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Responses of Rabbit Bone Marrow Cells to Clinoptilolite Natural Zeolite Biomimetic Coat on Nanoporous Titania Plate

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Abstract

Modified titanium surfaces are widely used in dental and orthopedic implants because they can improve osseointegration and promote the cell function. Biomimetic clinoptilolite natural zeolite coat was prepared successfully on the surface of the nano-porous titanium by anodization process. Novel natural zeolite coatis proposed to enhance biocompatibility of titanium. In vitro, results show that, rabbit bone marrow–derived mesenchymal stem cells (rBMSCs) has higher viability, cell proliferation, cell attachment, and alkaline phosphates (ALP) over 21 days period on the zeolite microstructure surface compared with uncoated titanium surface, indicating that the zeolite are highly biocompatible and non-cytotoxic coat. Results show that high corrosion resistance porous titania coated with zeolite has the superior ability of bone-like nodule formation, which can be used as a cancellous bone substitute. In conclusion, TiO_2 and ZNT are very promising and are better understanding of cell/biomaterial interactions. Their mechanisms can help in the development of more effective orthopedic and dental implants.

Key Words: *Natural zeolite, Nano-porous Titania, Stem Cells, Alkaline Phosphates.*

Introduction

Many of the important characteristics that must be displayed by the biomedical implants which used in bone surgeries such as total joint displacement, biocompatibility and corrosion resistance are most essential properties that must be tested to select the best (Advincula et al. 2007; Long & Rack 1998; Williams 2003). Titanium (Ti) is widely used for implant applications because it is readily available and has reasonable biocompatibility and corrosion resistance. In recent years, several researchers have tended to modify the surface topography of Ti by various methods (El-Shenawy et al. 2012; Mohsen et al. 2012; Fadlallah et al. 2013), in order to improve its corrosion resistance and biocompatibility.

Anodic oxidation treatment is one of the most important methods to modify the surfaces of Ti implants (Mohsen et al. 2012; Gad El-Rab et al. 2012). The effects of surface topography on tissue response *in vitro* and *in vivo* have been extensively studied over the last decade, and no consensus has been reached. Several authors have reported an association between modified implant surface topography and improved cell adhesion and proliferation findings (El-Shenawy et al. 2012; Lüdecke et al. 2013).

Zeolite is considered a promising coating material to alter the surface topography of titanium implant for its high efficiency medical applications such as drug carriers, antibacterial agents, haemostatic agents and medical diagnostic agents (Matsumura et al. 2003). Zeolite coatings act as a barrier between metal and corrosive medium to prevent corrosion (Montalvo et al. 2012; Mohsen et al. 2014). In addition, zeolite coatings have been shown high antimicrobial properties which make it a favorite in medical applications as a coat on implant surface to promote its capability, especially for bone replacement surgery (Cachinho & Correia 2008).

Bone marrow (BM) contains two different types of the progenitor cells, hematopoietic stem cells which are responsible for the production of the blood cells, and other cell type called mesenchymal stem cells (MSCs), which have capability to differentiate into the various cell lineages including osteoblast, adipose lineages and chondrocytes (Aubin 1999). MSCs were originally isolated from the bone marrow stromal cells but they have recently been identified also in other tissues, such as fat, epidermis, and cord blood (Gnecchi & Melo 2008).

Friedenstein et al. (1974) was the first who described MSC specifics to adhere for tissue culture plates, their characteristic fibroblast morphology and capacity to form colonies. Hunt et al. (1987) also described similar heterogeneous and fibroblastic appearance and a colony forming characteristic cells in the bone marrow.

Therefore, these cells were named as a plastic-adherent or colony-forming unit fibroblast (CFU-F) which is named as MSCor inducible pluripotent stem cells (iPSC) due their heterogeneous character and potential to differentiate into various cells phenotypes (Jiang et al. 2002). Those capabilities make MSCs valuable tools in regenerative medicine and tissue engineering fields.

Evaluations of biocompatibility through cell culture would have to be made using primary culture because the biomaterials interact with these kinds of cells after *in vivo* implantation (Deligianni et al. 2001). The cell culture system used in this study was rabbit bone marrow directed *in vitro* to form osteoblastic cells. This culture system contains mesenchymal stem cells (progenitor cells) that have the potential to differentiate into various cell types depending on the culture condition (Wilke et al. 1998).

