Archives of Razi Institute, Vol. 74, No. 4 (2019) 395-403

Original Article

Evaluation of Influence of Zeolite/Collagen Nanocomposite (ZC) and Hydroxyapatite (HA) on Bone Healing: A Study on Rabbits

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Received 15 May 2018; Accepted 30 June 2018
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ABSTRACT

Bone healing is still a great challenge in orthopedic surgery and clinical practice. There is a dearth of research investigating the effect of Zeolite/Collagen (ZC) nanocomposite on bone regeneration. In the present study, a critical segmental defect of the rabbit femur was repaired using defects in femurs repaired by ZC nanocomposite, and the effects were examined histologically. In total, 45 rabbits at seven months of age weighing 3.5 kilograms were utilized in this study. After making the bone defects, all animals were randomized into three groups (n=15). In a normal control group (NC), a defect was created, no intervention was made, and the skin incision was sutured. On the other hand, in the ZC group, the nanocomposite of ZC was placed into the created defect. In the hydroxyapatite group (HA), the hydroxyapatite was placed into the created defect. The samples were collected on days 15, 30, and 45 postoperatively and assessed histopathologically. The mean scores of the index of the union were compared and considerable alterations were observed in this regard in the experimental groups ($P<0.05$). The values of the index of spongiosa demonstrated that on day 15, it was the highest in the ZC group (2.2) and lowest in the HA and NC groups (0.6). Moreover, the values of the index of bone marrow demonstrated no noticeable alteration among the values of the index of bone marrow in the experimental groups ($P>0.05$). The findings of this study demonstrated that ZC nanocomposite might be considered for reconstruction in bone damages. It seems the ZC nanocomposite bears a crucial capability in the reconstruction of bone damages and might be used as a biological frame in bone damages.

Keywords: Bone Regeneration, Histopathological Evaluation, Nanocomposite, Zeolite/Collagen Nano Particles, Rabbit

Evaluation de l'Influence des Nanocomposites de Zéolite / Collagène (ZC) et d'Hydroxyapatite (HA) sur la Consolidation Osseuse: une Étude sur les Lapins

Résumé: La consolidation osseuse reste un défi de taille en chirurgie orthopédique en pratique clinique. La littérature relative aux effets du nanocomposite de zéolite / collagène sur la régénération osseuse est médiocre. Dans la présente étude, un défaut segmentaire critique du fémur de lapin a été réparé en utilisant des défauts des fémurs réparés par un nanocomposite de zéolite / collagène et les effets ont été examinés histologiquement. Quarante-cinq lapins âgés d'environ sept mois et d'un poids de 3,5 kg ont été utilisés. Après la création des

défauts osseux, tous les animaux ont été randomisés en trois groupes et chaque groupe comprenait 15 animaux Dans le groupe témoin normal (TN), un défaut a été créé et aucun l'intervention n'a été réalisée et l'incision de la peau a été suturée. Dans le groupe zéolite / collagène (ZC), le nanocomposite de zéolite / collagène a été placé dans le défaut créé. Dans le groupe hydroxyapatite (HA), l'hydroxyapatite a été placée dans le défaut créé. Les échantillons ont été prélevés aux jours 15, 30 et 45 ans et évalués histopathologiquement. Les scores moyens de l'indice d'union ont été comparés et des modifications considérables ont été observées parmi valeurs d'indice d'union dans les groupes expérimentaux ($p < 0,05$). Les valeurs de l'indice de spongiosité démontraient qu'au 15ème jour, il était le plus élevé dans le groupe zéolite / collagène (2,2) et le plus bas dans les groupes d'hydroxyapatite et de contrôle normal (0,6). Les valeurs d'indice de la moelle osseuse il n'y avait pas d'altération notable entre les valeurs d'indice de la moelle osseuse dans les groupes expérimentaux ($P > 0,05$). Les résultats de notre enquête ont démontré que la reconstruction des dommages osseuses par un nanocomposite zéolite / collagène pourrait être envisagée. Il semble que le nanocomposite zéolite / collagène possède une capacité cruciale dans la reconstruction des dommages osseuses et pourrait être utilisé comme un cadre biologique pour les dommages osseuses.
Mots-clés: Régénération Osseuse; Évaluation histopathologique; Nanocomposite, Nano Particules de Zéolite / Collagène, Lapins

