Original Article

Cytotoxic Effects and Osteogenic Activity of Calcium Sulfate with and without Recombinant Human Bone Morphogenetic Protein 2 and Nano-Hydroxyapatite Adjacent to MG-63 Cell Line

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Abstract
Objectives: The aim of this study was to assess the cytotoxic effects and osteogenic activity of recombinant human bone morphogenetic protein (rhBMP2) and nano-hydroxyapatite (n-HA) adjacent to MG-63 cell line.
Materials and Methods: To assess cytotoxicity, the 4,5-dimethyl thiazolyl-2,5-diphenyl tetrazolium bromide (MTT) assay was used. Alkaline phosphatase (ALP) activity and osteogenic activity were evaluated using Alizarin red and the von Kossa staining and analyzed by one-way ANOVA followed by Tukey's post hoc test.
Results: The n-HA/calcium sulfate (CS) mixture significantly promoted cell growth in comparison to pure CS. Moreover, addition of rhBMP2 to CS (P=0.02) and also mixing CS with n-HA led to further increase in extracellular calcium production and ALP activity (P=0.03).
Conclusion: This in vitro study indicates that a scaffold material in combination with an osteoinductive material is effective for bone matrix formation.
Keywords: Calcium sulfate; Cytotoxicity; Nano-hydroxyapatite; Recombinant human bone morphogenetic protein-2
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INTRODUCTION

Treatment of bone defects is a challenge in reconstructive surgery [1]. However, bone is among the few organs that can reconstruct itself after injury; but if the defect is large, the bone may not be able to remodel itself completely and cannot regain its mechanical function [2]. For treatment of bone defects, several methods have been suggested to replace or repair the lost tissue, such as the use of autografts, allografts, and xenografts [3]. Autograft is the "gold standard" for treatment of bone defects, but limited amount of bone can be procured from the donor site [4].

Materials used for reconstruction of bone defects should have standard characteristics such as osteogenic properties, biocompatibility, biodegradability, and affordability [5-7]. CS has recently been suggested as an alternative for reconstruction of bone defects during apicoectomy [8]. CS has been used in bone defects as an alternative for bone regeneration in orthopedic and maxillofacial surgeries for many years [9,10].

This material with its unique crystalline structure provides osteoconduction when placed in bone defects and is completely absorbed and replaced by the newly formed bone and reconstructs the anatomical structure of the area [11]. When CS is placed in bone defects, it begins to dissolve slowly, and releases calcium (Ca+) and sulfate (So42-) ions. The high extracellular calcium concentrations stimulate chemotaxis of osteoblasts, which leads to cell differentiation and cell growth. Furthermore, this ion increases the osteogenic activity of preosteoblasts by increasing ALP activity [12]. Therefore, CS is used as a bone substitute for filling the cystic cavity after removal of cyst, bone cavity [13], initial bone defects [14] or segmental bone defects, and prepares the site for bone grafting. As CS is moldable, absorbable [15], and histocompatible [16], it can be applied as bone graft substitute. The n-HA is a histocompatible ceramic formed at high temperatures. In fact, it is the crystalline form of CS [17] and its unique properties are due to its chemical similarity to the mineralized phase of bone [18]. Due to some properties of n-HA, such as high histocompatibility, n-HA is used as a bone graft substitute to achieve desired results. The n-HA is suggested as an excellent carrier for osteoinductive growth factors [17]. In comparison to pure n-HA, the mixture of n-HA and CS results in more accurate application of material to bone defects [19], and also provides higher primary stability in bone lesions [20]. BMP of TGF-β family is among the most important growth factors for increasing osteogenic properties [21]. BMP contains dimmers, which are cross-linked to one another by seven disulfide bonds [22]. BMPs are a well-known group of growth factors involved in the process of bone repair and have different functions. Insufficient BMPs may be one reason for non-healing of bone fractures [23]. These factors are the main regulators of differentiation and have chemotactic properties for osteoblasts [24]. They have been named for their ability to induce ectopic bone formation. BMP2 gene expression stimulates osteoblast differentiation from progenitor cells, osteogenic effects of BMP focus on immature and multipotent cells, and mature cells show no such reaction to BMPs [23]. In addition, it has been indicated that rhBMP2 increases osteoblastic parameters in bone marrow, such as ALP activity and also the synthesis of collagen [25]. CS seems to be a suitable carrier for BMP [26]. But the mixture of BMPs and resorbable ceramics, such as CS, induces osteogenesis. The ability to understand the interaction between bone-forming cells and biomaterials plays an important role in understanding the function of bone-graft substitutes. Therefore, to determine the cellular response to biomaterials, the effects of materials on osteoblasts should be investigated. Consequently, the cellular and molecular events involved in bone graft replacement can be elucidated. Despite many studies done on the significant role of CS as a bone graft substitute, the cellular and molecular mechanisms of CS with or without n-HA and BMP2 have not yet been completely understood [27].

