Evaluation the Efficiency of Novel Dextran-Chitosan/Nano-Hydroxyapatite Composite Scaffolds for Bone tissue Engineering

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Abstract: The efficiency of the three-dimensional scaffolds of dextran/chitosan/nano-hydroxyapatite (where, nHA has different concentrations of 0, 20, 30 and 40 Wt%) on the repair of bone defects in rats was investigated. The dissolution of the composite scaffolds was estimated by immersing the scaffolds in phosphate-buffered saline (PBS) solution till 28 day. In vitro cytotoxicity test of the composite scaffolds against baby hamster kidney (BHK) fibroblast cells was examined. A defect of 2 mm diameter in the femur bone was created in 25 white rats, which were divided randomly into five groups (n=5). Postoperatively at (9, 12 and 24 week), the bone healing was evaluated by X-ray and bone mineral density (BMD) was measured. The dissolution of the composite scaffold with 40 Wt% nHA demonstrated higher ionic release profile of calcium species. The cytotoxicity test showed that, the incorporation of nHA to Dex/CS composite scaffolds increases the viability of the cells. In vivo results showed that, the BMD of composite scaffold containing a higher concentration of HA nanoparticles reached 191.58 mg/cm³ after 24 week of implantation. The obtained results revealed that, the efficiency of the composite scaffolds to repair defect in the femur bone increases with increasing nHA content.

Key words: Nano-Hydroxyapatite · Dextran · Chitosan · Composite Scaffolds · Bone Tissue Engineering

INTRODUCTION

Bone is a mineralized tissue that assembles the body skeleton and is prone to modification over the whole life span. Large bone defects due to trauma, surgery or due to cancer treatment remains a big problem for orthopedic surgeons. Until now, the main approach for replacing missing bone is by using grafts. Alternatively, the field of tissue engineering aims to regenerate damaged tissues, instead of replacing them, by developing biological substitutes that restore, maintain or improve tissue function [1].

Owing to the very important significant role played by the scaffold material in the success of tissue engineering, a wide variety of biomaterials have been investigated for scaffold fabrication. Biodegradable ceramics drew wide attention as scaffold materials due to their high strength and bioactivity, yet their brittleness is considered a limitation [2]. Polymers, on the other hand, have a flexible nature that mimics that of normal tissues but they lack strength [3]. Both synthetic and natural polymers have been used for scaffold fabrication, with the latter type having the advantage of possessing chemical cues that are readily identified by cells which results in more cell adhesion.

Dextran is a neutral, hydrophilic, biocompatible and biodegradable polysaccharide, it has been shown to be a bone healing promoter. Even though hydrophilic materials like dextran have several advantages when used as scaffold materials, their major limitations are its slow mechanical strength and the inability to provide a surface that supports cell adhesion and spreading [4], which is a prerequisite for cell proliferation and osteogenesis in bone tissue engineering applications.

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Attempts have been made to overcome such drawbacks by combining the dextran with another polymer, such as chitosan (CS). CS has been suggested as apoor polymer for orthopedic applications, owing to its good biocompatibility [5], biodegradability [6] and inherent wound healing properties [7]. It also promotes cell adhesion due to its positive surface charge that induces electrostatic interactions with the negatively charged cell membranes [8]. Since bone is a natural composite comprising a polymer matrix reinforced with a ceramic material. Therefore, the ability of polymerceramiccomposite scaffolds to promote bone regeneration have been investigated. These composite scaffolds are believed to provide a more nature-like environment for cells and to combine the advantages of both polymers and ceramics [3].

There are many reports in literature have confirmed that chitosan- nanohydroxyapatite composite scaffolds suitable for cell ingrowth in bone tissue repair [9-11]. However, to the best of our knowledge, no study has been previously reported on the use of dextran/chitosan/nanohydroxyapatite (Dex/CS/nHA) for bone scaffolding. Therefore in the present study, three-dimensional scaffolds of Dex/CS/nHA were used to repair femoral bone defects in rat model. Also, invitro cytotoxicity and the behavior of composite scaffolds in PBS were also evaluated.

