IJVS 2017; 12(2); Serial No:27

IRANIAN JOURNAL OF VETERINARY SURGERY (IJVS)
WWW.IVSA.IR

Original Article

Effect of Zeolite and Zeolite/Collagen nanocomposite scaffolds on healing of segmental femur bone defect in rabbits

Darab Faraji[1], Alireza Jahandideh[1*], Ahmad Asghari[1], Abolfazl Akbarzadeh[2,3], Saeed Hesaraki[4]

Abstract

Objective- The present study was aimed to evaluate repair of a critical segmental defect of rabbit femur using the rabbit's femoral defects repaired by Zeolite and Zeolite/Collagen scaffolds

Design- Experimental Study

Animals- Forty-five mature male New Zealand white rabbits

Procedures- The animals were randomly divided into three groups of 15 animals each. In the first group (NC) the defect was made and with no treatment the wound was closed. In the second group (ZNC) the nanocomposite of zeolite was implanted into the defect. In the third group (Z/COL NC) the nanocomposite of zeolite/collagen was implanted into the defect. The periost and subcutaneous tissues were then closed primarily. The specimens were taken on days 15, 30, and 60 postoperatively and assessed histopathologically.

Results- Comparison of average scores of union index among groups showed that there was a significant difference between the mean scores of union in three groups ($p<0.05$). Descriptive statistics of spongiosa index showed that the highest point to the index was found in Z/COL NC group on day 15 (2.2) and the lowest point was found in both ZNC and NC groups (0.6). Descriptive statistics of Bone marrow index showed that there was not a significant difference between the mean scores of the index in three groups ($P>0.05$).

Conclusion and clinical relevance- The results of this study showed that zeolite and zeolite/collagen nanocomposite could be taken into consideration for grafting for bone fracture healing. It could be concluded that zeolite and zeolite/collagen nanocomposite bear a crucial capability in the reconstruction of bone defects and could be used as scaffold in bone fractures.

Key words: Bone regeneration; histopathological evaluation;nanocomposite, zeolite, zeolite/collagen, rabbits.

Received: 1 January 2018; **Accepted:** 17 Febraury 2018; **Online:** 6 March 2018

Introduction

The repair of bone defects in plastic and orthopedic surgery continues to pose a number of clinical problems. Although autologous bone has been the historical standard for these procedures as a source of reconstructive material, it has significant limitations. In particular, donor site morbidity, including pain, loss of function, and local injury in the harvesting procedure, and a limited supply of bone are among the most significant of these. The use of allograft also carries with it significant problems, including disease transmission, host rejection, and lack of osteoinductive properties.[1]

In an effort to address these problems, the search for an ideal material has led to the development of several reconstructive options to engineer new bone tissue. These include synthetic osteoconductive bone substitutes and osteoinductive compounds such as bone morphogenetic protein or demineralized bone powder and, more recently, osteoinductive calcium phosphate materials.[2-7] The development of synthetic resorbable scaffolds of either inorganic or polymer composition as carriers of progenitor cells of various tissues has facilitated the move toward this ideal material.[8-11] One of the main goals of bone tissue engineering is the design of a biodegradable porous material scaffold integrated with biological cells

[1]Department of Clinical Science, Faculty of Specialized Veterinary Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran
[2]Universal Scientific Education and Research Network (USERN), Tabriz, Iran
[3]Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[4]Department of Pathobiology, Faculty of Specialized Veterinary Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran
Corresponding author: Adress all correspondence to Alireza Jahandideh (DVM, DVSc). Email: dr.jahandideh@gmail.com

DOI:10.22034/ivsa.2018.112807.1133

and molecular cues able to guide the process of de novo tissue regeneration.[12,13] Biodegradable scaffolds are generally considered as indispensable elements for engineering living tissues. An ideal scaffold to be used for bone tissue engineering should possess characteristics of excellent biocompatibility, adequate pore size, controllable biodegradability and suitable mechanical properties.[14]

The silica-based materials like zeolite have taken great attention for their potential of improving the osteoconductivity of hydroxyapatite. These materials possess unique properties like nontoxicity, excellent biocompatibility, and in vivo biodegradability.[15] The silica based materials find applications most often as bone substitute material, implant coats and drug delivery systems.[16] Many biological studies involving silica-based materials have demonstrated that these materials can enhance the rate and quality of bone tissue repair.[17] Zeolite, classified with the crystalline aluminosilicates as a mesoporous material, is characterized by large surface area, rapid diffusion, adjustable porosity and high mechanical strength.[18] The non-cytotoxicity, biocompatibility and mechanical strength of the zeolite make it useful in the biomedical field as a bone graft material.[19] The literature is poor regarding effect of Zeolite and Zeolite/Collagen nanocomposite scaffolds on bone regeneration. In the present study, a critical segmental defect of rabbit femur was repaired using the rabbit's femoral defects repaired by Zeolite and Zeolite/Collagen scaffolds and the effects were examined histologically.

