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Response of MC3T3-E1cells on microroughen bioactive glass coated zirconia

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ABSTRACT

Background & Objectives: The objective of this study was to determine the cellular response of micro-roughened bioactive glass coated zirconia substrate (ZBR) and non roughen bioactive glass coated zirconia substrate (ZB), and compare them with uncoated zirconia substrate (Z). **Materials & Methods:** Surface micro-roughening was obtained using an Al2O3 sandblasting method. Abrasive blasting of zirconia coated bioactive glass produced an irregular finish with surface roughness average Ra 0.85 µm as determined by profilometer and scan electron microscope. Surface roughness of the samples in ascending order was ZBR>ZB>Z. Murine derived preosteoblast (MC3T3-E1) cells were seeded on the samples, and the cell morphology, growth, differentiation, were observed. Cell morphology was evaluated by means of scanning electron microscope (SEM), while cell proliferation and differentiation using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test and alkaline phosphates activity respectively. **Results:** The cell growth on all the samples continual increase with culturing up to 5days, showing good cell viability. However, there was no significant difference ($p>0.05$) with respect to the Z, ZB, and ZBR at day 5 at MTT assay. In particular, the alkaline phosphatase (ALP) activity of the cells was significantly higher on the ZB and ZBR than Z samples at both 7 and 14 days. **Conclusion:** Our findings demonstrate that bioactive glass coated surface was found to have better surface conditions to regulate bone cell differentiation

Key words: Bioactive glass; surface roughness; zirconia

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INTRODUCTION

Since the term osseointegration was defined as a direct - on the light microscopic level - contact between living bone and implant,[1,2] biomaterial and research on dental implant systems have increased significantly. Zirconia was introduced to implant dentistry as metal-free framework material. Due to its tooth like color and outstanding mechanical properties like high flexural strength and fracture toughness[3,4] zirconia implant have the potential to become an alternative to titanium implant. Additionally, zirconia displays the significantly reduced plaque affinity, shallower probing depths were observed,[5,6] and several animal studies have shown that osseointegration is approximately the same as titanium.[7,8] Many in-vitro and in-vivo studies have demonstrated that bioactive glass as a coating material for implant has promising results.[9,10] Consequently, the material has become a better choice to be used as a coating on zirconia implants.

Furthermore, recently it was observed that bioglasses stimulates expression of several genes of osteoblastic cell,[11] angiogenesis in vitro and in vivo.[12] In vitro, bioactive glass enhances osteoblast attachment, proliferation, differentiation and mineralization.[3]

However, little information exists on the different surface topography of bioactive glass coated zirconia substrate. So, the aim of present study was to

analyse the effect of osteoblastic response on roughened bioactive glass coated and non roughened bioactive glass coated zirconia substrate and compare them with uncoated zirconia substrate.

MATERIALS AND METHODS

Sample preparation: Total Seventy-five zirconia samples were prepared from pure grade powders (yttria stabilised ZrO2, Y-PSZ stabilized with 3 mol % Y2O3). Disks of 10 mm in diameter and 1 mm in thickness were prepared by die pressing method. After drying, all samples obtained were fired in laboratory kiln with the following thermal cycle, increased at the rate of 100ºC /h up to the final temperature of 1550ºC, steady temperature for 1h and cooling at the rate of 200ºC/h. All samples were homogeneous compact, without apparent porosity. The samples were rinsed in ethanol, and were ultrasonically cleaned with distilled water and air-drying.

The samples were randomly divided into three groups. Fifty Samples were coated with bioactive glass. The glass had the following composition: 45% SiO_2, 24.5% CaO, 21.7% Na_2O, 2.8% MgO and 6% P_2O_5. The bioactive glass was applied as enamel to the surface of zirconia samples. Application was performed by brushing the slurry in suitable concentration on the surface of sample. After drying the coated samples, were fired in a laboratory kiln at 1170ºC to obtain the vitreous coating. The procedure follows heating rate of 200ºC/h to the final temperature of 1170ºC, steady temperature for 1h and then followed by nature cooling (about 5ºC/min).