Cell and implant interaction *in vitro* is monitored by cell adhesion, proliferation, and differentiation, as well as the cell morphology can be applied (Ciolfi et al. 2003). Moreover, the adsorption of serum albumin on the implant surface (Deligianni et al. 2001); the synthesis of extracellular proteins and proteoglycans by cells cultured on the implant surface (Ciolfi et al. 2003); and methods of scanning, confocal, and electron microscopy, as well as X-ray diffractometric (Wang et al. 2004) were used to evaluate the interaction between the cells and biomaterials.

Recently, in our pervious study, we show that clinoptilolite natural zeolite coating can be deposited on anodized titanium surface to enhance its corrosion resistance (Mohsen et al. 2014). Therefore, in the present study we demonstrate that natural zeolite coatings deposited on nanoporous titania surface are biocompatible and can be better response of rabbit bone marrow cell culture by evaluating cell attachment, cell proliferation, total protein content, alkaline phosphatase (ALP) activity, and bone-like nodule formation than un-modified titanium surface.

Materials and Methods

Samples Preparation

Titanium foil, Ti (Sigma-Aldrich Chemise GmbH, Riedstr. 2D-89555 Steinheim 497329 970) with 0.25 mm thick, 99.7% metals basis was used as base material in this study. The exposed metal surface (area: 1 cm^2) of each specimen was ground with silicon carbide paper to 2000 grit, then washed in distilled water and rinsed with alcohol before anodization process (Gad El-Rab et al. 2012). The anodic oxidation treatment was used to prepare the first modify Ti surface known as nanoporous titania surface by anodize Ti in fluoride bath which consists of 1 M H_2SO_4 + 0.5 wt % NaF, for 20 min at 30 V in two electrode cell, where rectangular samples of titanium area 1 cm^2 were used as anode and a platinum plate measuring 3 × 3 cm^2 was used as cathode. Nano tiania coat from TiO_2 is produced and dominated by NT. Clinoptilolite natural zeolite mineral extracted from Miocene volcanic exposed 100 Km South Jeddah, at Harrat Shama Saudi Arabia. Coat is deposited on NT by adding 0.5 wt% from the purified raw zeolite material to the pervious fluoride bath, supplement with stirring at 60 °C. The obtained surface from this step is dominated by ZNT. Briefly, the order of deposition processes can summarize as: a) mechanical polishing of Ti sample; b) ultra- sonically cleaning and washing by acetone; c) anodizing process at 30 V for 20 min in fluoride bath to prepare NT sample and d) anodizing process with stirring at 30 V for 20 min at 60 °C in fluoride bath containing natural zeolite to prepare ZNT (Mohsen et al. 2014).

Surface Topography Characterization and Chemical Composition

The surface morphology and chemical composition of Ti, NT and ZNT samples were studied before and after cultured rabbit bone marrow–derived mesenchymal stem cells (rBMSCs) by scanning electron microscopy (SEM). Different titanium samples were removed from culture medium and left to dry to analyze using scan electron microscope, magnification 14 x up to 1000.000x.

Preparation of Bone Marrow Cells from Rabbit

Bone marrow cells were obtained from the femur of rabbit. The cells were cultured in collecting medium (100 mL DMEM, 15 mL fetal calf serum, 2 mL antibiotic and 1mL L-glutamine. The cultures were incubated in humidified atmosphere of 95% with CO2 incubator at 37 ºC. When the cells were grown to confluence (about 4 days), the media were removed and differentiated medium were added (22 mL Dulbecco's MEM, 21 mLHAMF12 medium, 0.5 mL antibiotic, 0.5 mL Dexamethane, 0.5 mL ascorbic, 0.5 mL β-Glycero-phosphate and 5 mL FCS). When the cells were changed to osteoblast cells (about four days), the medium had been changed. The trypsin-EDTA was added for 2 min at 37 ºC, when the cells were separated, the media was added to stop the reaction of trypsin. The cells were centrifuged at 900 rpm for 10 min. The supernatant were removed and 1 mL of medium was added to count the cell by heamocytometer, then the cells were divided into TC –plate 12 well (Park et al. 2007).

