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RESEARCH PAPER

Cytotoxicity and Genotoxicity Evaluation of Fluorapatite/Bioactive Glass Nanocomposite Foams with Two Various Weight Ratios as Bone Tissue Scaffolds: an in vitro Study

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Abstract: *The optimization of biomaterials biodegradation rate similar to tissue regeneration, is one of the main goals in the field of tissue engineering. However, the necessity to assess their possible toxicity is always considered. The aim of this study was cytotoxicity and genotoxicity evaluation of fluorapatite/bioactive glass (FA/BG) nanocomposite foams with two various weight ratios to determine the optimal composition. Nanocomposite foams were made by gel-casting method with FA and BG as precursors in two weight ratios (A and B). Nanocomposite foam extracts (CFEX) were prepared by shaking 100 mg/mL of each foam in a complete culture medium for 72 h in a shaker incubator at 120 rpm/37ºC. Saos-II cells were exposed to different concentrations of CFEXs for 24 and 48 h and then cytotoxicity and genotoxicity were evaluated by MTT and comet assay, respectively. Based on the MTT assay results after 24 h exposure, CFEX A at concentrations ≥75% and CFEX B at concentrations ≥50% had a cytotoxic effect, while after 48 h, both CFEXs showed similar cytotoxicity at concentrations ≥25%. According to the result of the comet assay, DNA damage increased with the increase of CFEXs concentration and exposure time. Both CFEXs showed significantly higher comet tails elongation scores at concentrations ≥50% and ≥25% after 24 and 48 h exposure, respectively. Both composite foams could be considered as a non-toxic candidate for tissue engineering at concentrations <25% which was less than FA50%/BG50% composite. Therefore, the composite with equal FA/BG proportion has priority if similar results are obtained in in vivo complementary experiments.*

Keywords: *Cytotoxicity and Genotoxicity, Fluorapatite, Bioactive glass, MTT assay, Comet assay.*

1. INTRODUCTION

Bone tissue damage and its substitution is a major challenge in orthopaedics and dentistry. Restrictions and complications in the application of natural bone grafts such as the site of surgery in the donor, immunological reactions, and disease transmission force researchers to make and use synthetic biomaterials. However, the possibility of biomaterials' toxicity and their safety in terms of cellular and genetic damage to the patients and clinicians should be examined [1]. According to ISO specifications, implant devices need to be examined by various tests such as cytotoxicity, subchronic systemic toxicity, skin irritation, intracutaneous reactivity, sensitization systemic toxicity, genotoxicity, chronic toxicity, and local effects prior to implantation [2].

In recent years, *in vitro* evaluations in the field of toxicology have received much attention as an alternative method to animals studies [3].

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Single Cell Gel Electrophoresis (SCGE) assays, are sensitive methods to evaluation cytotoxicity and genotoxicity, respectively [1, 2].

The structural, physical, and chemical similarities to subject tissue are the most important criteria for biomaterials. Calcium phosphate (CP) derivatives such as hydroxyapatite (HA) and bioactive glass (BG), due to their similarity to the chemical composition of natural bone, teeth and enamel, can provide a favourable environment for bone tissue regeneration [4, 5]. If the OH^- groups are completely replaced by F^-, fluorapatite (FA) is formed which is significantly more resistant to biodegradation, provide better protein absorption, and express better cell adhesion than HA [6, 7]. In dentistry, there is a lot of attention to glasses that, in addition to calcium and phosphate ions, release fluoride ions into the environment and can form FA [8-11].

The new challenge in biomaterials is to increase the body's self-regeneration capacity by stimulating repair-initiating genes at the site of injury or damage. A very important characteristic

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of BGs is that they have shown genetic control over osteoblasts. Ionic products resulting from the decomposition of BGs increase the proliferation of human osteoblasts and induce mRNA expression of insulin-like growth factor II and protein synthesis [12]. The combination of BG particles with apatite in a bone tissue scaffold creates special features such as enhanced bioactivity and mechanical properties with a chemical composition similar to human hard tissue, and an exceptional opportunity to fabricate bio-absorbable scaffolds with similar degradation as same as new bone formation [5].

Based on the results of our previous study comparing the cytotoxicity and genotoxicity of HA/BG and FA/BG nanocomposite foams both composites showed cytotoxic effects at concentrations ≥50% on Saos-II cells [1]. Since the superiority of the FA-containing composite has been confirmed in in vivo study [13], and on the other hand, due to the possibility of more toxicity in fluorine-containing compounds, the necessity for further investigations is felt. The aim of this study was cytotoxicity and genotoxicity evaluation of FA/BG nanocomposite foams with two different weight ratios (25% FA/ 75% BG and 75% FA/ 25% BG) to determine the optimal composition as a bone tissue scaffold.

