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Dexamethasone-loaded hydroxyapatite enhances bone regeneration in rat calvarial defects

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Abstract A combination of bioceramics and osteogenic factors is potentially useful for bone regeneration applications. In the present study, hydroxyapatite particles (HA) were loaded with dexamethasone (Dex) and then characterized using SEM and drug release study. The bone regeneration ability of Dex-loaded HA (Dex/HA) was investigated in a rat critical size bone defect using digital mammography, multislice spiral-computed tomography (MSCT) imaging, and histological analysis. The HA and Dex/HA showed nano and micro-scale morphology with a nearly homogenous distribution of diameter. In addition, about 90 % of the drug was released from Dex/HA over a period of three days. After 8 weeks of implantation in rat calvarial defects, no sign of inflammation or complication was observed at the site of surgery. According to digital mammography and MSCT, Dex/HA showed the highest bone regeneration in rat bone defects compared to those received drug-free HA. Histological studies confirmed these data and showed osteointegration to the surrounding tissue. Taking all together, it was demonstrated that Dex/HA can be used as an appropriate synthetic graft for bone tissue engineering applications. These newly developed bioceramics can be used as new bone graft substitutes in orthopaedic surgery and is capable of enhancing bone regeneration.

Keywords Hydroxyapatite · Dexamethasone · Calvarial bone · Rat · Tissue engineering

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Introduction

Bone damages and lesions caused by accidents, traumas or various diseases are of major important problems which can disturb the normal life of the injured person for a period of time. A patient suffering from these bone defects can be treated through different approaches [1]. The gold standard of treatments is autograft which can ideally contribute in the healing process of bone defects. However, limited amount of the graft and donor-site morbidity are the two major drawbacks of this procedure [2]. An alternative is using allografts in which the bone graft are achieved from another person and then transplanted in the patient. This approach also suffers from two major challenges including immunogenic rejection and the risk of disease transfer. Due to these limitations, the science of tissue engineering has emerged with the goal of developing organs, tissues, and synthetic materials ready for the recovery of their structure or function in the future [3–6]. Various synthetic grafts have been designed and developed to treat bone defects. Among these, calcium phosphate minerals such as hydroxyapatite (HA) are the most popular and common bone graft substitutes applied for filling the void spaces of defects. Osteoconductivity is considered as the most important characteristic of HA which contribute to guide the healing process of host bone via supporting its remodelling and repair. It can also efficiently bond to the surrounding host bone under a process termed as osseointegration [7, 8]. There are also some materials which are known to induce progenitors or stem cells toward osteoblastic lineage. This ability is termed as osteoinductivity which belongs to biomolecules such as bone morphogenetic protein- 2 (BMP-2). Dexamethasone (Dex) is also an inducing factor which stimulate the up-regulation of some major bone-related genes during osteogenesis. Bone has an

architecture mainly composed of inorganic phase (70 %) consisting of HA and an organic fraction consisting of 95 % Type I collagen [9]. So synthetic HA can greatly mimic the natural structure of bone while used as a bone graft substitute and enhance the process of healing in bone defects. To give HA the characteristic of osteoinductivity in addition to its inherent osteocondutivity, some researchers have loaded osteoinductive molecules such as BMP-2 onto the structure of HA [10, 11]. However, these studies are clinically limited due to the high cost of this protein and also its instability under fabrication procedures. Dex has been shown to be loaded in biomaterials such as biodegradable polymers to create osteoinductive grafts [12]. However, there is a limited knowledge about its effect on osteogenesis in vivo while loaded into a biomaterial. To the best of our knowledge, there is no study about the in vivo performance of HA particles loaded with Dex. Therefore, in the present study we loaded Dex into synthetic HA to provide a bone graft substitute which simultaneously exhibit osteoconductivity and osteoinductivity. This novel graft was demonstrated to efficiently heal rat calvarial bone defect compared to that observed in control group.