INTRODUCTION

Recently, tissue engineering has provided a promising method for repairing bone defects. Management of segmental bone defects due to trauma, inflammation or tumors remains a considerable challenge for orthopedic surgeons. The autograft, allografts and bone graft alternatives could be used as bone substitutes (Brydone et al., 2010). Their bone reconstruction capability is assessed because of their ability in ion osteogenicity, osteoconduction and osteroinduction (Mousavi and Rezaie, 2011). Osteocytes, osteoblasts and mesenchymal stem cells are the main sources of the osteogenicity of a bone graft. Osteoconduction is defined as a framework that induces osteocytes to develop on their surfaces. These characteristics of such scaffolds are very crucial to the reconstruction of damaged bone. Autograft is the most common form of these frameworks, which is the transplanting of bone tissue from one location to another in the same patient. The use of autograft is restricted by problems like pain, infection, scarring, blood loss, and donor-site morbidity (Laurencin et al., 2006; Jayakumar and Di Silvio, 2010; Zhang et al., 2012). The objective of bone reconstruction is the design of a biodegradable porous material scaffold integrated with biological cells and molecular cues able to guide the process of de novo tissue regeneration. Biodegradable scaffolds are generally considered as indispensable elements for engineering living tissues. A perfect framework to be utilized for bone reconstruction must bear properties of the best tissue compatibility, enough porosity, manageable biodegradability and appropriate mechanical properties (Arcos and Vallet-Regí, 2010). The silica-based materials like Zeolite/Collagen 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 (Bedi et al., 2012).The silica based materials find applications most often as bone substitute material, implant coating and drug delivery systems. Many biological studies involving materials based on silica have displayed that these materials may augment the rate and quality of reconstruction of bone (Kihara et al., 2011). Zeolite/Collagen, classified with the crystalline alumina-silicates as a mesoporous material, is characterized by large surface area, rapid diffusion, adjustable porosity and high mechanical strength. The non-cytotoxicity, biocompatibility and mechanical strength of the Zeolite/Collagen make it suitable in the biomedical field as a bone graft material. (Mousavi and

Rezaie, 2011). The literature is poor regarding effect of Zeolite/Collagen nanocomposite on bone regeneration. During this investigation, the rabbit femur defect was reconstructed using Zeolite/Collagen nanocomposite and the effects were examined histologically.

MATERIAL AND METHODS

Samples preparation and characterization. In this research Collagen, Type 1 solution from rat tail (Sigma-Aldrich), Hydroxyapatite (Sigma-Aldrich), Zeolite (Sigma-Aldrich), 3, 6 Dimethyl-1,4-dioxane-2, 5-dione(Acros), Glycolide (Sigma-Aldrich), Caprolactone (Aldrich) were selected for synthesis of Zeolite/Collagen nanocomposite. In order to evaluate the healing effect of Zeolite/Collagen nanocomposite, Forty-five mature male New Zealand white rabbits, 6–8 months and with an approximate weight of 3–3.5 kg were used.

Preparation and characterization of Zeolite/Collagen nanocomposite. Five percent solution of Zeolite 5 mL was added to 100 mL collagen solution, adjusted the pH of the solution to about 8.0 with NaOH solution stirred and mixed with a magnetic stirrer for 30min. After that, 2 mol hydroxyapatite, were dropped into the collagen– Zeolite mixture solution with the 10 ◦C temperature and pH=8, and stirred fully for 5 h and aged at 32 ˚C for at least 24 h. Then, it was centrifuged at 12000rpm for15 min, wastes the supernatant, and washed it with distilled water. After this process, the mixture was placed on frizz drying about 6 hours. A cylindrical Col–HA/Zeolite composite would be got. Then the final sample approved with FT-IR spectroscopy.

Study design and animals. This study was conducted on the basis on rules and regulations for animal handling review board of the Islamic Azad University, Faculty of Veterinary Medicine, adhering to the guide for care and use of laboratory animals; the study was accepted by the ethics committee. 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 entered in this investigation to decrease the genetic variability. The animals were housed separately (one rabbit per cage) and were fed with pellets that we prepared from the 'Araz fidar' Animal Feed Company. Pellets contain ingredients such as Soya Meal, Fish Powder, DI calcium Phosphate, Iron sulfate, Calcium Carbonate, Vitamin and Mineral Supplements, Methionine, Lysine, Multi Enzyme, Antioxidant, Growth Stimulant, Appetite Powder, and Other Supplements (Suckow et al., 2012). Animal houses were in regular environmental conditions at temperature of 18 ± 3 °C, humidity of 60 ± 5% and natural light/dark cycle (Richardson, 2008) .Lateral femoral osteotomies were performed surgically.