2. EXPERIMENTAL PROCEDURES

2.1. Preparation of Nanocomposites Foams and Their Extracts

Nanopowders of FA [$Ca_{10}(PO_4)6F_2$] and BG 58S (58% SiO_2, 36% CaO and 6% P_2O_5) made by sol-gel method were purchased from Nikceram Razi Co., Ltd. (Isfahan, Iran) and used as a precursor of the foams. The nanocomposite foams synthesized by the gel-casting method according to the previous study [14] with two various FA/BG weight ratios (A: 25% FA/ 75% BG, B: 75% FA/ 25% BG). The mixture of powders added 60 wt% to 1% TPP in deionized water, and mixed for 15 min. Then, a 7% agarose solution was added to the mixture and mixed at 130°C. Finally, 3% Tergitol was added to the suspension as the surfactant, and the foaming process was carried out by means of a 3-blade mixer at 80°C. Gelation was achieved by cooling the foam to 0°C. Then, the samples were removed from the molds, dried at room temperature and sintered at 1200°C.

To prepare the nanocomposite foams extracts, a suspension of 100 mg/mL autoclaved foams (which were crushed by hand mortar) in DMEM culture medium containing 10% FBS (Fetal Bovine Serum), penicillin (100 IU/ml), and streptomycin (100 µg/ml) were placed in a reciprocal shaker-incubator at 120 rpm/ 37ºC for 72 h. Then eluted solutions were centrifuged at 360 g for 10 min and the supernatant after filtration was considered as 100% saturated solution (CFEX). In addition to measuring the concentrations of calcium, phosphorus and silicate of CFEXs by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Varian 730-ES), the pH of various concentrations of each CFEXs (100, 75, 50, 25, 10, 5 and 1%) were measured by digital pH meter (HANNA, HI8424, Romania) [1].

2.2. In Vitro Cytotoxicity and Genotoxicity Evaluation of Nanocomposite Foams

Cell preparation: The Saos-II cell line was obtained from Pasteur Institute of Iran (Tehran, Iran) and cultured in DMEM culture medium supplemented with 10% FBS, penicillin (100 IU/mL), and streptomycin (100 µg/mL) in a humid atmosphere containing 5% CO_2 at 37°C. The culture medium was changed every two days and subconfluent cells were harvested from flask using 1 mL trypsin/EDTA (0.25, 0.02%) solution (Sigma-Aldrich, St. Louis, MO, USA) and seeded in a new flask, 96 well plate for MTT assay, or 24 wells plate for the comet assay.

2.2.1. Cytotoxicity evaluation

This experiment was conducted in triplicate. From the third passage, 6 × 103 cells/well were seeded in a 96-well plate. After 48 h, the supernatant was exchanged by various concentrations of CFEXs (100, 75, 50, 25, 10, 5 and 1%).

The complete medium without CFEX was considered as a negative control. The MTT assay was used to measure cell viability after exposure to CFEXs. After 24 and 48 h incubation, the media were removed and 50 µL MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 h at 37°C. Then 150 µL dimethyl sulfoxide (DMSO) were added to each well to dissolve the formazan crystals. The optical absorption was read at 570 nm wavelength and 630 nm as the reference wavelength, using an ELISA reader (Rayto RT-2100C). The results were reported as the percentage of control group

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optical absorption values mean.

2.2.2. Genotoxicity evaluation

This experiment was conducted in duplicate. From the third passage, 4 × 104 cells/well were seeded in 24-well plates. After 48 h, the culture medium was replaced by 1 mL of various concentrations of CFEXs (100, 75, 50 and 25%). The complete culture medium without extract was used as a negative control. After 24 and 48 h incubation, the cells were harvested from the bottom of the wells using trypsin/ EDTA solution and centrifuged at 360 g for 5 min. Finally, cells were suspended in PBS (without Mg^{2+} and Ca^{2+}) at a density of 1 × 106 cells/mL. The alkaline comet assay was used to assess DNA damage using the Singh protocol with minor modifications provided by Slamenova and Gabelova et al. [15]. In this method, 600 µL hot normal melting point agarose 1% in PBS (without Mg^{2+} and Ca^{2+}) was poured as a base layer on a microscopic slide and spread.

