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BIOAVAILABILITY

Encapsulated boron as an osteoinductive agent for bone scaffolds

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ABSTRACT

The aim of this study was to develop boron (B)-releasing polymeric scaffold to promote regeneration of bone tissue. Boric acid-doped chitosan nanoparticles with a diameter of approx. 175 nm were produced by tripolyphosphate (TPP)-initiated ionic gelation process. The nanoparticles strongly attached via electrostatic interactions into chitosan scaffolds produced by freeze-drying with approx. 100 μm pore diameter. According to the ICP-OES results, following first 5 h initial burst release, fast release of B from scaffolds was observed for 24 h incubation period in conditioned medium. Then, slow release of B was performed over 120 h. The results of the cell culture studies proved that the encapsulated boron within the scaffolds can be used as an osteoinductive agent by showing its positive effects on the proliferation and differentiation of MC3T3-E1 preosteoblastic cells.

Introduction

In recent years, mineral ions such as copper, strontium, zinc, cobalt and boron have emerged as potential therapeutic agents with their stimulating potential on human health especially on osteogenesis [1]. Among them, boron (B) is one of the trace elements which plays a crucial role in many life processes including embryogenesis, psychomotor skills, immune functions, wound healing, bone growth and maintenance [2]. Although boron is increasingly identified as an element that has many benefits especially on bone health [3], the exact mechanism of B on bone health is still unknown. However, it is known that B has an important role on the metabolism of calcium, magnesium, vitamin D and steroid hormones which have many indirect effects on bone [4]. The boron concentration of tissues and organs shows a heterogeneous distribution that indicates it has variable roles depending on the tissue type. It is estimated that majority of total B content in human body accumulates in bone and keratinous tissue [5] and thus it would be convenient to assume boron has an important role in hard tissues [6].

In recent studies, it is indicated that dietary intake of B plays an important role in bone mineralization and strength [7]. The recommended daily allowance of B has not been established, but no adverse effect has been indicated for excess of it, since B is rapidly excreted in the urine [8]. However, upper limits of dietary intake for B is determined as 20 mg/day and the potential lethal dose of boric acid has been reported as 15–20 g/day for adults and 3–6 g/day for infants [9]. Histological and histomorphometric analysis of in vivo studies showed that B deficiency causes an increase in bone fragility and has adverse effect on the repair of bone tissue. Demirer et al. [10] suggested that systemic administration of boron reduced inflammation and bone loss in periodontal disease of rats. Previous studies were performed by Gorustovich et al. [11,12], to determine the effects of B deficiency on bone healing and periodontal alveolar bone remodeling. Their results showed that dietary B deficiency (0.07 mg/kg) markedly reduce osteogenesis and inhibit formation of bone. Bone loss which can lead to osteoporosis and debilitating fractures has been treated by estrogen at post-menopausal women. In human studies, it has been reported that, boron might mimic estrogen and stimulates the production of estrogen, thus it may prevent osteoporosis by influencing calcium metabolism [13,14].

Limited number of studies that are investigating the effects of B in osteoblasts in cellular/molecular level are avaliable. Recently, Hakkı et al. [15], showed that viability of MC3T3-E1 preosteoblasts decreased at 1000 ng/mL boric acid, but increased expression of bone morphogenetic proteins, BMP-4, -6 and -7, were detected at 0.1, 1.0, 10 and 100 ng/mL B concentrations. In another study, the effect of different concentrations of B in the concentration range

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of 0–1000 ng/mL was evaluated on the osteogenic differentiation of bone marrow derived stem cells (BMSCs) [16]. It was reported that B can induce ostogenic differentiation of BMSCs by stimulating synthesis of osteogenic differentiation-related marker genes. Taşlı et al. [17] evaluated odontogenic and osteogenic differentiation and biomineralization of human tooth germ stem cells (hTGSCs) by measuring their mRNA expressions. They used sodium pentaborate pentahydrate (in brief sodium borate) as a boron source and their results proved that appropriate concentration of sodium borate in treated group showed significantly higher osteogenic activity when compared with control group.

In order to evaluate the osteogenic potential of B in bone tissue engineering, boron containing glass, glass-ceramic and ceramic materials have been synthesized. Wu et al. [13] developed boron-containing mesoporous bioactive glass (B-MBG) which releases dexamethasone to enhance the osteogenic differentiation of human osteoblasts. Their results showed that boron embedded MBG glass scaffolds led to a controllable release of boron ions and significantly improved the proliferation and expression of bone-related genes (*col1* and *runx2*) of osteoblasts. Gorustovich et al. [18] proved that boron-modified 45S5 bioactive glasses enhanced bone formation more than non-modified 45S5 glass when implanted into the intramedullary canal of rat tibiae. Recently, Haro Durand et al. [19,20] reported the in vitro and in vivo angiogenic effects of B released from a B-doped 45S5 bioactive glass. Xie et al. [21] investigated the role of vancomycin releasing borate glass for the treatment of chronic osteomyelitis in rabbit models and they showed effectiveness of this material for the elimination of osteomyelitis and stimulation of bone regeneration. Although all of the suitable properties of these materials, it was reported that crystallization of glasses caused the decreasing of their bioactivity and even turned a bioactive glass into an inert material. This is an important disadvantage that limits the application of bioactive glasses as scaffold materials [22].