Materials and methods

Preparation of HA and Dex-loaded HA particles

A femur of an adult bovine was boiled in water for 12 h and then it was cleaned and washed carefully to remove visible tissues and fats on the bone surface. The bone was then heated in an electric furnace to remove the internal organic content. The resulting solid specimens were ground and crushed with a mortar and pestle to produce a powder. Bone powders were then sterilized at 150 °C for 1 h, rinsed in distilled water and incubated in 1 % phosphoric acid. The prepared HA particles were then immersed in Dex solution (0.01 g/mL) for 24 h. They were then lyophilized and termed as Dex/HA for the rest of experiments. To investigate the drug release, Dex/HA particles were immersed in 2 mL phosphate-buffered saline (PBS) at 37 °C for up to 10 days under static conditions. At predetermined time intervals, the PBS solution was collected and replaced with fresh one. The amount of released Dex was measured using a UV–Vis spectrophotometer at 242 nm.

Scanning electron microscopy (SEM)

To investigate the morphology of HA particles, the powder was coated with gold using a sputter and characterized using a scanning electron microscope (SEM, LEO 1455VP, Cambridge, UK). Using image analysis software (ImageJ, NIH, USA), the particle diameter was determined using SEM images in which the mean diameter of 100 particles was measured.

Animal study and in vivo implantation

A total of $n = 15$ 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 using 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. Bioceramics were then implanted in critical-size calvarial defects and each defect was filled with an equal amount of materials. 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' ethical guidelines.

Digital mammography and multislice spiral computed tomography (MSCT) imaging analysis

After 8 weeks, the animals were euthanized, 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).

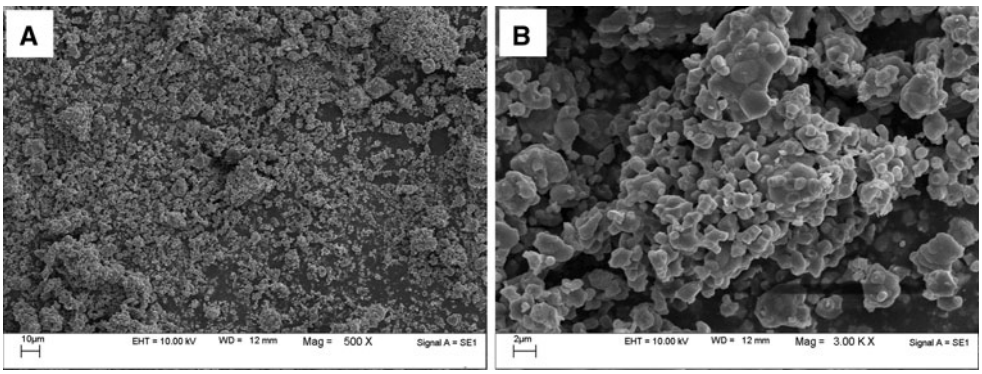

Fig. 1 Morphology of fabricated Dex/HA particles at ×300 (**a**) and ×3000 (**b**)

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 HA particles

The morphology of Dex/HA was investigated under SEM (Fig. 1). The particles shows almost a homogenous distribution with a diameter in the range of 500 nm to 5 μm. There was also no significant difference observed between the morphology of HA particles before and after Dex loading. The cumulative rate of Dex release form Dex/HA particles during a 10-day period is demonstrated in Fig. 2. It is obvious that about 90 % of the drug was released from Dex/HA over a period of three days.

In vivo bone regeneration

Gross examinations

No local or general implication was observed in any of the animals during the period of this study. All of them survived and no sign of scalp edema, infection, wound fester or effusion was detected at the site of surgery. The HA and Dex/HA implants were retrieved 8 weeks after surgery. In a gross view, no sign of inflammation was detected (Fig. 3). In addition, no sign of encapsulation or prominent foreign body reaction was observed. No spontaneous

