Effects of Graft-carrier Solutions on Osteoblast-like Cells with or without Beta-tricalcium Phosphate

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Submitted: 2019-03-29; **Accepted:** 2019-06-25; **Published Online:** 2019-07-11; **DOI:** *10.22037/rrr.v4i3.26000*

Introduction: The enhancement of osteogenesis by tissue engineering is a challenge in periodontal therapy. Several graft materials in conjunction with carriers, such as blood or saline, are used for this purpose. This study aimed to assess the effect of phosphate buffered saline (PBS), Hank's balanced salt solution (HBSS) and saline on the activity of MG-63 osteoblast-like cells in the presence and absence of beta-tricalcium phosphate (β-TCP). **Materials and Methods**: In this *in vitro* experimental study, MG-63 osteoblast-like cells were cultured in 10% PBS, HBSS and saline (10%) with and without β-TCP granules for 24 and 72 h and five days. At 24 and 72 h, cell viability and proliferation were assessed. Alkaline phosphatase (ALP) activity test was used to assess bone activity. The data were analyzed using SPSS version 20 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp) via one-way and two-way ANOVA ($P<0.05$). **Results**: Pairwise comparisons showed no significant difference in the viability of MG-63 cells at 24 h in the three solutions (with equal β-TCP content) or with the negative control group (complete culture). At 72 h, significant differences were only observed in the reduction of cell proliferation between 10% saline without β-TCP and 10% saline with β-TCP , and also between HBSS without β-TCP and HBSS with β-TCP ($P<0.05$). **Conclusion**: The three solutions did not induce ALP activity at 24 or 72 h and did not cause the formation of any calcified nodule at three or five days in MG-63 cells.

Keywords: Alkaline Phosphatase; HBSS; MG-63 Cells; Osteogenesis; Saline

Introduction

Alveolar bone augmentation is commonly performed prior to dental implants. Autografts have osteogenic properties, but their use is limited due to the need for a second surgical site harvesting (1) ,surgical cost (2) and possible impaired function of donor sites (3).

Tissue engineering with the triad of stem cells, scaffold and signaling molecules, have been used to enhance bone regeneration in recent years (3). Adhesion, proliferation, and differentiation of recipient site stem cells is mainly affected by physicochemical properties of the scaffold as an osteocondutive synthetic matrix (4). Thus, material properties and proper scaffold design are necessary to achieve the desired cell response (5). Hydroxyapatite and β-TCP are among the most commonly used synthetic materials in bone tissue engineering. With concomitant use of mesenchymal stem cells and morphogenic proteins, bone regeneration in critical sized defect may find a possible solution in near future. However the cost and difficulties in production may guide researcher to find an alternative to increase the rate of bone regeneration.

Advances in knowledge for fabrication of bone substitutes and grafts with osteoconductive properties have enabled the application of bone tissue engineering for periodontal purposes (6). Though, the procurement of stem cells with the potential to differentiate into active osteoblasts from the respective site and their loading on bone scaffolds remains clinically challenging. The introduction of signaling molecules or solutions is expected to provide an ideal environment for osteoblastic adhesion and growth, and enable interactions between cells and scaffolds or the matrix at their favorable osmolarity, pH, buffering capacity and the ionic charge (7). At present, most clinicians use saline or blood as a carrier medium for delivery of biomaterials to the graft site. Blood and its serum can serve as an ideal carrier for this purpose because it has a pH and osmolarity (290-300) similar to that of the extracellular bone matrix. Since obtaining patient's serum has some limitations, its use for periodontal regeneration is not yet practical and as such, some other solutions need to be introduced for this purpose. These solutions may be chosen among those prepared as a culture of bone cells with optimal pH, osmolarity, and ionic charge (8).

Carriers, such as Blood and saline, are commonly used to deliver graft materials to the defect site in routine surgical procedures. Blood components such as platelet showed their effectiveness in healing process. HBSS has a physiological osmolality and has a basis of physiological salt (9-11). It contains phosphate and thus, has a buffering capacity and maintains the pH within the physiological range. It is also non-toxic. Saline has physiological osmotic pressure but does not have a buffering capacity (9).

The possible effect of saline, HBSS, and PBS on proliferation and differentiation of osteoblasts and consequently bone regeneration has not been previously evaluated. Thus, this study aimed to assess the effect of PBS, HBSS, and saline on the morphology and differentiation of MG-63 osteoblast-like cells to determine the most efficient carrier for bone regeneration in the clinical setting.

