Original Article

Poly (lactide-co-glycolide) nanofibers coated with collagen and nano-hydroxyapatite for bone tissue engineering

Reza Tavakoli-Darestani, Gholamhossein Kazemian*, Mohammad Emami, Amin Kamrani-Rad

Department of Orthopedic Surgery, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Abstract

Background: A combination of polymeric nanofibrous scaffold and bioactive materials is potentially useful in bone regeneration applications.

Materials and Methods: In the present study, Poly (lactide-co-glycolide) (PLGA) nanofibrous scaffolds, fabricated via electrospinning, were initially coated with Type I collagen and then with nano-hydroxyapatite. The prepared scaffolds were then characterized using SEM and their ability for bone regeneration was investigated in a rat critical size bone defect using digital mammography, multislice spiral-computed tomography (MSCT) imaging, and histological analysis.

Results: Electrospun scaffolds had nanofibrous structure with homogenous distribution of n-HA on collagen-grafted PLGA. After 8 weeks of implantation, no sign of inflammation or complication was observed at the site of surgery. According to digital mammography and MSCT, PLGA nanofibers coated simultaneously with collagen and HA showed the highest regeneration in rat calvarium. In addition, no significant difference was observed in bone repair in the group which received PLGA and the untreated control. This amount was lower than that observed in the group implanted with collagen-coated PLGA. Histological studies confirmed these data and showed osteointegration to the surrounding tissue.

Conclusion: Taking all together, it was demonstrated that nanofibrous structures can be used as appropriate support for tissue-engineered scaffolds, and coating them with bioactive materials will provide ideal synthetic grafts. Fabricated PLGA coated with Type I collagen and HA can be used as new bone graft substitutes in orthopaedic surgery and is capable of enhancing bone regeneration via characteristics such as osteoconductivity and osteointegration.

Keywords: Electrospinning, bone, collagen, hydroxyapatite, tissue engineering

***Corresponding Author:** Gholamhossein Kazemian. Department of Orthopedic Surgery, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, Tel, Fax: +98(21)77558081. E-mail: aminkomrani@yahoo.com

Introduction

Procedures for reconstruction surgery and organ transplantation improve the quality of life, and in some cases save life. However they are accompanied with various complications. In most cases these procedures require either organ donation from a donor individual or tissue transplantation from a second surgical site [1]. Autografts exhibit the distinct advantage of histocompatibility without the risks of disease transfer and are still the gold standard for bone repair [2]. The major problem with allograft and xenograft organ transplantation is that there exists a drastic threat of disease transmission and immune responses, which can lead to the graft failure. With the limited availability of donor organs, the science of tissue engineering has

emerged with the goal of developing organs, tissues, and synthetic materials ready for recovery of their structure or function in the future [3-6]. Bone grafts have been used to fill bone defects caused by disease or trauma, such as bone fractures, infections, and tumours. In this regard, bone graft substitutes (BGS) are the field of increasing interest. One of the main characteristics of these substitutes is the vital capability to support the ingrowth of cells and newly formed bone tissue. Osteoconductivity and osteoinductivity are also considered as the significant and ideal characteristics of graft substitutes [7, 8]. With regards to the site of use and specific characteristics, BGS can be fabricated via different techniques. Electrospinning is a versatile, new and cost-effective method which has recently been used extensively for fabrication of tissue-engineered scaffold. Using this technique, porous scaffolds with nanofibrous structure can be constructed [9]. This topology can efficiently mimic the fibrous architecture of natural extracellular matrix (ECM) in various tissues and will enhance the process of regeneration and repair while applied as BGS [10]. Bone has an architecture composed mainly of inorganic substance (70%) consisting of hydroxyapatite and an organic fraction consisting of 95% Type I collagen [11]. Selecting a Biomimetic approach, an ideal BGS should mimic both physical and chemical characteristics of bone. In some recent works, our research group has used the nanofibrous scaffolds as a base for BGS and coated a bioactive material, such as nano-HA or Type I collagen, onto the surface of fibers to improve the characteristics of the scaffolds [12, 13]. As both HA and Type I collagen are present in bone, it seems that simultaneous coating of these materials will provide a very useful substrate for tissue engineering. Therefore, here we report a new nanocomposite structure consisting of PLGA nanofibers coated with Type I collagen and nano-hydroxyapatite particles for bone repair, employed in critical-sized rat calvarial defects.