In this study, we decided to assess the effects of CS with and without rhBMP2 and n-HA on primary osteoblast-like cells in terms of cytotoxic and osteogenic activities.

MATERIALS AND METHODS

Research groups

In this experimental study, the samples were divided into 4 groups: pure CS (group 1), CS+ rhBMP2 (group 2), CS+ n-HA (group 3), and CS+ rhBMP2+ n-HA (group 4). Also, normal culture medium and 6% sodium hypochlorite

(Merck, Darmstadt, Germany) were used as positive and negative control groups, respectively. All the above-mentioned groups were investigated in terms of cytotoxic and osteogenic activities of MG-63 osteoblast–like cells.

Preparation of materials

The materials used in this study were CS (Merck, Darmstadt, Germany), rhBMP2 (Abcam, Cambridge, UK), and n-HA (Sigma Aldrich, MO, USA). In groups 1 and 2, CS and n-HA were separately mixed with distilled water at a ratio of 1:1 and in groups 3 and 4, CS and n-HA were mixed with distilled water in a volume ratio of 35:65%. Then, all materials were cut into disc-shaped pieces with a diameter of 1mm and a height of 2mm and they were separately placed in a 96-well plate. Next, 0.00001 mol rhBMP2 was added to the wells related to groups 2 and 4. The discs were sterilized by gamma radiation (25 kGray). In addition, 100 mL of Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, MA, USA) and 10 mL of fetal bovine serum (FBS) (Gibco, MA, USA) were added into each well.

Cell culture

The human osteosarcoma MG-63 cell line purchased from the Pasteur Institute of Iran was cultured in a 75-cm^2 flask. The culture medium used was PRMI (Gibco, MA, USA), supplemented with 10% FBS (Gibco, MA, USA). The cells were then incubated at 37ºC with 5% CO_2 and were observed under a digital inverted microscope (Cetti, Madrid, Spain) to assess their morphology. After obtaining a confluent monolayer of MG-63 cells, they were detached from the flask using 25% trypsin/EDTA (PAA Laboratories Gesellschaft m.b.H., Pasching, Austria). Next, they were transferred into 15-mL tubes and centrifuged at 1500 rpm for 10 minutes. Finally, the supernatant was discarded. After homogenization, the Trypan Blue (TB) viability assay was performed and cells were passaged again.

Preparation of differential medium

The cells were cultured on the differential medium. The osteogenic activity was investigated with respect to cell viability using MTT assay.

MTT assay

For MTT assay, 10 mg of MTT powder (Sigma Aldrich, MO, USA) was dissolved in 1 cc of phosphate buffered saline (PBS) to prepare 5mg/mL of MTT stock solution. MG-63 cells were placed adjacent to the above-mentioned 4 groups for 12, 24, 48, and 72 hours and 40µL of the MTT solution was added into each well of a 96-well plate.