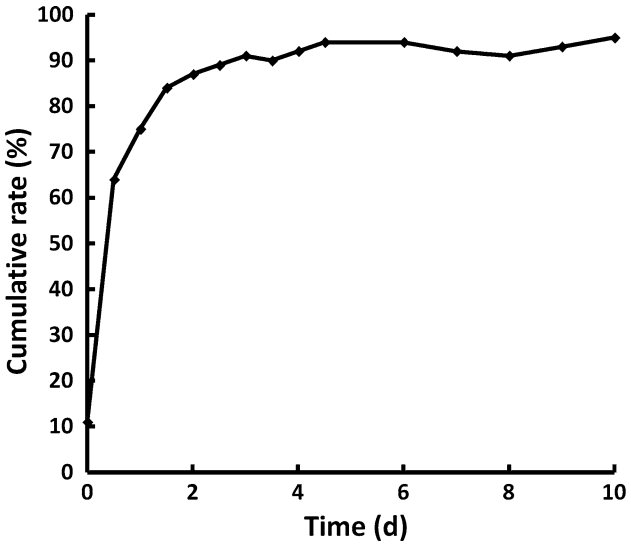

Fig. 2 The profile of Dex release from Dex/HA particles

mineralization and bone repair was observed in the control group at the end of study.

Evaluation of bone regeneration

To investigate the bone regeneration in rat calvarial defects, the specimens were retrieved 8 weeks after implantation. According to the digital mammography results (Fig. 4), no spontaneous regeneration was observed in the control group animals. In addition, almost 60 % of the defect healed after 8 weeks in rat calvarium implanted with HA. The amount of regeneration was significantly higher in the animals received Dex/HA compared to control animals. It was found that almost higher than 80 % of the defects regenerated during the period of implantation. These results were confirmed with MSCT which qualitatively demonstrated the higher reconstruction of bone defects in the animals received Dex/HA compared to HA and empty groups (Fig. 5). The specimens were also cross-sectioned and stained with H&E to investigate the histomorphology of the

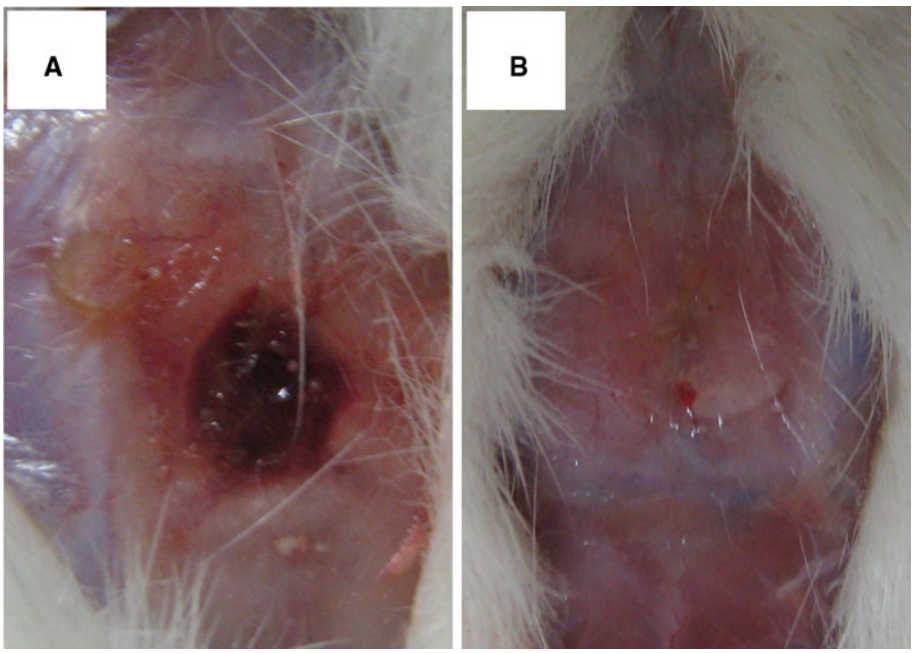

Fig. 3 Critical-size defect created in rat calvaria before (**a**) and after (**b**) 8 weeks of study with implanted bioceramics

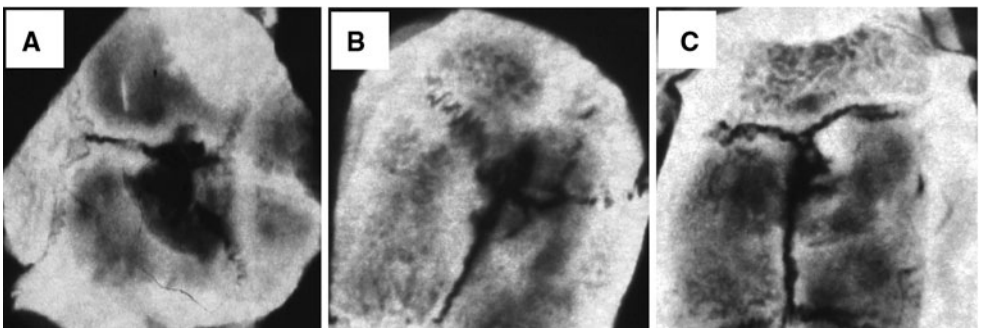

Fig. 4 Digital mammography images of the rat calvarial after 8 weeks of study: control (**a**), HA (**b**), and Dex/HA (**c**)