Surgical Procedures and grouping. Surgical method were performed following an IM administration of Ketamine 10% (ketamine hydrochloride, 50mg/kg), Rompun 5% (xylazine, 5mg/kg) (Longley et al., 2008). The hair was callipered from the surgical position and the skin was cleaned with iodinated surgical soap to keep the aseptic condition. Asepsis method was used during 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 circular hole was made in the femur bone. After making the bone defects, all animals were randomized into three groups and each group included 15 animals. In normal control group (NC) a defect was created and no intervention was made and the skin incision was sutured. In the zeolite/collagen group (ZC) the nanocomposite of zeolite/collagen was placed into the created defect. In the hydroxyapatite group (HA) the hydroxyapatite was placed into the created defect. 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 2ml per 4.5-kg dose of Ethanol containing 240mg pentobarbital per milliliter on days 15, 30, and 45 after surgery. After sacrifice, the left femur was harvested and fixed in 10% buffered formalin and then stored for histological examination.

Histological assessments. For histological examination, the obtained tissues were decalcified with 10% formic acid solution that was changed every day. The surgical specimens were submitted to decalcification and routine histological processing for slide preparation and then embedded in paraffin blocks. Then, 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.

Statistical analysis. Logged factors from specimens were evaluated with a 0-4 point histological grading scale to determine the quality of the union, appearance and quality of the spongiosa, as well as to evaluate the Bone marrow. The collected data were analyzed statistically by 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 values for index of union demonstrated that the highest point of the index union on day 15 was found in HA group and the lowest in NC group (0.2). The highest score on day 30 was found in and NC group (3.4) and the lowest one was found in the ZC and HA groups (2.5). The highest value of index of union was observed on day 45 in zeolite/collagen and hydroxyapatite groups. This value was observed to be lowest in normal control group (Table 1). Average values of the index of union were compared and considerable alterations were observed among the values of index of union in experimental groups (*p*>0.05). The values of the index of sponges demonstrated that on day 15 it was the highest in the zeolite/collagen group. (2.2) and it was the lowest in hydroxyapatite and normal control groups. (0.6). Regarding the values of index of spongiosa on day 30 there was no significant difference among groups (*P*>0.05). The values of the index of sponges demonstrated on day 45 they were the highest in zeolite/collagen group and in normal control and hydroxyapatite groups they were the lowest (Table 2). The values of index of Bone marrow demonstrated that there was not a noticeable alteration among the values of index of bone marrow in the experimental groups (*P*>0.05).

Table 1. Descriptive statistics of Union index in experimental groups. Data are expressed as Mean±SD

Groups	Days		
	15	30	45
NC	0.2 ± 0.45	3.5 ± 0.55	2.4 ± 0.55
ZC	3.5±0.55*	2.5 ± 0.55	3.5 ± 0.55
HA	2.5 ± 0.55	2.5 ± 0.55	3.5± 0.55

*P<0.05 *vs.* HA group

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

Groups	Days		
	15	30	45
NC	0.60 ± 0.55	1.60 ± 0.45	2.20 ± 0.45
ZC	3.50 ± 0.55*	1.60 ± 0.45	3.40 ± 0.55*
HA	1.60 ± 0.55	1.60 ± 0.45	2.20 ± 0.45

*P<0.05 *vs.* HA group

Findings of histological assessments. On the fifteenth day, histological observations showed that in NC group plentiful cartilaginous callus and mild primary woven bone were formed near the defect. In the ZC group histological observations on day 15 showed moderate cartilaginous callus and mild primary woven bone near the defect. In the HA group histological observations on day 15 showed well developed primary woven bone near the defect (Figure 1-Figure 3). The histological observations on day 30 showed that in NC group primary woven bone was formed in the defect. In the ZC group histological observations on day 30 showed thin lamellar bone spicules in the defect. In the HA group histological observations on day 30 showed that the thickest lamellar bones were remained from former control bone (Figure 4-Figure 6).