Materials and Methods

Chemicals

All chemicals were of research grade and zeolite nanoparticles were purchased from Sigma-Aldrich Company. The zeolite was natural and Mordenite type ($|Na_2,Ca,K_2)4(H_2O)_{28}|\ [Al_8Si_{40}O_{96}]$)

Preparation of zeolite/collagen nanocomposite scaffolds

Zeolite powder was added to HFIP with 3, 7, and 10 (wt%). The mixture was homogenized at 200 r/min to disperse zeolite into the solution. Then, the solution was mixed by a magnetic stirrer (WiseStir, Wisd, Germany) for an hour at 25°C to obtain the well-mixed zeolite/collagen suspension. Zeolite/collagen nanocomposite scaffolds were prepared by electrospinning of the suspension based on a method described by others.[20] Briefly, the suspension was added into a plastic syringe equipped with a needle with an inner diameter of 1.2 mm. The syringe was mounted on a syringe pump (TOP 5300, Intermedical, Japan) in which the needle was connected to a high-voltage power supply (HPPS-800NP, Bisotun,Iran). Under the 12–15 kV voltage, the fluid jet was injected at a rate of 1.0 mL/h, and the resultant fibers were collected on an aluminum foil which was put in the distance (13 cm) down from the needle. This method was done for all samples. When the electrospinning was finished at room temperature, the scaffolds were obtained. In order to evaporate the solvent completely, all the scaffolds were kept in a vacuum oven (Memmert, Germany) at 25°C with minimal moist and under the pressure for 24 h. Scanning electron microscopy (SEM) investigations were carried out using a Cambridge electronmicroscope, model Steroscan 360 (LEO, Cambridge, UK) to determine morphology of the nanocomposite (Fig 1). We did not perform EDX analysis of the nanocomposite to confirm the existence of Zeolite and Collagen in the nanocomposite structure. The cytocompatibility tests were not performed as well. These could be considered as limitations of the present study.

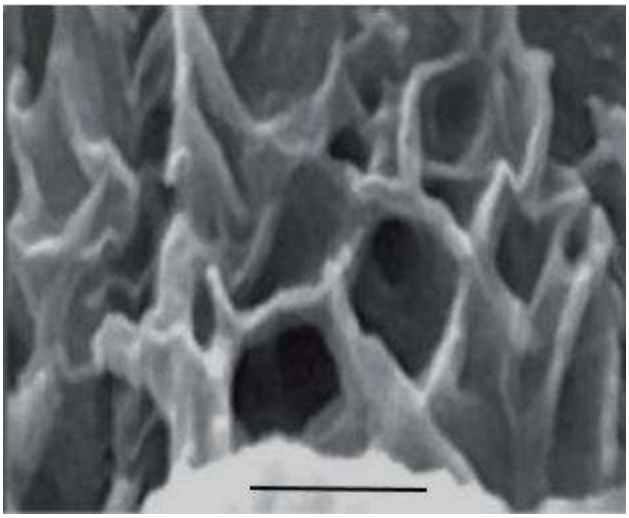

Figure 1. Scanning electron microscopy (SEM) photo of zeolite/collagen nanocomposite scaffold. Scale bar: 300 nm

Experimental design and animal models

Forty-five mature male New Zealand white rabbits, 6–8 months and with an approximate weight of 3–3.5 kg were included into the study. All animals were obtained from

the same source and used in this study in order to decrease the genetic variability. The animals were housed separately (one rabbit per cage) and maintained on standard pellet diet and tap water. Animal houses were in standard environmental conditions at temperature of 18 ± 3 °C, humidity of 60 ± 5% and natural light/dark cycle. Lateral femoral osteotomies were performed surgically. This investigation was approved by the institutional animal care and use committee (IACUC), at Islamic Azad University. Rabbits were randomized into three experimental groups of 15 animals each.