Viability Measurement and Proliferation Tests

The 2 mL of cells were diluted with 1 mL of media in each well. 20 µL of the diluted cells was taken and 20 µL of trypan blue was added to test the viability using the heamocytometer as well as proliferation of the cells was tested. Live cells were classified as viable (without intake of dye) or non-viable (with intake of dye). The viability was tested after one, two and three weeks of the immersing the plates. The assay for cell proliferation was used to monitor the number of cells over time.

Determination of ALP

The rabbit bone marrow cells were fixed for 30 sec in 10% formal-methanol at -4 ºC using 5 mg Naphthol AS-MX phosphate (DMF) with 0.25 mL of N,N-dimethyleformamide. After fixation, the cells had been

washed in tap water and left to dry. The cells was incubated in the mixture of 10 mL of 0.2 M Tris buffer (2.42 g Tris Base/100 mL) at pH 8.74, 4 mL of 0.1 M hydrochloric acid (HCl) and 26 mL of distilled water for 30 min. The substrate working solution consisted of 0.25 mL of DMF solution, 25 mL D.W. and 25 mL of Tris buffer. The cells placed into substrate working solution at 37 ºC for 30-60 minutes and the cells were stained in 0.1% neutral red for 7 min. Slides were checked microscopically after 30 min for the intense red color indicative of enzyme activity (Rosa &Beloti 2003).

Cell Attachment

For attachment evaluation, cells were cultured for 4 h on Ti or NT or ZNT discs. The culture medium was removed and the wells were washed three times with PBS at 37ºC to eliminate unattached cells. The adherent cells were then enzymatically (1 mM EDTA + 0.25% trypsin; Gibco) released from the Ti discs and counted using a hemacytometer. Cell attachment was expressed as percentage of the initial number of cells (Park et al. 2007).

Cell Proliferation

For proliferation evaluation, cells were cultured for 21 days on different Ti discs. The culture medium was removed and the wells were washed three times with PBS at 37ºC to eliminate unattached cells. The adherent cells were then enzymatically (1 mM EDTA + 0.25% trypsin; Gibco) released from the Ti, NT or ZNT discs three times, pooled, and counted using a hemacytometer. Data were used for calculating the doubling time as described by Patterson (1979).

Bone-Like Nodule Formation

After 21 days in culture, the cells were washed three times with PBS at 37ºC. The attached cells were fixed in 3% glutaraldehyde in 0.1 M sodium calcium codylate buffer for 2 h at room temperature and rinsed once in the same buffer. After fixation, the specimens were dehydrated through a graded series of alcohol and processed for staining with Alizarin red S (Sigma), that stains bone-like nodules rich in calcium. The specimens were evaluated using an image analyzer (Image Tool, University of Texas Health Science Centre, San Antonio, TX, USA) and the amount of bone-like nodule formation was calculated as a percentage of total Ti disc area.

Results and Discussion

Ti, NT and ZNT discs were immersed in differentiated cells. For attachment evaluation, cells were cultured for 2 days and mineralization in bone marrow–derived mesenchymal stem cells (BMSCs) induced toward osteogenic differentiation *in vitro* was cultured in the presence or absence of different titanium discs after 7 days. The cell viability was tested at different time intervals (7, 14 and 21 days). After 21 days, cell proliferation and ALP activity were evaluated as well as bone-like nodule formation.

Cell Culture

Isolation and culturing of BMSCs *in vitro* by plastic adherence capability of the BMSc, the cells suspension containing both stromal and hematopoietic cells was seeded in tissue culture flasks using α-DMEM. After 48 hours, many of the rounded as well as spindle shaped cells had attached to the base of the tissue culture flask (Fig. 1A).

The spindle shaped cells were attached to the bottom of the flask while the round cells remained suspended in the medium and were mostly eliminated from the culture with subsequent media changes. These cells began to proliferate after four days (Fig. 1B).

