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# Comparison of cell viability and morphology of a human osteoblast-like cell line (SaOS-2) seeded on various bone substitute materials: An *in vitro* study

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## ABSTRACT

**Background:** Many studies have shown favorable results following the use of different bone graft materials. The aim of the present study was to evaluate the biocompatibility of four different bone graft materials regarding cell viability and morphology of Human osteoblast-like cells (SaOS-2) *in vitro*.

**Materials and Methods:** The effects of Bio-Oss®, Tutodent®, Osteon®, and Cerasorb® were studied on the human osteoblast-like cell line to evaluate various parameters. Human osteoblast-like cells were seeded onto the mentioned bone substitute materials (BSMs). Cell differentiation; cell viability and alkaline phosphatase (ALP) activity of the seeded cells were evaluated by means of scanning electron microscopy, cell viability test and phase contrast microscopy Analysis of variance (ANOVA). Tamhane´s post-hoc, Kruskal-Wallis Test, and Dunn's Test were used. The results were considered to be statistically significant at $P<0.05$.

**Results:** The control group (SaOS-2 cells which were incubated in Dulbecco Modified Eagle Medium without any kind of bone graft materials) had the highest level of cell viability ($P<0.001$), followed by Tutodent®, Osteon®, Cerasorb®, and Bio-Oss®. There was no significant difference in MTT assay results between Tutodent® and the control group ($P=0.032$). All tested bone graft materials showed significantly higher ALP activity than the control ($P<0.001$). The Tutodent® group showed the best cell growth among all experimental groups, followed by the Osteon® group. The former had a higher spindle-like morphology with good attachment to the surface. Cells cultivated on the surfaces of the Cerasorb® and Bio-Oss® granules had more round morphologies.

**Conclusion:** This *in vitro* study demonstrated that all tested BSMs can provide good cell differentiation but a lower rate of proliferation.

**Key Words:** Alkaline phosphatase, bone graft, cell culture, cell morphology, cell viability, osteoblast

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## INTRODUCTION

Bone substitute materials (BSM) are commonly used for bone augmentation. Autogenous bone is considered the gold standard, although it has disadvantages such as limited availability, morbidity of the donor site, and insufficient biomechanical properties.[1,2] As a result of these potential problems, several allogeneic, xenogeneic, and synthetic BSMs have been developed. Many studies have shown favorable results following the use of different bone graft materials.[3-6] Clinical studies have mainly focused on the physical and chemical properties of these materials, such as particle size, porosity, and surface structure. Few studies have evaluated the physiological and histological behavior of BSMs. Biocompatibility and osteoconductivity are essential prerequisites for BSMs. *In vitro* studies have been a valuable method for evaluating cell interactions with the bone graft materials. The materials must provide a suitable environment for the attachment,

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proliferation, and differentiation of the cells.[7,8] Currently, most investigations use osteogenic cell lines to study cell interactions by adapting BSMs to the target cells *in vitro*.[7-11] Many studies have been conducted to evaluate the biocompatibility of different bone graft materials. Trentz *et al*.,[7] Mayer-wohlfart *et al.*,[10] and Herten *et al.*[12] investigated the biocompatibility of tutodent. Schmitt *et al.*,[8] Kübler *et al.,*[9] Acil *et al.,*[11] and Wiedmann-Al-Ahmad *et al.*[13] studied the behavior of cells seeded on Bio-Oss® and reported different results. The aim of the present study was to evaluate the biocompatibility of four different bone graft materials *in vitro*. The effects of Bio-Oss®†, Tutodent®‡, Osteon®¶, and Cerasob®r were studied on the human osteoblast-like cell line (SaOS-2) to evaluate various parameters.