The electrospun structure, composed of poly (D,L-lactic-co-glycolic acid) (PLGA) fibers [14], displays a morphologic similarity to the extracellular matrix (ECM) of natural tissue [15]. Such a structure meets the essential design criteria of an ideal engineered scaffold. Moreover, the addition of biodegradable nanofibers, such as PLGA, to bioactive structures allows a better manipulation and control over the shaping of various nanocomposites to fit bone defects [16-18].

Materials and Methods

Preparation of scaffolds

In this study, all scaffolds were fabricated via electrospinning. A 15% (wt/wt) solution of PLGA in DMF/tetrahydrofuran (THF) (Sigma-aldrich, USA) was prepared and a 5mL syringe was filled with the solution. The solution was fed into a 21-gauge needle via an extension tube by a syringe pump. The needle was located at a distance of 15 cm from a grounded collector, and a 15-`kV voltage was applied to this setup using a high-voltage direct-current power supply (Stem Cell Technology Research Center, Tehran, Iran). Because of application of a voltage between the needle and the collector, the solution droplets were forced to leave the needle and deposit on the cylinder in the form of ultrafine fibers. Having reached an appropriate thickness, the mat was detached from the collector and placed in a vacuum for evaporation of the residual solvent. The coating of collagen and n-HA was performed as described previously. Using a low-frequency plasma generator of 44 GHz frequency with a cylindrical quartz reactor (Diener Electronics, Ebhausen, Germany), Oxygen plasma treatment was performed on the PLGA scaffolds. In this process, pure oxygen was introduced into the reaction chamber at 0.4 mbar pressure, and the glow discharge was ignited for 5 min.

For collagen grafting, plasma-treated mats were cut into 1.5 cm diameter punches and immersed in EDC/NHS (Merck) solution (5 mg/ml) for 12 h. A 1 mg/ml collagen I solution was used to immerse scaffolds overnight. The scaffolds were then rinsed with distilled water. For n-HA coating, a 1% (w/v) solution of HA in de-ionized water was prepared after thorough dispersion in an ultrasonic bath for 20 min. To deposit these materials on the surface of nanofibers, collagen-grafted scaffolds were immersed individually in each aqueous solution overnight. After that, the mats were thoroughly rinsed with de-ionized water and dried in vacuum. All experiments were performed on the following groups: Untreated control group, pristine PLGA nanofibers (PLGA), PLGA grafted with

collagen I (PLGA-Col) and PLGA-Col coated with HA (PLGA-Col-HA).

Scanning Electron Microscopy (SEM)

To investigate the morphology of nanofibers, the surface of scaffolds was coated with gold using a sputter and characterized using a scanning electron microscope (SEM, LEO 1455VP, Cambridge, U.K.). Using image analysis software (image J, NIH, USA), the fiber diameter was determined using SEM images in which the mean diameter of 100 fibers was measured.

Animal Study and in vivo implantation

A total of n= 20 male Sprague Dawley rats (Razi Institute, Karaj, Iran) with an average weight of 200-250 g were housed 5 to a cage under standard conditions. Animals were anesthetized via intraperitoneal injections of ketamine (20 mg/kg) / xylazine (2 mg/kg) and inhalation of a mixture of 20% v/v isoflurane and propylene glycol. The surgical site was shaved and scrubbed with iodine. Then an incision was made in the sagittal plane across the cranium. To expose the calvarial bone, a full-thickness flap including the periosteum was reflected. Then using a saline-cooled trephine drill, a critical-size (8-mm-diameter) transosseous and circular defect was created on the cranium. Scaffolds were then implanted in critical-size calvarial defects and each defect was filled with a circular scaffold. For the control group, the defect was left empty. Finally, absorbable sutures were used to close the incisions. All animal experiments were performed in accordance with the Shahid Beheshti University of Medical Sciences's guidelines.