Surgical Procedures and animal grouping

Surgical procedures were done after an intramuscular injection of Ketamine 10% (50mg/kg) (Alfasan, The Netherlands) and Xylazine 2% (5mg/kg) (Alfasan, The Netherlands). The hair was callipered from the surgical site and the skin was cleaned with iodinated surgical soap. Aseptic technique was used throughout the surgical procedure. An incision of approximately 5 cm long was made along the medial right upper hind limb, and the mid diaphyseal surface of the femur was surgically exposed by blunt dissection. The periosteum was stripped from the bone using a periosteal elevator and an approximately a 6mm diameter – 5mm cylinder bilateral bone defect was created in the femur of one of the hind limbs. This osteotomy site was then irrigated with 0.9% saline, but periosteum around the osteotomy site was preserved and retracted with the overlying muscles. The osteotomy site was then treated according to the treatment protocol for each rabbit. After making the bone defects, all rabbits were marked with non-toxic color and randomly divided into three groups of 15 animals each. In the first group, Normal Control, (NC) the defect was made and with no treatment the wound was closed. In the second group, zeolite nanocomposite (ZNC) group, the nanocomposite of zeolite was implanted into the defect. In the third group (Z/COL NC) the nanocomposite of zeolite/collagen was implanted into the defect (Fig 2). The periost and subcutaneous tissues were then closed primarily. Antibiotics (penicillin G procaine 40,000 IU/kg IM, bid), dexamethasone (0.6 mg/kg, IM) and analgesic such as tramadol hydrochloride (5 mg/kg, IM, bid) were administered for three post-operative days. Experimental animals were kept in separate cages to prevent self-injury. After the procedure, daily observation was performed and evidence of infection or other abnormalities were noted. Five experimental subjects from each group were euthanized with an intravenous infusion of 2 mL per 4.5-kg dose of Euthanyl containing 240mg pentobarbital per milliliter on days 15, 30, and 60 postoperatively. After sacrifice, the left femur was harvested and fixed in 10% buffered formalin and then stored for histological examination.

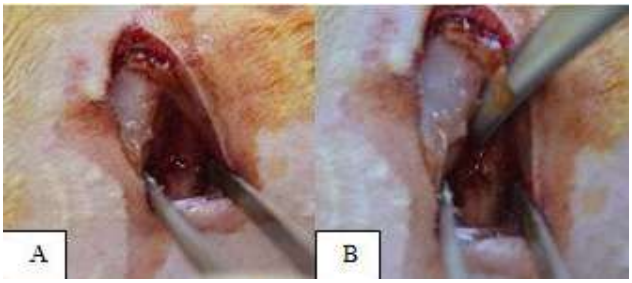

Figure 2. Intraoperative photos of (A) creation of defect and (B) implantation of the scaffold into the bone defect.

Histological assessments

For histological examination, the obtained tissues were decalcified with 10% formic acid solution that was changed daily. The surgical specimens were submitted to decalcification and routine histological processing for slide preparation and then embedded in paraffin blocks. Thereafter, they were sectioned at a thickness of 6 µm in a microtome using the largest diameter of the defect, stained with Trichrome, and analyzed under a light microscope by pathologist in a double-blind manner. Recorded factors from specimens were evaluated with a 0-4 point histological grading scale (Table 1) to determine the quality of the union, appearance and quality of the spongiosa, as well as to evaluate the Bone marrow that was based on the works of others.[21]

Statistical analysis

The collected data were analyzed statistically with one-way analysis of variance SPSS version 22 (IBM, Armonk, NY). The differences were statistically significant at $P < 0.05$.

Results

Findings of bone parameters indices

Descriptive statistics of union index showed that the highest point to the index union on day 15 was found in Z/COL NC group (1.8) and the lowest in the ZNC and NC groups (0.2).

Table 1. Histologic scoring system.

