditions with a temperature of 18 ± 3°C, a humidity of 60 ± 5%, and a 12-hour light/dark cycle. All rabbits were starved at least 6 hours before the surgical operation. Additionally, rabbits were thirsty 2 hours before the surgical operation.

Treatments

Animals were divided into four equal groups (n = 7 in each) as follows: group I (control; received no treatment), group II (treated with nHA scaffold), group III (treated with zeolite/collagen nanocomposite scaffold), and group IV (treated with autograft).

Surgical Operation and Treatments

The surgical procedures were carried out following intramuscular infusions of Ketamine 10% (ketamine hydrochloride, 50 mg/kg) and Rompun 2% (xylazine, 5 mg/kg). The surgical site was cleaned with an iodinated surgical soap after the hair was removed. The aseptic technique was used throughout the surgical procedure. An entry point around 5 cm long was made together with the lateral right upper rear appendage, and the mid-diaphyseal surface of the femur was surgically uncovered by limit dismemberment. The periosteum was stripped from the bone employing a periosteal lift and around a 5-6 mm breadth bone defect was made within the proximal femur of one of the hind limbs employing an uncommon moo circular orthopedic penetrate. The osteotomy site was hydrated with 0.9% saline, while the periosteum around the osteotomy site, as well as the overlying muscles, were protected and removed. The osteotomy sites of each rabbit were treated according to the protocol in place at the time.

Group I received no treatment or scaffolds. The defect was implanted with a nHA scaffold in the rabbits of group II. The defect was implanted with zeolite/collagen nanocomposite scaffolds in the rabbits of group III. Finally, autograft was used in the rabbits of group IV (the defect was filled with bits of the drilled area). After that, the subcutaneous and periosteal tissues were closed. Muscles of the area were sutured with Vicryl stitches 0-4, and at the end, the skin was closed with nylon 0-4 sutures. During the postoperative period, the patient was given antibiotics (penicillin G procaine 40 000 IU/kg IM, bid), dexamethasone (0.6 mg/kg, IM), and analgesics, including tramadol hydrochloride (5 mg/kg, IM, bid). To prevent self-injury, all experimental animals were kept in separate cages (1,2).

Radiologic Examination

As a result of the procedure, the subjects were monitored daily for signs of infection, inflammation, and other abnormalities. Skin sutures were removed 10 days after the surgical operation. Rabbits were then subjected to radiological examination. All examinations were prepared at days 0, 7, 15, 30, 45, and 60 after surgical operation. Each animal was x-rayed in a lateral decubitus position in lateral-medial and ventral-dorsal views. All animals were exposed with a target-to-film distance of 100 cm and an exposure time of 0.10 second at 40 kV and 1 mA. Each radiograph was photographed with a 12-megapixel Canon® digital camera. The photographs were analyzed using the Image J software version 1.46r (Wayne Rasband

National Institutes of Health, USA). We studied gap distance, density of gap, amount of cortical callus, amount of external callus, amount of internal callus, new bone formation, and soft tissue swelling in all groups by radiologic examination. Findings were presented as 0, 1, 2, 3, and 4 grades; zero was the time when no bone reaction was created and 4 was the time when the defect was filled. Adjusted path and Sandhu radiological scoring framework were displayed as follows:

- 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

Ultrasonographic Examination

Rabbits were subjected to two-dimension, color, and power Doppler ultrasonographic examination to study the presence of defect and occurrence of probable injuries surrounding the bone defect, determine the amount of creation of collateral arteries surrounding the bone defect, and measure pulsatility index, resistive index, maximum, average, and minimum blood flow velocity. All examinations were prepared at days 0, 7, 15, 30, 45, and 60 after surgical operation. The proximal part of the femur bone was used for color Doppler ultrasonographic examination.

To quantify the data obtained from an ultrasonographic examination, 1, 2, 3, 4, and 5 numbers were used;1 was the time when no bone healing was created and 5 was the time when the healing procedure was completed. Other numbers showed the degree of angiogenesis based on the number of Doppler signals received in the ongoing repair defect. The stage of fracture healing was determined via ultrasonography based on the echogenicity and organization of the tissue at the fracture site and localization of neovascularization. Vascularization was assigned a grade based on the mean of the number of Doppler signals from all assessed sites of one rabbit at one visit as follows: grade 0, 0 Doppler signals; grade 1, 0 to < 5 Doppler signals (red or purple); grade 2, 5 to < 10 Doppler signals (orange); and grade 3, > 10 Doppler signals (yellow).