## MATERIALS AND METHODS

### Cell culture

The human osteoblast-like cell line was purchased from the Pasteur Institute (Tehran, Iran). SaOS-2 cells were incubated in 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% Dulbecco Modified Eagle Medium (DMEM) and supplemented with 10% fetal bovine serum (Gibco®, Invitrogen™ GmbH, Karlsruhe, Germany). Incubation was conducted at 5% $CO_2$ at 37°C. The medium was changed three times during one week. The cells of the third passage were used for the experiment. The cells were washed with phosphate buffer solution (PBS) and detached with trypsin/Ethylenediaminetetraacetic acid (EDTA). A suspension of cells was prepared with a $2\times10^4$ cell density. Bone grafts including Bio-Oss®; Geistlich Bio-materials, Switzerland; Tutodent®, Microchips-Totugen Medical Gmbh, Germany; Osteon®, Dentium, Republic of Korea; and Cerasorb®, Curasan ag, Germany; in the form of granule with particle size between 0.25 to 0.5 mm (30 mg) were placed in 24-well plates and incubated with the culture medium for four hours. The prepared cells ($2\times10^4$ cell in 100 µl) were seeded on the bone graft materials in all wells containing the culturing medium. The control group contained the same amount of cell suspension, incubated in DMEM medium, without any of the BSMs. Each experimental group was divided into three groups to evaluate the cell morphology, cell viability, and alkaline phosphatase (ALP) activity.

### Cell proliferation and viability

Cell proliferation and viability were measured by the MTT (3-4,5-Dimethylthiazol2,5-diphenyltetrazolium bromide) assay. After day 15, the remaining medium was removed, and the cells were washed with PBS. Then, 100 µl of culture media and 10 µl of MTT solution were added to each well and incubated in a humidified atmosphere of 5% $CO_2$ at 37°C for 4 hours. After incubation, the cells were washed with PBS solution. Subsequently, 100 µl of isopropanol acid 4% and hydrochloric acid were added to each well, and the cells were incubated at room temperature for 10 minutes. The absorbance was measured by Elisa Reader (Anthos 2020 ver1.8, Anthos Lab Tec Instruments®, Austria) at 492 nm with 620 nm as reference.

### Investigation of cell morphology by scanning electron microscope

The cell growth and proliferation patterns were investigated by scanning electron microscopy 21 days after incubation. The cells were fixed with 2.5% glutaraldehyde for two hours and washed three times with PBS solution. One percent osmium tetroxide was used for the secondary fixation. After washing, dehydration of the samples was performed for 30 minutes through a graded ethanol series (in 50%, 70%, 90%, and 100% ethanol at 20 minutes each). Subsequently, the samples were subjected to sputter–coating with gold/palladium and were examined using a Vega–TEScan (Tescan USA Inc, USA) at 20 KV (it needs figures to represent scanning electron microscope (SEM).

### Alkaline phosphatase activity

An ALP kit (Sigma-Aldrich, Product No.85) was used to assay the ALP activity. After the cultivation period of 15 days, the SaOS-2 cells were fixed in 4% buffered formaldehyde, incubated for 15 minutes in an ALP staining solution (Sigma Deisenhofer, Germany) and stained with 6% hematoxylin. Phase contrast microscopy and software image J (g Image Processing Analysis in Java) were used to evaluate the ALP activity.

### Statistical analysis

All values are expressed as the mean±standard deviation. The statistical comparisons were made via analysis of variance (ANOVA). The Tamhane´s post-hoc test was used for evaluations of differences among the groups. The Kruskal-Wallis Test and Dunn's Test were used to compare the ALP activity. A software package (SPSS version 16, SPSS Inc, Chicago IL, USA) was used for the statistical analysis. The results were considered to be statistically significant at $P<0.05$.

## RESULTS

### Cell morphology

The morphology and the shape of the cells seeded on the surfaces of all bone grafting materials were examined by scanning electron microscopy. There were different patterns of growth and proliferation for the investigated materials. SEM images of the control group and four biomaterials seeded with SaOS-2 cells are shown in Figures 1-9. SEM revealed that all materials were covered by SaOS-2 cells. However, the appearance of cells varied with the surface characteristics of the different bone materials. The control group cells did not form a homogenous layer and appeared as round, separated cells. The Tutodent® group showed the best cell growth among all experimental groups. The SaOS-2 cells on the surface of the Tutodent® and Osteon® granules were elongated and had significantly higher spindle-like morphologies with good attachments to the surface. These features were more prominent in the Tutodent® group. In contrast, the cells cultivated on the surfaces of the Cerasorb® and Bio-Oss® granules had more round morphologies compare with the Tutodent® group. The worst results were observed for the Bio-Oss® group. The majority of cells, which were seeded onto the Bio-Oss®, produced a monolayer cell sheet and had a round morphology.