The plate was incubated at 37ºC with 5% CO_2 for 7 hours. After staining cells and observing them under an inverted microscope (Cetti, Madrid, Spain), the MTT solution was discarded and 40 µL of Dimethyl Sulfoxide cryopreservative in multi-dose format (DMSO) was added into each well. The optical density (OD) was read using an ELISA Microplate Reader at a wavelength of 570 nm.

Osteogenic activity

To show extracellular matrix calcification, osteogenic activity of the cells was evaluated using the von Kossa staining and Alizarin red staining. Assessment of ALP activity was performed as well.

Von Kossa staining

Von Kossa staining is a method for verification of osteogenic differentiation. In the current study, mineralized matrix production was visualized using this staining method. The samples were exposed to the above-mentioned 4 groups for 3, 7, 14, and 21 days; then they were rinsed with PBS (Sigma Aldrich, MO, USA) and fixed in 4% paraformaldehyde (Sigma Aldrich, MO, USA) for 20 minutes.

After washing with deionized water (DI water), 1% $AgNO_3$ (Gibco, MA, USA) was added into the flasks and the cells were exposed to UV radiation for 20 minutes.

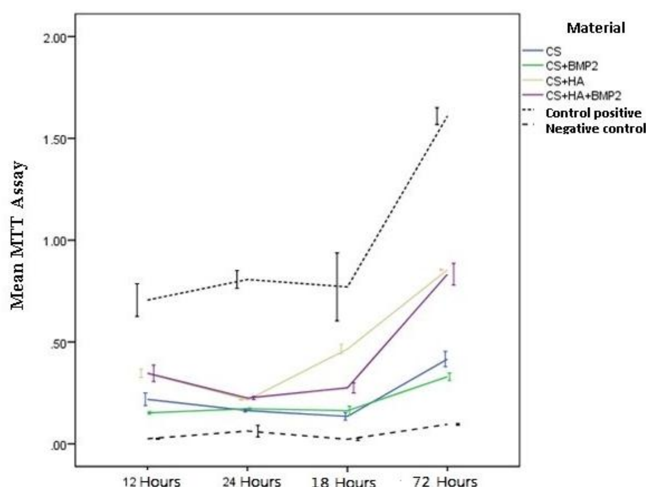

Diagram 1. The mean (and SE) optical density of MG-63 cells adjacent to the above-mentioned materials (pure CS, CS+ rhBMP2, CS+ n-HA, and CS+ n-HA+ rhBMP2) and positive and negative controls during 12, 24, 48, and 72 hours using the MTT assay.

After rinsing the samples with DI water for 3 times, they were washed with 5% sodium thiosulfate for 5 minutes in order to remove detached Ag^{+} ions. After rinsing again with distilled water, the area containing phosphate salts was detected following the appearance of black nodules.

Alizarin red staining

The above-mentioned materials in the 4 groups were placed adjacent to cultured MG-63 cell line for 3, 7, 14, and 21 days; thereafter, the cells were stained using Alizarin red. Next, their OD was read at 405 nm wavelength on an ELISA Microplate Reader.

After determination of OD, the amount of extra cellular calcium was calculated using the following formula:

OD = A / L

OD = Optical density

A = Absorbance

L = Thickness of sample

A = - Log (I / Io)

A = Absorbance

Io = Intensity of light before it enters the sample

I = Intensity of light that passed the sample (transmitted light)

Measurement of ALP activity

ALP enzyme is a hydrolyser enzyme found naturally in all body tissues. ALP enzyme catalyzes the hydrolysis of monophosphoric esters in basic medium and plays an important role in the calcification process to synthesize bone matrix and matrix protein. Consequently, the activity of ALP increases significantly during the production of bone matrix by osteoblasts; thus, it serves as a marker to show osteoblast activity in bone generation. To measure ALP activity, osteogenic medium, containing 10 mM β-glycerol-phosphate and 25 μg/mL ascorbic acid, was added to the culture medium. ALP activity was normalized to total protein concentration and measured at 280 nm wavelength with a NanoDrop ND-1000 spectrometer (NanoDrop Technologies Inc., DE, USA).

