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In vitro Adhesion and Proliferation of MG63 Osteoblast-Like Cell Line on Bio-Oss and Bone+B Xenografts

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Abstract

Background

This study aimed to assess the adhesion and proliferation of MG63 osteoblast-like cell line on Bio-Oss and Bone + B xenografts.

Methods

In this in vitro experimental study, MG63 osteoblast-like cells were added to the culture medium in wells of a microplate at a density of 5 x 10^4 cells/well. Each well contained 100 mg of either Bio-Oss or Bone + B xenograft particles. Cell adhesion to xenografts was evaluated after 24 hours under a scanning electron microscope (SEM) while cell proliferation was evaluated after 3 and 5 days by the methyl thiazolyl tetrazolium (MTT) assay. Data were analyzed by the Kruskal-Wallis, Mann-Whitney, and Wilcoxon tests (alpha = 0.05).

Results

Cell adhesion was higher in the control group (culture medium without xenograft powder) than the Bone + B and Bio-Oss groups; this difference was statistically significant for Bone + B ($P < 0.05$) but not for Bio-Oss ($P > 0.05$). Cell adhesion to Bio-Oss was slightly, but not significantly, higher than Bone + B ($P > 0.05$). At 3 and 5 days, cell proliferation was significantly higher in Bone + B and Bio-Oss groups compared with the control group, and also in Bio-Oss group compared with Bone + B group ($P < 0.05$).

Conclusions

This in vitro study showed comparable adhesion of MG63 osteoblast-like cells to Bio-Oss and Bone + B xenografts. However, cell proliferation was significantly higher in the Bio-Oss group, indicating that different xenografts have different effects on biological behavior of MG63 osteoblast-like cells.

Introduction

Bone regeneration following resorption due to tooth extraction, periodontal disease, or trauma has always been a challenge in dentistry which led to development of guided bone regeneration (GBR) techniques.[1] Different materials and techniques have been proposed to enhance new bone formation, improve the quality of the newly formed bone, and decrease the treatment time (especially for implant placement) in GBR. Also, attempts have been made to enhance the application of bone graft materials and improve their effects on biological processes involved in bone regeneration.[2, 3] Bone graft materials, also known as bone substitutes, are commonly used for GBR. Bone graft materials used for bone regeneration have different potentials for osteogenesis, osteoconduction, and osteoinduction.[4]

To date, several different bone graft materials including autografts, allografts, alloplasts, and xenografts with various levels of success have been used for regeneration of bone defects.[5]

Autografts can be harvested from intraoral or extraoral donor sites, and are the gold standard for GBR in the head and neck region since they possess osteogenic, osteoinductive, and osteoconductive properties altogether.[6] Nonetheless, they have drawbacks such as donor site morbidity, fast resorption, and the need for experience and expertise of the clinicians, which led the researchers to search for alternatives.[6] Allografts also have drawbacks such as limited availability, risk of disease transmission, fast resorption, immune rejection, infection, and technical problems in vascular grafts.[7] Alloplasts are more popular due to their higher safety and low cost. Calcium sulfate, calcium phosphate ceramics and cements, bioactive glasses, and their compounds are among the commonly used synthetic bone substitutes. Although they do not have some of the drawbacks of the aforementioned materials, they are not osteoinductive, and therefore, cannot be compared with autogenous bone in terms of quality and duration of bone regeneration.[8]

Considering the limitations of autografts, allografts and alloplasts, xenografts have attracted the attention of researchers and clinicians as the next best option after autografts for regeneration of bone defects.[7] Xenografts are bone substitutes with osteoconductive properties that serve as a scaffold to support new bone formation and its later replacement with the host tissue.[9] Xenografts can be derived from different species such as cows, pigs, camels, or ostriches; nonetheless, bovine bone is more commonly used for this purpose.[8] It undergoes thermal processes at a controlled temperature, which result in formation of an inter-connected porous system with 36% to 80% porosity rate. The particles have smooth borders and sufficient surface for the activity of osteoblasts. These properties significantly enhance bone regeneration and improve the quality of the newly formed bone.[8] Xenografts have been successfully used for regeneration of bone defects. Evidence shows that their rough surface topography enhances ectoblastic retention, cell proliferation, and formation of bone matrix on their surface.[10] Xenografts are currently among the most commonly used biomaterials for GBR around dental implants, sinus floor augmentation, and alveolar ridge augmentation.[11, 12]