### Cell proliferation and viability

The proliferation and viability of SaOS-2 cells on the bone graft materials are presented in Figure 1 and Table 1. There were significant differences between the tested bone graft materials and the control group. The control group had the highest level of MTT (0.528±0.049) at day 15 ($P$<0.001). The Tutodent®

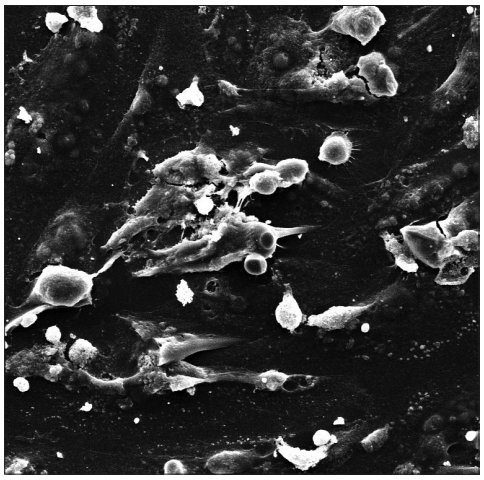

**Figure 1:** Scanning electron microscope of SaOS-2 cells in control group × 1000

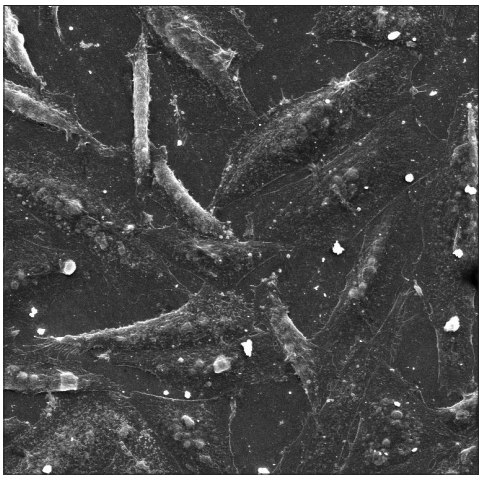

**Figure 2:** Scanning electron microscope of SaOS-2 cells seeded on Bio-Oss × 1000

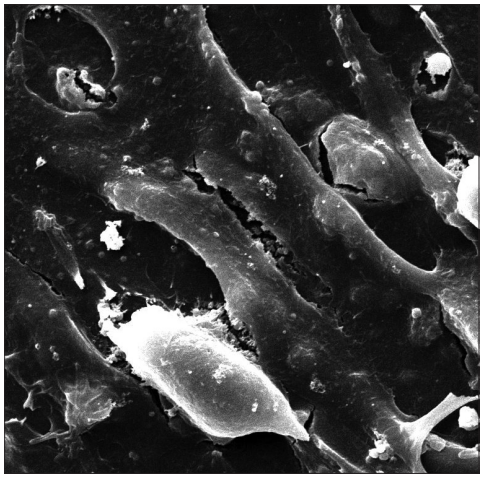

**Figure 3:** Scanning electron microscope of SaOS-2 cells seeded on Bio-Oss × 5000

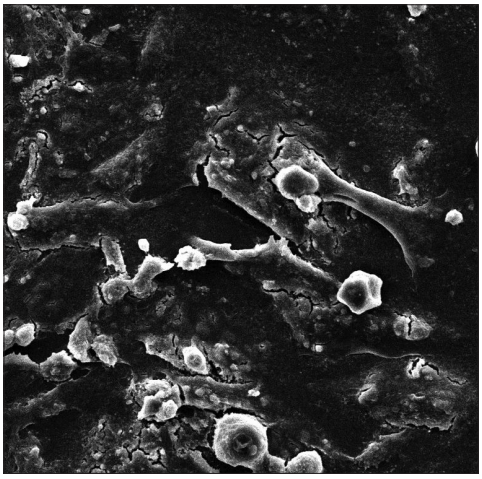

**Figure 4:** Scanning electron microscope of SaOS-2 cells seeded on Osteon × 1000

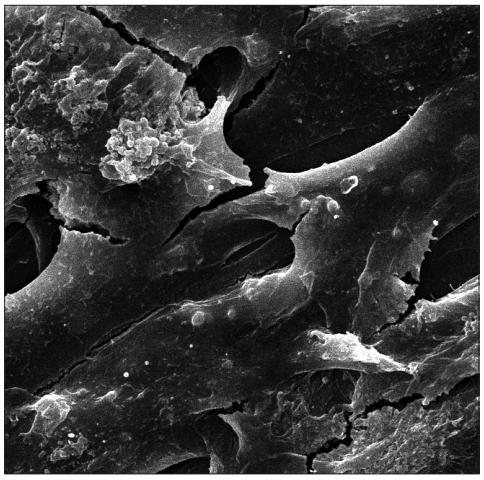