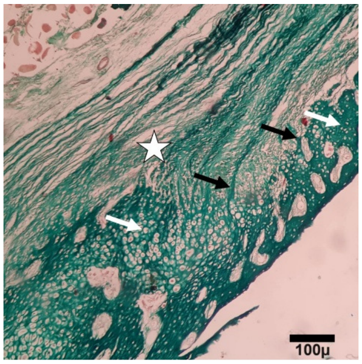

Figure 1. Microscopic section from the healing site of Control group on day 15 of healing shows abundant cartilaginous calus (white arrows) and mild primary woven bone (black arrows) near the defect. The retained granulation tissue (star) in the defect is shown (Trichrom x100).

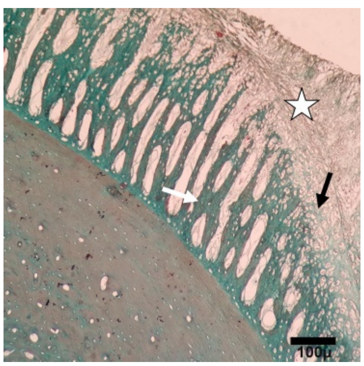

Figure 2. Microscopic section from the healing site of Control Zeolite - collagen treated group on day 15 of healing shows moderate primary woven bone and mild cartilaginous calus (black arrow) near the spicules of the prior Control bone (white arrow). The retained granulation tissue (star) in the defect is shown (Trichrom x100).

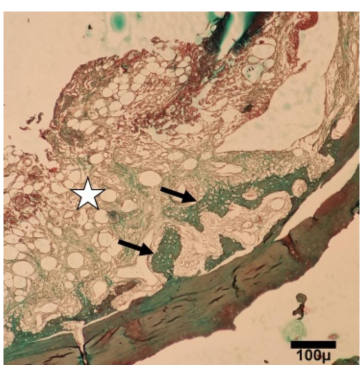

Figure 3. Microscopic section from the healing site of HA treated group on day 15 of healing shows well developed primary woven bone (arrows) near the defect. The retained granulation tissue (star) in the defect is shown (Trichrom x100).

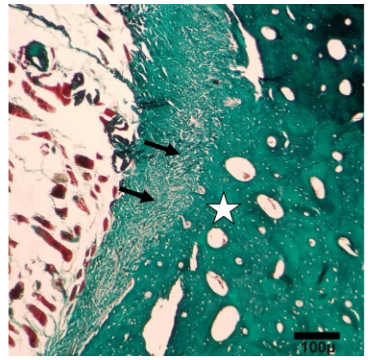

Figure 4. Microscopic section from the healing site of Control group on day 30 of healing shows primary woven bone (arrows) in the defect. The compact bone (star) around the defect is shown (Trichrom x100).

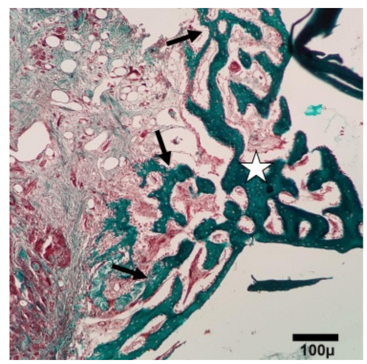

Figure 5. Microscopic section from the healing site of Zeolite - Collagen treated group on day 30 of healing. Thicker lamellar bones (arrows) are being to produce. The former bone (star) around the defect is shown (Trichrom×100).

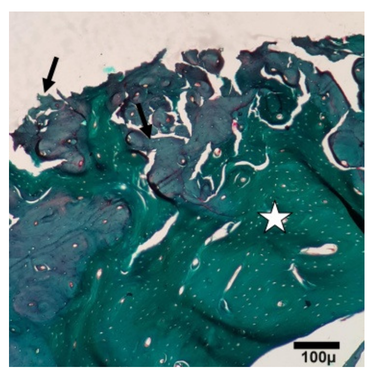

Figure 6. Microscopic section from the healing site of HA treated group on day 30 of healing. The Thickest lamellar bones (arrows) remained from former Control bone are shown. The former intact bone (star) around the defect is shown (Trichrom×100).