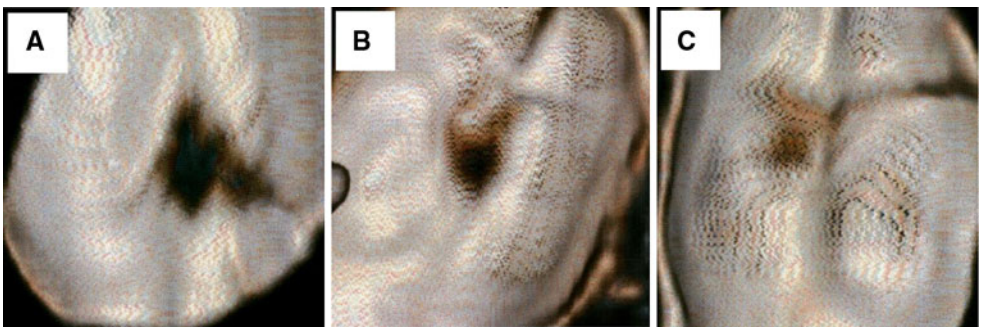

Fig. 5 MSCT images of the rat calvarial after 8 weeks of study: control (**a**), HA (**b**), and Dex/HA (**c**)

regenerated areas (Fig. 6). Neo-bone formation was detected in both HA and Dex/HA groups and no sign of bone tissue was observed in defects of control groups. Qualitatively, the regenerated bone was higher and more mature in the groups received Dex/HA compared to HA. The area of newly formed bone was also quantified and depicted in Fig. 7. Based on the MSCT and H&E quantified results, the highest amount of regeneration was observed in the animals received Dex/HA compared to HA and the control group.

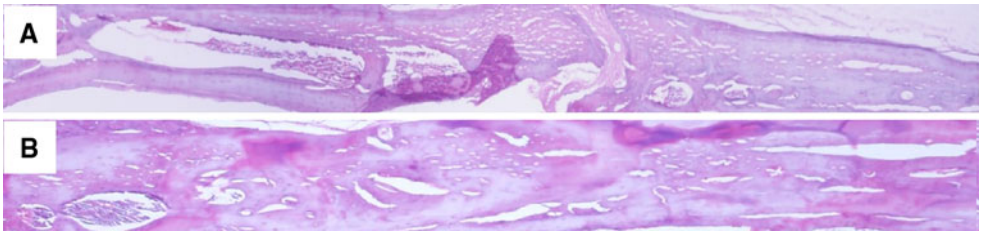

Fig. 6 Optical micrographs of the defects stained with H&E: HA (**a**), and Dex/HA (**b**)