Statistical Analysis

Results were analyzed using the Statistical Package for the Social Sciences (SPSS, V. 21) software (SPSS Inc., Chicago, IL, USA). A Kruskal-Wallis test was used to determine any significant differences between groups. A *P* value of 0.05 was considered statistically significant.

Results

Radiologic Evaluation

This study examined the effects of zeolite/collagen nanocomposite and nHA bone scaffolds on the healing process of rabbit femur bone defect using radiologic and ultrasonographic methods. Figure 1 shows the lateral and ventral-dorsal views of an experimental defect in rabbit femur bone at day 0.

Table 1 represents the amount of filling of the experimental bone defect based on days of measurement in the radiologic examination. There were no healing effects on days 0 and 7 in any groups. The highest and lowest amounts of healing were related to day 60 after surgical operation in animals treated with zeolite/collagen nanocomposite (3.85±0.377 mm) and control group (3.57±0.534 mm), respectively. The amount of healing in rabbits treated with zeolite/collagen nanocomposite in all the tested days was higher than other groups. In addition, the amount of healing was equal on day 60 after surgical operation in rabbits treated with autograft and nHA (3.71±0.487 mm).

Figure 2 represents the amounts of filling of experimental bone defect in all the studied groups on day 30 after surgical operation.

Table 2 represents the results of the Kruskal–Wallis test for evaluating the healing effects in four different groups. There was a statistically significant difference between the

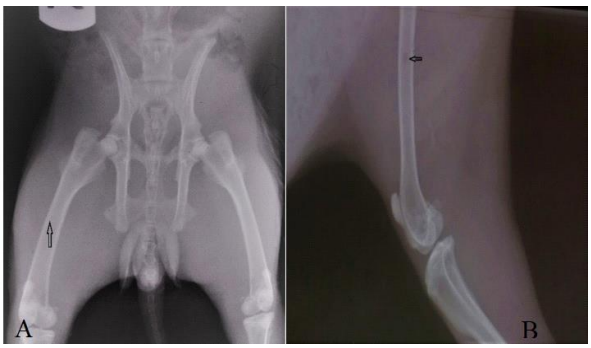

Figure 1. VD (a) and lateral (b) views of an experimental defect in the rabbit femur bone.

Table 1. Amount of Filling (mm) of the Experimental Defect Based on Days of Measurement in the Radiologic Examination

Groups	Days of Experiment					
	0	7	15	30	45	60
Control	-	-	1 ± 0.577	1.14 ± 0.377	2.28 ± 0.487	3.57 ± 0.534
Nanohydroxyapatite	-	-	1.14 ± 0.377	2.14 ± 0.690	2.71 ± 0.487	3.71 ± 0.487
Zeolite/collagen nanocomposite	-	-	1.71 ± 0.755	3.14 ± 0.377	2.85 ± 0.377	3.85 ± 0.377
Autograft	-	-	1 ± 0.577	1.28 ± 0.487	2.71 ± 0.487	3.71 ± 0.487

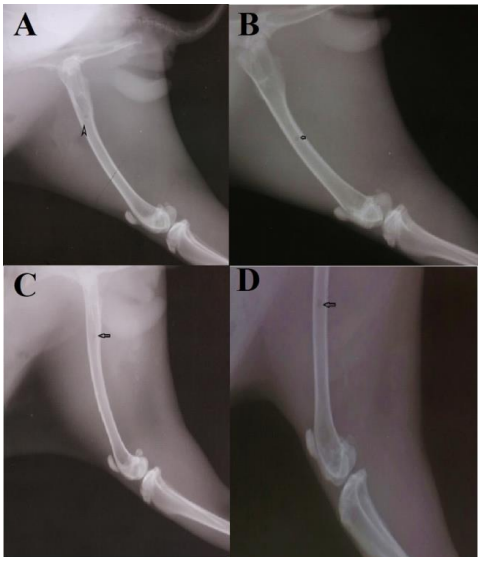

Figure 2. Amounts of filling of experimental bone defect in rabbits of autograft (A), nanohydroxyapatite (B), zeolite/collagen nanocomposite (C), and control (D) groups on day 30 after surgical operation.

four studied groups on day 30 postoperatively (P <0.05). However, there were no significant differences between the four studied groups on other days of the experiment.

Ultrasonographic Evaluation

Findings of the radiologic examination were confirmed using the ultrasonographic examination. Figure 3 represents the color ultrasonographic findings of an experimental defect in rabbit femur bone on day 0.

