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ORIGINAL ARTICLE

Boron mediated 2D and 3D cultures of adipose derived mesenchymal stem cells

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Abstract Boron (B), which is a beneficial bioactive element for human, has an increasing interest in tissue engineering for the last 5 years. However, the effective B concentration in cell culture is still unknown. The aim of the present study is to investigate in vitro osteogenic potential of mesenchymal stem cells, isolated from adipose tissue (AdMSCs), on boron containing 2D and 3D cell cultures. At first, the effects of B concentrations between 1 and 20 µg/mL were evaluated on the survival and osteogenic differentiation of AdMSCs cultured on 2D cell cultures. The 3D cultures were established by using chitosan (Ch) scaffolds prepared by freeze-drying and Ch scaffolds combined with hydroxyapatite (HAp) and B containing hydroxyapatite (B-HAp) that are produced by microwave-induced biomimetic method. The proliferation and osteogenic differentiation of AdMSCs on Ch, HAp/Ch and B-HAp/Ch scaffolds were investigated by in vitro cell culture studies. The results were evaluated with respect to cell viability, bone related ECM gene expressions, and cellular morphology. It was demonstrated that cellular functions of AdMSCs were enhanced by boron in both 2D and 3D cultures. Especially, B-HAp/Ch scaffolds, which have both osteoinductive and osteoconductive properties based on presence of B and HAp in its structure, promoted adhesion, proliferation and osteogenic differentiation of AdMSCs.

Keywords Boron · Chitosan · Hydroxyapatite · Adipose derived mesenchymal stem cells · Bone tissue engineering

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Introduction

Mesenchymal stem cells (MSCs) derived from bone marrow and adipose tissue, BMSCs and AdMSCs, respectively, are promising cell sources for bone tissue engineering. However, isolation process of bone marrow is highly invasive, painful and cell yield is low. The cells can be harvested from the hip bone or "iliac crest" where a large amount of stem cells are located (Aliborzi et al. 2016; Wang et al. 2017). On the other hand, adipose tissue represents an abundant and easily accessible source for MSCs. AdMSCs can be harvested by minimally invasive procedure and obtained in larger quantities than that of BMSCs (Kern et al. 2006). In order to isolate AdMSCs, adipose tissue may be collected during liposuction surgery as a waste material. Also, adipose tissue can be

harvested from different anatomic sites of body (e.g., abdomen, thighs, infrapatellar, visceral, and subcutaneous) (Rada et al. 2009). Moreover, Peng et al. (2008) indicated that AdMSCs showed the higher proliferation potential than that of BMSCs (Peng et al. 2008).

Boron (B) is an element that contains properties of both metals and nonmetals and it has a broad range of physiological effects on biological systems (Uluisik et al. 2018). Its importance and metabolic pathway in plants are well known for a long time. In a few studies, there are plausible predictions concerning boron mechanisms of action in humans (Nielsen 2014). Although its metabolic pathway in humans has not been determined definitely yet, several studies have indicated that dietary intake of boron has beneficial effects on various systems (Hunt 2012). In recent years, scientist have been interested in direct and different effects of B on cells and tissues. Hakkı et al. (2010) used MC3T3-E1 pre-osteoblastic cell line and Ying et al. (2011) used BMSCs to determine the effects of boron on osteogenic differentiation. According to their findings, effective concentration of B varies with cell source; however, it was reported that B stimulated osteogenic differentiation in both studies. Also, Movahedi Najafabadi and Abnosi (2016) observed that 6 ng/mL boric acid increased the osteogenic differentiation of BMSCs. Human tooth germ stem cells (hTGSCs) were evaluated in terms of osteogenic and odontogenic differentiation and 20 μg/mL NaB (sodium pentaborate pentahydrate) was found suitable for hTGSCs (Tasli et al. 2013). In another study, the effect of B on myogenic differentiation of AdMSCs was investigated. The results indicated that low dose of B could be useful in muscle regeneration (Apdik et al. 2015). The studies performed on 2D culture have clearly demonstrated that boron has favorable effect on the cells. In our previous studies, we showed the positive effect of B on osteogenic differentiation of MC3T3-E1 cells in 3D scaffolds (Gümüşderelioğlu et al. 2015). Afterwards, we synthesized boron containing hydroxyapatite (B-HAp) and B-HAp coated chitosan scaffolds (B-HAp/Ch) by microwave induced biomimetic method (Tunçay et al. 2017). Our results indicated that B-HAp coated chitosan scaffolds promoted the proliferation and ostogenic differentiation of MC3T3-E1 preosteoblasts more than the chitosan and HAp-coated chitosan scaffolds (Tunçay et al. 2017). Apart from these, there are a few studies investigating the effect of B on cells in 3D scaffolds. Wu et al. (2011) and Gorustovich et al. (2006) used different boron containing glass scaffolds. Their results showed that the presence of B enhanced osteogenic differentiation of human osteoblasts in vitro and bone formation in vivo. In another study, boron incorporated porous polylactide-co-glycolide (PLGA) scaffold was prepared and its positive effect on attachment, proliferation and osteogenic differentiation of AdMSCs were evaluated (Doğan et al. 2014).

Recent studies indicated that effective boron concentration differs from cell to cell, however, there is not any study which describes concentration dependent effect of B on AdMSCs. In this study, firstly, we investigated the effects of different concentrations of B on proliferation and osteogenic differentiation of AdMSCs in 2D cultures. In addition, the osteoinductive capacities of combined structure of boron with hydroxyapatite in the chitosan scaffolds (B-HAp/Ch), were evaluated on AdMSCs in 3D cultures.

Materials and methods

Materials

Medium molecular weight chitosan (75–85% deacetylation degree) derived from crab shell and its solvent acetic acid were purchased from Sigma (Germany) and Riedel–de–Haen (Germany), respectively. Boric acid (H_3BO_3) was obtained from BDH chemicals (England). Paraformaldehyde was obtained from Sigma-Aldrich.