Then 25 samples coated with bioactive glass were sandblasted with by 50 µm Al_20_3 at 2.5 bar, 90° against the surface, for 5s and 10mm away from substrate. The experimental zirconia samples were described below-

Z : zirconia samples with no surface modification (25 samples)

ZB : zirconia samples with bioactive glass coated (25 samples)

ZBR : zirconia samples with bioactive glass coated then sandblasted (25 samples)

Before use in cell culture experiments, all the specimens were ultrasonically cleaned with acetone and distilled water for 10 min, followed by sterilization in autoclave at 121ºC for 20 min.

Surface analysis:

The surface morphology of the samples was examined by scanning electron microscopy (SEM, Inspect F, FEI company, OR 98124-5793,USA). The surface roughness of the samples was measured from ten specimens of each group using a surface profilometer. Based on profilometric measurements average roughness (Ra) and peak to valley data (Rz) of different zirconia substrates were calculated. The ZB and ZBR samples obtained were then characterized by mean of Energy dispersive X-ray analysis (EDS) to identify the elemental composition of surface of samples.

All three groups of samples were soaked in an acellular SBF (simulated body fluid), having the same ion concentration as the human plasma. The composition of the solution is given in Table1. Each sample was soaked in 5ml SBF at 37°C, without stirring. After 14 days, they were removed from the solution, gently washed in distilled water, and dried at room temperature. Then samples were characterized by scanning microscopy (SEM) and compositional analyses (EDS), in order to observe the modification of their surface.

Cell culture:

MC3T3-E1 osteoblasts were selected to measure cell proliferation, cell morphology and alkaline phosphates activity. Osteoblast were grown in an incubator at 37ºC in humidified atmosphere of 5% C02 and 95% air, in α-modified Eagle's medium(α-MEM) containing 10% fetal bovine serum(FBS) and 1% penicillin /streptomycin with growth medium changed in every two days.

Cell morphology:

The Z, ZB, and ZBR substrates were placed in 24-well plate and seeded in density of 2 x 104 cells / ml. Cells morphology were observed on 3rd day. In designated time point, SEM observations were performed after fixing the cells with 2.5% glutaraldehyde, dehydrating them with graded ethanol (30%, 50%, 75%, 80%, 95%, and 100%), and critical point drying in CO2.

Cell proliferation:

The proliferation of the MC3T3-E1 cells on Z, ZB, and ZBR substrates was investigated by measuring

Table1. Molar Composition of SBF

Ion	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	HPO_4^-	SO_4^-
Concentration (mM)	142	5.1	1.5	2.5	148	4.2	1.0	0.5

number of cells after one, three, and five days of incubation. Cells were seeded onto the substrates at the density of 2 x 104cells/ml and cultured in a medium. The MTT test was performed to assess cell proliferation. At each time period, the disc were washed three times with PBS to remove unattached cells and transferred to new culture plates. The culture wells were incubated at 37ºC for four hours with 20µl reagent containing MTT(3-(4,5-Dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide) solution and 1ml culture medium. After discarding the supernatant, dark blue crystals of formazan were dissolved by adding 450 µl dimethylsulphoxide (DSMO) and after which 200 µl reaction solution was moved to 96- well plates, and the absorbance measured at 570 nm with a spectrophotometer.

Cell differentiation:

Alkaline phosphatase (ALP) is the early marker of osteoblast differentiation and relates to the production of a mineralized matrix.[13] For determination of the activity of ALP, the MC3T3-E1 cells were seeded onto to the substrates at the density of 2 x 104cells/ml. At seven, and 14 days of incubation, cell layers were washed with PBS and scraped off the samples. After clarifying the cell lysates by centrifugation, alkaline phosphatase activity was assayed according to the ALP kit (Merit Choice Bioengineering, Ltd, Beijing, China).

Statistical analysis:

The data were analyzed using SPSS16.0 for windows. As more than two groups were compared, statistical analysis was performed using a one-way analysis of variance (ANOVA) at the significance level of 5%. To find out which groups were different post hoc test, Tukey method was used for a comparison among specimens at a significance level of 5%.

RESULTS

Surface analysis:

Surface morphology and surface roughness:

When the disks were examined by SEM, the surfaces were found to be very different. Fig.1, 2, and 3 shows SEM image of surface microstructure. A smooth surface was observed on Z specimens (Fig.1), a part from minor surface irregularities due to sample processing. The ZB surfaces (Fig.2) had smooth homogeneous glass layer can be seen. Whereas, less homogenous roughen structure with edges and deep depressions were on were demonstrated in ZBR surfaces (Fig.3) due to sand blasting of Al_2O_3 particles.