After coagulation, 20 µL of the mentioned cell suspension in 80 µL of warm (< 40°C) low melting point agarose 1% in PBS was resuspended and immediately spread on the base layer and covered with a coverslip. The slides were placed on the ice pack for 3 min to coagulate the second layer, after which the coverslips were removed. Then the slides were immersed in a lysis buffer containing 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH= 10 and 1% freshly added X-100 at 4°C for 30 min. After that, the slides were washed with deionized water and transferred to an electrophoresis tank with an alkaline buffer containing 300 mM NaOH, 1 mM Na_2EDTA, pH= 13 and kept at 4°C for 40 min to DNA strands be unbraided. The electrophoresis was performed for 30 minutes at 19 v/300 mA. The slides were then removed and fixed with 70% ethyl alcohol after washing 3 times with deionized water. Finally, immediately before the microscopic examination, 20 µl of Ethidium Bromide 10 µg/mL solution in deionized water was poured on each sample and covered by a coverslip. The stained nuclei were observed under a fluorescent microscope (Nikon, Japan) at 200X magnification. Fifty nuclei of each sample were randomly examined and their comet tail elongation was scored (0-4) by an observer.

2.3. Statistical Analysis

Statistical analysis of data was performed using SPSS software version 22. To compare the toxicity of various concentrations of CFEXs at different time intervals and between two CFEXs, analysis of variance and post-hoc multiple comparisons were used. A p value< 0.05 was considered significant.

3. RESULTS AND DISCUSSION

Fig. 1 shows the morphology of Saos-II cells exposed to various concentrations of CFEXs after 24 and 48 h.

Mean ± SD of MTT assay's (Saos-II cells optical absorption) results and comparison of similar concentrations of two CFEXs cytotoxicity in each time interval and each CFEX between two different time intervals are shown in Fig. 2. According to the results (Fig. 1, 2), with increasing concentration and exposure time, both CFEXs showed increase in toxicity. After 24 h, concentrations ≥50% of CFEX A and concentrations ≥75% of CFEX B had significant higher toxicity than control (p value < 0.030 and p value < 0.008, respectively). After 48 h, both substances showed significant toxicity in concentrations of 25% and above in comparison to the control group (p value < 0.015 and p value < 0.036, respectively). There was no statistically significant difference in comparing the toxicity of similar concentrations of two CFEXs at the same time intervals, while most concentrations of each CFEXs after 48 h had higher toxicity than the similar concentration at 24 h. Theiszova et al. in a similar study to the present study in term of experimental procedures, while examining different concentrations of hydroxyapatite extract on NIH-3T3 cells, concluded that antiproliferative effects only have been seen at the highest concentration [3]. Swain et al. investigated the effects of cytotoxicity of porous hydroxyapatite gelatin polyvinyl alcohol scaffold on murine L929 cells and in agreement with the present study, observed good biocompatibility and cell viability at concentrations below 25% [15]. In an in vitro study on fluoride-containing bioactive glasses, Gentleman et al. demonstrated that whilst Saos-II cells proliferation was higher on low-fluoride-containing composites, markers for cell differentiation and mineralization were higher in samples with more fluoride contents, a likely effect of a combination of surface effects and ion release [16].