In cell culture studies, collagenase type I and Minimal Essential Medium Alpha Modification (α-MEM) were obtained from Biochrom (Germany). Fetal bovine serum (FBS), trypsin/EDTA solution, penicillin–streptomycin, Triton-X 100, bovine serum albumin (BSA), TGF-β3 (transforming growth factor-beta), indomethacin, insulin and IBMX (3-isobutyl-1-methylxanthine) were obtained from Sigma. Dulbecco's phosphate buffer solution (DPBS) and gentamycin were purchased from Lonza (Sweden). Amphotericin B and isopropanol were purchased from Hyclone (USA). Hexamethyldisilazane (HMDS) and formaldehyde were purchased from Merck. PrestoBlue®, which is used for cell vitality test, was obtained from Invitrogen (USA). DAPI (diamidino-2-phenylindole) and Alexa Fluor 488 Phalloidin were

purchased from Thermo Scientific (USA) and Invitrogen (USA), respectively.

Preparation of scaffolds

Porous chitosan scaffolds were fabricated by freeze-drying method (Tigli et al. 2007). Briefly, chitosan was dissolved in acetic acid solution and the solution containing 2% (w/v) chitosan was poured into the 24-well tissue-culture polystyrene dishes (TCPS, TPP Switzerland), and frozen at − 20 °C for 1–2 days. Then, the dishes were transferred into a freeze-drier (Christ, Germany) and lyophilized. After the scaffolds were completely dried, the scaffolds were stabilized by ethanol. Fabricated porous chitosan scaffolds were coated with biomimetic hydroxyapatite (HAp) and boron-doped hydroxyapatite (B-HAp) in the presence of ten times concentrated simulated body fluid (10xSBF) and boron containing 10 × SBF (B-SBF) by microwave irradiation (Milestone, Italy), respectively (Demirtas et al. 2015; Kaynak Bayrak et al. 2017; Tunçay et al. 2017). In brief, these scaffolds were immersed into 10x SBF or 10x B-SBF solutions and microwave was applied for 9 × 30 s at 600 W power (Kaynak Bayrak et al. 2017). In order to remove undesirable inorganic phases, scaffolds were rinsed with distilled water and ethanol after each coating. Then, all samples were frozen and lyophilized. Hence, three types of scaffolds were obtained: chitosan (Ch), HAp coated chitosan (HAp/Ch) and B-HAp coated chitosan (B-HAp/Ch) scaffolds. For cell culture studies, the scaffolds having approximately 9 mm diameter and 2 mm thickness were used.

Cell culture

Isolation and characterization of adipose derived MSCs

Mesenchymal stem cells (MSCs) were isolated from the adipose tissue of adult rats (2–5 months old). Adipose tissues were collected from the different localization such as subcutaneous, gonadal and surrounding kidney (perirenal fat), washed extensively with pre-warmed sterile PBS (pH 7.4) to remove contaminating debris and hematopoietic cells, and then cut into small pieces. Afterwards, the tissue fragments incubated with 0.075% (w/v) collagenase in PBS (pH 7.4) for 60 min at 37 °C for digestion. Then, collagenase was inactivated with PBS (pH 7.4) and centrifuged twice for 5 min at 1800 rpm, then at 1000 rpm. The supernatant was discarded, the cell pellet was suspended in growth medium (α-MEM supplemented with 15% v/v FBS, 0.8% v/v P/S, 0.4% v/v amphotericin B and 0.4% v/v gentamicin) and filtered through a 0.70 µm mesh filter (BD Bioscience). The filtrate was centrifuged at 1000 rpm and cultured in T25 flasks until they reached near confluence (Çapkın et al. 2012).

Viability and proliferation behaviors of cells were determined using PrestoBlue® at desired time points (at days 1, 3, 5, 7, 11 and 13). AdMSCs were seeded in 24-well plates at the density of 5×10^3 cells/cm^2. Briefly, the culture medium was aspirated, 400 µL of mix solution (10% PrestoBlue® reagent + 90% growth medium) was added to each well and incubated at 37 °C for 2 h. Then, 200 µL sample was taken from every well and transferred to a 96-well plate. The fluorescent signal was measured using a microplate spectrophotometer (ASYS-UVM340) with an excitation wavelength of 570 nm and an emission wavelength of 600 nm.

Cell morphology was detected with F-actin/nucleus (Alexa Fluor 488 Phalloidin conjugated anti-F actin and DAPI probe) staining. The cells were analyzed under fluorescent microscope (Olympus IX71, USA). Surface markers of the isolated AdMSCs were analyzed by flow cytometry. The cells at passage 4 were characterized using fluorescence-activated cell sorting (FACSAria II Cell Sorter, BD Biosciences) with MSCs specific and unspecific surface markers such as, CD90 (Thy-1), CD73 (5′-nucleotidase), CD105 (endoglin), CD45 (receptor-type tyrosine-protein phosphatase C), CD34 (hematopoietic progenitor cell antigen), CD11b (integrin αM), MHC class I and MHC class II surface receptors.

For osteogenic differentiation AdMSCs at passage 5 were induced in the presence of osteogenic medium which consists growth medium supplemented with 25 µM ascorbic acid, 5 mM β-glycerolphosphate and 10 nM dexamethasone. Calcium deposition in differentiated cultures was revealed by staining with ALP/von Kossa. For adipogenic differentiation AdMSCs were induced in adipogenic medium which consists growth medium supplemented with 5 µM insulin, 25 µM indomethacin, 0.25 µM dexamethasone, and 0.25 mM IBMX. Adipogenic differentiation was dedicated by the formation of lipid vacuoles. To

examine lipid accumulation, cells were stained with Oil Red O (Çapkın et al. 2012). For chondrogenic differentiation the cells were cultured in chondrogenic medium, which consists of growth medium supplemented with 10 ng/mL TGF-β3, 10^{-7} M dexamethasone and 25 μM L-ascorbic acid. The cell pellets were stained with Safranin O/Fast Green (Çapkın et al. 2012). Cells seeded in growth medium were used as a control group.