MATERIALS AND METHODS

Dex/CS/nHA Composite Scaffold Preparation:
Dextran-Chitosan composite scaffold was prepared via blending methods discussed in our previous work [12]. A dextran-chitosan aqueous solution of 8Wt% was prepared by dissolving dextran and chitosan powders(Sigma-Aldrich-USA)into acidified distilled water (3% acetic acid). Then, HA nanoparticles (nHA) powder was added with different proportions into a dextran-chitosan aqueous solution (0, 20, 30 and 40Wt%) with constant stirring overnight at room temperature to get a homogeneous mixture. After that, the mixture was moulded, frozen to freeze the solvent and lyophilized in a freezing dryer(SCANVAC, Denmark) at -90°C for 48h and a porous scaffold was achieved. These samples were symbolized by A, B, C and D, respectively.

Dissolution Test of the Composite Scaffolds: The pH values and the concentrations of ions released from the composite scaffolds into PBS solution (pH 7.4 at 37°C) were measured. PBS was prepared using the chemical reagentstabulated in Table 1 according to Motwani et al. [13]. The composite scaffolds (n= 3 for each concentration) were immersed in plastic tubes containing 50 ml of PBS. Ata predetermined times (1, 2, 4, 6, 8, 24, 48, 96, 192, 360 and 672 hour), 10 ml of the solution was taken out from each tube and stored at -20°C until analyzed for Ca and P ion concentrations. The pH of the PBS solution of the composite scaffolds was measured with a pH meter. The concentration of Ca and P ions released from the composite scaffolds into PBS solution was measured by UV/visible spectrometer (model, SP-2000UV) at a wavelength of 585 nm and 640 nm, respectively, with colorimetric method using a chemical kit.

In vitro Cytotoxicity Test: Cytotoxicity test was carried out using Animal Fibroblasts (BHK) cell line prepared in R&D sector, VACSERA–Egypt. Test scaffolds were serially diluted in a descending order and were dispensed to pre-cultured BHK cells. Negative cell culture control was included. Cell culture plates were incubated at 37°C in humidified chamber and CO₂ atmosphere (5%) for 24 h. Treatment media was discarded. Plates were washed with sterile PBS (ADWIA-Egypt) and 0.5 mg/ml MTT(3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazolium bromide) was dispensed to treated plates as 0.05 ml/well for 4 h, at 37°C (Sigma-Aldrich-USA). Plates were microscopically examined to detect the crystal formation in the treated cell cytoplasm. Dye was removed by phosphate buffer saline flushing. Crystals detected in the treated cells were dissolved using dimethyl sulfoxide (BDH, England) added as 0.05 ml/well for 30–45 min.

The absorbance was measured at 570 nm using ELISA reader (Dynatech, USA). Triplicate repeats were performed for each concentration and the mean optical density (OD) of test samples and control wells were recorded. Viability percentage was determined according to Zhang et al. [14].

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\text{cell viability(\%)} = \frac{OD_{570(\text{sample})}}{OD_{570(\text{control})}} \times 100
\]

In vivo Study: Twenty five adult male rats (230-280 g in weight) were kindly supplied by the Research Institute of Ophthalmology, (Giza, Egypt) and were used in this study. Throughout this study, animals were housed in individual cages, given free access to food and water and monitored regularly for signs of pain.
Animals were anesthetized with sodium thiopental with dose 40-45 mg/kg body weight given in intraperitoneal cavity and xyla-ject with dose 10 mg/kg. Using a slow speed drill, a hole with diameter 2 mm (critical bone defect in rats) is created in the femur bone on both sides. The hole is either left empty (n=5 animals) or filled with a composite scaffolds (n=5 animals) for each concentration. The scaffolds were washed with ethanol, dried at room temperature and cut into small pieces before implantation. Immediately following surgery, each animal was given an Analgesic and antibiotic for three days after the surgery. At the selected experimental periods (after 9, 16 and 24 week), the animals were scarified with an overdose of anesthesia and both femurs were extracted and cleaned of soft tissues and fixed in 10% formalin.

**Radiological Analysis:** Radiology measurements of bone density scanning are enhanced form of X-ray technology that is used to measure bone quality. It is a reliable and non-invasive method [15]. Radiological analysis was done to obtain bone density in the defect site.

