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Research Article

Analysis of different implant materials by using a stromal cell culture in the laboratory

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ABSTRACT

Objective: In this study, rabbit bone marrow cells were used to evaluate different titanium, tantalum and alloys for dental implant surfaces. Bone marrow stromal cells of femora from young adult rabbits produce bone-like mineralized tissue in culture. We evaluated three implant materials (pure titanium, tantalum and alloy of Ti-Al-Va), as to their ability to provide an environment for marrow cells to differentiate into osteoblasts and function as suitable for mineralized tissue formation.

Materials and methods: Rabbit bone marrow cells were cultured on the disc-shaped test substrates for 15 days. The culture medium was changed daily and examined for calcium and phosphate concentrations. After 15 days specimens were examined by different methods The DNA content, alkaline phosphatase (ALP) activity and aluminium (Al) content in culture were also studied.

Results: DNA measurement showed no difference between each material, but alkaline phosphatase activity and aluminium content in the culture on hydroxyapatite and glass-ceramics was higher than on Ti-Al-Va alloy and the control. All test substrates facilitated rabbit bone marrow growth of extracellular matrix formation.

Keywords: Titanium, Osteoblasts, Dental implant, Hydroxyapatite, bone marrow

INTRODUCTION

Rabbit bone marrow cells (RBM) shows various features of stem cell culturing. The growth of these cells could be significantly expanded in vitro and stimulated to the differentiation into manifold mesenchymal cell types (Woodbury et al. 2000). Basically, bone marrow cells (BMC) contains two different kinds of the ancestor cells, hematopoietic stem cells which are accountable for the formation of the blood cells through the process of hematopoiesis, and Mesenchymal Stem Cells (MSCs), having the potential to differentiate in to diverse cell pedigrees including adipose lineages and osteoblasts (Smajilagic et al. 2012).Osteoblasts are cells that respond to their substrate and depend on indications to maintain the appearance of osteoblasts. If the signals presented by the substrate are inadequate than osteoblasts appearance turns into fibroblast. Former studies have established that by the treatment of strong base, titanium might form bone-like mineral when immersed in stimulated body fluid (SBF). These properties of strong base and the alloys of titanium which are treated by heat suggest their possibility in the organization of medical devices (Lee et al. 2012).

For the production of dental implants Titanium and Titanium alloys are appropriate and biologically companionable materials which are proven by various research studies. Alloys of Titanium having maximum tensile potency near

about 900 MPa are frequently utilized in coating implants comprising of 4 mm width for the purpose of maintaining mechanical power of the material (Diniz et al. 2005). Titanium has been widely accepted as worldwide substance for permanent implants coating because of its biocompatibility (Almeida et al. 2005). Most of the presently applied oral titanium implant coatings are not suitably described with reference to their surface properties. This does not clearly indicate that whether the improvement in bone response is because of the roughness of the surface or the composition of the implant surface (Palmquist et al. 2010).

Glass ceramic systems has been widely developing their attention due to their excellent fissure confrontation to force of occlusion, linking strength in between the prepared surface of the tooth and ceramic and basic invention procedures by means of computer-aided design/computer-aided manufacturing (CAD/CAM) technology which facilitated dental surgeons to re-establish teeth by the use of glass-ceramics in a solitary meeting (Kang et al.2013).

MATERIAL AND METHODS

This study was conducted in the laboratory of Punjab medical and dental college and Punjab dental hospital, Lahore. This study is the investigation of implant materials (hydroxyapatite, glass-ceramics and titanium) using a rabbit bone marrow stromal cell culture because bone marrow of rabbit is exactly resembles with human and it is difficult to get the human bone marrow for experimental purpose.DNA content, alkaline phosphatase (ALP) activity and Tantalum content in culture were studied.

Specimen preparation

Four different types of plates for bone marrow cell culture were constructed by mechanical, chemical, and anodization methods. Three implant surfaces e.g. titanium, hydroxyapatite and glass-ceramics were examined in the present study. These plates were mechanically and chemically polished by sandpaper and H_3PO_4 respectively and were 10 mm in diameter and 1 mm in thickness. Once the treatment has been performed, every specimen was rinsed in acetone and non-ionic water and dried. Polished titanium plates were utilized as the control group.

Isolation of RBM Cells

There are numerous procedures for the isolation of rabbit bone marrow cells including plastic adherence process. In our study we have applied this process because of its ease of achieving cells, but on the other hand obtaining pure stromal cells is a bit difficult by this process. General testing has described the circumstances for the isolation, proliferation and differentiation of rabbit bone marrow (RBM) cells in vivo and in vitro. Nearly 50µl volume of bone marrow was attained from femora from young adult rabbits. By using narrow gauge needle fresh femora of young adult rabbits were eradicated, sanitized of soft tissue and eliminated of their marrow.