Effect of boron on AdMSCs in 2D culture

Boric acid (H_3BO_3) was prepared in ultrapure water at a concentration of 20 mg/mL as a main stock solution and pH was adjusted to 7.4 with NaOH solution. Then, the stock solution filtered through a 0.22 μm pore size filter (Sartorius AG, Germany). Other concentrations were obtained from the stock solution for further studies.

For 2D experiments, cells were seeded at the density of 5×10^3 cells/cm^2 in 24 well and 12 well-culture plates and statically cultured in growth medium for 24 h to allow the adherence of cells. Three different concentrations of boron between 1 and 20 μg/mL were prepared in growth medium. Then, growth media were changed to osteogenic differentiation media containing three different B concentrations. Osteogenic differentiation media without boron supplement were used as negative control.

For viability experiments, cells were allowed to adhere for 24 h in growth media and the media were changed to osteogenic media containing boron (1, 10, and 20 μg/mL). At desired time period, metabolic activity of the cells was measured using Presto Blue® reagent (Asghari Sana et al. 2017).

Real-time polymerase chain reaction (PCR) was used to determine the expression of several osteogenic differentiation-related marker genes such as *Col1A1*, *RunX2*, osteopontin (*Opn)* and osteonectin *(On)*. At each time point (days 7, 14 and 21), cells were lysed using QIAzol and further processed with the Qiagen miRNeasy Mini kit (Qiagen, Valencia, CA). Sampled RNA quantity (ng/μL) and integrity (260/280 absorbance ratio) were measured using a Nanodrop 2000 (Thermo Fisher Scientific Inc, Minneapolis, MN). Primer specific reverse transcription of RNA samples were performed using the Applied Biosystem, High Capacity cDNA Reverse Transcription Kit. Real-time PCR was carried out using 5x HOT FIREPol® EvaGreen® qPCR Supermix with Light Cycler Nano, Real Time PCR system (Roche). The sequences of primers specific for *Col1A1* was as follows: forward primer 5′-CAAGATGTGCCACTCTGACT-3′ and reverse primer 5′TCTGACCTGTCTCCATGTTG-3′ and for *RunX2* was as follows; forward primer 5′-GCATGG CCAAGAAGACATCC-3′ and reverse primer 5′-CCTCGGG TTTCCACGTCTC -3′ and for *On* was as follows; forward primer 5′-ACAAGCTC-CACCTGGACTACA-3′ and reverse primer 5′-TCTTCTTCACACGCAGTTT-3′ and for *Opn* was as follows; forward primer 5′-CACTTTCACTC-CAATCGTCCCTAC-3′ and reverse primer 5′-ACTCCTT AGACTCACCGCTCTTC-3′. The primer pairs for β-actin used as internal control to normalize the genes RNAs, was as follows; forward primer 5′-GTGCTATG TTGCCCTAGACTTCG-3′ and reverse primer 5′-GATGCCACAGGATTCCATACCC-3′. RT-PCR cycling conditions starting from initial denaturation 5 min at 95 °C followed by three step amplification for 45 cycles; denaturation at 95 °C for 15 s, annealing at 60 °C 20 s, and extension at 72 °C 20 s and then, by melting at 60 °C 20 s and at 95 °C 20 s, 0.1 °C/s.

Effect of boron on AdMSCs in 3D cultures

Fifty μL suspension of AdMSCs at a concentration of 2.5×10^5 cells/scaffold were seeded into Ch, HAp/Ch, B-HAp/Ch scaffolds. Cells were incubated in the presence of growth medium at 37 °C in 5% CO_2 atmosphere for 5 days. After 5 days of incubation, culture medium was replaced with osteogenic medium.

Proliferation behaviour and mitochondrial activity of cells on scaffolds were determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay at desired culture period. Briefly, the culture medium was aspirated, the scaffolds immersed in a mixture consisting of 600 μL serum-free α-MEM and 60 μL MTT solution in PBS and incubated for 3 h at 37 °C in 5% CO_2. Afterwards, mix solution was aspirated and 400 μL of 0.04 M HCl was added to each well to dissolve formazan salts. Then, 200 μL of the obtained purple solution were transferred to 96-well plates and the optical density was measured using a microplate spectrophotometer at a wavelength of 570 nm with the reference to 690 nm. Control samples, scaffolds without cells, were subjected to the

same procedure and values were subtracted from the values obtained from scaffolds with cells.

The adhesion behaviour and morphology of AdMSCs on scaffolds were observed by SEM (Zeiss Evo 50, Germany) analysis at the 7th, 14th and 21st days of culture (Tunçay et al. 2017). Real-time PCR was used to determine the expression of several osteogenic differentiation-related marker genes such as *Col1A1*, *RunX2*, osteopontin (*Opn)* and osteonectin *(On)*. At each time point (days 7, 14 and 28), cells from HAp/Ch and B-HAp/Ch were lysed using QIAzol and further processed with the Qiagen miRNeasy Mini kit (Qiagen, Valencia, CA). The real-time PCR steps were repeated, as given in Sect. 2.3.2.

Statistical analysis

Triplicate or quartet experiments were performed for each sample and statistical analysis was performed with Graph-Pad InStat and Prism 6 software. Unpaired *t* test, one-way and two-way ANOVA were carried out to determine the statistical significant differences among the groups, and *p values* less than 0.05 were considered significant.

Results and discussion

Bone tissue engineering aims to combine most suitable cells, biosignals and biomaterials to repair bone damage within the shortest time. From this point of view, based on our previous work, we focused on the effect of boron (B) as a biosignal on osteogenic differentiation of AdMSCs in 2D culture and 3D culture on the chitosan scaffolds.