Digital Mammography and Multislice Spiral Computed Tomography (MSCT) Imaging Analysis

After 8 weeks, the animals were euthanized, and scaffolds and host calvarial bone were recovered and fixed in 10% formalin. The samples were then radiographed under direct digital mammography equipment (Konica Minolta, Regius model 110HQ). The specimens were also scanned using a spiral high-resolution computed tomography (CT) system (Siemens, SOMATOM Sensation) in multislice mode. The radiograph images from digital mammography were scored by two independent radiologists. To quantify the level of bone regeneration via MSCT, a 9-mm circular region of interest was placed in each CT image. The area of newly formed bone was quantified relative to the original calvarial defect.

Histological assessments

Calvarial bone samples were fixed and decalcified in ethylenediaminetetraacetic acid/HCl and embedded in paraffin. Histological Sections (3-5 μm) were stained with hematoxylin and eosin (H&E) and the newly formed bone was examined under light microscopy and quantified using a computer-assisted Image-Pro Plus System (Media Cybernetics, Silver Springs, MD).

Statistical Analysis

All data were reported as mean ± standard deviation (SD). The statistical significance was determined by a Mann_Whitney U test as a nonparametric equivalent of an independent sample Student's t test. Simple one-way analysis of variance and its nonparametric equivalent (Kruskal_Wallis test) were used to compare the results among multiple groups. All analyses were performed using SPSS 17.0 software and the P value of <0.05 was considered as statistically significant.

Results

Morphology of scaffolds

The morphology of PLGA, PLGA-Col and PLGA-Col-HA was investigated under SEM (Figure 1). The nanofibers in all scaffolds showed a randomly oriented uniform morphology with an average diameter of 887±57 nm. After grafting of collagen, the average diameter of fibers did not significantly change. After coating of n-HA on the surface of collagen-coated PLGA nanofibers, a homogeneous distribution of nanoscale HA along the surface of the nanofibers was observed in PLGA-Col-HA. Energy dispersive spectroscopy (EDS) was also used to map Ca^{2+} ions and verify the coating of hydroxyapatite on the surface of nanofibers (Figure 2).

Evaluation of Bone Regeneration

For evaluation of bone regeneration after 8 weeks implantation, specimens from the calvarium of rats in all groups were prepared, fixed and used to determine the quantity of newly formed bone using Digital Mammography and MSCT. Figure 3 shows the images of digital mamography from the calvarium samples.