After the animals were scarified, femurs were extracted and X-rayed with cabinet X-ray machine (DigoraOptim, Finland), using a high contrast x-ray film at 70 kV, 10 mA for 0.06 sec.

**Statistical Analysis:** All the data were expressed as means ± standard deviation (SD) of a minimum of three replicates for each scaffold in each experiment. Statistical significance was evaluated with a non-parametric “Kruskal-Wallis Test”. A probability value less than 0.05 (p< 0.05) was considered statistically significant.

**RESULTS AND DISCUSSION**

**Composite Scaffolds Dissolution:** The biodegradation properties of the scaffolds are an important factor on the long term functionality of the bone graft. Fig. 1 (a, b) shows the cumulative release profile of Ca and P from the composite scaffolds B, C and D into PBS solution. It is well known that HA structure consists of Ca, PO₄ and OH groups closely packed together. The OH and PO₄³⁻ groups are responsible for negativity of HAsurface and Ca²⁺ ions form the positive group. When the composite scaffolds were incubated in PBS, there was dissolution of these ions from the scaffold.

After 24 hour of incubation, a sharp increase in the concentration of calcium ion was released from sample C compared with samples B and D as shown in Fig. 1a. The orientation of the hydroxyl and phosphate groups in hydroxyapatite provides itself to a negatively charged surface which in turn attractsthe positively charged Ca²⁺ ions. Therefore, the surface gains positive charge and further attracts the negatively charged OH and PO₄³⁻ ions from the PBS. This leads to formation of the apatite layer on the surface of the samples. The increase in the concentration of calcium ion released from sample C may be due to the dissolution of in situ synthesized calcium phosphate occurred at a greater rate than the reprecipitation on the surface of the sample, resulting in a net reduction in pH.

On the other hand, the concentration of P ions released from sample B is moderately higher than that released from sample C and D as shown in Fig. 1b. This may be due to the less concentration of Ca ions which responsible for the attraction of PO₄³⁻ ions on the surface of sample.

Further, the free calcium ions might have been simultaneously forming CaOH₂ as a precipitate. This precipitate decreases in OH⁻ ions which would result in a net increase in H⁺ ions and therefore a decrease in pH as shown in Fig. 2. The PO₄⁻ ions that were being released would likely form phosphoricacid (H₃PO₄), this also cause decrease in pH. This decrease in pH may have had an autocatalytic effect on the dissolution of more calcium phosphate, as calcium phosphate is known to degrade in acidic conditions.

**Cytotoxicity Evaluation:** The combination of physiochemical properties and biological activities of the implanted material is a critical factor in the success of a tissue scaffold, especially in bone tissue engineering. In vitro cytotoxicity study is a common technique used to evaluate the biomaterials. The toxicity of the prepared composite scaffolds was investigated by MTT-based assay on Baby Hamster Kidney (BHK) animal fibroblast cells. The cell viability after exposure to the composite scaffolds A, B, C and D for 24 hour is shown in Fig. 3. The obtained results showed that, at higher concentration (5 mg) of composite scaffold A the viability of the incubated cells was 75 % suggesting that composite scaffold (A) was toxic, while the cell viability was increased to approximately 90% as the dosage of composite scaffold A decreased to 3.125 mg. On the other hand, at higher concentration of composite scaffolds B, C and D the viability of the incubated cells was 80 %, which indicated that these composite scaffolds were not toxic. The results also showed that, the viability increases as the concentration of nHA increase, this might be due to he presence and proper distribution of HA in the scaffold [10]. From previous study, β-chitin/HA compositewas fabricated and enhanced cell growth.
Table 1: Amounts of reagents for preparing 1000 ml of PBS

<table>
<thead>
<tr>
<th>Order</th>
<th>Reagents</th>
<th>Amount (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaCl (MP Biomedicals-USA)</td>
<td>8.0</td>
</tr>
<tr>
<td>2</td>
<td>Na₂HPO₄ (MP Biomedicals-USA)</td>
<td>1.38</td>
</tr>
<tr>
<td>3</td>
<td>KH₂PO₄ (Genlabs)</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Table 2: The bone density measurements of composite scaffolds with respect to empty bone