Materials and Methods

Cell culture

This experimental study was conducted on MG-63 human osteoblast-like cells obtained from the Genetic Engineering and Biotechnology Research Center, Tehran, Iran. The obtained cell culture flask containing MG-63 human osteoblast-like cells was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, Spain), 100 IU/mL penicillin (Gibco, Spain) and 100 mg/mL streptomycin (Gibco, Spain). Thus, it was incubated (Memmert, Germany) at 37°C with 5% CO_2 and 95% humidity. After proliferation, the cells were passaged using trypsin-EDTA. The cell suspension was prepared from the fourth passage cells with a concentration of 20,000 cells/mL for use in the MTT assay and ALP activity test at 24 and 72 h

Preparation of the test and control solutions

Saline solution (0.9 g NaCl in 100mL of deionized distilled water; Sigma, France), PBS (one tablet in 500 mL of deionized distilled water; Invitrogen) and x10 HBSS stock (1cc of HBSS stock solution in 9cc of distilled water; Life Technologies, Gibco®, catalog number: 10977015) were prepared in 10%. To prepare 10% concentration of solutions, 4cc of each solution was added to 36cc of the DMEM in 15 mL falcon tubes (SPL CO. Pyeongtaek, South Korea). Before mixing with the culture medium, the solutions were sterilized using a 0.2 µm filter and were then added to sterile culture medium. To prepare osteogenic medium, 10 mM ascorbic acid, 50 mM beta-glycerophosphate and 10 mM dexamethasone (Iran Hormone®, Karaj, Iran) were added to the culture medium under sterile conditions; DMEM was prepared according to the manufacturer's instructions and sterilized using a 0.2 µm filter. First and foremost, 130 sterile 1.5 mL microtubes (SPL Co. Pyeongtaek, South Korea) were obtained and capped under a biological laminar flow hood (Azar Pars Tajhiz, Tehran, Iran) to maintain their sterility. The microtubes were weighed on a digital scale (Sartorius Co. Göttingen, Germany) with 0.0001g accuracy. Thereafter, three to five MBCP granules (Biomatlante Co. Vigneux-de-Bretagne, France) were added to the microtubes and were weighed again on a digital scale for the purpose of standardization of the weight of granules.

Cell proliferation

Cell proliferation was assessed using the MTT assay. For this purpose, 10% concentration of each solution (saline, PBS, HBSS) was added to six wells in three rows of a 96-well plate. A six-well row was allocated to the positive control group containing distilled water and a six-well row was allocated to DMEM. In each row, three to five β-TCP granules (standardized in terms of weight) were added to each well. Similar wells with the same concentration of solutions were also considered with no granules. At 24 and 72 h, the culture plates were evaluated under a light microscope (Nikon Co. Tokyo, Japan) at x4 and x10 magnifications to ensure the proliferation of cells in wells and no contamination. The overlaying culture medium and the β-TCP granules were removed from the wells and all wells were rinsed with PBS; 200 µL of the MTT salt diluted 1:10 with the culture medium was then added to each well and incubated for three hours at 37°C with 5% CO_2 and 98% humidity. After completion of incubation time, cells were evaluated under a microscope to ensure staining. The overlaying solution was then removed and 200 µL of dimethyl sulfoxide (Sigma-Aldrich Co. Missouri, France) was added to each well to dissolve formazan crystals. After four hours, the optical density of the solution was read using ELISA Reader (Apex Laboratory Equipment Co. Saint Paul, USA) at 570 nm wavelength and 620 nm wavelength reference filter. To determine the cell proliferation rate, the values obtained in each group were divided by the mean value of the negative control group and multiplied by 100. This was done for all groups.