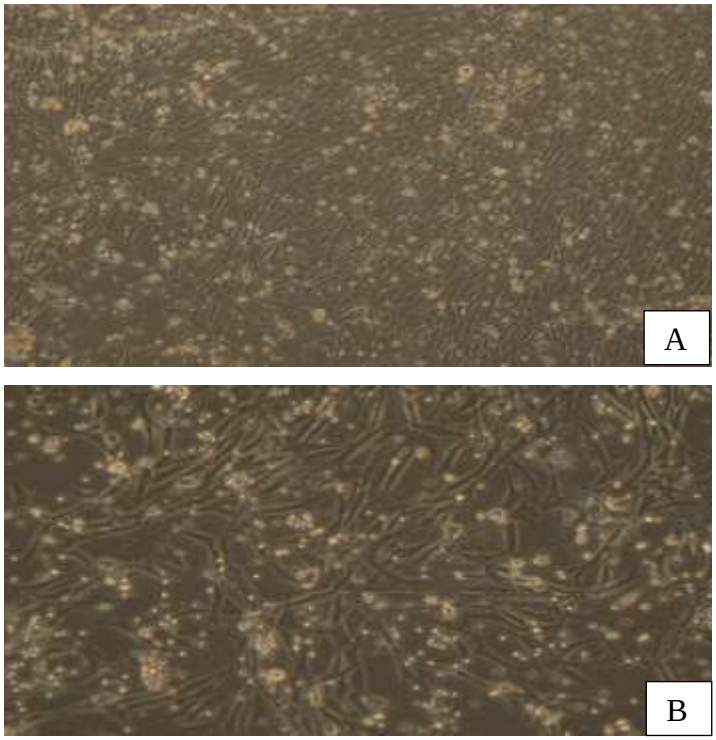

Fig. 1: Phase contrast microscopy images (10 x) representative of the morphological changes induced after 4 days of culture in osteogenic media. Control cultures showing spindle and proliferated cells morphology. A; the cells begin to change to spindle shaped stromal cells of the bone marrow attached to the floor of the flask, surrounded with rounded hematopoietic cells (at 2nd day after isolation). B; the spindle-like cells were attached to the bottom of the flask start to proliferated (increasing the number of osteoblasts, at 4th day of isolation).

The cells obtained by plastic adherence were cultured and serially passaged in the osteo inductive medium to differentiate into the osteocytes. The results obtained suggest that all the surfaces submitted did not affect the mesenchymal cell differentiation into osteogenic lineage cells when compared with smooth surfaces of Ti-plates.

Initial Cell Attachment, Viability and Proliferation

The cells attachment with different plates was presented in Fig. 2. Effects of different types of Ti-plates on viability of rabbit cells were performed. The results showed that there was insignificant difference between all types of plates as comparable to the negative control (cells without plates). The plates did not induce a cell viability reduction and an inhibition of cell growth resulting to have no toxic effects (Table 1). The viability of the control was 98% at zero time. After 21 days of immersing the plates with the differentiated cell, the viabilities were 90%, 93% and 92% for Ti, NT and ZNT-plates, respectively.

Table 1: Viability % of rabbit bone marrow cells grown for 21 days on Ti, NT and ZNT.