Statistical analysis

The quantitative variables (MTT, Alizarin red staining, and ALP) were reported as mean and standard deviation (SD) and were presented in the line charts with standard errors (SEs).

First the effects of BMP-2 and HA were evaluated by two-way ANOVA and because of the presence of interaction between the two mentioned factors in some situations, we decided to perform one-way ANOVA followed by

Tukey's post hoc test. P-values less than 0.05 were considered statistically significant.

RESULTS

In this study, the cytotoxic effects and osteogenic activity of CS with and without rhBMP2 and n-HA adjacent to MG-63 cell line were investigated and the following results were obtained.

Assessment of cell viability adjacent to the tested materials:

The results showed an increase in the number of cells (which had OD) in all groups over time (Diagram 1).

According to the analysis of the results, the difference in the mean optical density among the 4 groups was significant during 12, 24, 48 and 72 hours (P= 0.002 for 12 hours and P<0.001 for the other 3 time intervals). Pairwise comparisons (Table 1) showed that the cell viability adjacent to materials containing HA was significantly higher than that of the others (P=0.002).

In comparison to pure CS, addition of n-HA to CS led to an increase in the number of cells during all time periods, but adding rhBMP2 to CS in comparison with pure CS, did not cause a significant difference in the number of cells (Table 1).

The results of osteogenic assessment

As seen in figures, the area containing mineralized matrix was detected by the appearance of black nodules (von Kossa staining) (Fig. 1) and red color (Alizarin red staining) (Fig. 2).

Table 1. The mean optical density of MG63 cells adjacent to the above mentioned materials (pure CS, CS+ rhBMP2, CS+HA, and CS+HA+ rhBMP2), positive and negative controls, during 12, 24, 48, and 72 hours

Material	12 h	24 h	48 h	72 h	P-value
CS	0.22±0.05	0.16±0.01	0.13±0.03	0.42±0.06	<0.001
CS+rhBMP2	0.15±0.01	0.17±0.01	0.16±0.04	0.33±0.04	<0.001
CS+HA	0.35±0.04	0.22±0.00	0.47±0.04	0.85±0.01	<0.001
CS+HA+rhBMP2	0.35±0.07	0.22±0.01	0.28±0.04	0.83±0.09	<0.001
Positive control	0.71±0.14	0.81±0.08	0.77±0.29	1.61±0.07	0.001
Negative control	0.03±0.00	0.06±0.05	0.02±0.01	0.10±0.01	0.02

Table 2. P values of post hoc test for multiple comparison of optical density.

		CS+BMP2	CS+HA	CS+HA+BMP2
12 hours	CS	0.39	0.05	0.05
	CS+BMP2		0.005	0.005
	CS+HA			1.00
24 hours	CS	0.66	0.001	<0.001
	CS+BMP2		0.003	0.001
	CS+HA			0.66
48 hours	CS	0.81	<0.001	0.009
	CS+BMP2		<0.001	0.03
	CS+HA			0.001
72 hours	CS	0.34	<0.001	<0.001
	CS+BMP2		<0.001	<0.001
	CS+HA			0.97