The results of profilometric surface roughness assessment are listed in Table 2. Ra and RZ value increased as follows: Z< ZB< ZBR. Ra and Rz values of Z and ZB where significantly lower than ZBR ($P<0.05$).

Energy-dispersion X-ray analysis (EDS)

The results of EDS analysis (Table3) made on ZB and ZBR samples surface, confirmed that surface of both ZB and ZBR are coated with bioactive glass with almost similar surface composition i.e. sandblasting had only changed its surface

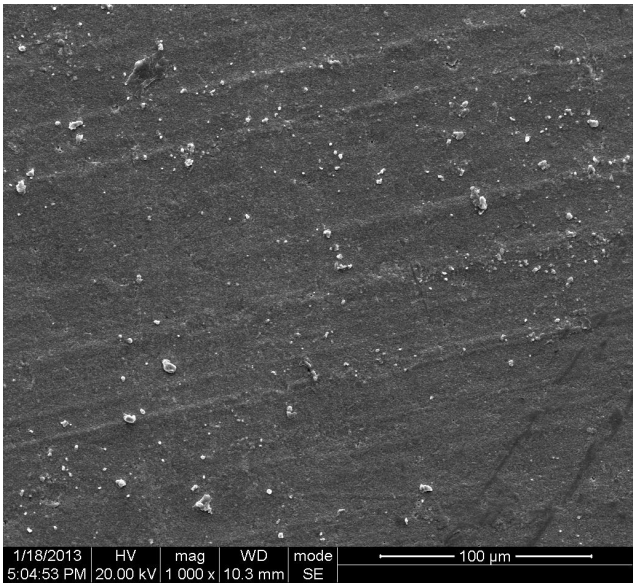

Fig.1 SEM image of Z surface.

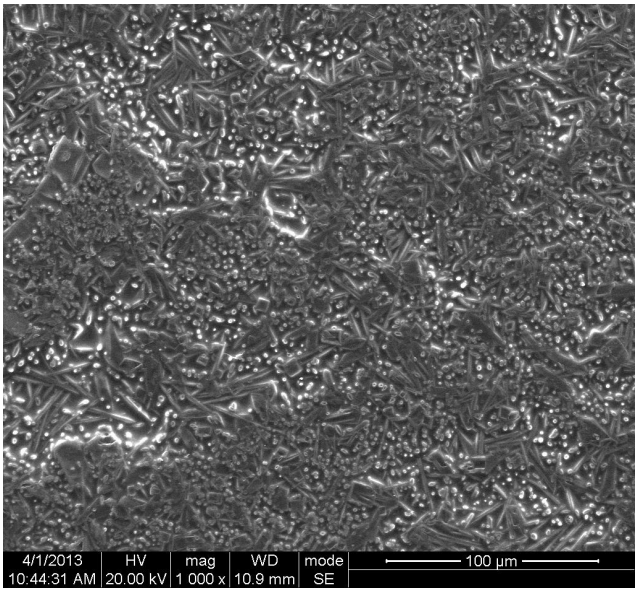

Fig. 2 SEM image of ZB surface.

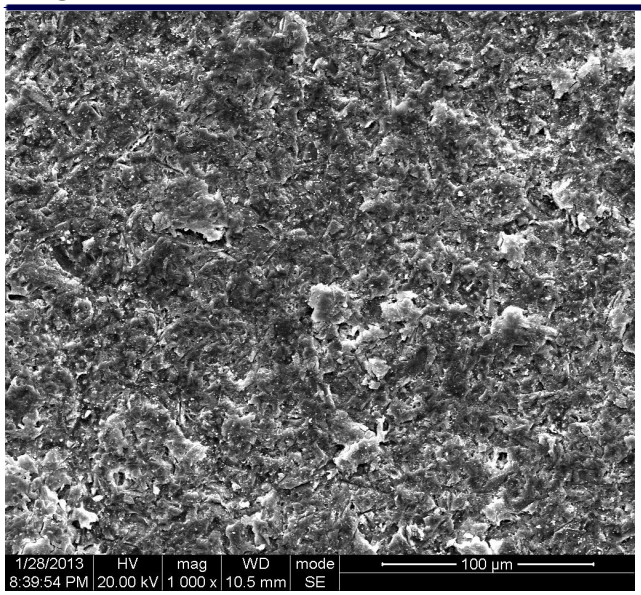

Fig.3 SEM image of ZBR surface.