Disk type	Days		
	7	14	21
Ti	98	95	90
NT	95	95	93
ZNT	96	94	92

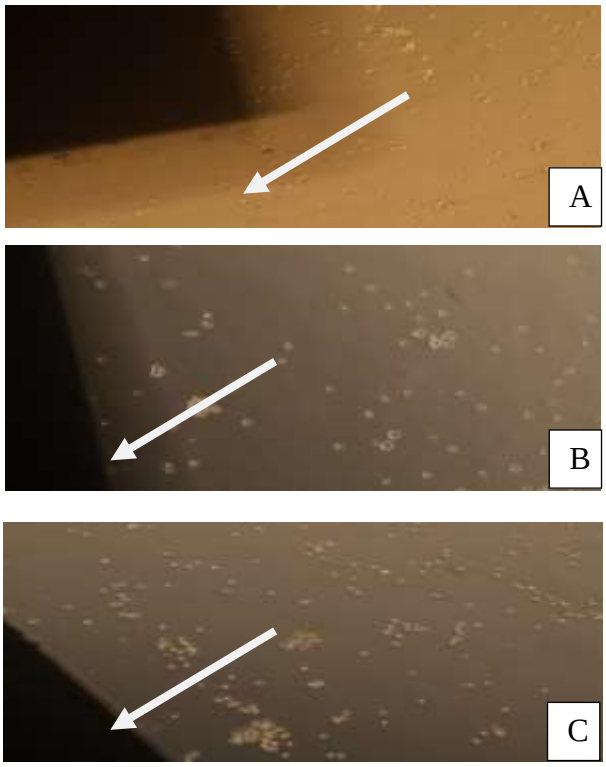

Fig. 2: Phase contrast microscopy images (10X) representative of the morphological changes of osteoblasts and the proliferation after 21 days of immersing the different Ti-plates in the osteoblast. The cells attachment with Ti, NT and ZNT disks were presented as A, B and C, respectively.

Cell proliferation was analyzed for the different groups by determining the number of cells adhered to the different types of titanium surfaces at 24, 48 and 72 h after plating. The number of cells per well was obtained by counting viable cells in a hemocytometer using the trypan blue exclusion method. Cell counts per group for all time intervals did not show any significant difference.

The results showed that all discs depending on the surface structure, allowed cell attachment, cell proliferation, and osteoblastic differentiation expressed as both ALP activity and bone-like nodule formation at different time periods. The surface condition of the biomaterial is an important factor for implant acceptance in vital bone (Heitza & Norment 1974). Blasting the surface with particles of a material other than that of the implant itself altered the surface composition and affect biocompatibility (Rosa et al 2012). The surface characteristics of biomaterials include surface topography, charges, components and chemical states. Most studies focused on surface topography (Gnecchi & Melo 2009; Olefjord & Hansson 1993). It is generally accepted that rough, textured and porous surfaces could stimulate cell. Bone-like nodule formation related to surface characters did not show a marked difference between the plates.

Alkaline Phosphatase (Alp) Activity

ALP activity is a relative marker of osteoblastic differentiated. The level of this enzyme in culture grown in standard medium present low during two week but after 3 weeks the activity of the enzyme increased. Count the number of colonies expressing ALP (red colonies) and the number of differentiated colonies is colorless. The red color was indicated for the formation of mineralization. Control cultures presented no signs of mineralization. After 7 days from immersing the Ti-plate showing low stain of ALP (Fig. 3A) and by increasing the time to 14 and 21 days, the culture cells were starting to form colonies and showed deposition through culture and a positive staining, respectively (Figs. 3B and 3C). After 7, 14 and 21 days

of immersing the NT-plate, less of staining cells appeared, then a large number of staining cells increased and formed colonies and finally most of cells take a positive stain (Figs 3D-3F). Positive staining colonies appear as a significant degree during the different time of ZNT immersion in culture (Figs. 3G-3I).