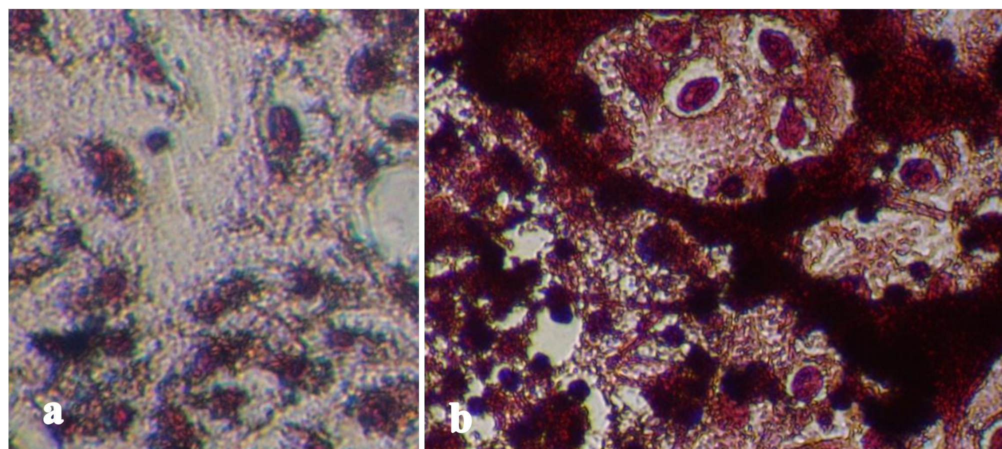

Fig. 1. (a) The von Kossa staining was performed to detect mineralized matrix after exposure to materials in the 4 groups. **(b)** The area containing mineralized matrix was detected by the appearance of black nodules.

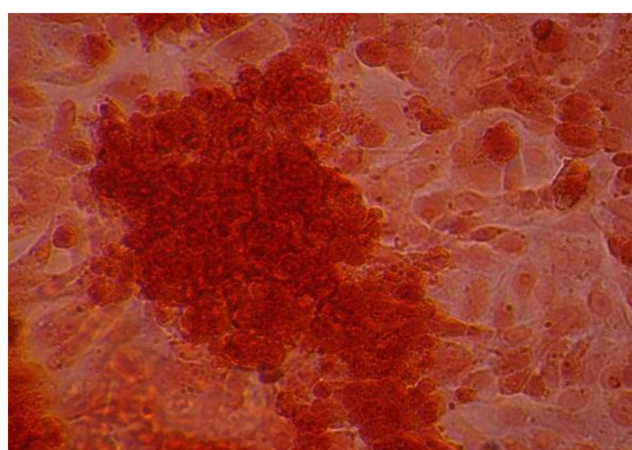

Fig. 2. The Alizarin red staining was done to show mineralized matrix after exposure to the materials in the 4 groups. The area containing mineralized matrix was detected by the appearance of red color.

Quantitative assessment of Alizarin red staining

The results showed that the extracellular calcium content increased in all groups with time except for pure CS and negative control groups (Table 3). There was a significant difference in terms of extracellular calcium among the 4 groups at all time-intervals except for day 7 (P<0.001 for day 3, P=0.22 for day 7, P=0.03 for day 14 and P=0.006 for day 21).

Quantitative assessment of ALP activity

The results revealed that ALP activity increased in all groups over time (Diagram 3). According to the analysis of the results, there were significant differences among the 4 groups at days 3 (P<0.001), 7 (P=0.008), 14 (P=0.001) and 21 (P<0.001).

DISCUSSION

The purpose of this study was to assess the cytotoxic effects and osteogenic activity of CS with and without rhBMP2 and n-HA on osteoblasts. CS can be mixed with other substances. Recently, n-HA was mixed with CS to form n-HA/CS mixture with improved efficacy and minimal disadvantages.