**Figure 5:** Scanning electron microscope of SaOS-2 cells seeded on Osteon × 5000

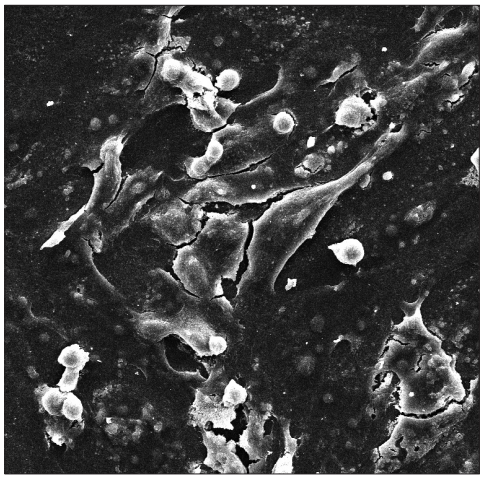

**Figure 6:** Scanning electron microscope of SaOS-2 cells seeded on Cerasorb × 1000

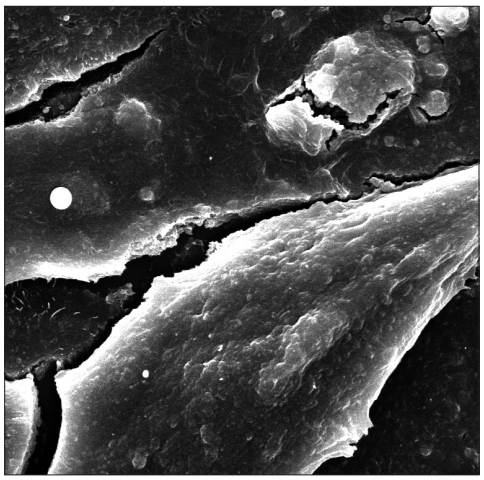

**Figure 7:** Scanning electron microscope of SaOS-2 cells seeded on Cerasorb × 5000

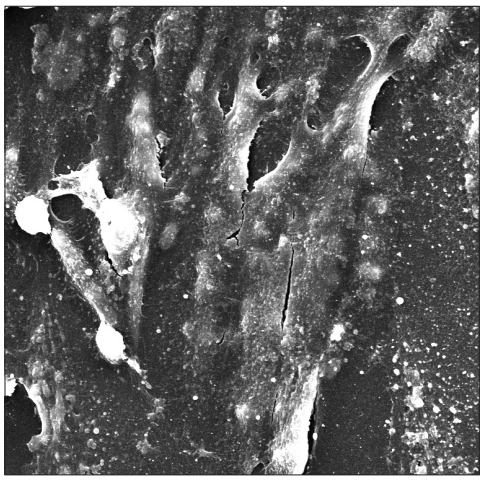

**Figure 8:** Scanning electron microscope of SaOS-2 cells seeded on Tutodent × 2000

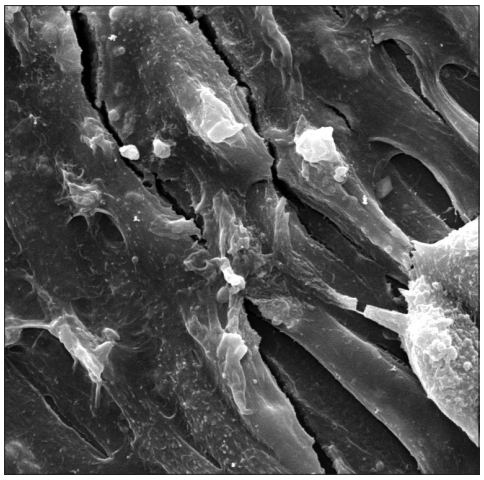

**Figure 9:** Scanning electron microscope of SaOS-2 cells seeded on Tutodent × 5000

**Table 1: Cell viability measured by MTT test**

| Bone graft | No. | Mean | SD | Min | Max | *P* value |
|---|---|---|---|---|---|---|
| Totudent | 12 | 0.348 | 0.026 | 0.30 | 0.38 | *P*<0.001* |
| Osteon | 12 | 0.262 | 0.046 | 0.23 | 0.29 | |
| Cerasorb | 12 | 0.256 | 0.087 | 0.20 | 0.37 | |
| Bio-oss | 12 | 0.222 | 0.04 | 0.17 | 0.29 | |
| Control | 4 | 0.528 | 0.049 | 0.49 | 0.60 | |