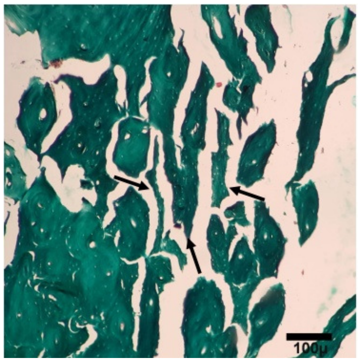

Figure 7. Microscopic section from the healing site of Control group on day 45 of healing. Lamellar bone specules (arrows) are thinner than others (Trichrom×100).

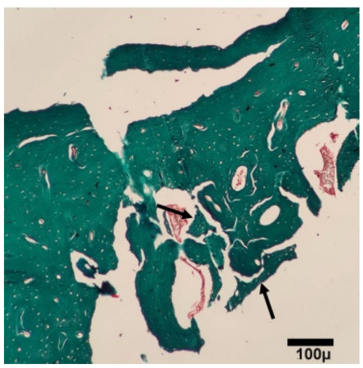

Figure 8. Microscopic section from the healing site of Zeolite – Collagen treated group on day 45 of healing. Lamellar bones (arrows) are being to produce (Trichrom×100).

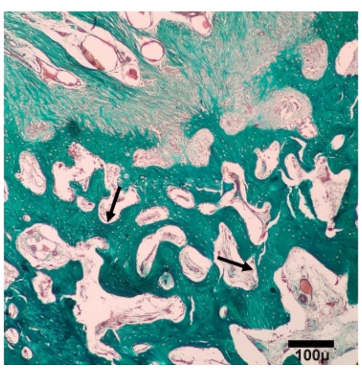

Figure 9. Microscopic section from the healing site of HA treated group on day 45 of healing. Lamellar bone specules (arrows) are thicker than others (Trichrom×100).

The histological observations on day 45 showed that in NC group lamellar bone spicules were thinner than others. In the HA and ZC groups histological observations on day 45 showed that lamellar bones were producing (Figure 7-Figure 9).

DISCUSSION

A favorable bone substitute must bear biological activities like osteo-conduction, osteo-induction and osteogenic characteristics Hence, some scientists employed materials of synthetic stuffs with osteoinductive properties to achieve the goal (Erol et al., 2012). Cerosium that is a ceramic and plastic made composite has been utilized for mandibular bone damages(Liu et al., 2015).Commonly, composite frameworks are produced by the use of a various kinds of matrices like polymer/ceramics, ceramic/metals and polymer/metals(Reichert et al., 2011). Recently, frameworks based on polymer/ceramic have been investigated. Other investigators have studied calcium phosphate/polyesters for producing scaffolds. In laboratory investigations, it has been demonstrated that cell-viability, proliferation and osteogenic differentiation occurs in these scaffolds that are important in regeneration of damaged tissue (Li et al., 2009). This study aimed to evaluate the effect of zeolite/collagen nanocomposite scaffolds in bone healing of the femural defect in rabbit. Histopathological evaluation was performed on days 15, 30, and 45 after surgery. It seems that quantity of newly formed lamellar bone in the healing site in ZNC group was better than onward compared to ZNC group after 45 days in the newly emerging field of tissue engineering, nanostructures as scaffolds have been developed (Khang et al., 2010). In this field, nanofibers have taken more attention because of their identical structure to the extracellular matrix. They can bear the variety of various porous architectures and a high surface area to volume ratio. Nanotechnological methods are favorable for medical applications. In specifically, the development of electrospinning is really crucial for healthcare applications because of its a rapidity, easy, and affordability in methods for fabricating nanostructured materials appropriate for numerous biomedical usage like tissue engineering (Linh et al., 2010). Zeolites are bio-ceramic materials, and bio-ceramics are also used as bone tissue engineering scaffolds with similar bone tissue properties. In 2014, for bone scaffolds, biomaterials zeolite Y composted in various weight percentages with hydroxyapatite, and by taking the MTT test showed that the survival of cells in the scaffold containing 10% zeolite was better than the rest of the percentages (Bedi et al., 2012; Iqbal et al., 2014). According to a study done at the University of Texas at 2012, zeolites can prevent osteoclast activity and generally osteoporosis.(Banu et al., 2012). American researchers at the University of California also showed that zeolites generally increase osteoconductivity, osteoinductivity and osteointegration in bone tissue engineering. (Keeting et al., 1992; Bedi et al., 2012; Auerbach et al., 2003). Other suitable properties of zeolite in the application of bone scaffolds include the following: increased osteoblast growth and excretion, increased production of TGFβ in the osteoblast, osteocondiac stimulation, increased TGFβ-related mRNA, and increased its release (Beachley and Wen, 2010)(Beachley and Wen, 2010) (Beachley and Wen., 2010).The morphology, nucleation, and growth of the hydroxyapatite have been demonstrated to be influenced by zeolite/collagen content in vivo investigations. The improved biological activity of these materials is associated with the existence in the silanol group on the composite matrix that helped the formation of an appetite layer (Kamitakahara et al., 2007).There are mechanisms that involve in Ca ion exchange from the surface of a composite with protons from the simulated body fluid ending up the formation of the silanol groups on the surface that based on the report of others the silanol group did not directly combine with the Ca ions (Ceyhan et al., 2007). The latter group separates into the negatively charged species which bind to the Ca ions in the simulated body fluid to form amorphous calcium silicate. It has been reported that the calcium silicate continuously absorbs the positive ions until interacts with the phosphate ions in the simulated body fluid to form the amorphous calcium phosphate layer on the surface, that finally becomes the crystalline apatite. It has been clarified that zeolite/collagen composites are able to support and improve the growth of the hydroxyapatite (Chou et al., 2005). Descriptive statistics of union index in experimental groups in the present study showed that on day 30 and 45 zeolite nanocomposite made a better improvement in the index that could be because of stimulation of the composite to form the amorphous calcium phosphate layer on the surface. This was also verified by histological assessments that lamellar bones were being to produce in Zeolite – collagen treated group on day 45 of healing. Findings of descriptive statistics of spongiosa index of our study also revealed improved index in zeolite nanocomposite implanted animals. This may have resulted in an inability of the healing bone to chemically bond and be integrated into the living bone through the formation of HA. The findings of the histological assessments of the present study showed that zeolite nanocomposite implanted animals on day 15 moderate primary woven bone and mild cartilaginous callus and on day 30 thicker lamellar bone spicules were present in the defect.