Table 3 represents the amount of angiogenesis in the experimental bone defect based on days of measurement in the ultrasonographic examination. There was no angiogenesis on day 0 in any group. The highest and lowest levels of angiogenesis were related to rabbits treated with zeolite/collagen nanocomposite (1.28 ± 0.487 mm) and control group (0.85 ± 0.377 mm) on day 60 after surgical operation, respectively. Also, the angiogenesis levels in

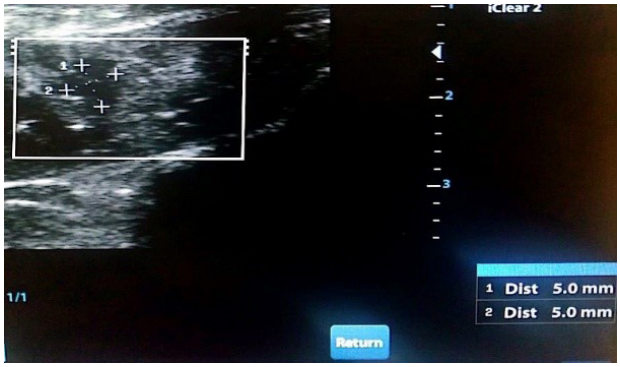

Figure 3. Color Ultrasonographic Findings of an Experimental Defect in Rabbit Femur Bone on Day 0.

rabbits treated with zeolite/collagen nanocomposite in all the tested days were higher than other groups. The angiogenesis levels on day 60 after surgical operation were equal in rabbits treated with autograft and nHA (1.14±0.377 mm). The highest angiogenesis levels were seen on day 30 after surgical operation in all the studied groups.

Figure 4 represents the angiogenesis levels of an experimental bone defect in all the studied groups on day 45 after surgical operation.

Table 4 represents the results of the Kruskal–Wallis test for the evaluation of the amounts of angiogenesis of 4 different groups. The amount of sig for the 45th day after the surgical operation was lower than 0.05 which showed statistically significant differences for the amount of angiogenesis of experimentally defect between four studied groups (P<0.05). Diversely, there were no significant differences between the 4 studied groups on other days of the experiment.

Discussion

Nowadays, many scientists are trying to invent suitable biomaterials that can improve the bone healing process.

Table 2. Results of the Kruskal–Wallis Test Regarding the Evaluation of the Amounts of Healing in the Radiologic Examination

Days	Groups	Number of Rabbits	Mean Score	Test Statistic	df	*P* Value
15	Control	7	12.29	5.603	3	0.132
	Nanohydroxyapatite	7	13.79			
	Zeolite/collagen nanocomposite	7	19.64			
	Autograft	7	12.29			
30	Control	7	7.86	19.939	3	0.001
	Nanohydroxyapatite	7	16.79			
	Zeolite/collagen nanocomposite	7	24.14			
	Autograft	7	9.21			
45	Control	7	9.50	5.400	3	0.145
	Nanohydroxyapatite	7	15.50			
	Zeolite/collagen nanocomposite	7	17.50			
	Autograft	7	15.50			
60	Control	7	12.50	1.350	3	0.717
	Nanohydroxyapatite	7	14.50			
	Zeolite/collagen nanocomposite	7	16.50			
	Autograft	7	14.50			

Table 3. Amount of Angiogenesis (mm) in the Experimental Defect Based on Days of Measurement in the Ultrasonographic Examination

Groups	Days of Experiment					
	0	7	15	30	45	60
Control	-	0.85±0.377	2.14±0.377	2.85±0.377	2±0.577	0.85±0.377
Nanohydroxyapatite	-	1.14±0.377	2.28±0.487	3.14±0.377	2.14±0.377	1.14±0.377
Zeolite/collagen nanocomposite	-	1.28±0.487	2.28±0.487	3.28±0.487	3±0.577	1.28±0.487
Autograft	-	1.14±0.377	2.14±0.377	3.14±0.377	2.14±0.377	1.14±0.377

Table 4. Results of the Kruskal–Wallis Test for Evaluating the Angiogenesis Levels in the Ultrasonographic Examination