It has been reported that application of Bio-Oss for sinus floor augmentation results in formation of 39% new bone after 6 months, which is comparable to 40% new bone formation at 6 months following the application of autografts.[13] A prospective 5-year study by Özkan et al.[14] demonstrated that one-stage sinus floor augmentation by using bovine bone graft resulted in adequate quality and quantity of bone for predictable implant placement. It has been clinically confirmed that application of Bio-Oss, as a valuable bone substitute, results in good-quality new bone formation and guarantees a long-term survival rate after dental implant surgery.[13]

A noteworthy issue in synthesis of xenografts is to eliminate all the organic components without compromising their natural biological properties. Moreover, not only the xenogeneic antigens, but also all cells and organic compounds should be eliminated as much as possible.[9]

Natural porous microstructure, optimal calcium/phosphorous ratio, favorable biocompatibility, high mechanical strength, and strong osteoinductive properties are among the advantages of xenografts. High cost, time-consuming manufacturing process, and ethical issues are among the drawbacks of xenografts. Different manufacturing processes of xenografts can affect their biological, mechanical, and physical properties, which should be taken into account in clinical practice.[8]

Osteoblasts are imperative for bone remodeling, and play a fundamental role in bone tissue regeneration. Osteoblasts proliferate on the surface of biomaterials and respond to external signals; this process is known as cell-material interaction. Cell adhesion is the first step of this interaction, which depends on the expression of cell adhesion molecules. Dynamic contact of cells with the extracellular matrix regulates cellular physiological processes such as growth and proliferation, cell cycle, migration, and differentiation.[15]

Bone substitutes initially serve as a scaffold for space maintenance, enabling the penetration and proliferation of bone cells. Thus, the host response to scaffolds is a determining factor for successful GBR.[16] Some scaffold properties such as their chemical and phasic composition, pore size, and cross-linking affect cell proliferation and osteogenesis.[1]

The osteoconductive property of bone substitutes depends on the adhesion of osteoblasts to their surface, and their subsequent migration and proliferation along the scaffold. Accordingly, the adhesion of osteoblasts is highly important. Cell adhesion is the first step of cell-material interaction and its quality affects subsequent cell functions.[17]

To the best of the authors' knowledge, the adhesion and proliferation of MG63 osteoblast-like cells to Bio-Oss and Bone + B have not been previously compared. Thus, this study aimed to assess the adhesion and proliferation of MG63 osteoblast-like cell line on Bio-Oss and Bone + B xenografts.

Materials and Methods

This in vitro, experimental study was conducted on MG63 osteoblast-like cell line and Bio-Oss (Geistlich Pharma, Wolhusen, Switzerland) and Bone + B (NavaTeb Pars, Tehran, Iran) xenografts. The study protocol was approved by the ethics committee of the university (IR.IAU.KHUISF.REC.1401.398).

Sample size:

The sample size was calculated to be 8 in each group according to a previous study [18] assuming alpha = 0.05, beta = 0.2, and study power of 80% to detect a difference by maximally 46% of the standard deviation.

Cell preparation:

Second-passage MG63 osteoblast-like cell line was obtained from the Rouyan Institute, Isfahan, Iran. The cells were placed in a cell culture flask containing Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 11% fetal bovine serum, penicillin, streptomycin, and amphotericin B with 100 µg/mL concentration. The cells were cultured in an incubator at 37°C and 5% CO2 under humid conditions. After reaching 90% confluence, the cells were transferred to a larger culture plate under a hood and the overlaying medium was removed. The cells were rinsed twice with phosphate buffered saline (PBS) without magnesium and calcium, and trypsin was then added to the cells. The cells were incubated at 37°C for 3 minutes and after detachment of cells from the bottom of the plate. Next, culture medium containing serum was added to the cells to neutralize trypsin. The cells were subsequently centrifuged and the supernatant was discarded. The cell sediment was suspended in a small amount of culture medium. Some cells were then transferred to a new labelled flask and incubated.