Culturing of RBM Cells

For the purpose of rabbit bone marrow cell culturing and basal medium, we follow the standard procedure derived from original protocol of Maniatopoulos et al. the basal medium according to that protocol is supported by Minimal Essential Medium (a-MEM), enriched with antibiotics, 15% nonactivated of heat fetal bovine serum (FBS) and antifungal agents. FBS addition can enhance variation in culture features depending upon the batch numbers provided by multiple suppliers. (Atala et al. 2002)

After 5 days of culture substitution, cells were removed using 0.01% trypsin solution and embedded on the test substrates. A small proportion of the cultured cells are impulsively osteogenic, and thus non adherent cells in culture populations can be completely eliminated. To produce high yields of osteogenic cells the use of dexamethasone and the specific concentrations of the culture medium additions was very helpful.

Determination of Alkaline Phosphatase (ALP) Activity

By the treatment of the cultures with 0.1% triton solution, the ALP activity determination was performed in bone marrow cell culture. Biochemical assay was carried out by p-

nitrophenyl phosphate hydrolysis at pH 10.3 in alkaline buffer by applying the process for 30 min at 38ºC.

DNA Content Analysis

The amount of DNA was concluded after 15 days of cell culture to achieve the report about cellular quantification. Cell culture implants were subjected to incubation and then washed with PBS two times. The cells lysation was performed using 1 mM MgCl2 and 10 mMTris-HCl at pH 8.5, with continuous slight shaking for 30 min. The amount of DNA was determined using a DNA assay kit.

Measurement of Al and P content

For the measurement of calcium and phosphate content in the culture medium atomic absorption spectrometry (AAS) was considered. After pre-incubation period of 36 h Ti-HA specimens were recovered from the multiple substrates.

Histochemical assays

For the purpose of histochemical staining, bone marrow cell cultures fixation was performed with 1.5% glutaraldehyde solution in 0.15 mol/l sodium cacodylate buffer and after that washed with distilled water (Coelho et al. 2000).

ALP staining

For ALP staining a mixture is prepared in Tris buffer having pH=10, containing 2 mg/ml of Na-α-naphtyl phosphate and 2 mg/ml of fast blue RR salt. Bone marrow cell cultures were incubated for 1hour in the dark along with the prepared buffer mixture followed by washing the samples with water. A brown to black stain indicates the presence of ALP in culture.

Microscopy

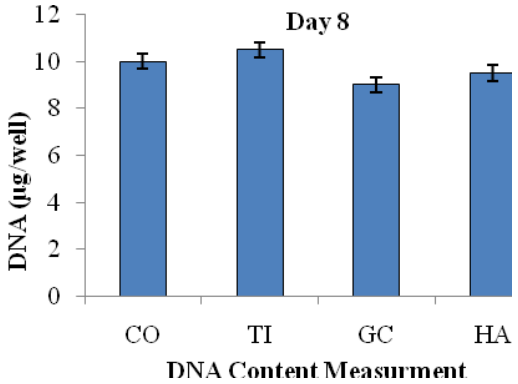
After 15 days of culture, Co, Ti, GC and HA specimens were examined by microscopy, scanning electron microscopy, dispersive X-ray analysis and morphometry of the cell-covered substrate surface.For the purpose of scanning electron microscopy, bone marrow cultured cells were fixed in glutaraldehyde (4%) solution and then washing step was performed by 0.1 M PBS. After washing, dehydration with isopropanol solution was performed followed by drying (Handschel et al. 2011). Once drying has done, the cultured specimens were directly place on a carbon pad of a SEM-holder

Statistical evaluation

For the purpose of statistical evaluation one-way analysis of variance (ANOVA) with Fisher's test was employed for several evaluations between multiple samples. The level of significance was at (P) <0.05 and results were summarized as means of standard error deviation.

RESULTS

Biomaterials are those materials which are suitable for living tissues and do not provide any harm to the living tissue. Impenetrable bunch of many-sided cells were appeared at various spaces on the culture plates at 11th day of the culture and these bunch or cluster of cells produced a node-like structure. These mineralized forms of node-like structures steadily increased near day 15, and visualization of these structures von Kossa stain is utilized.



