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ORIGINAL ARTICLE

Fabrication and Usage of a Nanocomposite Scaffold in Segmental Bone Healing: An Animal Model Study

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Abstract

Objective- The loss of bone fragments, often due to trauma, infection, mass loss, or even complete bone regeneration after complicated fractures, is one of the constant challenges in medicine and veterinary medicine. The aim of this study was to fabricate and use a nanocomposite scaffold in segmental bone healing in rabbits.

Design- Experimental Study

Animals- Forty adult male New Zealand male rabbits

Procedures- The animals were randomly divided into four groups of 10 animals each. On femur of each rabbit a bilateral 6 mm diameter defect was created. In the first group (control), no substance was used, in the second group, hydroxyapatite, in the third group, nanocomposite tri-calcium phosphate (TCP) and in fourth group, autograft was used to fill the defect. Bone specimens were harvested for histopathological evaluations on days 15 and 60 for evaluation of four indices of union, spongiosa, cortex and bone marrow.

Results- The results of using nanocomposite tricalcium phosphate in comparison with other groups were significantly different in all cases.

Conclusion and Clinical Relevance- It could be admitted that nanocomposite tri-calcium phosphate scaffold had a positive effect on the healing process and showed satisfactory bone strength, therefore, it could be widely used in orthopedic surgery as well as tissue engineering.

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1. Introduction

Bone tissue engineering is a rapidly developing research area with many clinical applications, such as in orthopedic defects, low back pain, osteoporosis, osteoarthritis, bone infection or tumors, as well as oral, maxillofacial, craniofacial, orthopedic, reconstructive, trauma, and neck and head surgery.[1–3] Scaffolds have been of interest to replace allografts and autografts, the gold standard for treating bone defects.[4–7] When designing scaffolds for bone tissue engineering, biodegradability, biocompatibility, bioactivity, osteoinductivity, and osteoconductivity play an important role in tissue regeneration. Among the materials, metals are suitable for load-bearing applications due to their favorable mechanical properties, while bioceramics show excellent biocompatibility due to their chemical composition being similar to the mineral phase of bone tissue.[8–10] However, both metals and ceramics are generally poorly degradable in vivo. Biodegradable and bioresorbable synthetic polymeric scaffolds consisting of poly α-hydroxy esters and their copolymers, such as poly lactic acid (PLA), poly glycolic acid (PGA), poly ε-caprolactone (PCL), and poly lactic-co-glycolic acid (PLGA), have been widely used in various tissue engineering applications including bone tissue regeneration.[11–12] Collagen offers low immunogenicity, a porous structure, permeability, good biocompatibility, and biodegradability and has functions to regulate the morphology, adhesion, migration, and differentiation of cells.[13,14] Tricalcium phosphate (TCP) is a tertiary calcium phosphate also known as bone ash [$Ca_3(PO_4)$]. It serves as a rich source for calcium and phosphorus, which can be easily assimilated and absorbed. Beta-TCP is highly biocompatible and creates a resorbable interlocking network within the defect site to promote healing.[15,16]

The present study was aimed at evaluation of bone regeneration using the rabbit's femoral defects repaired by the nanocomposite TCP/collagen scaffolds in two time points of 15 and 60 days post operation.

2. Materials and Methods

Fabrication and Characterization of TCP/Collagen Nanocomposite

TCP was synthesized by calcining amorphous calcium phosphate. Collagen (Sigma-Aldrich, St. Louis, US) was used for the collagen matrix, and aqueous solution of glutaraldehyde (Sigma-Aldrich, St. Louis, US) was used as a cross-linking agent. All other reagents and solvents are of analytical grade and are used as received. Aqueous alkali solution (pH = 12) was obtained by dissolving sodium hydroxide into deionized water. For preparation of TCP/collagen nanocomposite, collagen suspension was prepared in aqueous alkali solution (pH = 12) at room temperature. Then, TCP powder was slowly added into the collagen suspension (2:1) while stirring, after a homogenous suspension was formed, the glutaraldehyde solution was added as a cross-linking agent. The mixture froze in a refrigerator for 5 h at -40° C. Porous composites were obtained after further lyophilization. The scaffold was characterized using Field emission scanning electron microscope, size distribution curve and Fourier transform infrared (FTIR) spectroscopy (Figures 1 and 2).