The aim of the present study is to develop boron-containing polymeric scaffolds by embedding boric acid encapsulated nanoparticles into the chitosan scaffold which was fabricated by freeze-drying method and to evaluate the effect of released boron on the osteogenic activities of MC3T3-E1 preosteoblasts seeded into the scaffolds.

Materials and methods

Materials

Medium molecular weight chitosan, derived from crab shell, was purchased from Sigma–Aldrich (Seelze, Germany). The degree of deacetylation and molecular weight based on viscosity of the chitosan is 75–85% and 190–310 kDa, respectively. Acetic acid was obtained from Riedel de Haen (Seelze, Germany). Boric acid (H_3BO_3) (purity: 99.9%) and sodium hydroxide (NaOH, pH controlling agent) were purchased from Merck (Darmstadt, Germany). Mannitol (spairing agent) was obtained from British Drug Houses Ltd (Poole, UK). Tween 80 (suspending agent), sodium tripolyphosphate (TPP, cross-linking agent) and phosphate buffer saline (PBS, pH 7.4) tablets were obtained from Sigma–Aldrich.

In the cell culture studies, Minimum Essential Medium-alpha modification (α-MEM), fetal bovine serum (FBS), Dulbecco's phosphate buffer solution (DPBS) and trypsin-EDTA solution were used and they were obtained from Sigma (Seelze, Germany). Penicillin-streptomycin was obtained from Biological Industries (Beit HaEmek, Israel). β-glycerol phosphate, L-ascorbic acid and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. Isopropanol and HCl were obtained from Aklar Chemistry (Ankara, Turkey) and Merck, respectively. Hexamethyldisilazane (HMDS) and glutaraldehyde were purchased from Sigma. Triton X-100 (Sigma), 2-amino 2-methyl-1,3 propanediol (Sigma–Aldrich) and $MgCl_2{\cdot}6H_2O$ (Merck) were used for the determination of alkaline phosphatase (ALP) activity. Silver nitrate ($AgNO_3$) was purchased from Sigma. Trizol and chloroform which are used for RNA isolation were obtained from Invitrogen (CA, USA) and Merck, respectively.

Preparation of chitosan scaffolds

Freeze-drying method was used to fabricate porous chitosan scaffold samples [23]. Briefly, chitosan solution (2%, w/v) was dissolved in 0.2 M acetic acid solution and filtered to eliminate the impurities. The solution was poured into the each well of 24-well tissue-culture polystyrene dishes (TCPS, TPP Switzerland), and frozen at −20 °C overnight. Then, the dishes were transferred into freeze-drier (Christ, Germany) and lyophilized at −80 °C for four days. After the scaffolds were completely dried, they were kept in 96% (v/v) ethanol for overnight and 70% (v/v) ethanol for 1 h in order to stabilize the chemical structure. For cell culture studies, the scaffolds having approx. 9 mm diameter and 2 mm thickness were used (dry weight of each scaffold is 51 ± 5 mg).

Preparation of boric acid encapsulated chitosan nanoparticles

Chitosan nanoparticles were prepared by ionic gelation between TPP and chitosan, in which the positively charged amino groups of chitosan interact with the negatively charged TPP [24]. Briefly, chitosan was dissolved in 1% (w/v) acetic acid solution to obtain chitosan concentration of 0.75% (w/v). Tween 80 (0.5%, v/v), as a resuspending agent, was added into chitosan solution to prevent particle aggregation. Boric acid (17 mg) and mannitol (9 mg) were added into the 1 mL chitosan solution. Then, pH of chitosan solution was raised to 4.6–4.8 by adding 1 N NaOH. TPP was dissolved in distilled water to obtain 0.5 mg/mL TPP solution. All solutions were filtered through a 0.22 μm polyethersulfone membrane (Millipore, USA). Nanoparticles were formed by dropping TPP solution into boric acid-mannitol containing chitosan solution with a volumetric ratio of 2:1 (v/v) (chitosan:TPP) under magnetic stirring at room temperature. The formation of boric acid-mannitol encapsulated chitosan nanoparticles started spontaneously via the TPP initiated ionic gelation mechanism.

Preparation of boron containing scaffolds

Boric acid encapsulated chitosan nanoparticles were loaded into the chitosan scaffolds by embedding method. For this purpose, 50 μL of nanoparticle solution containing ~100 μg boron was injected into the porous scaffolds and then, scaffolds were freeze-dried. This procedure was repeated three times. Therefore, 300 μg boron was loaded into the each scaffold. In addition, the same amount of boron in free form was loaded into the each scaffold by embedding. Therefore, we prepared three types of chitosan scaffolds: blank chitosan scaffolds (chitosan); boron-containing nanoparticle loaded chitosan scaffolds (chitosan-Np) and boron embedded chitosan scaffolds (chitosan-B).

Characterization studies

Boron content of chitosan nanoparticles were determined quantitatively by inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin Elmer Optima 4300DM) analysis. Boron containing nanoparticle solution was poured into a Petri dish and dried at 37 °C for 3 days. Then, nanoparticles in the Petri dish were dissolved using 5% (v/v) acetic acid to determine