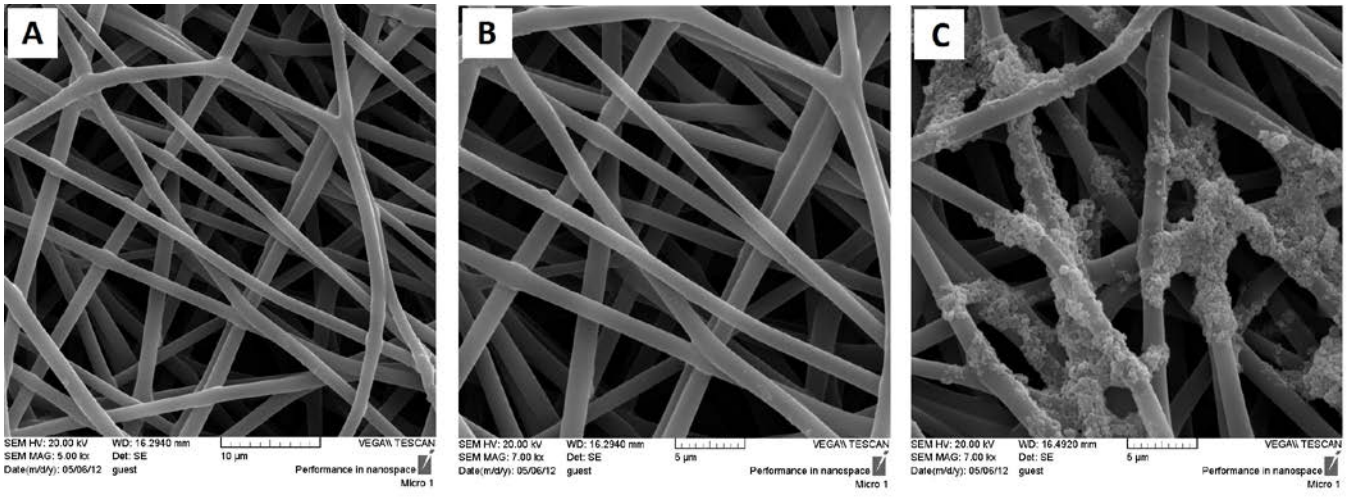

Figure 1

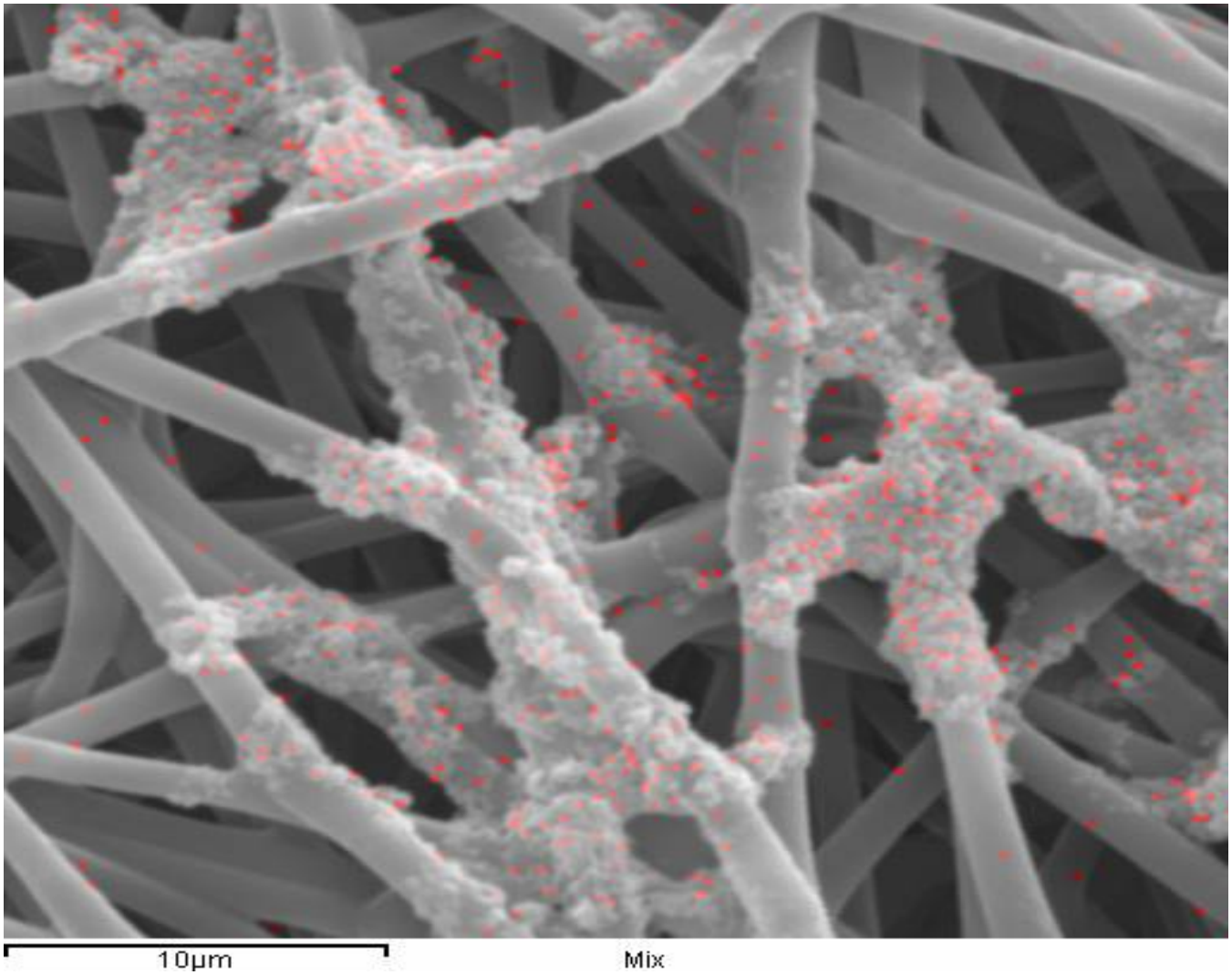

Figure 2

From a qualitative view, ossification was observed in both groups which received PLGA-Col and PLGA-Col-HA. There was no sign of regeneration in untreated control and the group with PLGA scaffold. These observations were confirmed by a quantitative analysis of images. It was demonstrated that there was no significant amount of bone regeneration in PLGA group compared to untreated group. However, new mineralized tissue was observed in groups that received PLGA-Col and PLGA-Col-HA ($P < 0.05$). In addition, it was found that the ossification was significantly higher in groups that received PLGA-Col-HA scaffold compared to PLGA-Col. Cranial-CT was also used to investigate ossification in rat bone defects (Figure 4). Bone regeneration was quantified in the area of newly formed bone by the imaging software. The results were similar to that obtained from Digital Mammography (data not shown).

Figure 5 depicts histological staining of specimens obtained from rat calvarium and regenerated bone in deferent groups can be compared from these results. The area of newly formed bone was also quantified by scoring the images of histological staining. The highest amount of newly formed bone was observed in the rats receiving PLGA-col-HA scaffolds ($P < 0.05$). The amount of bone repair was also higher in PLGA-Col group compared to control groups. No significant difference was observed in the amount of ossification in rats which received PLGA scaffolds compared to untreated control.