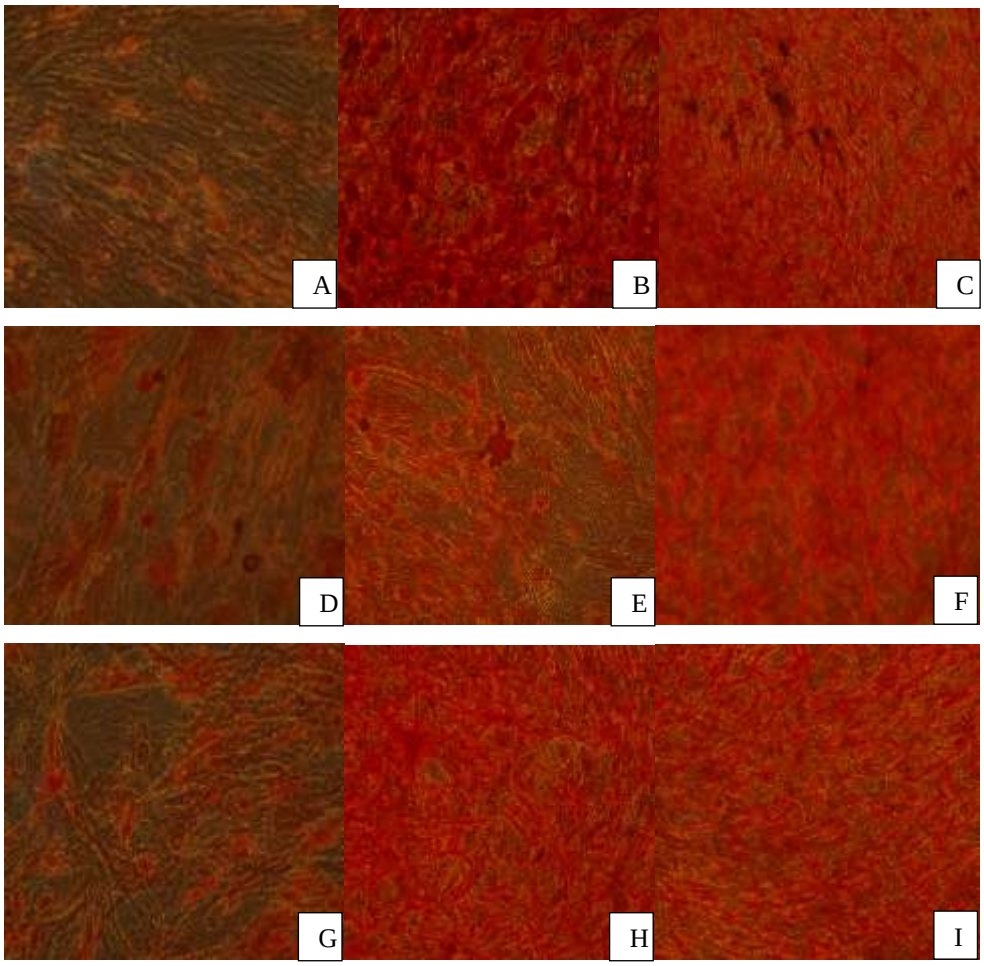

Fig. 3: ALP activity is a relative marker of osteoplastic differentiated in rabbit osteoblast. The level of this enzyme in culture grown in standard medium present low during two week but after 21 days the activity of the enzyme increased. Count the number of colonies expressing ALP (red colonies) and the number of differentiated colonies are colorless. Matrix mineralization was evident in the form of calcium nodules (stained red) in the cultures under osteogenic conditions. Control cultures presented no signs of mineralization. (A) After 7 days from immersing the Ti-plate showing low stain of ALP. (B-C) After 14 and 21 days from immersing the Ti-plate, starting to form colonies and showed deposition through culture and appositive and a positive staining, respectively. (D-F) After 7, 14 and 21 days of immersing the NT- plate showing less of staining cells appear, then, a large number of staining cells and begin to form colonies and finally most of cells take a positive stain, respectively. (G-I) After 7, 14 and 21 days of immersing showing low stain of ALP, start the formation of colonies and finally, increasing the positive staining colonies appear. Magnification 10 x.

ALP activity increased in cells cultured on rough surfaces. This seems to be a general characteristic of osteoblastic cells because it has been reported using MG63 cells (Prockop 1997), embryonic chick osteoblasts (Boyan et al. 1998) and in the present study. Normalizing the ALP activity and number of cells eliminates the effect of proliferation on this parameter, so the observed difference among surfaces could result from both a higher differentiated number of cells and a higher cellular activity (Park et al. 2007).

Cell Bone Growth

Fig. 4 represents the SEM for the different plated before immersion and after 21 of immersion in rBMSCs which induced toward osteogenesis. Rabbit bone-marrow stromal cells cultured on ZNT implants are well spread on the substrate and actively interacted with it. Cellular interaction was observed inside the implant. In implants with irregular pores, cells grew both on the surface and in the depth. Thus, cells exhibited more adequate interactions with irregular pore NT and ZNT implants *in vitro* and hopefully the same interaction will be true in tissues after the implantation of the prosthesis into the organism. Therefore, these biomaterials specially the NT and ZNT are very promising and better understanding of cell/biomaterial interactions. A possible limitation of the present study refers to the difficulty in comparing our results with other studies available in the literature, due to the lack of characterization of the new surface tested (ZNT) and the knowledge about the cell and tissue reactions obtained with different surface treatment options. Further investigations are in progress in order to deepen these results and to yield a complete interpretation of these events.