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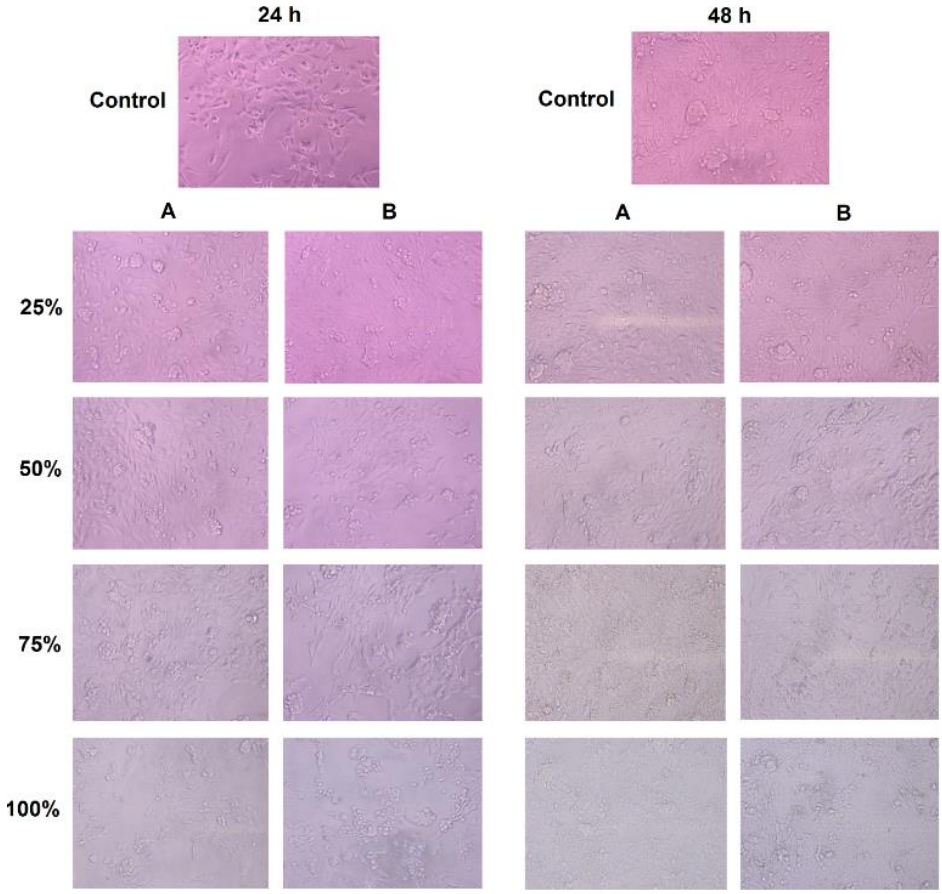
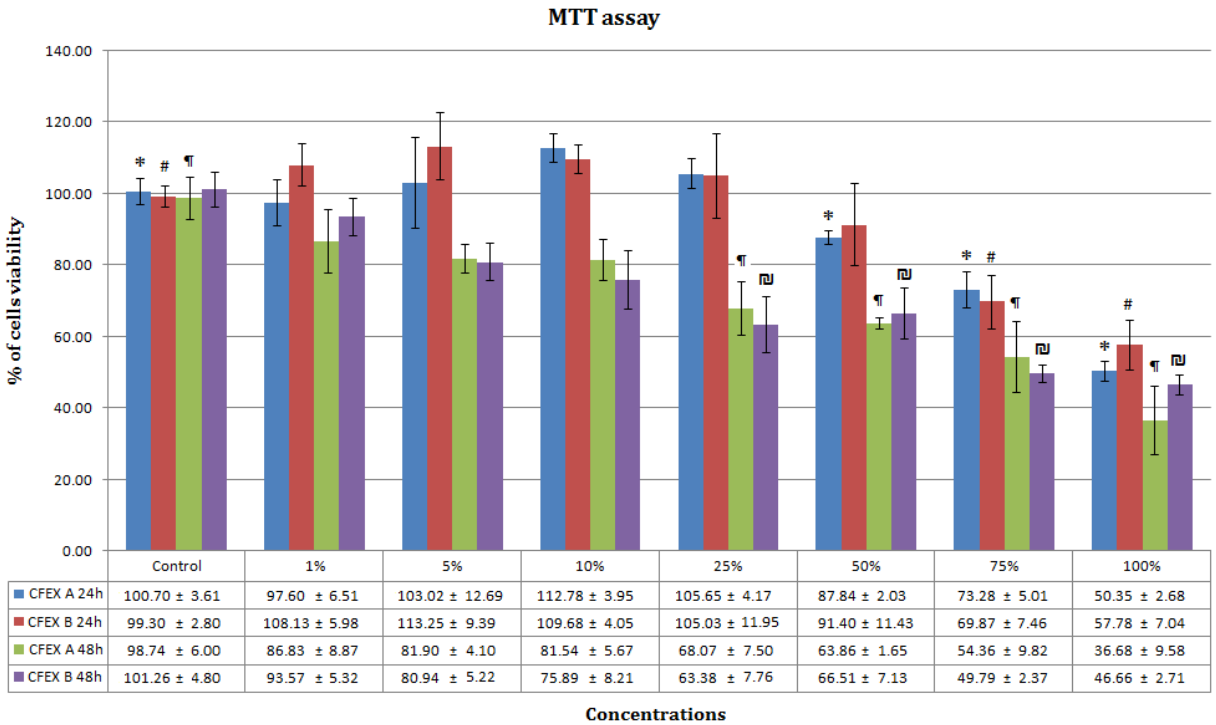


Fig. 1. Morphology of the Saos-II cells exposed to different concentrations of CFEXs after 24 and 48 h (magnification: ×200).