Results of an in vitro study has also verified the ability of the zeolite/collagen composites to support and accelerate the growth of the hydroxyapatite (Chou et al., 2005). In conclusion, the results of this study showed a promising potential for ZNC nanocomposite to be used widely in grafting for bone fracture healing. Nevertheless, two main facts should be considered in this regard. Primarily, biodegradation, reaction of body and other factors affecting bioceramic's capability is under the influence of body's environment. Therefore, human trail studies are strongly encouraged. The another fact in concern of ZNC nanocomposite and even HA is finding the best porosity amount and tridimensional structures of these bioceramics that would work in the best interest of defective bone. In conclusion, it seems that ZNC nanocomposite has an important role in the reconstruction of bone defects and can be used as scaffold in bone fractures.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

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).

References

Arcos, D., Vallet-Regí, M., 2010. Sol–gel silica-based biomaterials and bone tissue regeneration. Acta Biomaterialia 6, 2874-2888.

Auerbach, S.M., Carrado, K.A., Dutta, P.K., 2003. Handbook of zeolite science and technology, CRC press.

Banu, J., Varela, E., Guerra, J.M., Halade, G., Williams, P.J., Bahadur, A.N., *et al.*, 2012. Dietary coral calcium and zeolite protects bone in a mouse model for postmenopausal bone loss. Nutrition research 32, 965-975.

Beachley, V., Wen, X., 2010. Polymer nanofibrous structures: Fabrication, biofunctionalization, and cell interactions. Prog Polym Sci 35, 868-892.

Bedi, R.S., Chow, G., Wang, J., Zanello, L., Yan, Y.S., 2012. Bioactive Materials for Regenerative Medicine: Zeolite-Hydroxyapatite Bone Mimetic Coatings. Advanced Engineering Materials 14, 200-206.

Brydone, A.S., Meek, D., Maclaine, S., 2010. Bone grafting, orthopaedic biomaterials, and the clinical need for bone engineering. Proc Inst Mech Eng H 224, 1329-1343.