Table 2. Roughness parameters of the surfaces.

	R_a µm	R_z µm
Z	0.23 (0.20)[a]	2.19 (0.19)[a]
ZB	0.54 (0.19)[b]	5.38 (0.55)[b]
ZBR	0.85 (0.05)[c]	7.29 (0.42)[c]

Data are presented as mean (SD) for n=10.Statistical significance was observed on all three surfaces (p<0.05).Different superscript letters indicate statistical significance at each parameter.

Table 3. EDS (Atomic%) of ZB and ZBR surface

	O	Si	Ca	P
ZB	54.26	21.32	15.04	2.89
ZBR	56.71	20.56	14.92	2.01

topography without changing the surface elemental composition.

The in vitro bioactivity of the Z, ZB and ZBR surface was observed by immersion in SBF for upto 14 days. Fig.4 shows SEM images of the ZB and ZBR samples after 14 days of immersion in SBF. There were no noticeable changes on the surface of Z samples, whereas ZB and ZBR samples were covered with some precipitates Fig.4 (a,b). EDS analysis on both ZB and ZBR surface reveals Ca/P ratio of 1.52 and 1.58 respectively which is close to the theoretical value for apatites (1.67).

Cell morphology

MC3T3-E1 cell seeded on to the smooth surface of Z and ZB, exhibited a flattened morphology with smooth cell extensions in contact with the surface Fig.5 (a, b). Conversely, more elongated/spindle shaped and thicker cells were present on the rougher surfaces Fig.5(c). Cells seeded on ZBR projected filopodia, creating individual contacts with the surface and anchoring points for cell adhesion.

Cell proliferation

The cell proliferation was assessed using an MTT method, as presented in Fig.6. The MTT test showed an increase with increasing culturing time for all groups. Only at day 1, ZB and ZBR showed significant increase in cell proliferation than Z samples ($P<0.05$), however no significant difference was found between ZB and ZBR ($P>0.05$). Although, on day 3 and day 5 appeared to be higher in the order ZB>ZBR>Z, the difference was not significant ($p>0.05$).

Alkaline phosphatase activity

ALP, which is an indicator of earlier osteoblast differentiation and mineralization, was measured at

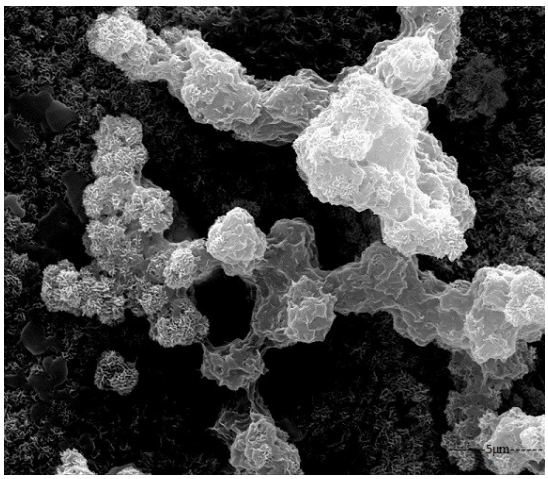

Fig.4 a

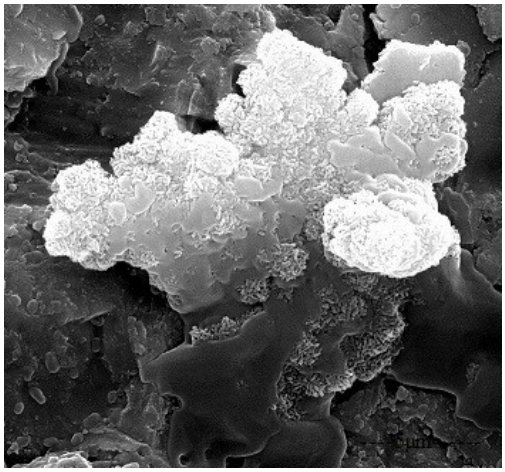

Fig.4 b

Fig.4 SEM image of the ZB(a) and ZBR(b) surface after immersion in SBF for 14days.