	Control	1%	5%	10%	25%	50%	75%	100%
CFEX A 24h	100.70 ± 3.61	97.60 ± 6.51	103.02 ± 12.69	112.78 ± 3.95	105.65 ± 4.17	87.84 ± 2.03	73.28 ± 5.01	50.35 ± 2.68
CFEX B 24h	99.30 ± 2.80	108.13 ± 5.98	113.25 ± 9.39	109.68 ± 4.05	105.03 ± 11.95	91.40 ± 11.43	69.87 ± 7.46	57.78 ± 7.04
CFEX A 48h	98.74 ± 6.00	86.83 ± 8.87	81.90 ± 4.10	81.54 ± 5.67	68.07 ± 7.50	63.86 ± 1.65	54.36 ± 9.82	36.68 ± 9.58
CFEX B 48h	101.26 ± 4.80	93.57 ± 5.32	80.94 ± 5.22	75.89 ± 8.21	63.38 ± 7.76	66.51 ± 7.13	49.79 ± 2.37	46.66 ± 2.71

Fig. 2. Effect of various concentrations of CFEXs on Saos-II cell viability after 24 and 48 h incubation. (Identical signs indicate significant difference with Control, error bars indicate standard deviations).

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Borkowski et al. in the study of cytotoxicity of fluorapatite ceramics made by the sol-gel method reported the high survival rate of pre-osteoblast cells and an increase in the proliferation rate of osteogenic cells incubated with the extract of this ceramic [17]. The results of the study conducted by Wei et al. did not show any toxic effect of dental cement's extract containing fluorapatite particles at concentrations of 25 to 200 mg/mL [18]. However, the amount of fluorapatite in their composition was much lower than in the present study.

The fluorescent microscopic fields of Saos-II cells' nuclei (comets) exposed to different concentrations of CFEXs depicted in Fig. 3.

It is observed that DNA damage and consequently the tail length elongation of comets increased with increasing CFEXs concentration and time of cells exposure. The mean ± SD of scores that have been given to comets of cells exposed to the various concentrations of CFEXs are presented in table 1.

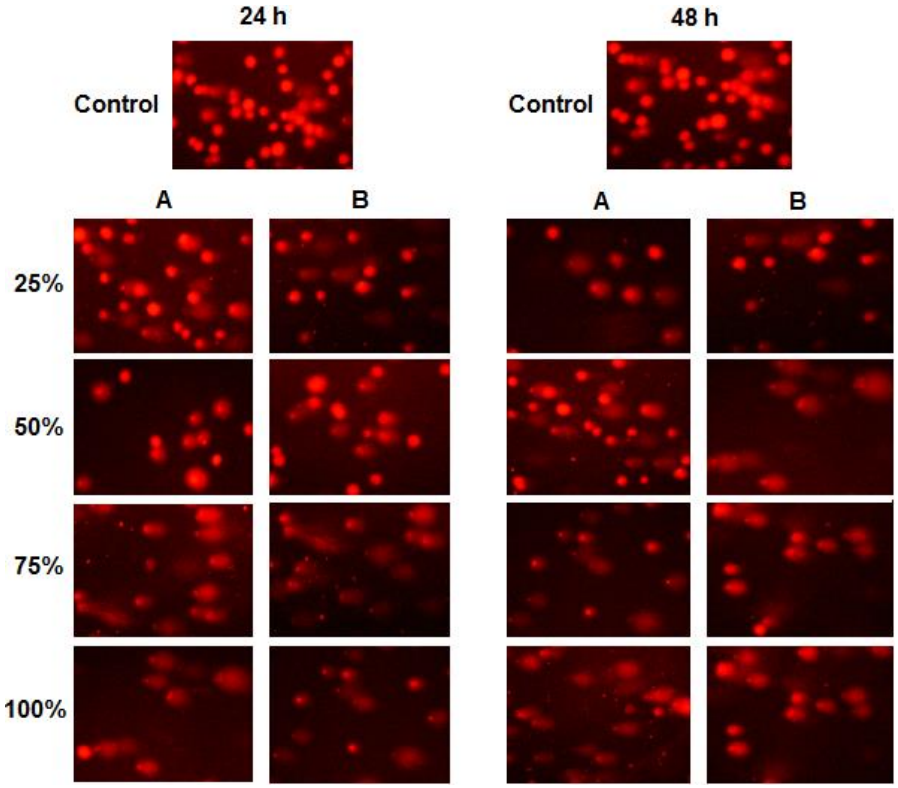

Fig. 3. Fluorescent microscopic views of the Saos-II cells after 24 and 48 h exposure to various concentrations of CFEXs (comet assay; magnification: ×200).

Table 1. Mean ± SD of comet scores of the Saos-II Cells after 24 and 48 h exposure to various concentrations of CFEXs (comet assay).