Days	Groups	Number of Rabbits	Mean Score	Test Statistic	df	*P* Value
7	Control	7	11.29	3.793	3	0.285
	Nanohydroxyapatite	7	14.93			
	Zeolite/collagen nanocomposite	7	16.86			
	Autograft	7	14.93			
15	Control	7	13.50	0.818	3	0.845
	Nanohydroxyapatite	7	15.50			
	Zeolite/collagen nanocomposite	7	15.50			
	Autograft	7	13.50			
30	Control	7	11.29	3.793	3	0.285
	Nanohydroxyapatite	7	12.93			
	Zeolite/collagen nanocomposite	7	16.86			
	Autograft	7	14.93			
45	Control	7	11	11.924	3	0.008
	Nanohydroxyapatite	7	12.36			
	Zeolite/collagen nanocomposite	7	22.29			
	Autograft	7	12.36			
60	Control	7	11.29	3.793	3	0.285
	Nanohydroxyapatite	7	14.93			
	Zeolite/collagen nanocomposite	7	16.86			
	Autograft	7	14.93			

Bone has an extraordinary regenerative capability, but a significant amount of bone damage or development of a contrary microenvironment, including revision surgeries, severe trauma, growing deformities, and tumor resection can delay this ability. Using bone tissue engineering to heal bones has a great therapeutic potential. A perfect and applied bone graft material should have osteo-inductive, osteo-conductive, and osteogenic features. Consequently, some investigators used a combination of osteo-inductive organic agents and synthetic biomaterials to achieve better outcomes(17).

The current research was conducted to study the effects of zeolite/collagen nanocomposite and nHA bone scaffolds on the healing process of rabbit femur bone defect using radiologic and ultrasonographic examinations. Bone healing effects of the zeolite/collagen nanocomposite were compared with nHA, autograft, and control groups. Both radiologic and ultrasonographic examinations confirmed that the bone filling and angiogenesis levels in rabbits treated with zeolite/collagen nanocomposite were higher than other studied biomaterials. It seems that the quantity of newly formed lamellar bone in the healing site in the zeolite/collagen nanocomposite group was better than other treatments after 60 days.

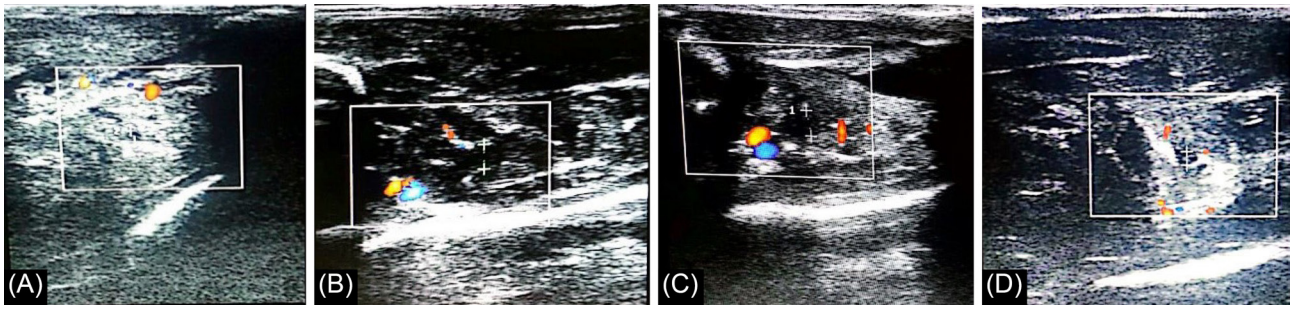

Figure 4. Ultrasonographic findings of the angiogenesis levels of an experimental bone defect in rabbits of control (A), nanohydroxyapatite (B), zeolite/collagen nanocomposite (C), and autograft (D) groups on day 45 after surgical operation.

Several similar investigations have been conducted on the effects of zeolite, collagen, and nHA bone scaffolds on the healing procedure of bone defect in animal models. As a biomaterial and drug delivery vehicle, nHA has been widely used as an orthopedic biomaterial due to its chemical and structural similarity to bone mineral. Researchers have demonstrated that nHA-based biomaterials exhibit minimal or no toxicity or inflammatory response when used for bone regeneration. Studies have been conducted to investigate the efficacy of nHA as a delivery system for bone regeneration. Additionally, it is still unclear whether combinations of proteins, antibiotics, or other bioactive molecules can further improve osteogenesis in vivo. In various animal models with large or critical-sized bone defects, open fractures, or methicillin-resistant *Staphylococcus aureus* (MRSA)-induced osteomyelitis, nHA induced bone regeneration. Drugs or bioactive molecules like bone-morphogenetic protein-2, vancomycin, calcitriol, dexamethasone, and cisplatin can be conjugated with nHA to enhance its osteogenic properties. Therefore, nanomaterials based on nHA can be used to promote bone regeneration in vivo (18).