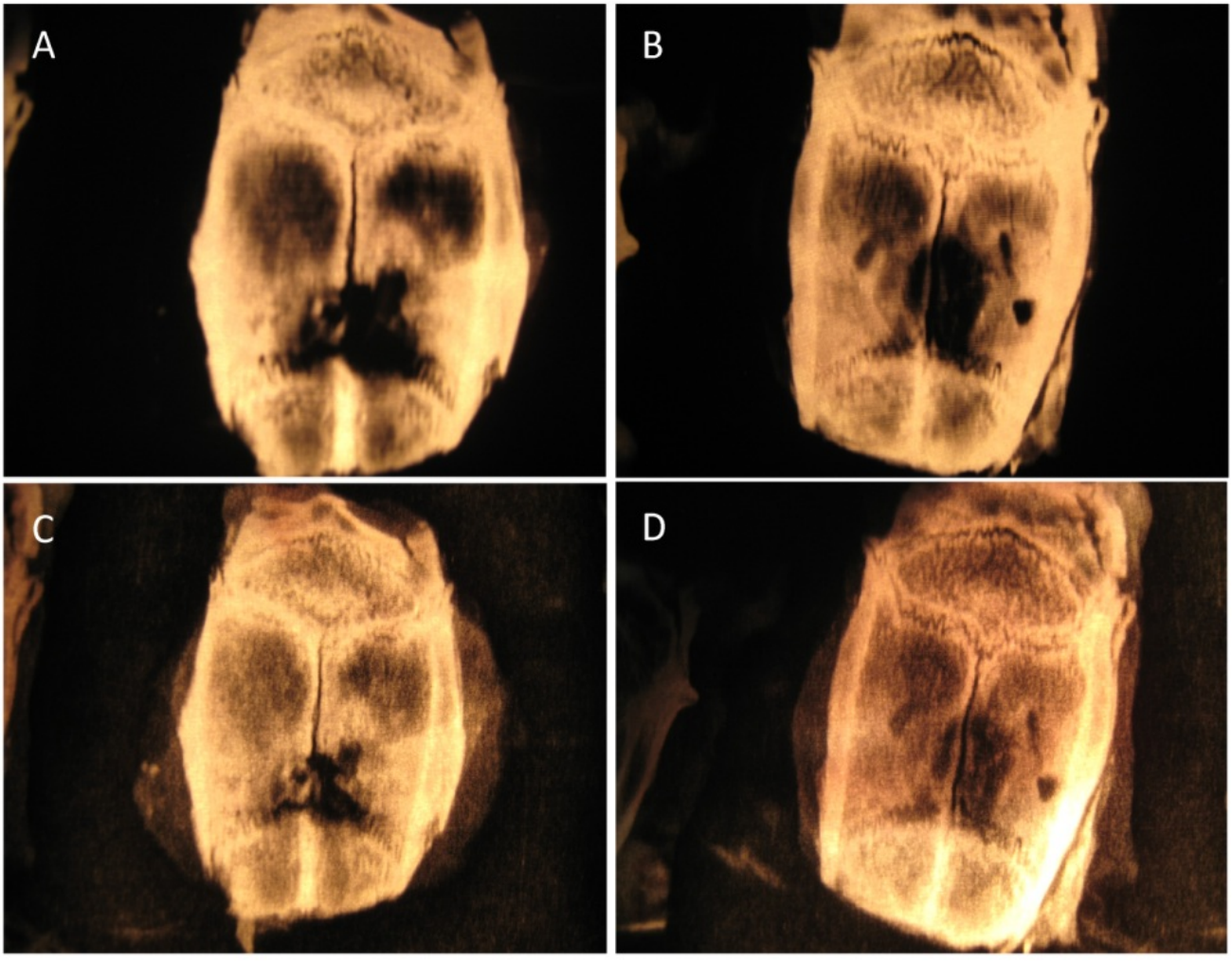

Figure 3

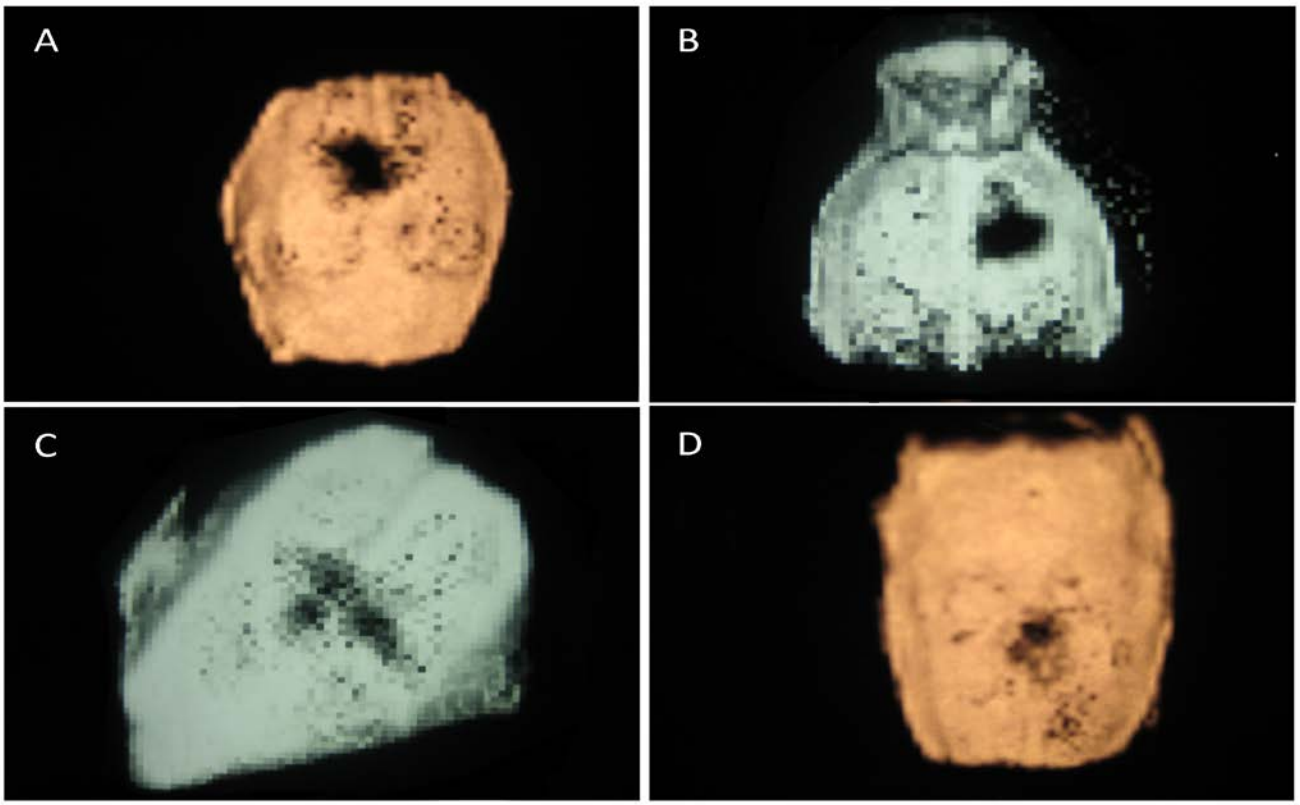

Figure 4

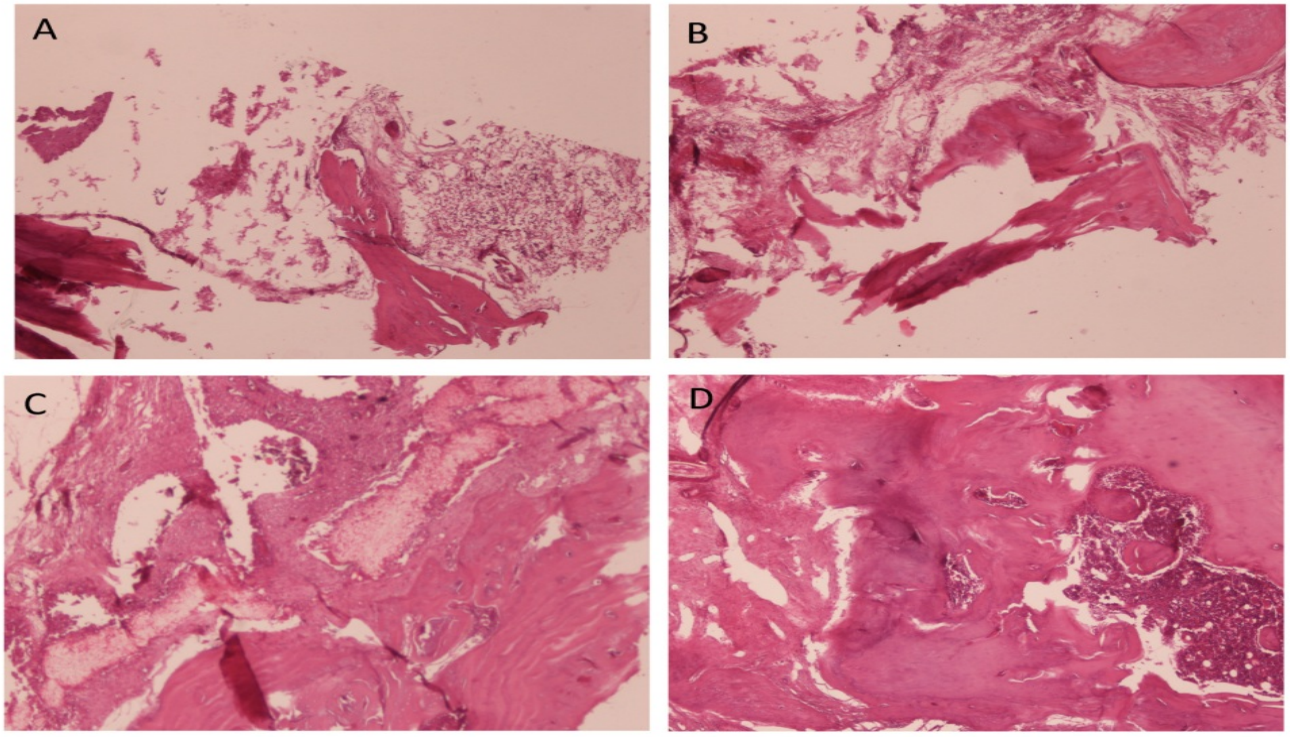

Figure 5

Discussion

Autografts and allografts are the classic treatments for filling void defects caused by trauma, accidents or diseases. However, drawbacks such as a limited supply and donor-site morbidity for autografts and immunorejection and risk of disease transfer for allografts have forced the researchers to look for synthetic materials as bone grafts [19]. An ideal BGS should have properties such as osteoconductivity, bioactivity and appropriate mechanical properties. Bone autografts naturally have these characteristics with an excellent quality [20]. Therefore, it seems that mimicking the physical and chemical properties of natural bone tissue, is an efficient strategy to construct tissue-engineered scaffolds. In the present study, we fabricated PLGA nanofibers via electrospining and coated them simultaneously with Type I collagen and HA and evaluated their performance for bone regeneration *in vivo*.

Bone tissue has a complex structure composed of highly ordered collagen fibers which are surface-mineralized with hydroxyapatite crystals [11]. A biomimetic approach guided our research groups to fabricate nanofibers and coated them with Type I collagen and HA to prepare an ideal BGS. In our recent work, PLLA nanofibers were grafted with collagen and were demonstrated to enhance in osteogenic differentiation of stem cells *in vitro* [12]. In another work, these electrospun nanofibers were coated with HA and the enhancement of differentiation