<table>
<thead>
<tr>
<th>Time (week)</th>
<th>Nine</th>
<th>Sixteen</th>
<th>Twenty four</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty bone</td>
<td>92±2.160</td>
<td>94.33±8.167</td>
<td>103±5.830</td>
</tr>
<tr>
<td>A</td>
<td>----</td>
<td>----</td>
<td>112.62±4.80</td>
</tr>
<tr>
<td>B</td>
<td>105.05±7.18</td>
<td>108.92±8.31</td>
<td>118.83±10.42</td>
</tr>
<tr>
<td>C</td>
<td>105.71±5.14</td>
<td>124.11±4.81</td>
<td>177.68±7.64</td>
</tr>
<tr>
<td>D</td>
<td>106.33±6.02</td>
<td>138.57±6.62</td>
<td>191.58±13.26</td>
</tr>
</tbody>
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Fig. 1: Ca and P cumulative ions release concentrations (ppm) from composite scaffolds B, C and D into PBS solution
obtained composites with HA and it was found that HA causes adsorption of more protein and consequently results in better cell attachment to the scaffold [16]. The MTT analysis has indicated that the developed scaffolds B, C and D were cytocompatible and contain no toxic leachable. In vivo test using rats as an animal model was also used in our study to evaluate the cytotoxicity of the prepared composite scaffolds.

**In vivo Study:** Rats recovered quickly from the surgery, walked freely and continued to gain weight throughout the observation periods. No wound or implant infection are detected, wound healing in the grafted areas is good, no postoperative swelling and no wound abscess formation. All the animals which received samples B, C and D are alive and healthy to the end of the experiment. On the other hand, approximately 85% of animals which received sample (A) died in the next day of the surgery.

**Radiological Evaluation:** Bone mineral density (BMD) is one of the most important factors to measure bone quality. It is a biophysical parameter of critical experimental importance and has been used in poultry production as a tool to assess bone quality because it is a reliable and non-invasive method [15].
The femora of rats were used to measure the BMD after nine, sixteen and twenty four week of implantation. Fig. 4 shows the radiographs of empty defect bone group (a) and bones with defect site filled with composite scaffolds A, B, C and D (b, c, d and e), respectively after 24 week of implantation. It is observed from the figures that as the amount of nAH increases, it is difficult to recognize the defect site because the hole is closed with newly formed bone.

Table 2 shows the bone density values for the composite scaffolds with respect to that of the empty defect bone after 9, 16 and 24 week of implantation. When evaluating the BMD after 24 weeks of implantation, there were statistically significant differences between the empty defect bone and the defects that received the composite scaffolds A, B, C and D ($p<0.001$). The results also indicated that the composite scaffold A (containing polymer only) promoted an increase in BMD compared to the empty defect bone but remained significantly lower than that observed with other composite scaffolds B, C and D. The BMD of composite scaffold D reached 191.58 mg/cm³ after 24 week of implantation.

From the above results, it could be concluded that mineralization has been delayed in empty defect bone in comparison with that filled with the composite scaffolds. This indicates that composite scaffolds of Dex/CS/nHA are suitable for filling defects and that mineralization has been favored in the presence of the material.

Previous study investigated the bone mineral density of nano-hydroxyapatite Pullulan/dextran composite macroporous material [17]. They showed that, a 3 mm diameter defect in femoral condyle of rat will heal in some cases when left empty without scaffold, whereas a 5 mm diameter defect was consistently fail to heal. They also showed that polymer composite only without nHA induces less mineralized tissues than polymer+ nHA.

**CONCLUSION**

In this study, it was found that Dex/CS/nHA composite scaffolds had good compatibility and were non toxic toward BHK fibroblast cells. MTT analysis revealed that the viability increases as the concentration
of nHA increases. Furthermore, composite scaffold D demonstrated high efficiency to repair femur defect in rat model. It can be concluded that Dex/CS/nHA is a suitable composite scaffold that can be applied greatly in the field of bone tissue engineering.

REFERENCES