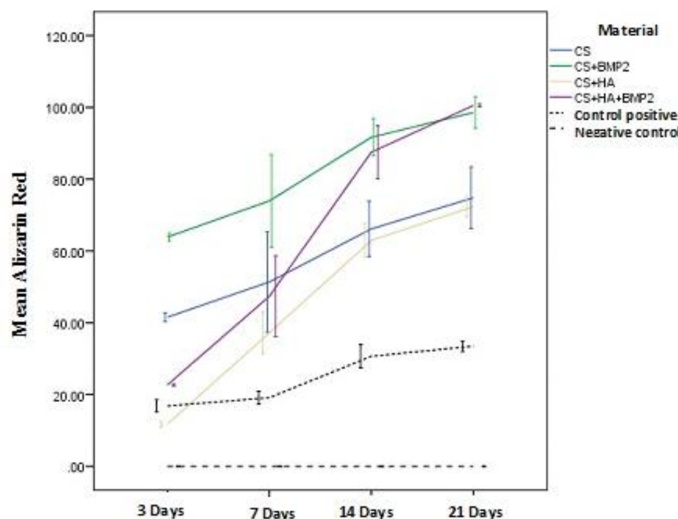

Diagram 2. The mean (and SE) extracellular calcium in osteogenic medium (µg) at 3, 7, 14 and 21 days

The n-HA cements are hard and brittle; thus, it is not easy to use them for clinical applications [2]. The n-HA degrades slowly and it takes a lot of time for the n-HA to be replaced with bone [28].

Therefore, to overcome the above-mentioned disadvantages, CS was added to n-HA to make n-HA/CS mixture and it seems that the short-comings of CS and n-HA were eliminated as n-HA neutralizes the adverse effects of CS

Table 3. Comparison of the mean ALP activity among all groups in osteogenic medium (IU/L) at 3, 7, 14. 21 days

	3rd day	**7th day**	**14th day**	**21st day**	**p-Value**
CS	37.83±1.33	43.33±2.08	45.33±3.51	56.50±1.38	<0.001
CS+BMP2	42.33±1.21	53.33±4.16	56.33±3.51	67.67±1.03	<0.001
CS+HA	37.00±0.89	46.67±9.07	48.00±2.65	57.83±1.17	<0.001
CS+HA+BMP2	38.67±1.21	64.00±4.00	69.33±7.37	87.83±2.32	<0.001
Positive control	12.67±0.52	14.33±2.52	11.67±2.08	16.67±1.03	<0.001
Negative control	0.50±0.55	4.67±1.53	5.33±1.15	5.50±1.22	<0.001

Table 4. Pvalues of post hoc test for multiple comparison of ALP activity between groups

		CS+BMP2	**CS+HA**	**CS+HA+BMP2**
3 days	CS	<0.001	0.62	0.62
	CS+BMP2		<0.001	<0.001
	CS+HA			0.10
7 days	CS	0.19	0.88	0.007
	CS+BMP2		0.48	0.16
	CS+HA			0.02
14 days	CS	0.08	0.89	0.001
	CS+BMP2		0.02	0.04
	CS+HA			0.002
21 days	CS	<0.001	0.47	<0.001
	CS+BMP2		<0.001	<0.001
	CS+HA			<0.001

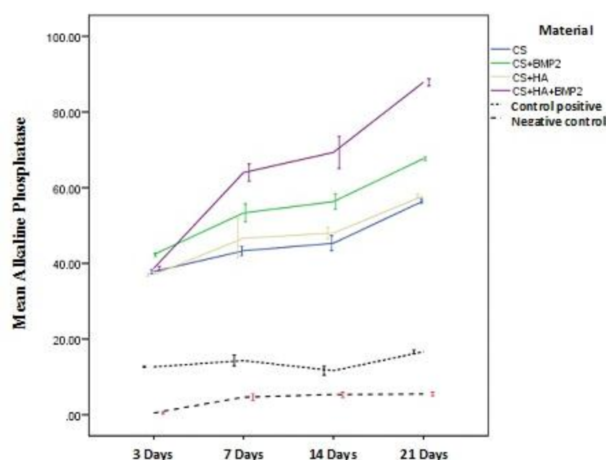

Diagram 3. Comparison of the mean (and SE) ALP activity among all groups in osteogenic medium (U/g protein) at different time points

such as acidifying the environment due to its rapid solubility causing temporary in situ cytotoxicity [29]. In this study, CS and n-HA were mixed at a volume ratio of 35:65%. Some studies have indicated that in order to obtain osteoconductivity, n-HA should comprise 20% of the total weigh.