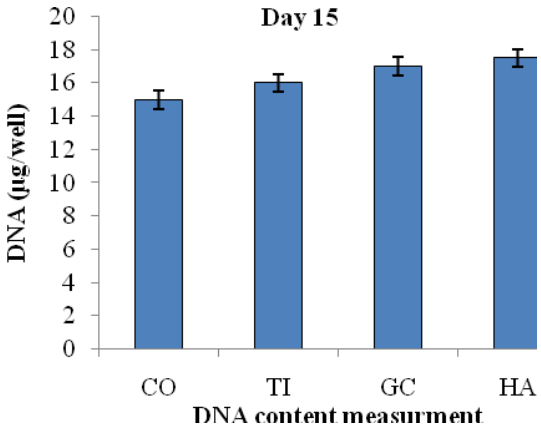


Figure 1: DNA content in culture at 8th day **Figure 2:** DNA content in culture at 15th day

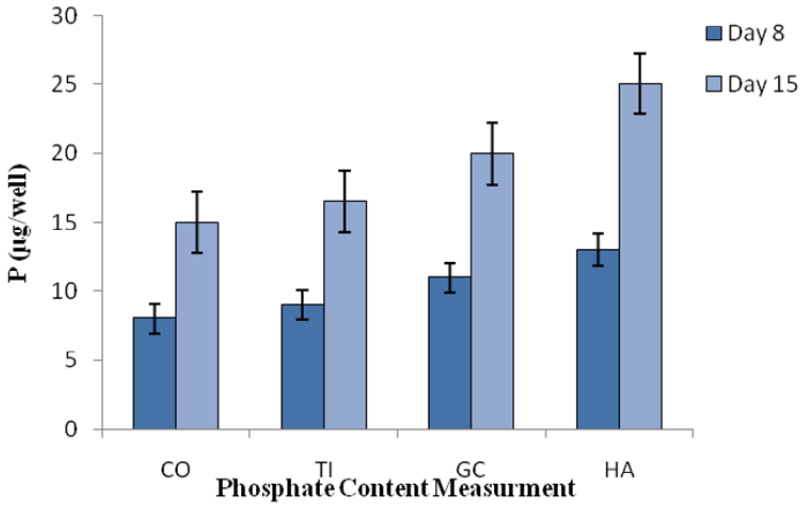


Figure 3: Phosphate content measurement in culture at 8th and 15th day

DNA content in each plate illustrate increased amount of DNA in equivalence on each culture plate at 8th and 15 day of culture implantation (Figure1, Figure2). Our study showed that theDNA content measurement showed no difference in HA, GC and Ti material. There was no significant difference in cell growth on each material**.** The amount of DNA at day 8 in each well for all the four plates is: for control plate 10µg/well, for Titanium 10.5µg/well, for Glass-ceramics it is 9µg/well and for Hydroxyapatite it is 9.5µg/well. Whereas at day 15 the amount of DNA on each plate is: for control plate 15µg/well, for Titanium 16µg/well, for Glass-ceramics it is 18µg/well and for Hydroxyapatite it is 18.5µg/well.