Ethical Consideration, Design, Animal Grouping and Surgery

The present study was approved by the Islamic Azad University Research Committee, Tehran Science and Research Azad University. In this study, we used 40 mature male New Zealand white rabbits, 6–8 months of age and with an approximate weight of 3–3.5 kg. All animals were obtained from the same source 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 the temperature of 18 ± 3° C, a humidity of 60 ± 5%, and 12 h light/dark cycle. Right lateral femoral osteotomies were performed surgically. Surgical procedures were done after an

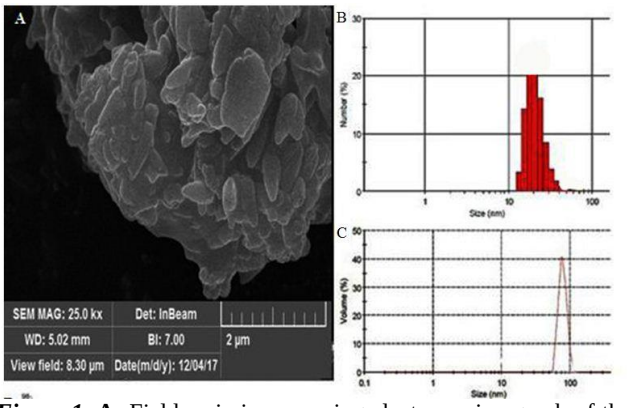

Figure 1. **A:** Field emission scanning electron micrograph of the tricalcium phosphate (TCP)/collagen nanocomposite. **B:** Size distribution of the nanoparticles based on numbers. D) Size distribution of the TCP nanoparticles based volumes.

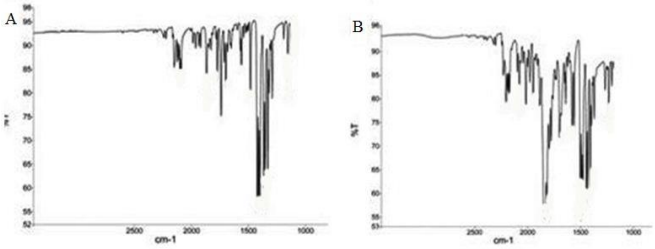

Figure 2. **A:** Fourier transform infrared (FTIR) spectrum of the TCP nanoparticles. B: FTIR spectrum of the tricalcium phosphate/collagen nanocomposite.

intramuscular injection of ketamine 10% (50 mg/kg, Alfasan, Holland) and xylazine 5% (5 mg/kg, Alfasan, Holland). The hair was removed from the surgical site and the skin was cleaned with iodinated surgical soap. Aseptic technique was used throughout the surgical procedure. An incision approximately 5 cm long was made along lateral 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 6 mm diameter, 5 mm cylinder bilateral bone defect was created in the femur of the right hind limb via drilling a hole in the diaphysis –for example in the lateral- medial direction. This osteotomy site was then irrigated with 0.9% saline solution, 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. The animals were randomly divided into 4 groups of 10 animals each. In the first group (control), no substance was used, in the second group hydroxyapatite (HA) (Merck KGaA, Darmstadt, Germany), in the third group nanocomposite tricalcium phosphate (TCP)/collagen, and in fourth group autograft was used to fill the defect. Bone specimens were harvested for histopathological evaluations on days 15 and 60 for evaluation of four indices of union, spongiosa, cortex and bone marrow.

Histopathology

For histological examination, the obtained tissues were decalcified with 10% formic acid solution which was replaced daily. The surgical specimens were submitted to 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. Samples on day 15 were stained by hematoxylin and eosin (H&E) stain since at this time evaluation of inflammation phases were considered and on day 60, the samples were stained by trichrome stain because the grade of osteoclasting was important at this times. Then, they were observed under a light microscope. Recorded factors from specimens were evaluated with a 12-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 based on a scoring system used in a previous study (Table 1).[12]

Statistical Analysis

The collected data were analyzed statistically with one-way analysis of variance using SPSS software version 22 (SPSS Inc., Chicago, US).

3. Results

Descriptive Statistics and Histopathological Findings

The highest point of the index union on day 15 was from the nanocomposite TCP/collagen group and the lowest

points belonged to the control and HA groups. (Table 2). Among the days on which the samples were obtained while the average score of union index demonstrated a significant difference in the HA and the control groups ($p < 0.05$). Such significant difference was not shown in the nanocomposite TCP/collagen group. Average scores union on days 15 and 60 showed that the highest union score belongs to the nanocomposite TCP/collagen group, while the HA and the control groups were in the second and the third place, respectively.

Descriptive statistics of spongiosa index were demonstrated that the highest scores on day 15 belonged to the nanocomposite TCP/collagen group, and the control group and HA group were both lower. On day 60, nanocomposite TCP/collagen group gained the highest score compared to groups 1 and 2 (Table 3). Among the days on which the samples were obtained average scores of spongiosa index demonstrates significant difference in all four groups ($p < 0.05$). Average scores of spongiosa on days 15 and 60 showed that spongiosa score in group 3 was significantly higher than groups 2 and 3 ($p < 0.05$), while the HA and the control groups were in the second and the third level, respectively.