*Significant (ANOVA test), SD: Standard Deviation

group had a significantly higher level of MTT than the other tested materials ($P<0.001$). The Tutodent® group was followed by the Osteon® and Cerasorb® groups; the Bio-Oss® group had the lowest level (0.222±0.04) [Figure 10]. There were no significant differences among the Osteon®, Cerasorb®, and Bio-Oss® groups. There was no significant difference for the results of the MTT assay between the Tutodent® and the control group. Post hoc tests (Tamhane's T2) were used to determine statistical differences between the means. The results of the post hoc tests are shown in Table 2.

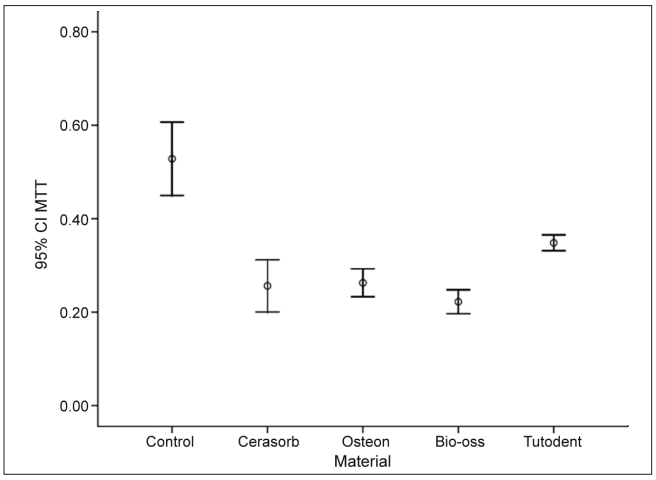


**Figure 10:** Error bar of means and 95% confidence interval of cell viability measured by MTT test

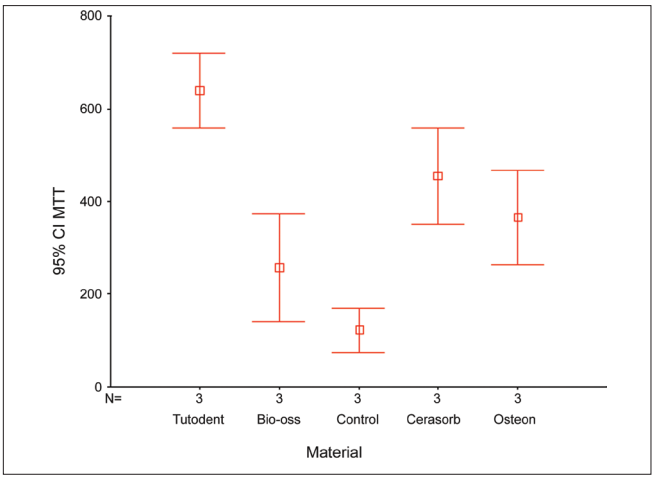


**Figure 11:** Error bar of means and confidence interval of alkaline phosphatase activity of tested biomaterials on day15