Category	Point
Union	
No sign of union	0
Fibrous union	1
Osteochondral union	2
Bone union	3
Complete reorganization	4
Spongiosa	
No sign of cellular activity	0
Early bone formation	1
Active new bone formation	2
Reorganized spongiosa formation	3
Complete reorganized formation	4
Cortex	
Absence of cortex	0
Early detection	1
Initiation of formation	2
Reorganization in majority	3
Complete organization	4
Bone marrow	
Not available	0
Detection of fibrinous material	1
Defect occupying more than half	2
Fully occupying the red B.M	3
Adult type fatty marrow	4

The highest score on day 30 was found in both Z/COL NC and NC groups (3.4) and the lowest one was found in the ZNC group (1.4). The highest point on day 60 was found in both Z/COL NC and NC groups (4). The lowest point was found in the NC group (2.4) (Table 2). Comparison of average scores of union index among groups showed that there was a significant difference between the mean scores of union in three groups (p<0.05). The average scores union on days 15, 30, and 60 showed that the highest point of union score was found in Z/COL NC group. Descriptive statistics of spongiosa index showed that the highest point to the index was found in Z/COL NC group on day 15 (2.2) and the lowest point was found in both ZNC and NC groups (0.6). Regarding the score of spongiosa index on day 30 there were no significant difference among groups (P> 0.05) (Table 3). Descriptive statistics of Bone marrow index showed that there was not a significant difference between the mean scores of the index in three groups (P>0.05).

Table 2. Descriptive statistics of Union index in experimental groups. Data are expressed as Mean±SD.

Groups	Days		
	15	30	60
NC	0.2 ± 0.45	3.5 ± 0.55	2.4 ± 0.55
ZNC	0.2 ± 0.45	1.4 ± 0.55	4.0 ± 0.45
Z/COL NC	1.8 ± 0.80*	3.5 ± 0.55*	4.0 ± 0.45

*P<0.05 *vs.* ZNC group.

Table 2. Descriptive statistics of Spongiosa index in experimental groups. Data are expressed as Mean±SD.

Groups	Days		
	15	30	60
NC	0.60 ± 0.547	1.60 ± 0.45	2.20 ± 0.45
ZNC	0.60 ± 0.547	1.60 ± 0.45	3.5 ± 0.55
Z/COL NC	2.2 ± 0.80*	1.60 ± 0.45	3.5 ± 0.55

*P<0.05 vs. ZNC group.

Findings of histological assessments

The histological observations on day 15 showed that in NC group abundant cartilaginous callus and mild primary woven bone were formed near the defect. In the ZNC group histological observations on day 15 showed moderate cartilaginous callus and mild primary woven bone near the defect. In the Z/COL NC group histological observations on day 15 showed moderate primary woven bone and mild cartilaginous callus near the spicules of the prior control bone (Fig 3). The histological observations on day 30 showed that in NC group primary woven bone was formed in the defect. In the ZNC group histological observations on day 30 showed thin lamellar bone spicules in the defect. In the Z/COL NC group histological observations on day 30 showed thicker lamellar bones (Fig 4). The histological observations on day 60 showed that in NC group lamellar bone spicules were thinner than others. In the Z/COL NC and ZNC groups histological observations on day 60 showed that lamellar bones were producing (Fig 5).

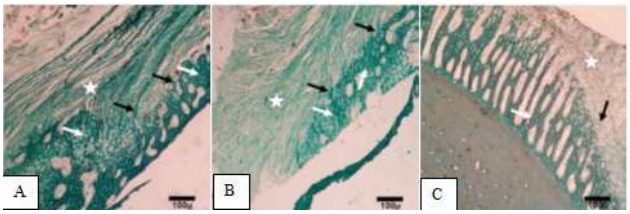

Figure 3. Microscopic section from the healing site of experimental groups on day 15 of healing site. (A) NC group shows abundant cartilaginous callus (white arrows) and mild primary woven bone (black arrows) near the defect. The retained granulation tissue (astrisk) in the defect is shown. (B) ZNC group shows moderate cartilaginous callus (white arrows) and mild primary woven bone (black arrows) near the defect. The retained granulation tissue (asterisk) in the defect is shown. (C) Z/COL NC group shows moderate primary woven bone and mild cartilaginous callus (black arrow) near the spicules of the prior Control bone (white arrow). The retained granulation tissue (star) in the defect is shown (Trichrom staining). Scale bar: 100 µm