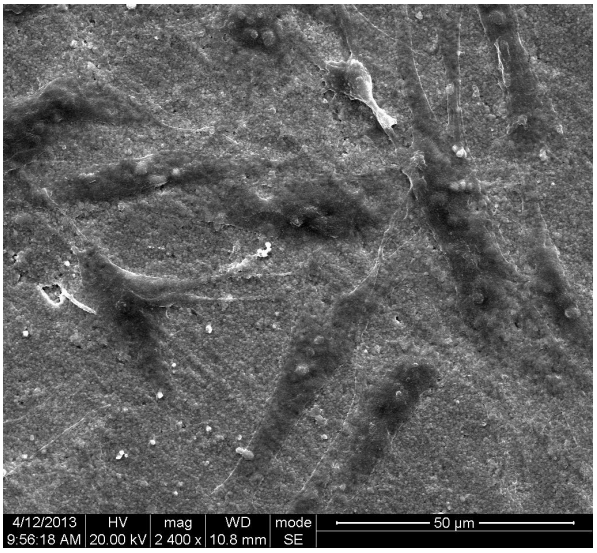

Fig. 5 a

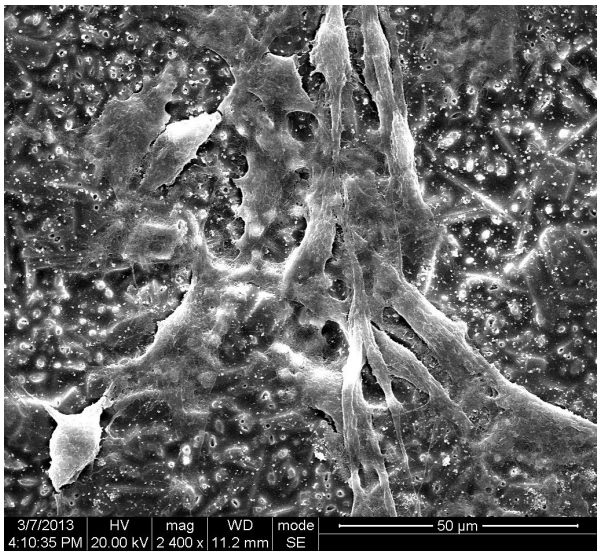

Fig. 5 b

Fig. 5 c

Fig.5 SEM image of MC3T3-E1 cells cultured on Z (a), ZB (b), andZBR (c) surfaces.

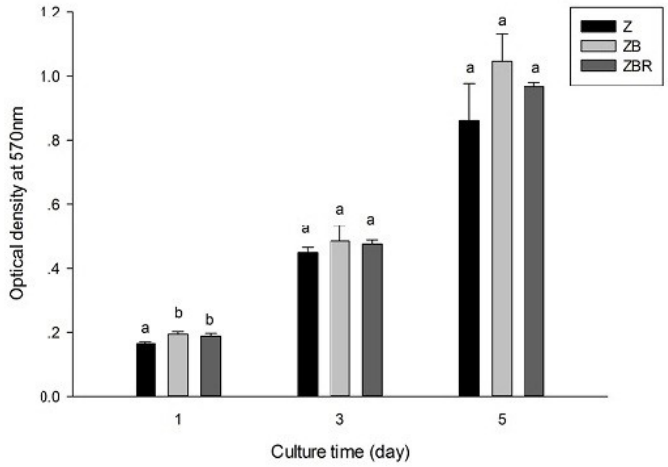

Fig.6 Cell proliferation of MC3T3-E1 cells on Z, ZB, and ZBR surfaces, n=5 . Identical letter indicates no significant difference at each culture times($p>0.05$). There is no significant difference between three surfaces at 3 and 5 days ($p>0.05$).

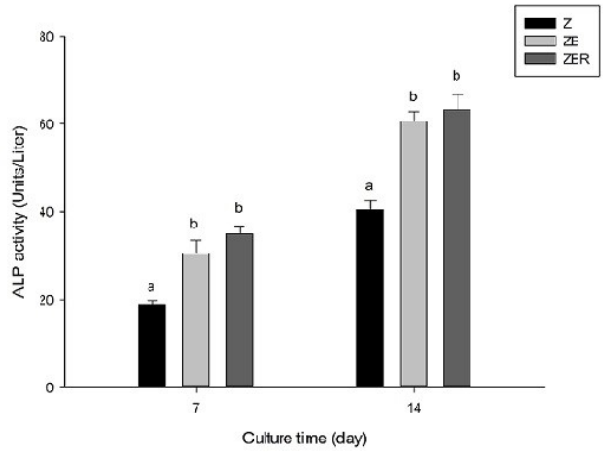

Fig.7. ALP activity of MC3T3-E1 cells on Z, ZB, and ZBR surfaces, n=5.
Identical letter indicates no significant difference at each culture times ($p>0.05$).
The expression of ALP activity on ZB and ZBR surfaces were significantly higher than Z surface ($p<0.05$) at 7 and 14 days, no significant difference between ZB and ZBR surface ($P>0.05$).