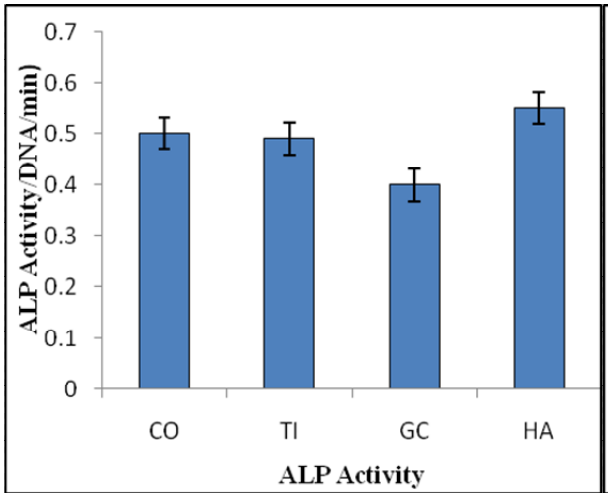


Figure 4: ALPactivity measurement in culture at 8th day

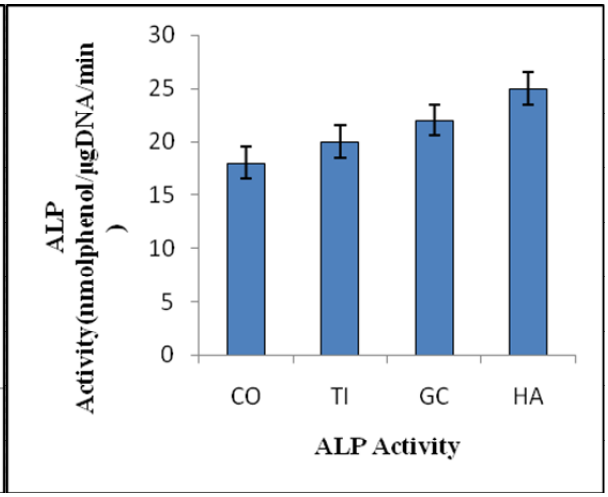


Figure 5: ALP Activity measurement in culture at 15th day

Alkaline phosphate activity estimation showed that at day 8 the control culture had low ALP activity in contrast with HA and GC. Ti revealed no significant increased in ALP activity at day 8. ALP activity for control culture at day 15 was significantly increased and Ti showed ALP activity at the same level as that of control (Figure 3, Figure 4). As compared to control HA at day 15 showed an elevated level of ALP activity whereas GC has higher level at day 15 as compared to day 8. ALP activity was also visualized by staining and brown to black stain indicates the presence of ALP in culture (Figure 5).

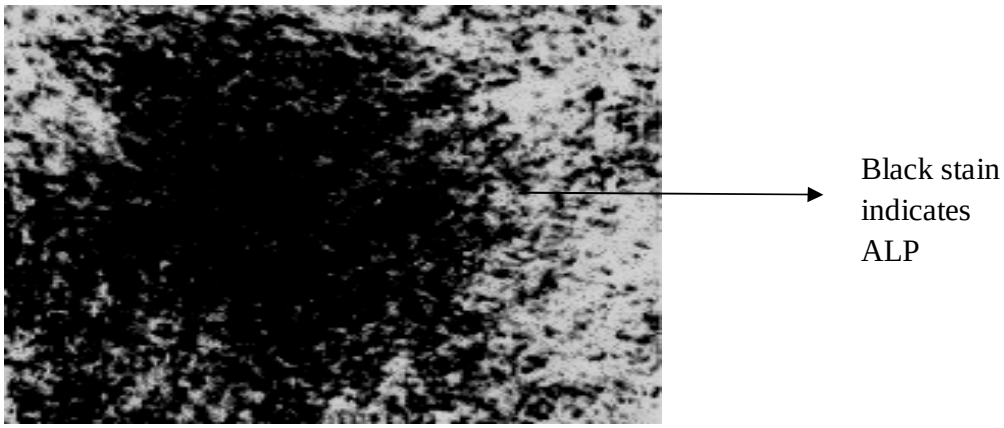


Figure 6: ALP staining in the culture of rabbit bone marrow stromal cells

Phosphate content

The Ca concentration in the control culture was very minute at day 8 whereas, Ti revealed a bit higher amount of Ca content as compare to Control. By the comparison of the cultures at day 8 and 15 reveals the difference in calcium content (Figure 8). The Phosphate concentrations in the culture medium for HA were moderately higher at day 8 and day 15, whereas, in case of GC the phosphate content was slightly higher at day 8 and significantly at day 15. For the measurement of Ti culture medium phosphate activity was same as that of control at day 8 and day 15 and was low as compare to GC and HA (Figure 8). Calcium and phosphate concentration was also measured by Von Kossa technique of staining. Red and black stain indicates presence of calcium and phosphate in the cell culture respectively (Figure 10,Figure 11).