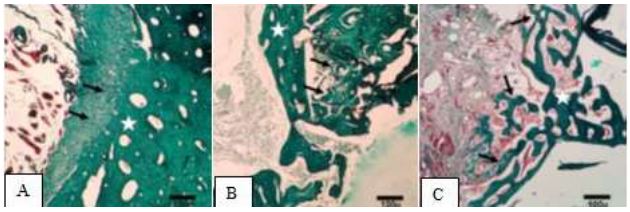

Figure 4. Microscopic section from the healing site of experimental groups on day 30 of healing site. (A) NC group shows primary woven bone (arrows) in the defect. The compact bone (asterisk) around the defect is shown. (B) ZNC group shows thin lamellar bone spicules (arrows) in the defect. The compact bone (asterisk) around the defect is shown. (C) Z/COL NC group shows that the thicker lamellar bones (arrows) are being produced (Trichrom staining). Scale bar: 100 µm

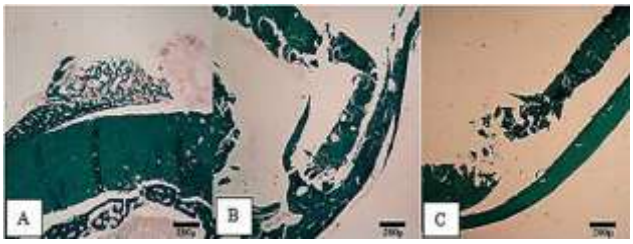

Figure 5. Microscopic section from the healing site of experimental groups on day 60 of healing site. (A) NC group shows that the lamellar bone spicules (arrows) are thinner than others. (B) ZNC group shows that the lamellar bones (arrows) are thick. (C) Z/COL NC group shows that the lamellar bones (arrows) are thick (Trichrom staining). Scale bar: 250 µm

Discussion

An ideal bone graft substitute should have osteoconductive, osteoinductive, and osteogenic properties.[22] As a result, some investigators used mixtures of synthetic

biomaterials and osteoinductive organic agents to achieve better results.[23] This study aimed to evaluate the effect of zeolite and zeolite/collagen nanocomposite scaffolds in bone healing of the femural defect in rabbit. Histopathological evaluation was performed on days 15, 30, and 60 after surgery. It seems that quantity of newly formed lamellar bone in the healing site in Z/COL NC group was better than onward compared to ZNC group after 60 days.

Ceramics such as hydroxyapatite (HA), calcium phosphates, and bioactive glasses have been attracted special attention, due to their excellent biocompatibility along with their osteoconductive

and osteoinductive properties.[24] Depending on their composition, particle size and production process, ceramics can have various degrees of bioactivity, which is the ability to chemically bond and be integrate into living bone through the formation of HA.[25]

However, these materials are brittle and have low mechanical stability, making them unsuitable for load bearing applications.[26] Synthetic polymers have great advantages because of their structure, composition and, consequently, their properties can be tailored to specific needs.[27,28] They are more ductile than ceramics and some can, in their solid form, reach mechanical compression strength close to that of cortical bone.[27]

New bone tissue engineering methodologies and progress in nanotechnology have triggered the use of nanostructures as scaffolds for the purpose of tissue engineering.[29,30] Among the various nanostructures, nanofibers are very attractive for the biomedical applications, since they present a similar fibrous structure to that of natural ECM. The nanofibers can be organized into various porous architectures and possess a high surface area to volume ratio.[31,32] Nanotechnological approaches have a great potential for medical applications. In particular, the development of electrospinning is very important for health care applications since it is a relatively quick, simple, and cost-effective method for producing nanostructured materials desirable for many biomedical applications such as tissue engineering. [33,34] However, most of the studies under the in vitro settings that used polycaprone scaffolds in bone healing were successful. The results of an *in vitro* bioactivity demonstrated that the morphology, nucleation, and growth of the hydroxyapatite were apparently affected by the zeolite content. The enhanced bioactivity of the composites could be attributed to the presence of the silanol group on the composite matrix, which could have helped in the apatite layer formation.[35] The overall mechanism involves the exchange of Ca ions from the composite surface with protons from the simulated body fluid resulting in the formation of the silanol groups on the surface. It has been proposed that the silanol group does not directly combine with the Ca ions.[36] Initially, the silanol group dissociates into the negative charged species which combine with the Ca ions in the simulated body fluid to produce amorphous calcium silicate. It has been noted that the calcium silicate continuously takes up the positive ions until it begins to interact with the phosphate ions in the simulated body fluid to ultimately produce the amorphous calcium phosphate layer on the surface, which eventually becomes the crystalline apatite.[37] Results of an in vitro study has also verified the ability of the zeolite composites to support and accelerate the growth of the hydroxyapatite.[38]

In conclusion, the results of this study showed that zeolite and zeolite/collagen nanocomposite could be taken into consideration for grafting for bone fracture healing. Biodegradation, reaction of body and other factors affecting bioceramic capability the influence of body environment should be taken into consideration. It could be concluded that zeolite and zeolite/collagen nanocomposite bear a crucial capability in the reconstruction of bone defects and could be used as scaffold in bone fractures.