Properties of B-HAp and B-HAp/Ch scaffolds

Chitosan scaffolds with $\sim$ 100 μm interconnected pores, were prepared by freeze-drying technique. In order to enhance mechanical and bioactive properties of the scaffolds, they were coated with HAp or B-HAp through the aid of microwave energy by using 10xSBF and 10xB-SBF, respectively. Physicochemical properties of HAp, B-HAp and composite scaffolds were reported in our previous publication (Tunçay et al. 2017). In brief, B-HAp has $1.15 \pm 0.11\%$ (w/w) boron content, 1.40 (w/w) Ca/P ratio, $4.30 \pm 0.07\%$ (w/w) carbonate content, 30 ± 4 nm rod-like morphology and bone-like amorphous structure. Morphology of Ch, HAp/Ch and B-HAp/Ch were visualized by SEM and given in Fig. 1. For B-HAp/Ch scaffolds, swelling ratio and porosity was found to be 28.9 ± 1.4 and $85.7 \pm 2.6\%$, respectively. Each scaffold contains 20 μg boron.

Cell culture studies

Isolation and characterization of AdMSCs

AdMSCs exhibit multipotency, meaning they retain the ability to differentiate into a number of mesodermal linages. The multipotency of these cells has increased interest in tissue engineering and regenerative medicine. The initial methods to isolate cells from adipose tissue were leaded by Rodbell and collagues in the 1960s (Rodbell 1964). Since that time, several groups developed and refined procedures for cell isolation.

In the presented study, we used enzymatic digestion for isolation of AdMSCs from rat adipose tissue. Our preliminary works demonstrated that enzymatic digestion is most suitable method than explant cell isolation for adipose tissue. Isolated AdMSCs were cultured, and attached cells expanded easily in vitro and exhibit a fibroblast-like morphology as shown in Fig. 2. To determine population doubling time and specific cell growth rate, we obtained a growth curve using PrestoBlue®. Doubling time and specific cell growth rate were found 44 h and 0.0157 h^{-1}, respectively. It is known that the doubling time of mesenchymal stem cells between 40 and 48 h in early passages (Izadpanah et al. 2006; Tropel et al. 2004).

Flow cytometry was performed to determine the surface protein expression on undifferentiated AdMSCs. Dominici et al. (2006) reported that MSCs must express CD105, CD73 and CD90 and must lack expressions of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA class II. Kern et al. (2006) compared MSCs from different sources. Their results demonstrated that expression of CD106 was less for AdMSCs when compared the other tissues derived stem cells. The results of Strem et al. (2005) indicated that AdMSCs also expressed high levels of CD54 compared with bone morrow MSCs. According to our characterization results, AdMSCs expressed the typical MSC marker proteins CD29, CD90, CD54 and CD106. Furthermore, expression of the endothelial

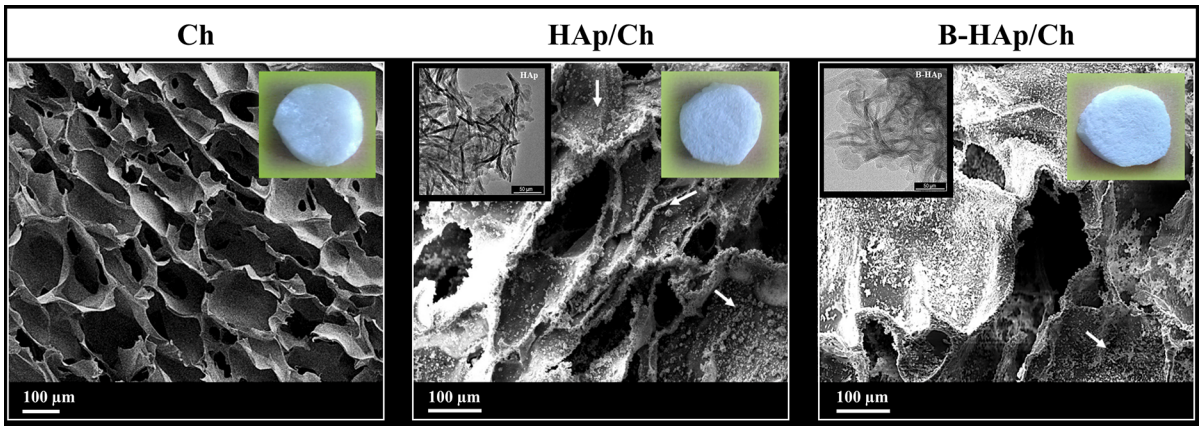


Fig. 1 SEM images of Ch, HAp/Ch and B-HAp/Ch scaffolds. Upper left images are TEM photographs of HAp and B-HAp particles. White arrows show HAp and B-HAp particles on the scaffolds. Upper-right images are photos of scaffolds

lineage and hematopoietic lineage markers CD45 and CD11b were lack of expression (Fig. 2).