of stem cells cultured on their surface was shown again [13]. Here, we combined these two bioactive materials, Type I collagen and HA, as a coating layer on the surface of nanofibers, and investigated their effect on bone regeneration of calvarial defect in a rat model. There are several research reports wherein electropsun nanofibers have been used for bone tissue engineering application [21-24]. There are also some reports about the coating of bioactive materials onto nanofibers and their characterization *in vitro* [25, 26]. But none of these has reported the *in vivo* performance of nanofibrous scaffolds coated with Type I collagen and HA. The PLGA-Col-HA scaffolds showed a nanofibrous structure with interconnected pores. This architecture is appropriate for osteointegration because of efficient bone bonding and ingrowth of surrounding tissue [27]. The HA particles were also attached to the surface of PLGA-Col after several rinsing. It is explained by the electrostatic interaction between Ca ion in HA and acidic side chains of collagen layer [28]. Upon retrieval of implanted scaffold, the appearance of the defect sites was normal and no sign of inflammation was observed. This finding demonstrated the biocompatibility of the scaffolds and was confirmed by histological results. In our study, X-ray radiology and CT were used simultaneously to evaluate ossification in bone defects in rats. The results obtained from these methods were comparable to each other. First of all, PLGA nanofibers alone did not enhance regeneration compared to that in untreated control. This is in concordance