BMEX	Time	Control	25%	50%	75%	100%	P value
A	24h	0.34±0.66	0.46±0.61	0.68±0.77	1.08±0.85	1.52±1.16	<0.001
B		0.34±0.66	0.56±0.67	0.78±0.84	1.26±0.92	1.46±1.03	<0.001
	P value	1	0.478	0.593	0.295	0.867	
A	48h	0.54±0.68	0.78±0.74	1.42±0.93	2.14±1.03	2.46±1.13	<0.001
B		0.54±0.68	1.06±0.91	1.44±0.86	2.12±1.37	2.32±0.94	<0.001
	P value	1	0.128	0.888	0.934	0.474	
A	24h	0.34±0.66	0.46±0.61	0.68±0.77	1.08±0.85	1.52±1.16	<0.001
	48h	0.54±0.68	0.78±0.74	1.42±0.93	2.14±1.03	2.46±1.13	<0.001
	P value	0.063	0.022	<0.001	<0.001	<0.001	
B	24h	0.34±0.66	0.56±0.67	0.78±0.84	1.26±0.92	1.46±1.03	0.002
	48h	0.54±0.68	1.06±0.91	1.44±0.86	2.12±1.37	2.32±0.94	<0.001
	P value	0.063	0.005	<0.001	<0.001	<0.001	

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The relationship between the increase in number and elongation of comets with the concentration of CFEXs (Table 1 and Fig. 3, 4) was in agreement with the results of similar studies on biomaterial genotoxicity conducted by Tavakoli et al. [2], Jontava et al. [19], and Seyedmajidi et al. [1]. After 24 h of exposure, concentrations ≥50% of both CFEXs showed significant differences in comparison with the control group (p value <0.018 and p value <0.014, respectively). After 48 h, the significant difference progressed to the concentration of 25% in CFEX B. There was no significant difference in genotoxicity between the two studied nanocomposite foams in each time interval, but in all examined concentrations of each CFEX, genotoxicity after 48 h exposure was significantly higher than 24 h (Fig. 4 and Table 1).

The concentration of calcium, phosphorus and silicate of the CFEXs and the pH of various concentrations (1-100%) of CFEXs has been shown in Fig. 5.

Although the amount of BG, which contains silicate, was different in the two studied composites, the amount of released Si from the two composite foams was almost identical and not sufficient to cause toxicity (< 15 ppm). The amount of calcium in both CFEXs increased compared to the complete culture medium. In contrast, phosphorus decreased, which this declension was greater in CFEX B.

The pH of the plain complete culture medium as control, was higher than 7.2-7.4 (the optimum pH for mammalian cell growth) due to the lack of CO_2 in the incubator during CFEX preparation [1]. Decrease in phosphorus could be the reason of pH increase because of the loss of phosphorus buffering properties and its ratio to Ca which in CFEX B is lower than CFEX A. Increase of the medium pH was directly in relationship to increase of CFEXs concentrations, which was higher in CFEX B.

The optimization of the bioactive and biodegradable compounds which is used in the manufacturing of cellular scaffolds so that could degrade at the same rate of tissue regeneration is one of the main goals of tissue engineering. However, the necessity to assess the possible toxicity of these compounds is always considered. In the study of Mansoorifar et al. with the aim of optimizing the different amounts of fluorine substitution in the structure of fluorhydroxyapatite, the adhesion and density of MG-63 cells increased with increasing of fluorine content [20]. The compound replaced with 75% fluorine in the apatite structure was determined to be the optimal compound in terms of biocompatibility and the compound containing 100% was the best compound in terms of corrosion resistance [20].