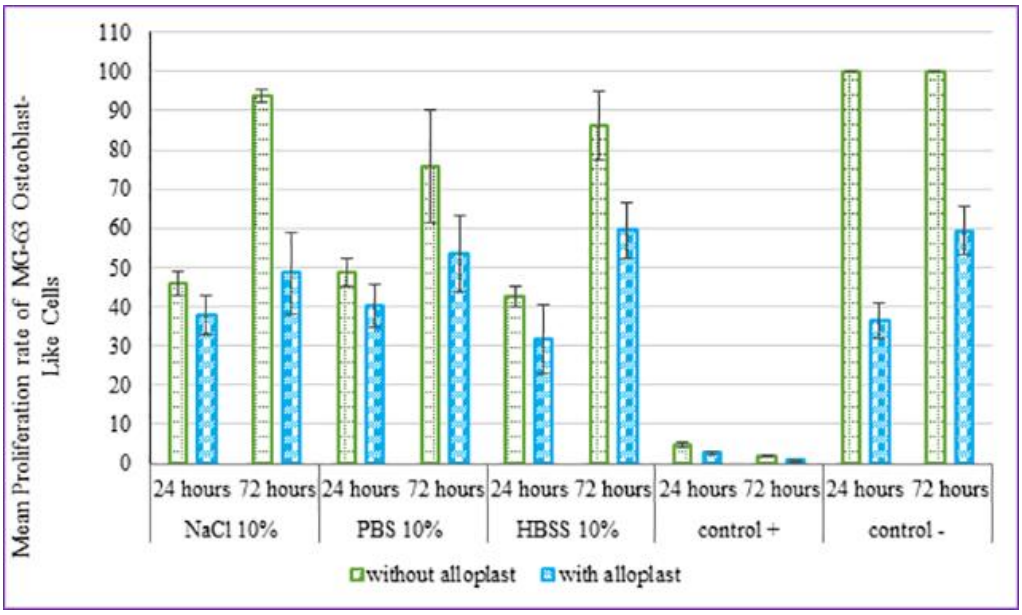

Figure 1. Mean proliferation rate of MG-63 osteoblast-like cells with or without β-TC.

ALP activity test

For the ALP activity test (to indicate osteoblastic differentiation), 10% concentration of each solution (saline, PBS, HBSS) was added to three wells in three rows of a 96-well plate. A three-well row was allocated to the positive control group, which contained osteogenic medium and a three-well row was allocated to the negative control group containing complete culture medium. Three to five β-TCP granules were added to each well in each row. Subsequently, 24 and 72h cultures were used for measurement of ALP activity using a special kit (Biovision Inc. California, USA). The overlaying culture medium was removed from the wells, cells were lysed using lysis buffer and the substrate was added to the wells (two para-nitrophenyl phosphate tablets were mixed with 4.5 mL of lysis buffer). Optical density was measured at 405 nm wavelength using ELISA reader.

Statistical analysis

Kolmogorov-Smirnov test was used to assess the normal distribution of data. One-way ANOVA was used to compare five groups in terms of the mean cell proliferation (MTT) and ALP activity. Two-way ANOVA was used to assess the effect of time and type of intervention (group) on cell proliferation and ALP activity. Type one error (α) was considered as 0.05 with 95% confidence interval. $P<0.05$ was considered statistically significant. Since the results of ANOVA was statistically significant, Games-Howell multiple comparisons test was used for pairwise comparisons of the groups.

Results

Proliferation of MG-63 osteoblast-like cells in the groups

MG-63 cells proliferation at 24 h with or without β-TCP showed statistically significant difference between groups (ANOVA, $P<0.001$) (Figure 1). Pairwise comparisons of the groups using Games-Howell test showed that at 24 h, 10% saline, 10% PBS and 10% HBSS in the presence or absence of β-TCP experienced a significant increase in cell proliferation as compared with the positive control groups ($P<0.05$). Also, 10% saline, 10% PBS and 10% HBSS with and without β-TCP experienced a significant reduction in cell proliferation as compared with the negative control group without β-TCP ($P<0.05$). Only 10% PBS without β-TCP showed a significant increase in cell proliferation as compared with the negative control with β-TCP ($P<0.05$). At 72 h, 10% saline, 10% PBS and 10% HBSS in the presence or absence of β-TCP experienced a significant increase in cell proliferation as compared with the positive controls with and without β-TCP ($P<0.05$). Also, as compared with the negative control without β-TCP, 10% saline with and