Also, to better meet the biological requirements of bone repair materials, nHAp can be modified by loading relevant growth factors, proteins, peptides, and other bioactive molecules. In 2021, it was reported that the mechanical properties and biocompatibility could be effectively improved. However, conventional physicochemical modification methods are complicated and can affect the biological activity of nHAp (19).

Mohseni et al (1) reported that on days 15, 30, and 45 after surgical operation of femur bone defect in rabbit, the amount of newly formed lamellar bone and the rate of bone formation in rabbits treated with tricalcium phosphate/collagen nanocomposite were higher than nHA and control groups. They concluded that the application of tricalcium phosphate/collagen nanocomposite was useful for the reconstruction of bone defects and it could be performed as a scaffold in bone fractures. Faraji et al (2) reported the higher effects of zeolite/collagen than zeolite scaffolds on the healing procedure of the rabbit bone defect. The presence of thick lamellar bones in the trichrome staining of the femur bone defect of the rabbits treated with zeolite/collagen nanocomposite was another finding of their survey. The researchers concluded that zeolite and zeolite/collagen nanocomposites could be used as bone grafts in fracture healing.

As a result of their excellent biocompatibility and osteoconductive and osteo-inductive properties, ceramics such as hydroxyapatite have received a great deal of attention in recent years. Ceramics can have numerous grades of bioactivity regarding their conformation, particle size, and production procedure, which is the capability to chemically bond and be assimilated into the living bone through the formation of nHA. In this regard, nHA is stiff and has little mechanical constancy, making it inappropriate for load-bearing applications (20,21).

Findings of previous studies established that the growth, nucleation, and morphology of the nHA were deceptively exaggerated by the zeolite content. Improved bioactivity of the nanocomposites, especially zeolite and nHA, could be due to the attendance of the silanol group on the matrix, which facilitates the formation of the apatite layer. In addition, silanol groups are formed by exchanging Ca ions from the surface of nanocomposite with protons from simulated body fluid (22, 23). The effect of collagen as a scaffold is also significant in the formation of bone defects. An in vivo collagen scaffold is valuable for cells grown on collagen, the main structural protein in the body of living organisms (24). Additionally, scaffolds made of collagen possess brilliant biocompatibility and adequate mechanical features and have gained excessive achievement in bone healing (24). Several investigations approved the functional features of collagen scaffolds alone (25) and with calcium-phosphate (26), hydroxyapatite (27) zeolite (2), and chitosan (28).

Scaffold biomaterials applied in bone formation should have the subsequent standards (29). At first, they should permit osteoblast attachment; since these cells are anchorage-dependent need a sympathetic matrix for survival. Second, they should prepare a suitable condition for the growth and function of osteoblast. Third, they should permit the proliferation of vascular tissue to certify the survival of transplanted cells. Fourth, they should have biodegradable features with high decomposability properties. The final step of processing them into three-dimensional irregular forms should be possible. All these features can be found in the zeolite/collagen nanocomposite bone scaffolds designed by authors.

Conclusions

In this study, we identified a considerable effect of zeolite/collagen nanocomposite bone scaffolds compared to hydroxyapatite in the healing procedure of experimental rabbit femur bone defect using radiologic and ultrasonographic examinations. Zeolite/collagen nanocomposite showed potential as a graft for healing femur bone defects. The bone filling and angiogenesis levels in rabbits treated with zeolite/collagen nanocomposite scaffolds were higher than rabbits treated with nHA, autograft, and the control group. In conclusion, zeolite/collagen nanocomposite scaffolds are vital in the reconstruction of bone defects and could be used in the treatment of bone fractures. Furthermore, radiology and ultrasonography are efficient techniques to estimate the healing procedure of femoral bone defect.

Authors' Contribution

Negar Javadian and Abbas Veshkini: Conceptualization, Methodology, Software. Alireza Jahandideh: Data curation, Writing- Original draft preparation. Abolfazl Akbarzadeh & Ahmad Asghari: Visualization, Investigation.

Conflict of Interests
The authors did not declare any potential competing conflicts of interest.

Ethical Issues
This study was approved by the Ethical Council of the Research Deputy of the Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran (No. IR.IAU.SRB.REC.1396.12, data: 2018/2).

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