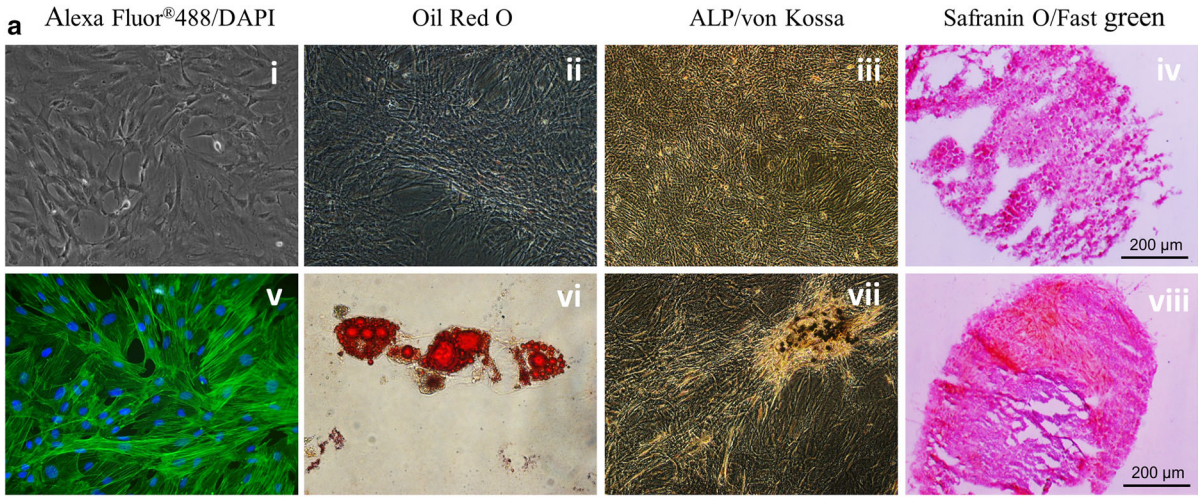
Differentiation is a complex process accompanied by coordinated changes in cell morphology, hormone sensitivity and gene expression. Differentiation of the cells can be directed by the addition of specific cocktails of chemical inducers or cytokines (Bunnell et al. 2008). In this study, we optimized content of the differentiation media according to the cell response. When we used the same concentrations given in the literature, we observed toxic effects on the cells. Thus, we used half amounts of differentiation inducers, except dexamethasone and TGF-β3, reported in the literature. Osteogenic potential of AdMSCs determined by ALP/von Kossa staining to identify calcified mineral deposits in the extracellular matrix (ECM) after 28 days of differentiation. Induced group demonstrated a dark mineralized bone matrix compared with the control group (Fig. 2). Adipogenic differentiation ability of AdMSCs was indicated by the presence of lipid vacuoles identified by oil red O staining. After 20 days of differentiation, there was evidence of lipid droplet accumulation that could only be detected in adipogenic induced group compared with control (Fig. 2). Chondrogenic differentiation capacity of AdMSCs was shown with Safranin O/Fast Green staining of frozen sections obtained from pellet cultures after 28 days induction. There were much more ECM synthesis and collagen production in induced group when compared to unstable pellets in control group (Fig. 2).

Effect of boron on AdMSCs in 2D culture

The goal of this part of study was to investigate the effect of boron treatment on the osteogenic potential of AdMSCs in 2D culture environment. As mentioned above, it is known that boron has beneficial effect on osteogenic differentiation of cells. However, the ideal concentration of B to induce osteogenic differentiation of AdMSCs has not been determined yet. Therefore, at first we investigated multiple concentrations of B for AdMSCs to evaluate cell response in 2D culture. We decided to use a wide range of B concentrations (1, 10 and 20 µg/mL). According to our observations, B has not any toxic effect on AdMSCs even in the highest concentration.

The results of PrestoBlue® analyses showed that the cell viability increased in each group with time. Even so, in control group, the absorbance value was nearly the same after the 3rd day of culture. On day 1, 1 µg/mL B induced proliferation when compared with control ($p < 0.05$). Unlike our expectation, there was no significant difference between control and B treatment groups until the day 7 (Fig. 3a). On day 7, as well as almost all B treatment groups were statistically significant than control group, 1 µg/mL B treatment group were relatively effective on proliferation ($p < 0.01$).

Gene expressions of osteogenic cells specific to *Col1A1*, *RunX2*, *Opn* and *On* were confirmed by RT-PCR (Fig. 3b). It was remarkable that 20 µg/mL B concentration enhanced the expression of *Col1A1* at day 7 ($p < 0.05$). Also, the expression of *Col1A1* was



CD marker	positive cells %
CD29	99.64
CD90	92.02
CD54	99.40
CD106	52.74
CD45	0.20
CD11b	0.18
MHC I	49.94
MHC II	1.11

b

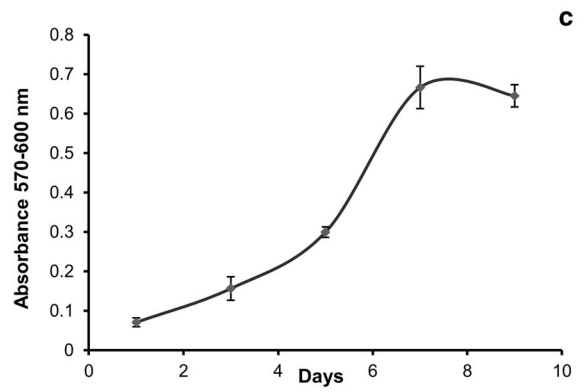


Fig. 2 **a** Immunofluorescence staining of AdMSCs with Alexa Fluor 488®/DAPI (i-control, v-staining). Multilineage differentiation capacity of AdMSCs: cells were incubated in the presence of specific differentiation agents. Differentiation into the adipocyte lineage was demonstrated by staining with Oil Red O (ii-in control medium, vi-in adipogenic medium). ALP/von Kossa staining shows the presence of calcium deposition and bone nodules (iii-in control medium, vii-in osteogenic medium). Staining with Safranin O/Fast Green demonstrates the deposition of proteoglycans (iv-in control medium, viii-in chondrogenic medium) **b** Flow cytometric analysis of CD marker expression of AdMSCs **c** Growth curve of AdMSCs at passage 4

higher at 20 µg/mL B treatment than control group at day 14 ($p < 0.05$). On the last day, *Col1A1* expression was significantly high at 10 µg/mL B treatment group compared to control group and 1000 ng/mL B treatment group ($p < 0.01$). Our results showed that the expression of *RunX2* was significantly high on 20 µg/mL B treatment group for the 7th, 14th and 21st day compared to control group ($p < 0.01$, $p < 0.05$ and $p < 0.01$, respectively). On day 7, *RunX2* expression was significantly high at 20 µg/mL B treatment group when compared to 1 µg/mL B treatment group ($p < 0.01$). On day 21, 10 and 20 µg/mL B treatment groups were higher than control ($p < 0.01$) and 1 µg/mL B treatment group ($p < 0.01$ and $p < 0.01$, respectively). Expression levels of *Opn* was not interpretable; however, there was a significant decrease in 10 and 20 µg/mL B groups in all day compared to control group. However, 1 µg/mL B treatment group was higher than control group on day 14 ($p < 0.01$). As show in Fig. 3b, expression levels of *On* were nearly same at day 7 and 14 for each group. On day 21, expression of *On* at 10 µg/mL B treatment group was higher than control group ($p < 0.01$). Also, 10 µg/mL B treatment group was higher than 1 µg/mL B treatment group ($p < 0.05$).