On the other hand, these studies have shown that in this mixture, increasing the ratio of n-HA to CS causes a decrease in its stiffness and brittleness [28]. We used a 35:65% ratio to retain both osteoconductive properties and stiffness. One of the problems of utilizing BMPs for stimulating bone formation is the type of

Table 5. The mean extracellular calcium in osteogenic medium (µg) at 3, 7, 14 and 21 days

	Day 3	Day 7	Day 14	Day 21	P value
CS	41.55±2.12	51.36±24.26	66.15±13.36	74.81±14.93	0.12
CS+BMP2	63.86±1.97	73.94±22.47	91.66±8.93	98.56±7.60	0.04
CS+HA	11.73±1.32	37.19±9.97	62.91±8.27	72.38±4.96	<0.001
CS+HA+BMP2	22.65±0.49	47.38±19.37	87.51±12.84	100.52±0.72	<0.001
Positive control	16.84±2.99	19.13±3.17	30.69±5.55	33.49±2.57	0.001
Negative control	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	

Table 6. Pvalues of post hoc test for multiple comparison of extracellular calcium between groups

		CS+BMP2	CS+HA	CS+HA+BMP2
	CS	<0.001	<0.001	<0.001
3 days	CS+BMP2		<0.001	<0.001
	CS+HA			<0.001
	CS	0.53	0.82	0.99
7 days	CS+BMP2		0.18	0.41
	CS+HA			0.92
	CS	0.09	0.98	0.16
14 days	CS+BMP2		0.05	0.97
	CS+HA			0.10
	CS	0.04	0.99	0.03
21 days	CS+BMP2		0.03	0.99
	CS+HA			0.02

carrier used to transport the BMP into the bone because the properties of carrier can affect the release and substantivity of BMP2, and this can impact on clinical outcomes [23].
Several studies have investigated the cytotoxicity of both pure CS and the mixture of CS and other materials in vivo and in vitro. In our study, the cells were placed directly adjacent to the tested materials and OD of living cells was measured over time and this method was performed according to the international standard guidelines (ISO 10993-Part 5) for in vitro testing. The results indicated that during 12, 24, 48, and 72 hours, the growth of MG-63 cells occurred adjacent to the following materials 1) pure CS, 2) CS+ rhBMP2, 3) CS+ n-HA, and 4) CS+ rhBMP2+ n-HA. This finding is similar to the results of Lazary et al [30], Finkemeier [31] and Najjar et al [32]. In our study, during 24 and 48 hours, the ascendant rate of cell growth decreased in comparison with other time periods. Studies have indicated that local cytotoxic properties in the initial hours and days following exposure to the above mentioned materials were due to the solubility and release of sulfate ions and the resultant pH [29,6]. This can be one of the reasons for low cell growth during 24 and 48 hours. The results demonstrated that adding n-HA to CS caused a significant increase in cell growth during 24 and 48 hours. Two in vitro studies done by Nilsson et al, [20] and Krisanapiboon et al [29] reported similar results. Zamanian et al [33] investigated the biological properties of n-HA/CS nanocomposite and the results showed an improvement in growth and proliferation of fibroblasts in culture medium in comparison to when pure CS had been utilized. The reason for this effect is mainly based on the similarity to mineralized phase of bone, which results in good biocompatibility and also due to the role of n-HA in preventing the effect of CS on acidifying the medium [29,33]. To investigate osteogenic effects of materials used in this study, the assessments of extracellular calcium ion concentration and ALP activity were carried out. To detect mineralized extracellular matrix, Alizarin red staining and von Kossa staining were used. Extracellular calcium and the extension of mineralized area increased over time. Different studies have shown that high extracellular calcium concentration is a chemotactic factor for osteoblasts and has positive effects on growth and differentiation of osteoblasts and osteogenic activity [34]. Also, calcium ion increases the osteogenic activity of pre-osteoblasts through increasing ALP activity [30]. Some studies have reported that calcium ions exert the above-mentioned effects to increase ALP activity via SNAD3, which is a transcription factor involved in bone repair and formation [12,30]. Presence of calcium ions in the medium inhibits osteoblast activity and balances the induction of osteoblastic bone formation [35]. Therefore, the increase of extracellular calcium concentration may inhibit osteoblast activity and shifting the balance toward new bone formation, and this is similar to the analytical phase occurring in the bone remodeling process [36]. In this study, the result of Alizarin red staining showed that extracellular calcium increased in all groups as time passed. Pairwise comparison of the studied groups revealed that the increase of extracellular calcium was significantly smaller (P=0.02) in CS+n-HA group in comparison with CS+ rhBMP2 group on day 3.
Adding rhBMP2 to CS and the mixture of CS and n-HA did not cause a significant increase in extracellular calcium concentration on days 3 and 7, but the reverse was true at 14 and 21 days. Studies have shown that rhBMP2 is a major regulator of differentiation and has chemotactic effects on osteoblasts [24]. BMP2 gene expression stimulates osteoblast differentiation and has a regulatory role in the transcription of osteoblast differentiation and intercellular signals [23]. There are different in vitro and in vivo studies on the role of rhBMP2 in inducing osteogenic activity by Wang et al [1], in 2011 and Yamazaki et al [36], in 1988. Considering the role of rhBMP2 in increasing ALP activity