7 and 14 days of cell incubation on the Z, ZB, and ZBR surface, and results are shown in Fig.7. It showed that ALP activities of MC3T3-E1 cells cultured on both ZB, and ZBR were significantly higher than that on Z substrate on both 7 and 14 days. In particular, cell culture on ZBR substrates were found higher than those on ZB substrate. However, no significant differences were found between ZB, and ZBR substrate both in 7 and 14 days.

DISCUSSION

Bioactive glasses are recognized as being highly biocompatible materials. Their surface reactivity makes them osteoconductivity when placed in bone tissue.[14] Bosetti et al[9] had investigated bioactivity of two different bioactive glass layers on zirconia substrate. They concluded that both bioactive glasses enhance zirconia integration with bone cells.

In the present study, the response of MC3T3-E1 cells cultured on zirconia substrate with roughened and non roughened surface of bioactive glass coated were evaluated, and compared with uncoated zirconia substrate. MC3T3-E1 cells were chosen in this study due to their high level of osteoblastic differentiation, mineralized extracellular matrix (ECM) deposition, and a similar behavior to primary osteoblasts, which altogether make them widely used model for in vitro.[15]

Representing SEM image of MC3T3-E1 cell culture on roughen bioactive coated substrate, cells are elongated/spindle shaped and thicker cells with many filopods. Some studies have suggested that the existence and creation of more cellular filopodia creates areas to which a bigger surface area of the cell can adhere and this, in turn, may promote faster and higher amount of mineral-like nodule formation in the longer term.[10]

Regarding cell proliferation (MTT test), no significant difference were observed between test materials and control. By observing the present data it postulates that the observed surface roughness range (Ra0.22–0.85µm for the three different groups) may not significantly alter the cell behavior at the MTT test. As regards to the bioglass coated group, it can be concluded that its chemical effect, such as ionic release is also not considered to have significant impact on cell proliferation. In the studies9 that reported an increase cell proliferation, the samples are first preconditioned in simulated body fluid. In this study, samples were not preconditioned before culture, so cell proliferation may not have been as apparent as in other studies. The result of some studies[16,17] were similar to those obtained in this study.

In this study, no significant difference in ALP activity was observed between bioactive glass coated zirconia substrates. However, the roughened bioactive glass coated substrate had mean ALP activity values higher than those of non roughened bioactive glass zirconia substrate. So, these data indicate the increase in the surface roughness to 0.85 µm of bioactive glass coated on zirconia substrate is not sufficient to enhance cellular activity in significant level. Contradictory, data were found in other studies where rough surfaces have been shown to promote osteoblastic maturation and protein production.[17,18] Rosa et al,[17] concluded that for titanium Ra ranging from 0.80 µm to 1.90 µm would optimize both intermediary cellular response such as proliferation, ALP activity, and total protein content. The contradictory result of present experiment might be due to difference in material used in study.

San Miguel et al,[18] demonstrated that the formation of a rough carbonated hydroxyl apatite layer on bioactive glass porous scaffolds enhanced osteoblast maturation in vitro. The discrepancy between the present data and the data of San Miguel et al studies may be attributable to surface modification techniques, where they used biological apatite which was created in vitro. Overall, the bioactive glass coated zirconia surface was found to have better surface conditions to regulate bone cell differentiation in-vitro. The limitations of this study was that, the zirconia samples were not roughened which could have increased one more comparison group. This study could be further extended with more number of samples and in-vivo studies.

CONCLUSION

The data in this study demonstrated that :

- Bioactive glass coated surface was found to have better surface conditions to regulate bone cell differentiation.
- The surface roughness to 0.85 µm of bioactive glass coated on zirconia substrate is not sufficient to enhance cellular activity in significant level.
- Though not significant, micro roughened surface had better cellular activity as compare to non roughened surface substrate.

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