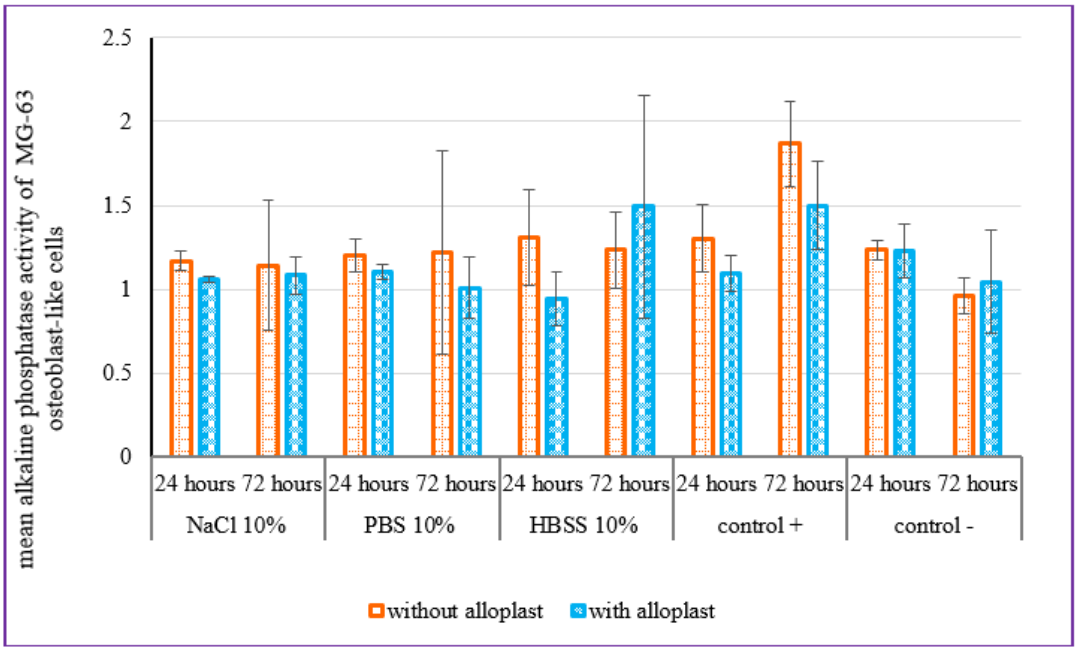

Figure 2. Mean alkaline phosphatase activity of MG-63 osteoblast-like cells with or without β-TCP

without β-TCP, 10% FBS with β-TCP and 10% HBSS with β-TCP experienced a significant reduction in cell proliferation (P<0.05). Also, as compared with the negative control with β-TCP, only 10% saline without β-TCP and 10% HBSS without β-TC experienced a significant increase in cell proliferation (P<0.05).

Cell proliferation was significantly lower in 10% saline without β-TCP as compared with 10% saline with β-TCP and also, in HBSS without β-TCP as compared with HBSS with β-TCP at 72 h (P<0.05). From 24 to 72 h, cell proliferation significantly increased in 10% saline without β-TCP as compared with 10% saline without β-TCP. Also, it increased in HBSS with β-TCP as compared with HBSS with β-TCP (P<0.05).

Based on the results of the two-way ANOVA, the effect of type of solution, presence/absence of β-TCP and time alone on the proliferation rate of MG-63 cells was statistically significant (P<0.001). In addition, the interaction effects of the solution with β-TCP (P<0.001), the solution with time (P<0.001) and β-TCP with time (P=0.004) on the proliferation of MG-63 cells were significant.

ALP activity of MG-63 osteoblast-like cells in the groups

Figure 2 shows the ALP activity of MG-63 cells at 24 and 72 h with and without β-TCP. Based on the results of ANOVA, a significant difference existed in ALP activity of MG-63 cells in different groups (P=0.048). However, pairwise comparisons of groups in terms of ALP activity with Games-Howell test showed no significant difference between the experimental and positive and negative control groups with and without β-TCP in 24 or 72 h (P>0.05). Based on the to two-way ANOVA, the effect of type of solution alone on the ALP activity of MG-63 cells was statistically significant *(P=0.02)*. The interaction effect of type of solution and time on the proliferation rate of MG-63 cells was also significant (P=0.02).

Discussion

The present study assessed the effect of PBS, HBSS, and saline on the activity of MG-63 osteoblast-like cells in the presence and absence of β-TCP.