Ceyhan, T., Tatlier, M., Akçakaya, H., 2007. In vitro evaluation of the use of zeolites as biomaterials: effects on simulated body fluid and two types of cells. Journal of Materials Science: Materials in Medicine 18, 1557-1562.

Chou, Y.F., Huang, W., Dunn, J.C., Miller, T.A., Wu, B.M., 2005. The effect of biomimetic apatite structure on osteoblast viability, proliferation, and gene expression. Biomaterials 26, 285-295.

Erol, M.M., Mourino, V., Newby, P., Chatzistavrou, X., Roether, J.A., Hupa, L., *et al.*, 2012. Copper-releasing, boron-containing bioactive glass-based scaffolds coated with alginate for bone tissue engineering. Acta Biomater 8, 792-801.

Iqbal, N., Kadir, M.R.A., Mahmood, N.H.B., Yusoff, M.F.M., Siddique, J.A., Salim, N., *et al.*, 2014. Microwave synthesis, characterization, bioactivity and in vitro biocompatibility of zeolite–hydroxyapatite (Zeo–HA) composite for bone tissue engineering applications. Ceramics International 40, 16091-16097.

Jayakumar, P., Di Silvio, L., 2010. Osteoblasts in bone tissue engineering. Proc Inst Mech Eng H 224, 1415-1440.

Kamitakahara, M., Ohtsuki, C., Miyazaki, T., 2007. Coating of bone-like apatite for development of bioactive materials for bone reconstruction. Biomed Mater 2, R17-23.

Keeting, P.E., Oursler, M.J., Wiegand, K.E., Bonde, S.K., Spelsberg, T.C., Riggs, B.L., 1992. Zeolite A increases proliferation, differentiation, and transforming growth factor beta production in normal adult human osteoblast-like cells in vitro. J Bone Miner Res 7, 1281-1289.

Khang, D., Carpenter, J., Chun, Y.W., Pareta, R., Webster, T.J., 2010. Nanotechnology for regenerative medicine. Biomed Microdevices 12, 575-587.

Kihara, T., Zhang, Y., Hu, Y., Mao, Q., Tang, Y., Miyake, J., 2011. Effect of composition, morphology and size of nanozeolite on its in vitro cytotoxicity. J Biosci Bioeng 111, 725-730.

Laurencin, C., Khan, Y., El-Amin, S.F., 2006. Bone graft substitutes. Expert Rev Med Devices 3, 49-57.

Li, X., Xie, J., Lipner, J., Yuan, X., Thomopoulos, S., Xia, Y., 2009. Nanofiber scaffolds with gradations in mineral content for mimicking the tendon-to-bone insertion site. Nano Lett 9, 2763-2768.

Linh, N.T.B., Min, Y.K., Song, H.Y., Lee, B.T., 2010. Fabrication of polyvinyl alcohol/gelatin nanofiber composites and evaluation of their material properties. Journal of Biomedical Materials Research Part B: Applied Biomaterials 95, 184-191.

Liu, Y., Chan, J.K., Teoh, S.H., 2015. Review of vascularised bone tissue-engineering strategies with a focus on co-culture systems. J Tissue Eng Regen Med 9, 85-105.

Longley, L., Fiddes, M., O'Brien, M., 2008. Anaesthesia of Exotic Pets, Elsevier Saunders.

Mousavi, G., Rezaie, A., 2011. Biomechanical Effects of Calcium Phosphate Bone Cem ent and Bone Matrix Gelatin Mixture on Healing of Bone Defect in Rabbits. World Appl. Sci. J 13, 2042-2046.

Reichert, J.C., Wullschleger, M.E., Cipitria, A., Lienau, J., Cheng, T.K., Schutz, M.A., *et al.*, 2011. Custom-made composite scaffolds for segmental defect repair in long bones. Int Orthop 35, 1229-1236.

Richardson, V.C., 2008. Rabbits: health, husbandry and diseases, John Wiley & Sons.

Suckow, M.A., Stevens, K.A., Wilson, R.P., 2012. The laboratory rabbit, guinea pig, hamster, and other rodents, Academic Press.

Zhang, Q., Tan, K., Ye, Z., Zhang, Y., Tan, W., Lang, M., 2012. Preparation of open porous polycaprolactone microspheres and their applications as effective cell carriers in hydrogel system. Materials Science and Engineering: C 32, 2589-2595.