Table 1. Histological scoring system based on Shafiei-Sarvestani *et al.* [12]

Groups	Point
Union (the highest score is 4)	
No sign of union	0
Fibrous union	1
Osteochondral union	2
Bone union	3
Complete reorganization	4
Spongiosa (the highest score is 4)	
No sign of cellular activity	0
Early bone formation	1
Active new bone formation	2
Reorganized spongiosa formation	3
Complete reorganization spongiosa	4
Bone marrow (the highest score is 4)	
Not available	0
Detection of fibrinous material	1
Defect involving more than half	2
Fully involving the red bone marrow	3
Adult type fatty marrow	4

The descriptive statistics of bone marrow index indicated that the control group has the lowest score by day 15 of the healing process and was lower in the nanocomposite TCP/collagen and HA groups compared to group 4. On day 60 of the healing process, the score the nanocomposite TCP/collagen group was significantly higher than groups 2 and 3 ($p < 0.05$). Among the days on which the samples were obtained average scores of bone marrow index shows significant difference in all groups during this study ($p < 0.05$) (Table 4).

Table 2. Descriptive statistics of union index in experimental groups.

Groups	Day 15	Day 60
Group 1 (control)	1(1-1)	2(2-2)
Group 2 (HA)	1 (1-1)	3(3-3)
Group 3 (TCP/collagen)	3(2-3) *	4(3-4) *
Group 4 (Autograft)	4(3-4)	4(4-4)

* $p < 0.05$ *vs.* other groups 1 and 2.

Table 3. Descriptive statistics of spongiosa index in experimental groups.

Groups	Day 15	Day 60
Group 1 (control)	1(1-1)	2(2-2)
Group 2 (HA)	1 (1-1)	3(2-3)
Group 3 (TCP/collagen)	3(1-3) *	4(3-4) *
Group 4 (Autograft)	3(3-3)	4(4-4)

* $p < 0.05$ *vs.* other groups 1 and 2.

Table 4. Descriptive statistics of bone marrow index in experimental groups.

Groups	Day 15	Day 60
Group 1 (control)	1(1-1)	2(1-2)
Group 2 (HA)	1 (1-1)	3(1-3)
Group 3 (TCP/collagen)	3(2-3)*	4(2-4) *
Group 4 (Autograft)	4(3-4)	4(3-4)

* $p < 0.05$ *vs.* other groups 1 and 2.

On the day 15, numerous capillary buds were present at the healing site of the control group. The healing site of the HA group demonstrated a fibrous tissue while the healing site from the nanocomposite TCP/collagen group showed a primary bone formation. Microscopically, the healing site of the control group on day 60 of healing showed bone

deposition within cartilage and primary ossification. In the healing site of the HA group on day 60 filling of the defect by primary bone was evident. While the healing site of nanocomposite TCP/collagen group at this time showed that the defect is filled with lamellar bones. Advanced stages of remodeling and consolidation and development of Haversian system is seen in repaired tissues. The quantity of newly formed lamellar bone in the healing site of nanocomposite TCP/collagen is more than that of the HA and the control groups. Histopathological evaluation was performed on the days 15 and 60 after surgery. On these days, the quantity and rate of bone formation in the healing site in nanocomposite TCP/collagen group was better than HA and control groups and the quantity of newly formed lamellar bone in the healing site in the nanocomposite TCP/collagen group was more than that of the HA and control groups (Figure 3).