The maximum proliferation of MG-63 cells at each time point occurred in the negative control group without β-TCP. The negative control group without β-TCP only contained complete culture medium and it was expected to show maximum proliferation of MG-63 cells due to its constituents. Cell proliferation was slightly less in experimental groups of saline, PBS and HBSS, which may be

due to dilution of nutrients in the culture medium, such as necessary amino acids, vitamins, and microelements, following the addition of these solutions to the standard culture medium. Cell proliferation rate significantly decreased following the addition of β-TCP granules to the negative control medium. This finding may be attributed to rapid dissolution of β-TCP bioactive granules in HA/β-TCP compound and the release of Ca2+ and PO43- ions in physiologic solutions (12). The released ions may react with other ions present in the culture media and produce compounds that affect the tonicity, pH or buffering capacity of the medium. Another possible explanation is that biphasic β-TCP compounds may have different dissolution dynamics as compared with HA/ β-TCP (13).

Similarly, Kim *et al.*, in their study, showed deposition of unique small granules on biphasic calcium phosphate (BCP) granules one week after their immersion in HBSS. By an increase in immersion time in HBSS, granules gradually merged to form a dense layer on the surface of BCP granule. Proliferation rate at 24 h with BCP was lower than that in the control group (14). The lower proliferation rate of cells in the media containing β-TCP can be attributed to the fast release of Ca2+ and PO43- (indicative of fast dissolution), which significantly decrease the accessible level of ions required for proliferation of bone cells and thus, its use is limited in the clinical setting (14). In the present study, at 24 h, cell proliferation was not significantly different among PBS, HBSS and saline (P>0.05), which may be due to the fact that these solutions are all balanced salt solutions with similar inorganic ions such as sodium, potassium, calcium, magnesium and chloride. Thus, they have similar effects on cell proliferation, buffering capacity and maintenance of optimal pH (15).

The ability of blood in the human body to neutralize non-volatile acids with no change in pH can be attributed to the alterations (volatility) of an acid, CO2, and the ability of the respiratory system to eliminate excess CO2 (16).

In bone grafts, storage solutions require physiological osmolarity, the presence of compounds preserving osteogenic cells, and physiological pH. Cell growth often occurs in osmolarity of approximately 230-400 mOsm/kg and efficient osmolarity is between 290-330 mOsm/kg (9).

Balanced solutions have been introduced to maintain the viability of cells under suitable conditions for growth and proliferation in short periods of time rather than to induce growth or differentiation of cells in the culture medium (9). HBBS contains calcium, magnesium, and glucose, which are required for viability of cells. Thus, it plays an important role in maintaining the osmotic balance and acidity of the storage medium and also provides solutions and ions necessary for cell viability (8). The osmolality of HBSS is 270-290 and it has a pH of 7.2. This solution has a physiological osmolality and a physiological salt basis (5, 14, 17). Also, the acidity of this solution is within an acceptable range for cell proliferation (between 6.6-7.8) (10).

It has a buffering capacity due to the presence of phosphate in its composition and thus, maintains the pH within the physiological range. However, a previous study reported that HBSS had a very low buffering capacity (15). It has been designed to maintain cells under viable conditions and is not intended to induce growth or proliferation of cells in long-term (9).

Saline has physiological osmotic pressure; however, its pH is not stable due to the absence of a buffering mechanism. Moreover, it does not have any nutritional value (9). In a study by Kanai *et al.*, the osmolarity of saline was reported to be 285 mOsm/kg with a physiological pH of 4-6.4. They showed that 60 min of incubation of bone marrow cells of rats in HBSS before culture better maintained cell proliferation as compared with incubation in saline. Their viability test showed that the ratio of viable cells after treatment with saline was 60% as compared with HBSS, indicating that short-term treatment with saline negatively affected bone marrow cells. They used 60% saline for a short period of time, while in the present study 10% saline was used for 24 and 72 h. Also, they used bone marrow cells, while MG-63 osteoblast-like cells were used in the present study.

In the current study, cell proliferation increased from 24 to 72 h in all three solutions; however, the difference in this regard only between 10% saline without β-TCP and HBSS with and without β-TCP was significant (P<0.05). Thus, this may be attributed to the effect of time on 10% saline without β-TCP or HBSS with and without β-TCP. Pautke *et al.*, immunohistochemically compared three osteosarcoma cell lines namely MG-63, Saos-2 and U2 OS, and observed that MG-63 cells did not show ALP activity (16).