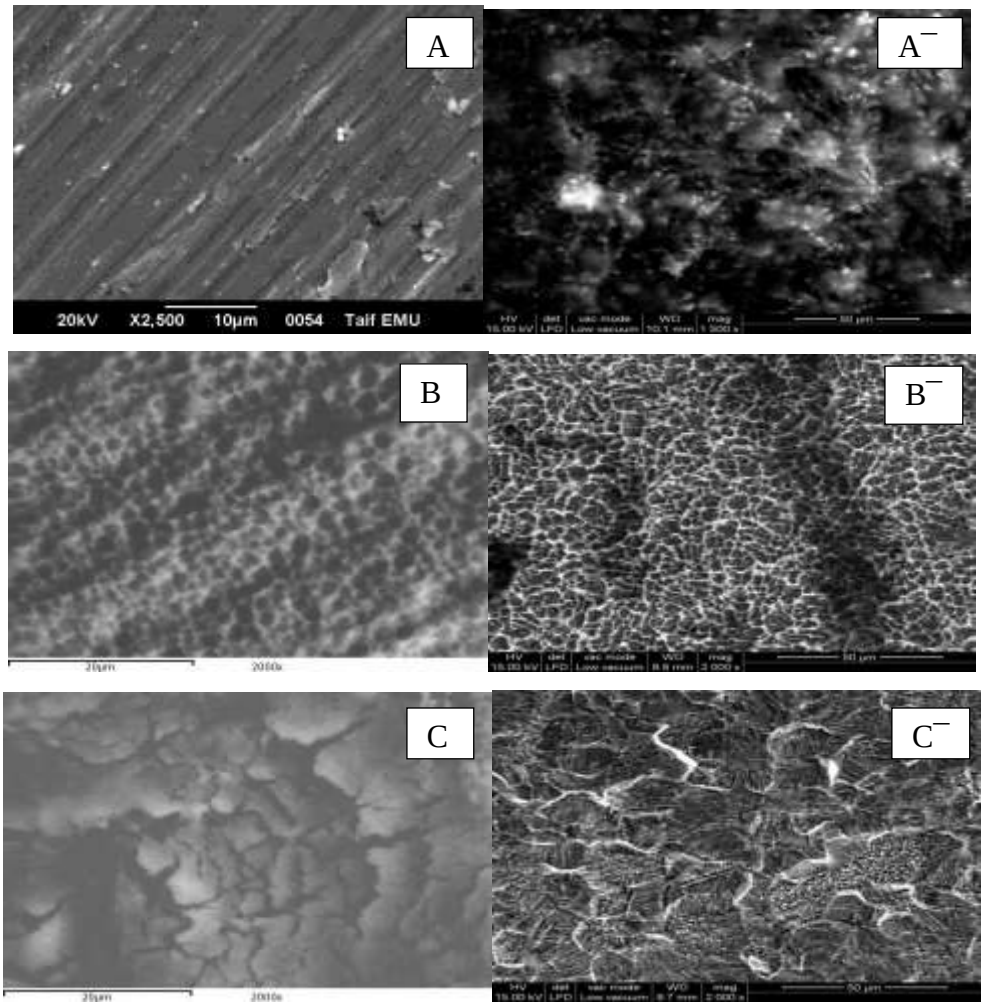

Fig. 4: SEM of polished Ti (A), NT (B) and ZNT (C) deposited (Magnification A was 2500 X while B and C were 2000 x).Scanning electron microscopy images of rabbit osteoblast-like cells grown for 21 days on titanium (A^{-}), NT (B^{-}) and ZNT (C^{-}). The osteoblasts with dorsal ruffles showed close to each other, connected by "lopodia and disposed in multilayers. Some round cells with numerous blebs on their membrane were present. On the NT (d) substrate, cells in mitotic division were observed. (Magnification was 1500 x for A and 2000 x for B and C as well as the scale 50 µm).

Conclusions

We successfully prepared a new biomimetic coat from cliniopetolite natural zeolite on titania surface. The natural zeolite coating has notably up-regulated osteoblastic cells, which subsequently enhanced cell viability and differentiation on titanium. Simple biomimetic coat treatment seems to be an effective way to further enhance the biocompatibility of titania surfaces. The results suggest that the natural zeolite coat is effective in producing a more osteoconductive implant surface, making it suitable for the applications in dentistry and orthopedics.

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Declaration of Interest

There is no conflict of interest.

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