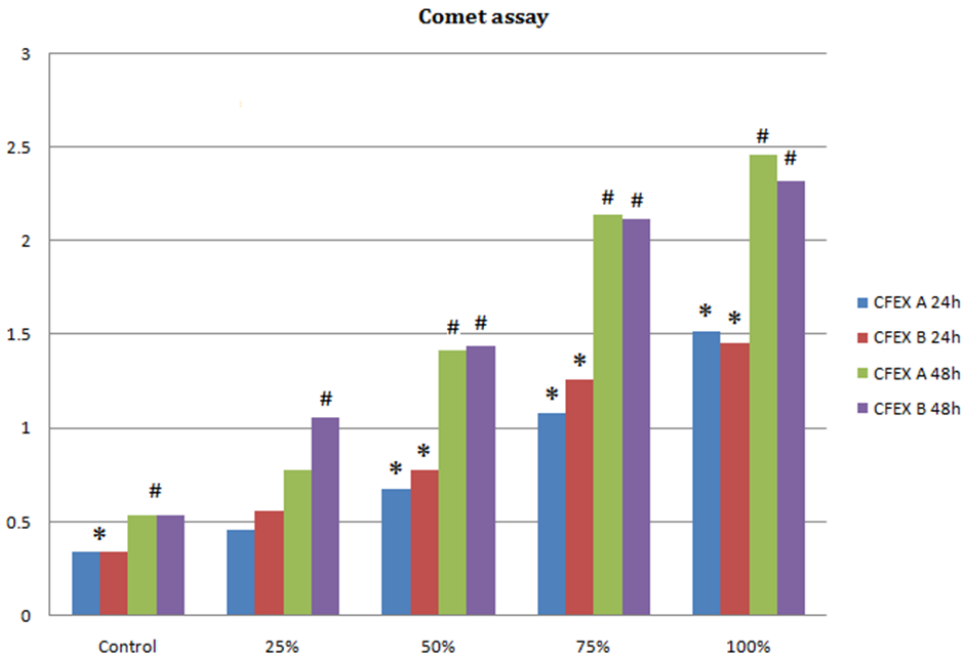

Fig. 4. Comets' elongation scoring results after 24 and 48 h Saos-II cells exposure to various CFEXs concentrations (comet assay). (Identical signs indicate significant difference with Control).

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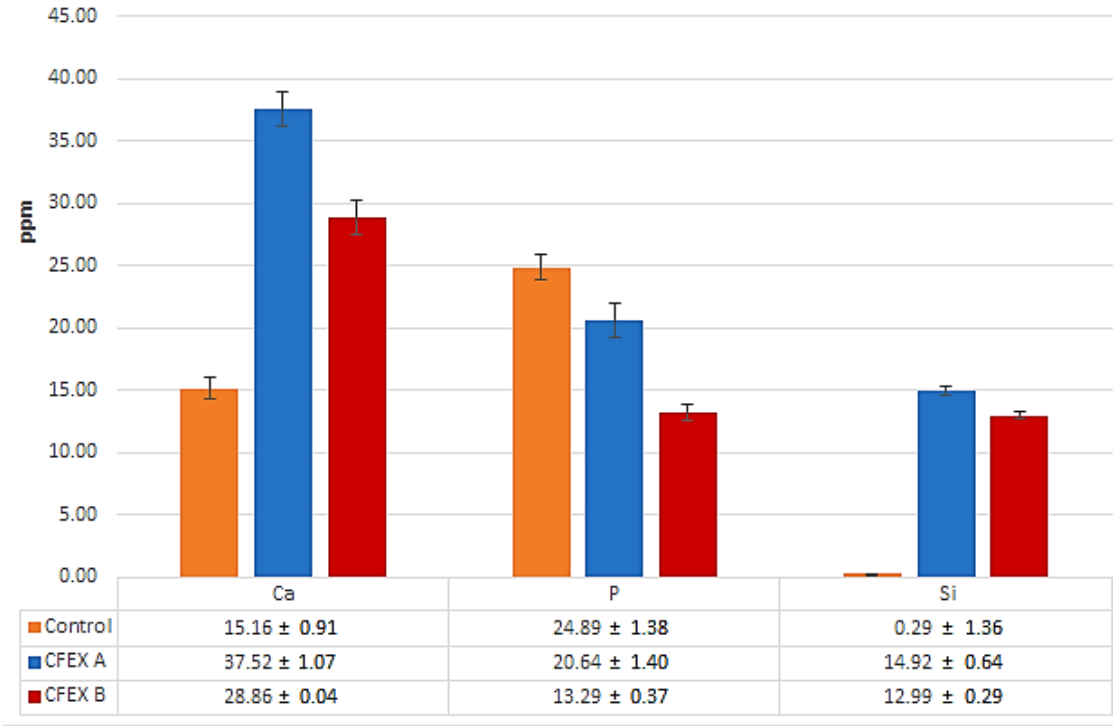
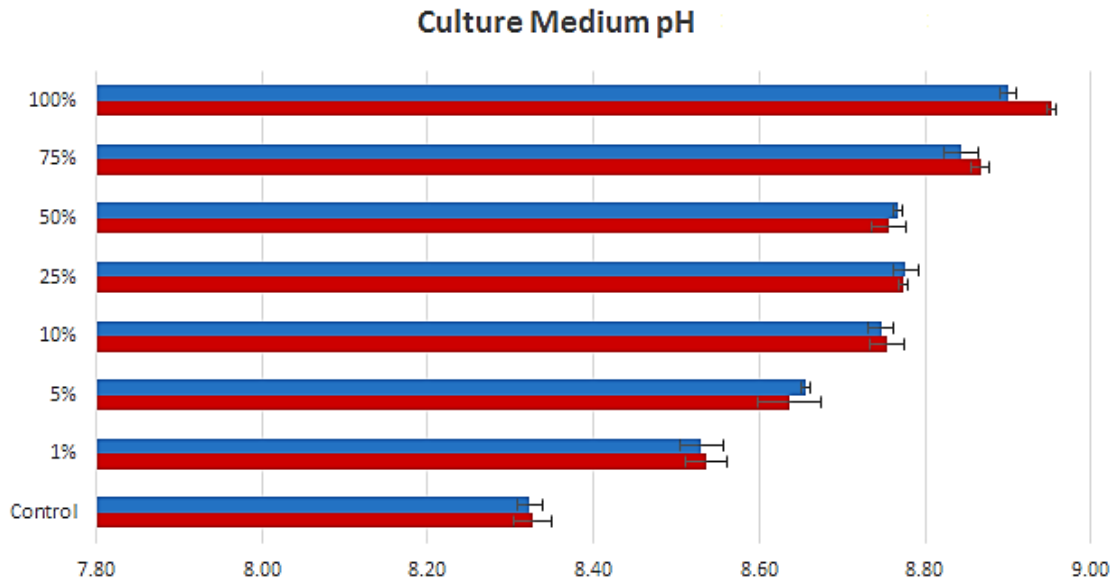