Furthermore, Park *et al.*, in their study, evaluated the ALP activity of MG-63 cells on Aln scaffold containing BCP and reported an increase in ALP activity by increasing the incubation time from three to seven and 10 days (18). On the other hand, the absence of ALP activity of MG-63 cells observed in the present study may be attributed to the

assessment time point. An increase in ALP activity of cells might have been seen in longer periods of time.

Type I collagen is expressed during the initial phase of proliferation and biosynthesis of the extracellular matrix, while ALP is expressed in the post-proliferation phase and maturation of extracellular matrix. The expression of osteopontin, osteocalcin, and sialoprotein occurs at the end of the third phase upon mineralization of extracellular matrix (12). Thus, the absence of ALP activity was justifiable in the present study since only the ALP activity at 24 and 72 h was assessed.

Kanai *et al.* measured the ALP activity of bone marrow cells in rats after 60 min of incubation in HBSS or saline before culture. The results showed that ALP activity of HBSS was significantly higher than that of saline after 10 and 14 days of culture (9).

Also, in the present study, the proliferation of cells in all solutions with β-TCP (PBS, HBSS, saline, positive and negative controls) was less than that in solutions without β-TCP.

Phosphate buffered saline, HBSS, and saline with β-TCP experienced a significant reduction in cell proliferation as compared with the negative control without β-TCP ($P<0.05$). This finding may be attributed to the presence of β-TCP or the passage of time. However, the aforementioned groups were not significantly different in this regard at 24 h. In the present study, calcified nodules were only formed in the positive control group without β-TCP. The reason for the absence of formation of calcified nodules may be due to the type of MG-63 cells because these solutions did not show ALP activity in ALP test (15). Also, the saline solution does not contain calcium or chemical agents required for bone formation. In the positive control group without β-TCP, the osteogenic medium was used. It appears that osteocytes initiate the secretion of sclerostin only after maturation of mineralized bone matrix, as reported by Irie *et al.*

Bioactivity of β-TCPs in a physiological environment is a part of dynamic events that highly depends on biological compound (12). In the present study, β-TCP was used as an osteoconductive agent. It has a shorter dissolution time and thus, may not be able to provide a suitable surface for attachment of immature osteoblasts during the desired time period. In a study by Sanchez-Salcedo *et al.*, it was shown that immersion of β-TCP/HA scaffolds in SBF caused significant changes in the chemical composition of SBF under both static and orbital stirring conditions. The concentration of calcium and phosphorus after 15 days of immersion in SBF remained constant due to the slight dissolution of more soluble phase, that is, the β-TCP phase. Thereafter, the chemical composition reaches a plateau with a similar concentration of calcium and phosphorus ions under static and orbital stirring conditions. These ions form nuclei on the surface of BCP and are gradually crystallized into apatite. It should be noted that high dissolution of β-TCP provides an adequate source of ions for the formation of apatite nuclei (12). This scaffold does not only protects the cells but also produces signals that induce osteogenic differentiation.

Researchers have shown that biomaterials containing calcium and phosphate moieties (CaP, the main constituent of native bone) can induce osteogenic differentiation of progenitor and stem cells and can enhance bone formation in vivo. Osteogenicity and osteoinductivity of the mineral CaP depend on factors, such as the potential of CaP ions and absorption and the release of osteoinductive growth factors such as bone morphogenetic proteins. Studies have shown that extracellular PO43- plays an important role in the induction of osteogenic differentiation of human mesenchymal cells by regulating the mitochondrial phosphate content and ATP synthesis (13).

Conclusions

The results of this study showed no significant difference in proliferation of MG-63 cells in different solutions at 24 h. At 72 h, significant differences were observed in cell proliferation between 10% saline with and without β-TCP and also between HBSS with and without β-TCP. As compared with the negative control without β-TCP, PBS, HBSS, and saline with β-TCP experienced a significant reduction in cell proliferation. None of the solutions in this study could induce ALP activity or calcified nodule formation in the medium containing MG-63 cells at the designated time points.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. This research was conducted in School of Dentistry, Shahid Beheshti University of Medical Sciences.

Conflict of Interest: 'None declared'.

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Please cite this paper as: Shamsoddin E, Fallah M, Esmaeilnejad A, Golabgiran M, Houshmand B. Effects of Graft-carrier Solutions on Osteoblast-like Cells with or without Beta-tricalcium Phosphate. Regen Reconstr Restor 2019;4(3):*91-97*. Doi: 10.22037/rrr.v4i3.26000.