Interaction of cells with bone substitutes:

All wells of a 24-well plate were coated with 200 µL of agar (0.1%) except for the control wells to prevent attachment of cells to the bottom of the well. Next, 100 mg xenograft particles were added to each well under a sterile hood and the plate was refrigerated for 24 hours. Xenografts were rinsed with 500 µL of PBS twice. Next, 5×10^4 MG63 cells were added to each well, and the plate was incubated at 37°C for 24 hours under humid conditions and in presence of 5% CO2. Sampling was performed on days 1, 3 and 5. [19] The following groups were evaluated:

Group 1: MG63 cells + Bio-Oss xenograft

Group 2: MG63 cells + Bone + B xenograft

Control group: MG63 cells in the culture medium with no bone substitute

Assessment of cell adhesion:

In the present study, 50,000 cells were exposed to powders and allowed 24 hours for the maximum adhesion to occur. The methyl thiazolyl tetrazolium (MTT) assay was used for evaluation of cell adhesion. Initial sampling for assessment of cell adhesion to xenografts was performed after 24 hours. [18, 20, 21] MTT was added after 24 hours and the plate was incubated for 3 hours. The culture medium was removed before addition of MTT, and the xenografts were rinsed with PBS three times to remove the unattached cells. Attachment of cells to the bottom of the plate in the control group was ensured under an inverted microscope (Sunny China) at ×40 magnification.

Assessment of cell proliferation:

The MTT assay was used for this purpose as explained for assessment of cell adhesion except that incubation was performed for 3 and 5 days.[19] The overlaying medium was discarded and 200 µL of the culture medium containing 0.5 mg/mL MTT solution was added to each well at 3 and 5 days, and after 3 hours of incubation at 37°C, 100 µL of the contents of each well of a 24-well plate was transferred to three wells of a 96-well plate. The optical density (OD) of the solutions was read by an ELISA Reader (Fluostar Optima, BMG Lab Technologies, Ortenberg, Germany) at 450 nm wavelength. A standard curve was used to quantify the number of cells. Each test was repeated 8 times. It should be noted that three wells in the 96-well plate contained agar alone to calculate the OD of agar, and subtract it from the OD values of the wells to calculate the pure OD of the samples.[1]

Scanning electron microscopy (SEM):

Prior to addition of MTT, samples of both xenografts were initially fixed with 2.5% glutaraldehyde for 2 hours, and rinsed with double-distilled and autoclaved water 5 times for 20 minutes. They were then dehydrated with 25%, 50%, 75%, and 95% alcohol (each concentration once) for 5 minutes, followed by 100% ethanol for 10 minutes. The specimens were then dried in a desiccator for 48 hours. They were then mounted on an aluminum plate and placed in a sputter-coater (Hummer) for 20 minutes to sputter-coat a thin layer of gold with 15 nm diameter on the surface of materials. Next, they underwent SEM (FE-SEM; Hitachi S4160, Tokyo, Japan) at x3000 and x6000 magnification and photographed (to ensure cell adhesion to bone powder).

Statistical analysis:

The non-parametric Kruskal-Wallis test was used for comparison of the three groups. Pairwise comparisons were performed by the Mann-Whitney test. The Wilcoxon signed rank test was applied to analyze the effect of time (3 and 5 days) on cell proliferation. All statistical analyses were carried out using SPSS version 23 at 0.05 level of significance.

Results

Cell adhesion:

Table 1 presents the mean cell adhesion in the three groups. The three groups had no significant difference in cell adhesion (P = 0.18). Pairwise comparisons showed that cell adhesion was higher in the control group (culture medium without powder) than the Bone + B and Bio-Oss groups; this difference was statistically significant for Bone + B (P = 0.03) but not for Bio-Oss group (P = 0.48). The difference between Bio-Oss and Bone + B groups was not significant (P = 0.27).

Table 1
Mean cell adhesion in the three groups

Group	Mean	Std. deviation
Bio-Oss	0.99	0.04
Bone^{+}B	0.92	0.06
Culture medium (control)	1	0.0

Cell proliferation:

Table 2 presents the mean cell proliferation in the three groups. Within-group comparison of cell proliferation at 3 and 5 days by the Wilcoxon test revealed no significant difference in cell proliferation between 3 and 5 days in neither the Bio-Oss (P = 0.1) nor the Bone + B (P = 0.1) group.

Table 2
Mean cell proliferation in the three groups

Group	Day 3		Day 5	
	Mean	**Std. deviation**	**Mean**	**Std. deviation**
Bio-Oss	2.06	0.19	2.68	0.13
Bone^{+}B	1.02	0.02	1.23	0.06
Culture medium (control)	1	0.00	1	0.00

Comparison of the three groups revealed significant differences in cell proliferation among them at both 3 (P = 0.02) and 5 (P = 0.02) days. Pairwise comparisons by the Mann-Whitney test at 3 days revealed significantly higher cell proliferation in the Bio-Oss than the control (P = 0.03) and Bone + B groups (P = 0.03). Cell proliferation in the Bone + B group was also significantly higher than that in the control group (P = 0.03). At 5 days, significantly higher cell proliferation was noted in the Bio-Oss than the control (P = 0.03) and Bone + B groups (P = 0.04). Cell proliferation in Bone + B group was also significantly higher than that in the control group (P = 0.03).