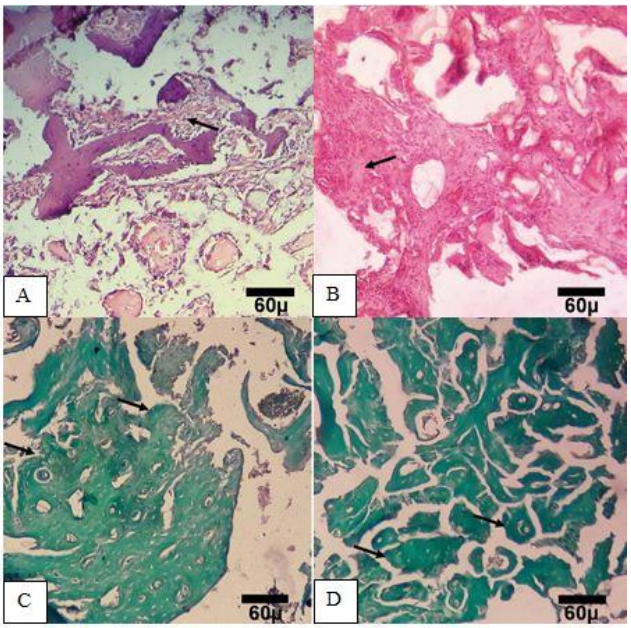

Figure 3. **A** and **B**: Microscopic section from the healing site of experimental groups on day 15 of healing site (H&E staining). **A:** Group 1 shows abundant cartilaginous callus and mild primary woven bone (arrow) near the defect. The retained granulation tissue in the defect is shown. **B:** Group 3 shows well developed primary woven bone (arrow) near the defect. The retained granulation tissue in the defect is shown. **C** and **D:** Microscopic section from the healing site of experimental groups on day 60 of healing site (trichrome staining). **C:** Group 1 shows that the lamellar bone spicules are thinner than others. **D:** Group 3 shows that the lamellar bones (arrows) are being produced.

4. Discussion

Bone has a remarkable regenerative ability but a considerable amount of bone loss or development of an adverse microenvironment can hinder this capacity, such as in cases of severe trauma, developmental deformities, revision surgeries, and tumor resection.[17,18] Bone tissue engineering holds the promise of great therapeutic potential.[18] This study aimed to evaluate the positive effects of nanocomposite TCP/collagen scaffold in comparison with hydroxyapatite scaffold in bone healing of the femoral defect in rabbits. In this study, it seems that on days 15 and 60 post-surgery, the quantity and the velocity of bone formation in the healing site of the nanocomposite TCP/collagen group were better than HA and control groups. Additionally, it appears that the quantity of newly formed lamellar bone in the healing site of the nanocomposite TCP/collagen group was more than that of the HA and control groups after day 60.

Nanostructures such as nanofibers and nanoparticles represent an interesting class of tissue engineering scaffolds with great potential for bone regeneration. The widely used nanocomposite constituents are hydroxyapatite crystals which are 20–80 nm long and 2–5 nm thick, and collagen fibrils with diameters of less than 500 nm. Nanofibers are extra cellular matrix (ECM) mimicking scaffolds that are characterized by high porosity with a wide range of pore-sizes, high surface area, unusual surface properties, and morphological similarity to native bone ECM.[19-21] Nanofiber scaffolds with interconnecting porous structures provide high surface area for cell attachment, growth, and differentiation as well as nutrient transport. In addition, nanoparticle-based materials have received significant attention for bone tissue engineering applications.[22]

A number of studies have documented the favorable responses of bone cells toward synthetic nanofibers or nanophase particles. It has been demonstrated enhanced osteoblast adhesion and proliferation on polymeric

nanofiber scaffolds that exhibit mimicry of the ECM.[23] The extracellular mimetic structure increased protein adsorption as well as to the selective adsorption of cell adhesion proteins such as fibronectin and vitronectin on the nanostructures compared to structures with solid pore walls.[23] Furthermore, the nanofiber scaffold environment has been shown to support mesenchymal stem cells to differentiate along the osteogenic lineage.[24] Nanophase materials composed of alumina, titania, and hydroxyapatite with grain sizes of < 100 nm have been shown to elicit favorable osteoblast and osteoclast activity compared with micrometer-sized particles of the same materials.[25,26]

There are several reported techniques to fabricate nanofiber scaffolds with unique properties including phase separation, self-assembly and electrospinning .[27-29] Among them, electrospinning holds great promise due to the ease of fabrication, efficient control over the process, and easy scale-up.[27] Zeleny first studied the electrospinning process which was initially known as electrostatic spinning in 1914.[30] It was not until 1934 that Formhals *et al*. reported the first patent describing the process of developing polymeric nanofibers via electrospinning.[31] Fibers with diameters ranging from few nanometers to several micrometers can be obtained via electrospinning process.[32] Unlike the other two processes, electrospinning allows for the development of complex structures such as aligned nanofibers. In addition to providing a high surface area for cell adhesion and proliferation, aligned nanofibers offer the ability to guide the orientation of cytoskeletal proteins of cells.[33-35]

The results of the present study indicated that nanocomposite TCP/collagen satisfied all the aforementioned tissue engineering scaffold criteria for bone regeneration. It seems that TCP/collagen nanocomposite has an important role in the reconstruction of bone defects and can be used as scaffold in bone fractures. Nanocomposite TCP/collagen granules exhibited a reproducible bone-healing potential. It seems that TCP/collagen nanocomposite has an important role in the reconstruction of bone defects and can be used as a scaffold in bone fractures.

Conflict of Interests

None.

Acknowledgments

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