Acknowledgments

The authors are thankful for technical assistance of the staff of the Universal Scientific Education and Research Network (USERN), Tabriz, Iran. This work was a part of a dissertation of first author (D.F) submitted as a Partial Fulfillment of the Degree of Doctor of Veterinary Science (DVSc).

Conflict of interests

None

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نشریه جراحی دامپزشکی ایران
سال۲۰۱۷ ، جلد ۱۲ (شماره ۲)، شماره پیاپی ۲۷

چکیده

تاثیر داربست نانوکامپوزیت زئولیت و زئولیت/کلاژن بر التیام نقیصه قطعه‌ای استخوان ران خرگوش

داراب فرجی[۱]، علیرضا جهان دیده[۱*]، احمد اصغری[۱]، ابوالفضل اکبرزاده[۲،۳]، سعید حصارکی[۴]

[۱] گروه علوم درمانگاهی، دانشکده علوم تخصصی دامپزشکی، واحد علوم و تحقیقات، دانشگاه آزاد اسلامی، تهران، ایران
[۲] شبکه جهانی آموزش و پژوهش های علمی، تبریز، ایران
[۳] مرکز تحقیقات کاربردی دارویی، دانشگاه علوم پزشکی تبریز، تبریز، ایران
[۴] گروه پاتوبیولوژی، دانشکده علوم تخصصی دامپزشکی، واحد علوم و تحقیقات، دانشگاه آزاد اسلامی، تهران، ایران

هدف- مطالعه حاضر به هدف ارزیابی ترمیم نقیصه قطعه ای استخوان ران خرگوش توسط داربست نانوکامپوزیت زئولیت و زئولیت/کلاژن صورت گرفت

طرح- مطالعه تجربی

حیوانات- ۴۵ خرگوش نر بالغ سفید نیوزلندی

روش کار- حیوانات به طور تصادفی به سه گروه ۱۵ تایی تقسیم بندی شدند. در گروه کنترل نرمال نقیصه ایجاد شده بدون درمان بسته شد. در گروه دوم نانو کامپوزیت زئولیت در داخل نقیصه قرار داده شد. در گروه سوم نانو کامپوزیت زئولیت/کلاژن در داخل نقیصه قرار داده شد. پرده ضریع و بافت زیر جلدی بسته شد. نمونه ها در روز های ۱۵، ۳۰ و ۶۰ بعد از عمل اخذ شدند و تحت ارزیابی آسیب شناسی قرار گرفتند.

نتایج- مقایسه میانگین مقادیر شاخص ترمیم در بین گروه ها نشان داد که اختلاف معنی داری بین گروه ها وجود دارد ($p<0.05$). مطالعات آماری توصیفی شاخص اسپونژیوزا بیانگر بالاترین میزان شاخص در گروه سوم در روز ۱۵ به میزان ۲/۲ و کمترین آن در گروه دوم به میزان ۰/۶ بود. مطالعات آماری توصیفی شاخص مغز استخوان در بین گروه های آزمایشی تفاوت معنی داری نشان نداد ($P>0.05$).

نتیجه گیری و کاربرد بالینی- نتایج این مطالعه نشان داد که نانو کامپوزیت زئولیت و زئولیت /کلاژن را میتوان برای گرافتینگ در شکستگی های استخوانی مدنظر قرار داد. میتوان نتیجه گیری نمود که نانو کامپوزیت زئولیت و زئولیت /کلاژن واجد قابلیت مهمی در بازسازی نقایص استخوانی بوده و میتوان آنها را به عنوان داربست در شکستگی های استخوانی مدنظر قرار داد.

کلمات کلیدی- ترمیم استخوان، ارزیابی هیستوپاتولوژیکی، نانوکامپوزیت، زئولیت، زئولیت/کلاژن، خرگوش