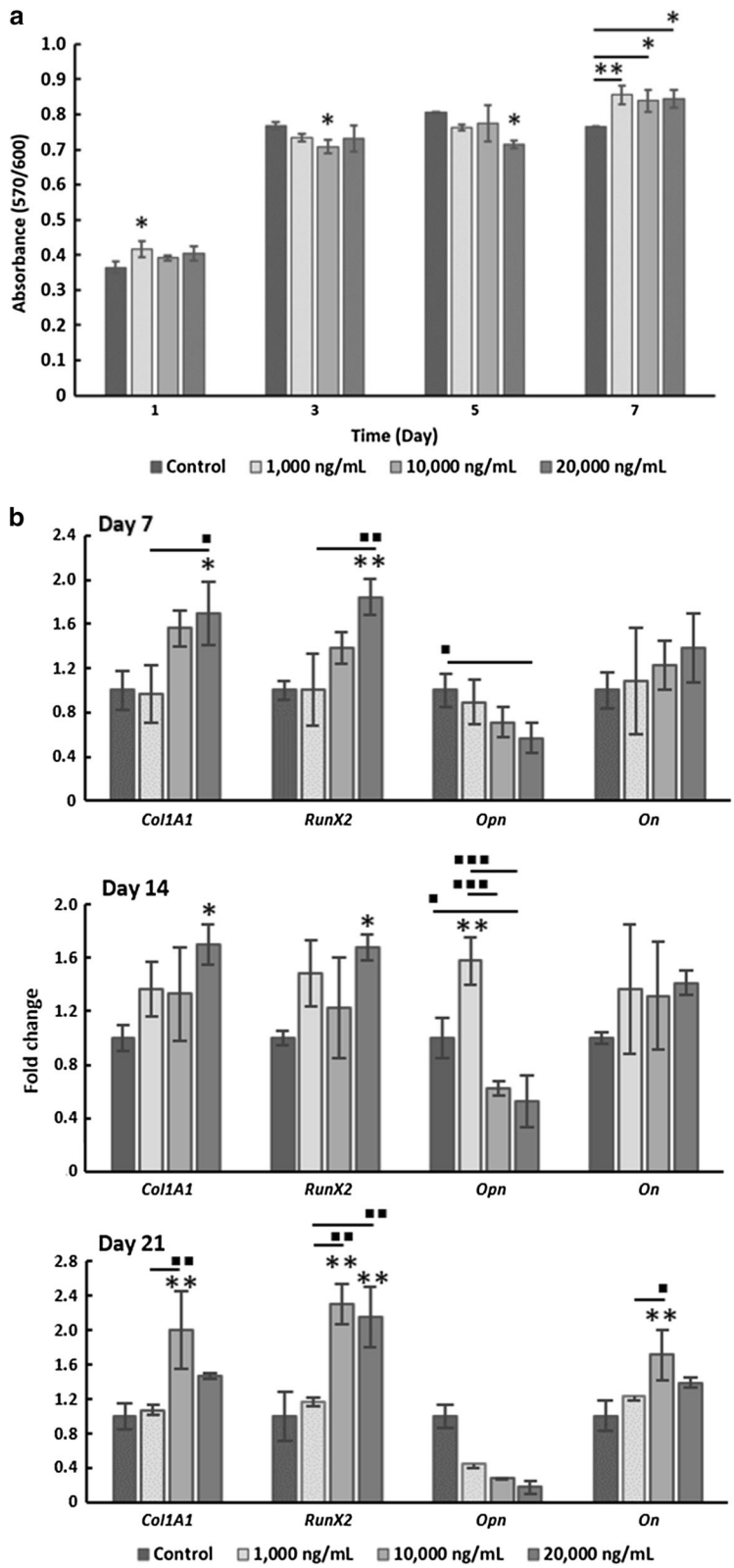


Fig. 3 **a** Presto Blue® results of AdMSCs cultured with the different concentrations of boron. **b** quantitative gene expression analysis by RT-PCR for collagen type I (*Col1A1*); *RunX2*; osteopontin (*Opn*); osteonectin (*On*) at the 7th, 14th and 21st days of culture. The y-axis represents the gene expressions normalized to β-actin. Statistically significant differences are denoted by symbols: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ when control group is 0 ng/mL boron; $\blacksquare p < 0.05$, $\blacksquare\blacksquare p < 0.01$, $\blacksquare\blacksquare\blacksquare p < 0.001$ when we compare each group with every other group

In conclusion, it is not possible to define the most effective concentration of B with these outputs for osteogenic differentiation and proliferation of AdMSCs in 2D cultures. However, it was obviously seen that B promotes osteogenic differentiation of AdMSCs. Also, even in the highest concentration, we did not observe any toxic effect of B on the cells.

Effect of boron on AdMSCs in 3D culture

Cell behavior and morphology on scaffolds were observed by SEM analysis. As seen in Fig. 4a, a few number of AdMSCs attached onto Ch scaffolds and exhibited rounded shape. Yeh et al. (2014) demonstrated that MSCs on chitosan membrane formed spheroids while the cells exhibited fibroblast like morphology on TCPS. It was concluded that it was related with calcium-associated genes regulation on chitosan. In contrast, a high number of AdMSCs attached onto HAp/Ch and B-HAp/Ch scaffolds as seen in Fig. 4b, c. It is known that cellular responses primarily depend on physical and chemical characteristics of the scaffolds. Surface properties of the scaffolds has a great effect on cell adhesion and spreading (Beşkardeş and Gümüşderelioğlu 2009). Due to the relatively homogenous HAp and B-HAp coating on scaffolds, cells attached and spread on both the HAp/Ch and B-HAp/Ch scaffolds.