SEM results:

Figures 1 to 4 show SEM micrographs of cell adhesion to xenograft materials at x3000 and x6000 magnifications.

Discussion

This study assessed the adhesion and proliferation of MG63 osteoblast-like cell line on Bio-Oss and Bone + B xenografts. Cell adhesion and proliferation on the extracellular matrix substrate are critical for tissue engineering.[22, 23] Cell adhesion to the substrate surface occurs in a four-stage process which includes adhesion of glycoproteins to the substrate surface, cell contact, attachment and spreading on

the surface. Cell migration and proliferation occur after these four stages.[24] In the present study, cell adhesion was higher in the control group (culture medium without powder) than the Bone + B and Bio-Oss groups; this difference was statistically significant for Bone + B ($P < 0.05$) but not for Bio-Oss ($P > 0.05$). Also, Bio-Oss showed slightly but not significantly higher adhesion than the Bone + B group. It should be noted that high adhesion in the control group indicates the ability of the cells to adhere to surfaces.

Esfahanian et al.[1] compared the behavior of periodontal ligament stem cells following exposure to different bone graft materials, and observed significantly greater cell adhesion to xenografts, compared with allografts and alloplasts. They attributed such a high cell adhesion to the rough surface topography and presence of variable-size pores on the surface of xenograft particles as shown on SEM micrographs. Significant capillary effect and hydrophilicity of the xenografts were also mentioned as other factors contributing to the higher adhesion capacity of xenografts.[1] Adhesion of cells to both xenografts in the present study indicated their optimal biocompatibility.

The present results regarding cell proliferation revealed significantly higher cell proliferation in both Bone + B and Bio-Oss groups than the control group at both 3 and 5 days. Stephan et al.[25] evaluated the attachment and proliferation of human osteoblasts exposed to bovine bone and reported results similar to the present findings. Also, Açil et al.[10] reported similar results regarding the adhesion and proliferation of osteoblasts. Ayobian-Markazi et al.[26] compared Bio-Oss, Tutodent, and Cerasorb regarding viability of SaOS-2 osteoblast-like cells. They reported higher cell viability in the control (no bone substitute) group. However, in the present study, significant differences were noted between the two xenograft groups with the control group, which can be due to differences in bone substitutes or type of cells used. Turhani et al.[27] demonstrated a reduction in proliferation of human osteoblasts cultured on Bio-Oss and added that low cell density of the primary culture was a limitation of their study.

A wide range of bone substitute densities have been used in the literature, ranging from 8 to 100 mg.[28] Miron et al.[28] evaluated the effect of bone substitute density on cell behavior and showed that application of high density of graft materials covering the bottom of the wells or culture plates entirely (over 100 mg per well) would result in over-estimation since cells adhere not only to the materials but also to the plastic bottom of the wells.[28] In the present study, 100 mg of each xenograft was used in each well, such that the bottom of the wells was completely covered with the material. Moreover, the well bottom and walls were coated with agar to prevent cell adhesion, and obtain more accurate results. Over-estimation of cell proliferation due to higher density of materials is probably due to increased available surface for cell adhesion and proliferation.[25]

In the present study, cell proliferation in both xenograft groups was greater on day 5 than day 3, but this difference was not significant. At both 3 and 5 days, cell proliferation was significantly greater in the Bio-Oss group than the Bone + B group. Khojasteh et al.[29] showed that Bio-Oss improved cell proliferation and differentiation. Jiang et al.[30] indicated that surface wetting and water sorption affect the molecular movement and biological behaviors of cells such as their proliferation. Thus, differences

between Bio-Oss and Bone + B can be due to their different water sorption properties. Lafzi et al.[4] compared biological reactions of dental pulp stem cells to three xenograft powders: a type derived from camel, Bio-Oss, and Bone + B. They observed higher cell proliferation in the camel xenograft group, and concluded that it probably has osteoinductive properties. Information regarding residual proteins in xenografts and their biological behavior is controversial.[31] According to some studies, the residual proteins in hydroxyapatite have osteoinductive properties.[32–35]. Kübler et al.[31] showed that different bone graft materials have different effects on the pattern of proliferation of human osteoblasts in vitro.[31] Also, it has been demonstrated that chemical composition, and geometrical and structural properties of scaffolds such as their pore size, porosity rate, and cross-linking affect biological responses.[35]

This study had an in vitro design, and complex cell interactions that take place in vivo cannot be perfectly simulated in vitro. Thus, the results should be generalized with caution to the clinical setting. Also, only one cell type and one time point (for cell adhesion) were assessed in the present study. Future studies are required on other cell types over longer periods of time. Moreover, cell differentiation and mineralization on Bone + B should be investigated in future studies.