and also in bone matrix calcification, the increase of extracellular matrix calcification resulting from rhBMP2 can be attributed to the increase of ALP activity. The results of our study showed that ALP activity increased over time. Increased ALP activity in presence of CS was shown in an in vitro study by Lazary et al [30]. In a study done by Winn et al, [3] on cytotoxicity and osteogenic potential of OsteoSet (a type of CS) in osteoblast cell line, a decrease in ALP activity occurred in the osteogenic cell line at days 4 and 7 and maintained for 16 days. The reason for this difference was due to the different types of culture medium. On the other hand, adding rhBMP2 to pure CS and n-HA/CS mixture causes an increase in ALP activity during all time periods, and this was in accordance with the results of an in vivo and in vitro study by Wang et al [1] on the properties of rhBMP2-loaded CS. In another study, Takuwa et al investigated the effects of BMP2 on ALP activity and collagen synthesis in osteoblastic MC3T3-E1 cells and found that BMP2 increased ALP activity in these cells [26]. This finding is generally consistent with the results of a study by Thies et al, on the effects of rhBMP2 on W-20-17 stromal cells [37]. Adding n-HA to CS causes an increase in ALP enzyme activity, but it was not significant in comparison to pure CS. Parsons et al, [38] in an in vivo study showed that mixing n-HA with CS promotes bone growth. Also Najjar et al [32] showed that the highest rate of bone formation was due to the n-HA/CS mixture. Stubbs et al. considered the effects of n-HA/CS mixture as a substitute for autograft materials for reconstruction of tibial bone defects in an animal model (rabbit) and observed that CS significantly caused osteogenesis in comparison to n-HA/CS mixture [39]. The results of in vitro studies cannot be generalized to the clinical setting due to the complex cellular interactions in the human body. In vitro studies use static conditions while in vivo studies apply dynamic ones, which result in elimination of the tested materials from the site. This is one of the main differences between in vivo and in vitro conditions and is responsible for the difference in results.

CONCLUSION

In this study, the mixture of CS, n-HA, and rhBMP2 was more effective in terms of bone matrix formation, but there was no significant difference in terms of cytotoxicity among the three groups of pure CS, CS+ n-HA, and CS+ n-HA+ rhBMP2, especially after 72 hours. Overall, this study indicates that a scaffold material in combination with an osteoinductive material is effective for bone matrix formation.

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