### Table 2: Post hoc test results of MTT between bone graft materials

| Groups | *P* value |
|---|---|
| Totudent-Control | 0.032 |
| Totudent-Osteon | <0.001 |
| Totudent-Bio-oss | <0.001 |
| Totudent-Cerasorb | 0.04 |
| Bio-oss-Cerasorb | 0.93 |
| Bio-oss-Control | 0.002 |
| Bio-oss-Osteon | 0.28 |
| Cerasorb-Control | <0.001 |
| Cerasorb-Osteon | 1.00 |
| Osteon-Control | <0.001 |

### Table 3: Alkaline phosphate activity of cells seeded onto tested biomaterials

| Alkaline phosphatase activity | No. | Mean | SD | Min | Max | *P* value¶ |
|---|---|---|---|---|---|---|
| Tutodent | 3 | 637.3 | 32.5 | 600 | 660 | <0.001 |
| Osteon | 3 | 364.3 | 40.7 | 321 | 402 | |
| Bio-Oss | 3 | 256.6 | 47 | 208 | 302 | |
| Cerasorb | 3 | 455.0 | 41.5 | 408 | 487 | |
| Control | 3 | 121.6 | 19.5 | 103 | 142 | |

¶Kruskal Wallis test, SD: Standard deviation

### Alkaline phosphatase activity

The differentiation of SaOS-2 cells was evaluated by histological staining of osseous ALP in cells seeded onto four tested materials. All of the tested bone graft materials showed significantly higher ALP activities compared with the control group [Figure 11 and Table 3]. Cells seeded onto Tutodent had the highest ALP activity (637±32.5), followed by Cerasorb® (455±41.5), Osteon® (364±40.7), Bio-Oss® (256.6±47), and the control group (121.6±19.5).

## DISCUSSION

In this experimental study, we used an *in vitro* culture of SaOS-2 cells to investigate the growth behavior of cells seeded onto four different BSMs. In previous investigations of cell behavior seeded onto BSMs, *in vitro* cell culturing has been shown to be valuable.[7-11] It has been shown that the adherence of osteoblasts to bone graft materials is necessary for bone formation and that surface morphology of the bone graft has an essential role in cell attachment.[14] In the present study, we used ALP activity and cell morphology as markers for osteoblastic differentiation. The highest differentiation was observed for cells seeded onto Tutodent®, followed by Osteon®, Cerasorb®, Bio-Oss®, and the control group. SaOS-2 cells on the surfaces of the Tutodent® and Osteon® granules were elongated and had more spindle-like morphologies with good attachment to the surfaces. In contrast, cells cultivated on the surfaces of the Cerasorb® and Bio-Oss® granules had rounder morphologies. Mayer–Wohlfort *et al*.[10] showed that on the surface of Tutodent®, cells were flat and well spread out but that the cells seeded onto α-Tricalcium phosphate (TCP) had better cell morphologies. Cells also had long extensions that covered distances in excess of 10 μm. Trentz *et al*.[7] reported that after 14 days, allogeneic and xenogeneic SDCB (solvent-dehydrated cancellous bone) discs were completely covered with a dense homogenous layer of human osteoblasts with a spindle-like morphology. Tutodent® has residual proteins in its structure; it has been reported that residual proteins in hydroxyapatite have osteoinductive properties.[15] Kübler *et al*.[9] reported that the cells in Bio-Oss showed poor differentiation

and were not attached. According to the authors, the relatively smooth surfaces of the Bio-Oss granules do not support cellular attachment. It has been shown that the morphology and roughness of the surface affect the attachment and differentiation of the osteoblasts and may enhance cell spreading as well.[16]

Our results showed that Bio-Oss suppresses the proliferation and differentiation of osteoblasts. The experimental study performed by Wiedmann-Al-Ahmad *et al*.[13] and Schmitt *et al*.[8] showed a low proliferation rate for Bio-Oss, which is in agreement with our findings. In contrast to our findings, however, Acil *et al*.[11] showed good results for the cultivation of primary osteoblasts onto Bio-Oss®. The different results, according to Herten *et al*.,[12] "could be due to different protocols since the BSM was in blocks and not granular, the cells were seeded in a higher density. In line with the present study, Aybar *et al*.[17] showed that Cerasorb® did not have significant differences with the control group in supporting osteoblast proliferation. One reason for the observed differences among the tested bone graft materials may be the differences in roughness and porosity. In bone grafts, attachment and proliferation of the osteoblasts are important factors for bone formation. Cell growth and proliferation depend not only on the chemical composition of the graft but also on the roughness and porosity of the surfaces. The amount of porosity and pore sizes could affect cell growth and morphology.[18] A second reason may be the effect of these materials on the pH of the medium because the release of phosphate ions from the materials could suppress cell growth.[19]