On the 7th day of culture, cells spreaded broadly on composite chitosan scaffold with fibroblast-like

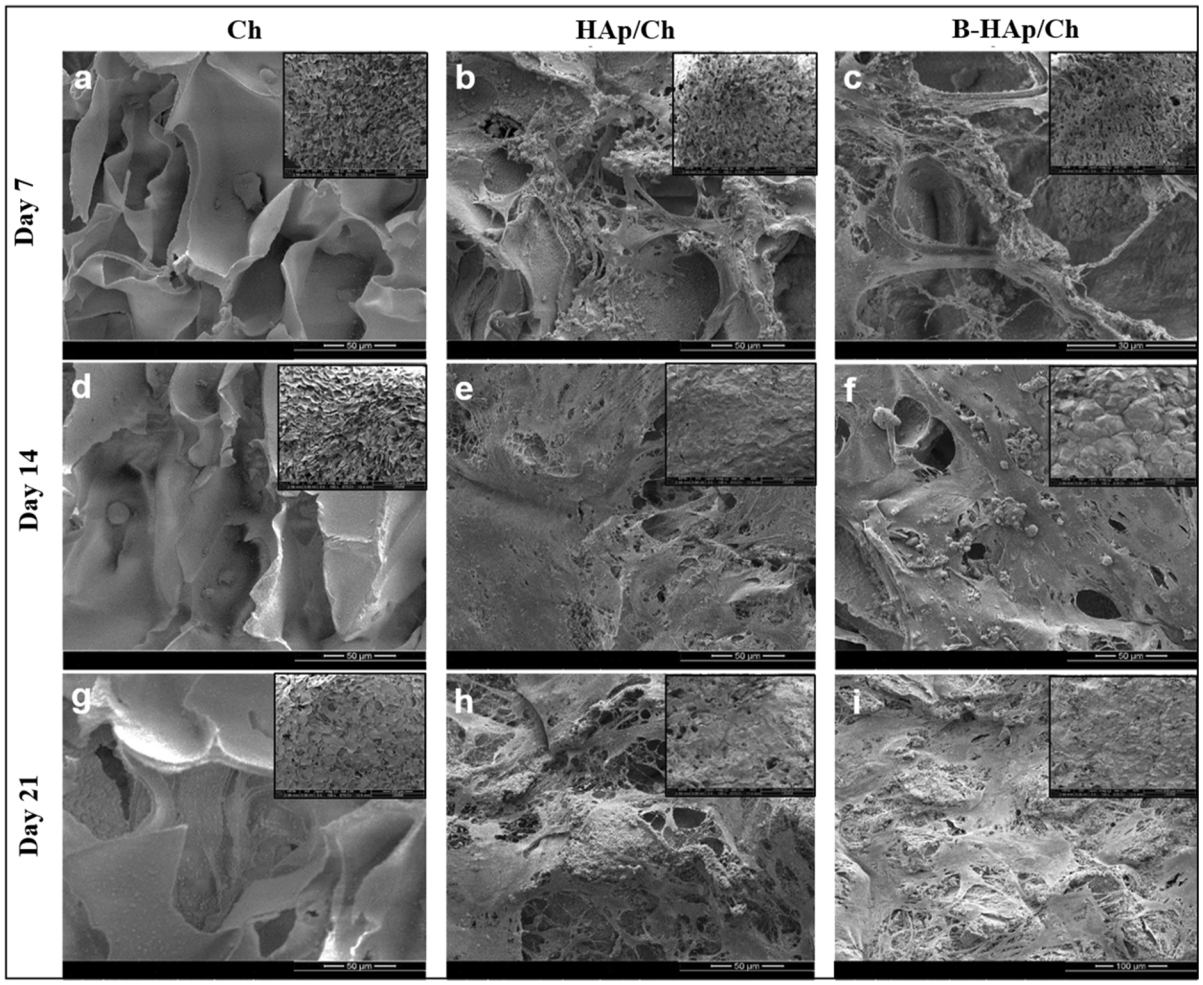


Fig. 4 SEM images of cells on Ch, HAp/Ch and B-HAp/Ch scaffolds at the 7th, 14th and 21st days of culture **a** 1000× **b** 1000× **c** 3000× **d** 1000× **e** 1000× **f** 1000× **g** 1000× **h** 1000× **i** 500×. Thumbnails on the right corners represent an overview of the scaffolds (100×)

morphology and interact with each other (Fig. 4b, c). They proliferated and migrated through the interconnected pores of these scaffolds with time (Fig. 4e, f, h, i). Having open pore structure is an important feature since a good nutrient and oxygen diffusion, which affects the cell distribution on scaffold. On the 14th and 21st days of culture, multiple cell layers and fibrillar ECM were observed (Fig. 4e, f, h, i). The composite scaffolds surface was almost completely covered by multiple layers of cells with ECM and also calcium rich globular aggregations that the markers of calcification were observed (Fig. 4f). Formation of mineral deposits indicates the osteogenic differentiation of AdMSCs. Comparison of SEM results concluded that the most secreted ECM and calcium phosphate mineralization were observed on B-HAp/Ch scaffolds.

Proliferation of ADMSCs on scaffolds was investigated by MTT assay and the results were given in Fig. 5a. After seeding cells, culture maintained in control medium for 5 days to support cell attachment before differentiation. The results revealed that metabolic activities of cells on HAp/Ch and B-HAp/Ch scaffolds increased with time. The lowest mitochondrial activity was determined for Ch scaffolds as expected. On the other hand, proliferation of AdMSCs on B-HAp/Ch was significantly higher than the other groups (Fig. 5a).