Conclusion

This in vitro study showed comparable adhesion of MG63 osteoblast-like cells to Bio-Oss and Bone + B xenografts. However, cell proliferation was significantly higher in the Bio-Oss group, indicating that different xenografts have different effects on biological behavior of MG63 osteoblast-like cells.

Declarations

Competing Interests

The authors have no competing interests to declare that are relevant to the content of this article.

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No funding was received for conducting this study.

Author Contribution

All authors have made substantial contributions to conception and design of the study. Mozhdeh Bourbourshirazi Mahmoudi, Ehsan Rafiei, have been involved in data collection and data analysis. Mozhdeh Bourbourshirazi Mahmoudi, Ehsan Rafiei, Maryam Ostadsharif have been involved in data interpretation, drafting the manuscript and revising it critically and have given final approval of the version to be published.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request

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Figures

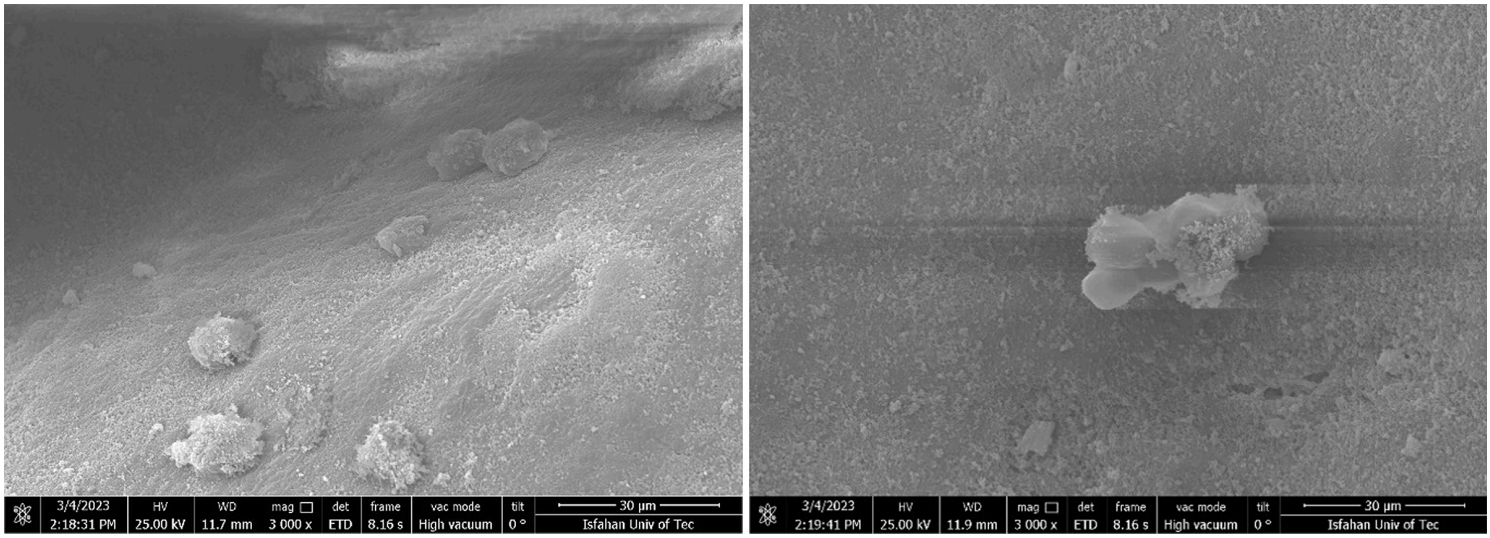


Figure 1

Bone+B xenograft at x3000 magnification



Figure 2

Bone+B xenograft at x6000 magnification



Figure 3

Bio-Oss xenograft at x3000 magnification



Figure 4

Bio-Oss xenograft at x6000 magnification