	Ca	P	Si
Control	15.16 ± 0.91	24.89 ± 1.38	0.29 ± 1.36
CFEX A	37.52 ± 1.07	20.64 ± 1.40	14.92 ± 0.64
CFEX B	28.86 ± 0.04	13.29 ± 0.37	12.99 ± 0.29



	Control	1%	5%	10%	25%	50%	75%	100%
CFEX A	8.32 ± 0.02	8.53 ± 0.03	8.66 ± 0.01	8.75 ± 0.02	8.78 ± 0.02	8.77 ± 0.01	8.84 ± 0.02	8.90 ± 0.01
CFEX B	8.33 ± 0.02	8.54 ± 0.03	8.64 ± 0.04	8.75 ± 0.02	8.77 ± 0.01	8.76 ± 0.02	8.87 ± 0.01	8.95 ± 0.01

Fig. 5. Mean ± SD of Ca, P, and Si ions concentrations in CFEXs and the pH of various concentrations of CFEXs. (error bars indicate standard deviations).

In our study, the OH^- groups in hydroxyapatite were completely replaced with F^-, which was finally combined with bioactive glass in a ratio of 1 to 3 and vice versa, and Saos-II cells were exposed to different concentrations of their extracts in complete culture medium.

One of the characteristics of nano-size biomaterials is high specific surface area which naturally increases their reactivity and performance in comparison to micro size ones.

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Consequently, this feature can accelerate the healing process and ossification when uses as bone substitute biomaterials. Some studies have investigated the biocompatibility and cytotoxicity of bioceramics containing bioactive glass, fluorapatite and their derivatives.

The results of a similar study which was conducted by Kazuz et al. in the investigation of biocompatibility of fabricated beta-tricalcium phosphate-based composite cement containing nano fluorapatite for use as a dental canal filler showed no cytotoxicity effect on MRC-5 human fibroblast cells in MIT and DET tests [21]. Manafi et al. studied on cytotoxicity of fluorapatite-bioactive glass S53P4 nanocomposite with 10, 20, and 30% fluorapatite by MTT assay and concluded that cellular responses were increased by incorporation of 10 and 20% fluorapatite, but with increasing to 30%, cell survival was decreased [22].

According to the international standard of medical equipment ISO-10993: 5, materials with 25% or less toxicity are considered practically non-toxic [21]. Therefore, the materials used in this study at concentrations <25% can be considered non-toxic. Based on the agreement between the results between the two methods of using OpenComet software and the visual scoring method in our previous study [1] was very good (above 0.75), in this study the comets were evaluated and scored visually. Since the possible mechanism of toxicity of two studied composites is changes in the concentration of calcium and phosphate ions along with the pH of the culture medium, composite B resulted in greater toxicity because of further pH change. Considering more toxicity of these composites than the composite with equal proportions of FA and BG, it can be said that equal amounts of FA and BG may prevent from the culture medium key elements (such as calcium and phosphorus) disturbance and further pH changes.

4. CONCLUSIONS

The possible mechanism of toxicity on the investigated composites is the disturbance between the balance of key elements in the culture medium due to their biodegradation and consequent changes in pH. Both composites could be considered as non-toxic candidates for use in tissue engineering at concentrations less than 25%. According to results using composite with equal FA/BG proportions has priority if similar results are obtained in *in vivo* complementary experiments.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this article.

ETHICS APPROVAL STATEMENT

This study was approved by Babol University of Medical Sciences Ethics Committee #IR.MUBABOL.HRI.REC.1399.085

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