In the present study, RT-PCR method was used to assess mRNA transcript expression of AdMSCs on scaffolds at the 14th and 28th days of culture. The following osteogenic differentiation genes *Col1A1*, *RunX2*, *On* and *Opn* were selected as differentiation markers. The results of RT-PCR for relative mRNA expressions were shown in Fig. 5b and the results were represented as fold increases of expression on HAp/Ch scaffolds at the 14th day.

Col1A1: On day 14th, no significant difference was observed between HAp/Ch and B-HAp/Ch while significant increase of *Col1A1* expression was obtained on B-HAp/Ch on day 28th (Fig. 5b). Hakki et al. (2010) showed a significant increase in *Col1A1* mRNA expression for MC3T3-E1 cells treated with boric acid. In the other study about effect of different concentrations of NaB on human tooth germ stem cells, a significant increase in mRNA levels of *Col1A1* expression was obtained for the cells treated with NaB (Demirci et al. 2014.

RunX2: As seen in Fig. 5b, there is a clear difference between *RunX2* levels of two group

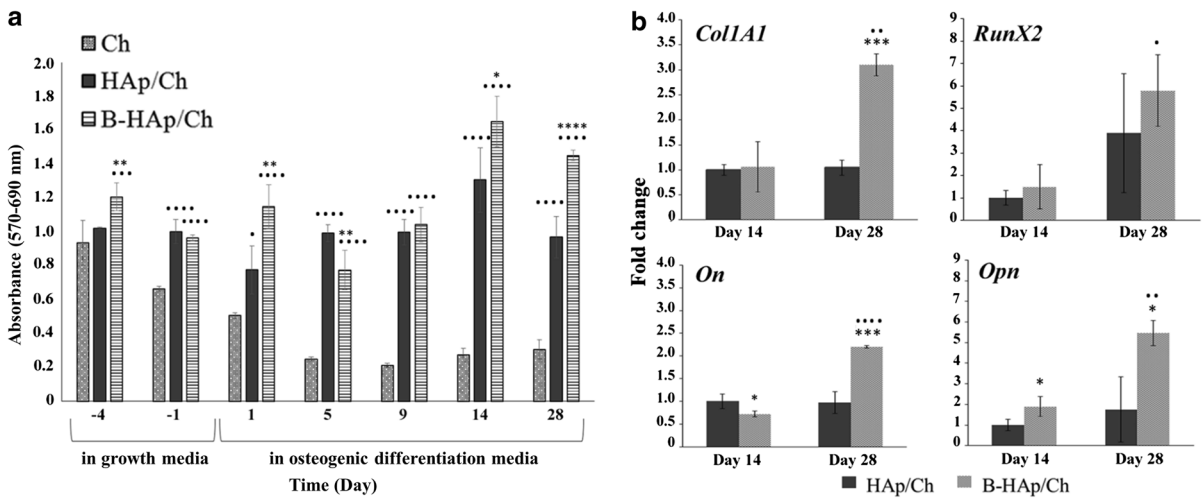


Fig. 5 **a** MTT results of AdMSCs cultured on Ch, HAp/Ch and B-HAp/Ch scaffolds (statistically significant differences n = 3, •$p < 0.05$, ••$p < 0.01$, •••$p < 0.001$ ••••$p < 0.0001$ when control group is Ch, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ when control group is HAp/Ch), **b** Quantitative gene expression analysis by RT-PCR for collagen type I (*Col1A1*); RunX2; osteonectin (*On*); osteopontin (*Opn*) at the 14th and 28th days of culture. The y-axis represents the gene expressions normalized to β-actin. Data are expressed as the mean value of triplicate samples an error bars as the standard deviation. Statistically significant differences are denoted by symbols: •$p < 0.05$, ••$p < 0.01$, •••$p < 0.001$, ••••$p < 0.0001$ when we compare the same group at different times, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ when we compare each group with the other group at the same time

scaffolds during culture period. Likewise, comparing these groups in itself against the time, expression levels reached the highest value on the 28th day of culture. Wu et al. (Wu et al. 2011) indicated that boron containing bioactive glass scaffolds enhanced RunX2 gene expressions. Also, Hakkı et al. (2010) noted that B (< 100 ng/mL) in the low concentrations increased RunX2 gene expressions. In this study, increase of *RunX2* expression with time can be explained by tardy osteogenic differentiation of stem cells relatively.

Osteonectin: According to our RT-PCR results, there was no significant difference for *On* expressions level between HAp/Ch and B-HAp/Ch groups at the day 14th. Also, *On* expression in HAp/Ch showed no difference with increasing culture time. Only at the 28th day, a significant increase was seen in B-HAp/Ch group when compared with the 14th day results (Fig. 5b). Taşlı et al. (2013) noted that On expression of hTGSCs treated with different concentrations of sodium borate was significantly increased compared with control group.

Osteopontin: RT-PCR results signed that *Opn* expressions increased on the 28th day of culture in both groups. Comparing *Opn* expressions of both groups in itself against the time, expression in HAp/Ch group on day 28 was slightly higher compared to the 14th day, while a significant difference existed in B-HAp/Ch with increasing culture time (Fig. 5b). We obtained similar results for MC3T3-E1 cells on B-HAp/Ch scaffolds in our previous study (Tunçay et al. 2017).

Conclusion

In this study, we have reported that boron and boron containing scaffolds have a significant effect on cell viability and osteogenic differentiation of AdMSCs on 2D and 3D cultures. Even though, it is not possible to define the most effective concentration of B with these outputs for osteogenic differentiation and proliferation of AdMSCs in 2D cultures, it was obviously seen that B promotes osteogenic differentiation of AdMSCs. In addition, even in the highest concentration, it is not observed any toxic effect of B.

B-HAp/Ch scaffolds affects adhesion, proliferation and osteogenic differentiation of AdMSCs. The results suggested that the B-HAp/Ch scaffolds were more beneficial for the osteogenic diffentiation of AdMSCs than Ch and HAp/Ch scaffolds.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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