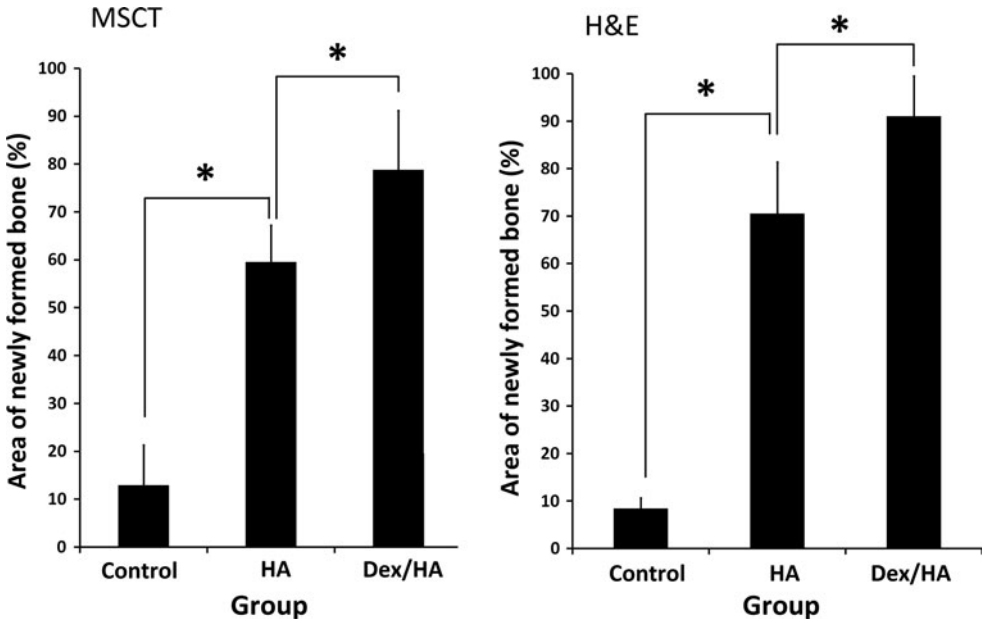

Fig. 7 Area of newly formed bone resulting from the quantification of MSCT and H&E data. The significant difference ($P < 0.05$) has been shown between the groups indicated by asterisks

Discussion

An ideal bone graft substitute should exhibit important characteristics such as osteoconductivity, osteoinductivity and the ability of osseointegration into host tissue [13]. The osteoconductivity of bioceramics have been shown in many studies in vitro and in vivo. Among these materials, HA is one of the most common and important graft substitute because of its bioactivity, bone-bonding ability and osteoconductivity. Most importantly, its structure greatly resemble the inorganic phase of natural bone extracellular matrix [14]. In addition, a number of studies been performed in order to improve the guided bone regeneration properties of HA via manipulation of its physical and mechanical properties such as crystal structure, porosity and surface chemistry [15, 16]. Herein, for the first time, we loaded Dex in HA particles and demonstrated their improved bone healing capabilities. Dex is a synthetic glucocorticoid which has been shown to induce in vitro osteogenesis of stem cells in combination with ascorbic acid and beta-gycerol phosphate [17]. Release of Dex from different types of biomaterials and its effect on the process of osteogenesis has been investigated. Nuttelman et al. [18] covalently attached Dex to polyethylene glycol gels and showed that its release induced human mesenchymal stem cells (hMSC) toward osteogenic lineages. In two other studies, Dex-loaded scaffolds have been demonstrated to induce ectopic bone formation while seeded with adipose tissue-derived stem cells [19] and hMSC [20]. In a recent study, Hong et al. [21] loaded HA granules with Dex by the immersion method. However, they did not evaluate the effect of this release on the process of osteogenesis. Herein, for the first time, we have prepared Dex-loaded HA particles and investigated their ability for bone regeneration in rat calvarial defects. The loading of Dex onto HA particles did not influence the morphology of HA as found by SEM images. According to the results, it was found that there was no difference between the in vivo performance of HA and Dex/HA in regard to gross morphology and inflammation. This is indicative that Dex did not influence the biocompatibility and host tissue response of HA. In the drug release studies, a burst release of Dex was observed over the initial two-day period. This initial burst may reduce the effectiveness of Dex in the process of bone reconstruction in calvarial defects. However, the main finding of the present study was the higher regeneration

of bone defects which received Dex/HA compared to HA. This superiority was demonstrated and confirmed via digital mammography, MSCT and histopathology of bone specimens. Interestingly, Dex was shown to enhance the bone healing effect of HA in rat calvarial defects. This is in accordance with the osteogenic induction characteristics of Dex in vitro. Therefore, loading the different biomaterials with Dex can lead to an ideal bone graft substitute which can be applied for efficient bone regeneration of defects and damages.

Conclusions

In this study, we efficiently loaded HA with Dex to improve its osteoconductive properties for bone regeneration applications. It was demonstrated that HA/Dex could heal bone defects higher than that observed with HA alone. The Combination of Dex with other biomaterials holds promising potential for improving their bone grafting-related properties such as osteocondution and osteoinduction.

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