All of the tested materials led to decreases in the cell viability of SaOS-2 cells. The results showed that these materials, despite good effects on cell differentiation, decreased cell proliferation. The control group had the highest cell viability rate, followed by the Tutodent®, Osteon®, Cerasorb, and Bio-Oss® groups. In contrast to our results, Herten *et al*.[12] observed the highest cell viability for Tutodent, followed by the control group and Cerasorb. Bio-Oss showed low cell viability in that study. It has been shown that in the presence of Bio-Oss, the cell viability of osteoblasts would be decreased.[9,13] As mentioned previously, Acil *et al*.[11] reported good cell viability results for Bio-Oss. Biphasic calcium phosphates (BCPs) consist of a mixture of hydroxyapatite and beta-tricalcium phosphate and are recommended as alternatives to autogenous bone grafts for implant dentistry.

Alcaide *et al*.[20] cultured SaOs-2 cells onto HA/β-TCP discs and reported good biocompatibility and high percentages of viable cells on the discs. Saldana *et al*.[21] observed that the cell viability of human mesenchymal cells cultured onto BCP did not decrease after four days. The better results for Osteon compared with those of Cerasorb could be due to the higher solubility of the β-TCP compound, which may have facilitated subsequent bone growth in the remnants of the hydroxyapatite particles. However, we found that the cells cultured onto Osteon, a BCP, had statistically lower cell viability compared with the control group. The different results in our study may be due to different protocols, such as the duration of culturing, bone graft materials, and methods used to evaluate cell viability.

Schmitt *et al*.[8] reported similar results following culturing of bovine osteoblast-like cells onto various BSMs. The authors observed the highest proliferation in the control group. Bio-Oss had the lowest rate of proliferation among the investigated materials. Moreover, SEM analysis showed that the Bio-Oss cells were sporadically attached and appeared to be flattened with long extensions.

SaOS-2 cell differentiation was evaluated by the histological staining of osseous ALP. Our study showed that all tested materials had higher ALP activities compared with the control group. Tutodent had the highest ALP activity; this finding was in agreement with the morphologic finding that more differentiation of cells to the osteoblastic phenotype was associated with more ALP activity. In line with the present study, Herten *et al*.[12] observed that Tutodent®, followed by Cerasorb®, had the highest ALP activities. Mayer-Wohlfort *et al*.[10] reported that SaOS-2 cells seeded onto α-TCP had more ALP activity than cells seeded onto Tutodent® (Solvent Dehydrated bone). They also found that cells seeded onto α-TCP had better osteoblastic phenotypes, which can explain their higher levels of ALP activity. Kubler *et al*.[9] reported that the highest ALP activity was related to the control group without any kind of bone graft material. The differences in the results could be due to the various cell lines and methods used to evaluate ALP activity.

Bone graft materials, depending on chemical properties and their impact on Ca ion concentration, can affect cell differentiation and bone formation.[21] Among the investigated materials, Tutodent® (due to the lack of calcium deficiency in its microstructure) seemed to prevent Ca2+ uptake, allowing the development of a functional osteoblast phenotype and ALP activity.

## CONCLUSION

This *in vitro* study demonstrated that xenogeneic hydroxyapatite (Bio-Oss, Tutodent®) and synthetic BSM (Osteon®, Cerasorb®) can provide good cell differentiation but lower rates of cell proliferation. Based on the chemical and physical properties, the different materials have varying levels of impact on the proliferation and differentiation patterns of osteoblastic cell lines *in vitro*. Structural and chemical characteristics of BSMs play a significant role on the behavior of osteoblasts. As such, in the selection of specific BSMs for clinical application, their impact on the proliferation and differentiation of osteoblasts should be considered.

## ACKNOWLEDGMENT

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