with our previous study [29]. However, surface-modified PLGA scaffolds significantly promoted bone healing in other two groups, PLGA-Col and PLGA-Col-HA. These findings suggest that the existence of bioactive materials such as collagen and HA can transfer the characteristics of osteoconductivity to nanofibrous scaffolds. In addition, HA coating on the surface of collagen coated nanofibers synergistically enhanced bone regeneration during the period of study. HA-coated scaffolds have been shown to be biocompatible and osteoconductive. As reported by others, when implanted in bone defects, these coated constructs were also demonstrated to be able to improve the process of regeneration compared to that guided by uncoated scaffolds [30, 31]. To confirm the bone regeneration and evaluate the quality of the repaired bone, histological study was observed on the fixed specimens. Interestingly, the quantified data of histologic staining was in concordance with X-ray radiology and CT results. Taking all together, our data suggest that LGA nanofibers coated with both Type I collagen and hydroxyapatite may offer a new and exciting scaffold for efficient regeneration of critical size bone defects.

Conclusions

In this study, we demonstrated that nanofibrous structures can be used as an appropriate base for tissue-engineered scaffolds. In addition, PLGA nanofibers coated simultaneously with Type I collagen and HA exhibit osteoconductivity and hold promising potential for bone tissue engineering applications.

Conflict of interest

None declared

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