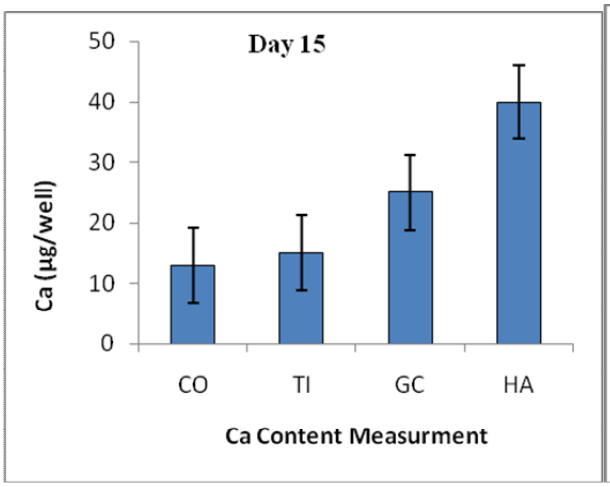


Figure 7: Calcium content measurement culture at day 15

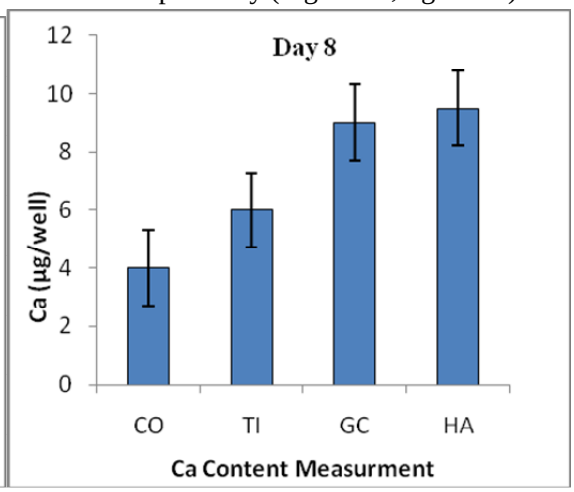


Figure 8: Calcium content measurement in culture at in day 8

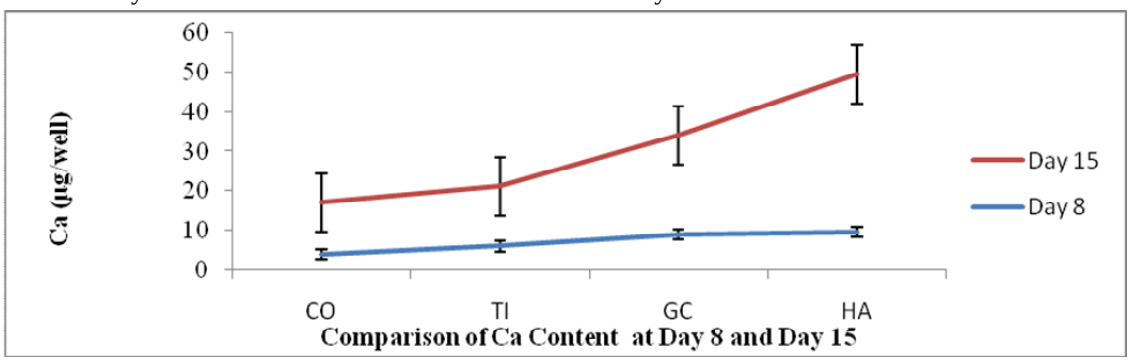


Figure 9: Calcium content Comparison in culture at day 8 and day 15

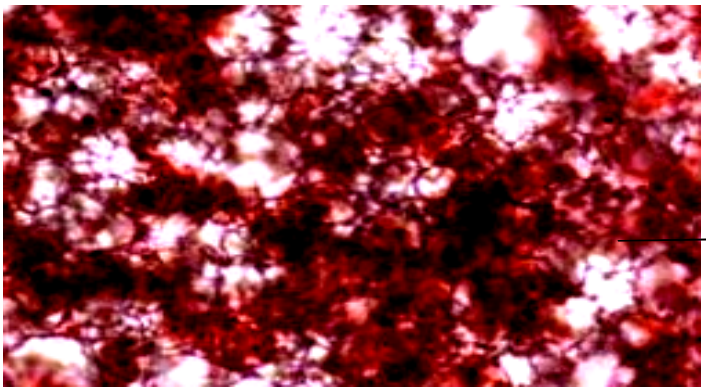


Figure 10: Von Kossa Technique for Calcium (Ca) Staining in rabbit stromal cells

Figure 11: Von Kossa Technique for Phosphate (P) staining in rabbit stromal cells

DISCUSSION

In this rabbit bone marrow cell culture, osteoblastic differentiation would occur, and these osteoblasts generate extracellular mediums resulting into mineralized nodules formations. DNA content measurement in culture medium demonstrated that each cell grew equally. The amount of DNA at day 8 in each well for all the four plates is: for control plate 10µg/well, for Titanium 10.5µg/well, for Glass-ceramics it is 9µg/well and for Hydroxyapatite it is 9.5µg/well. Whereas at day 15 the amount of DNA on each plate is: for control plate 15µg/well, for Titanium 16µg/well, for Glass-ceramics it is 18µg/well and for Hydroxyapatite it is 18.5µg/well.

For osteoblast formation and differentiation, ALP activity expression was made under consideration and the highest level of ALP activity indicates the increase amount of osteoblast differentiation. ALP activity for control culture at day 15 was significantly increased and Ti showed ALP activity at the same level as that of control (Figure 3). As compared to control HA at day 15 showed an elevated level of ALP activity whereas GC has higher level at day 15 as compared to day 8. Both GC and HA revealed higher ALP activity on the culture at 8^{th} and 15^{th} day. It clearly illustrated that GC and HA both could support osteoblastic differentiation. At days 8 and 15 activity of alkaline phosphatase (ALP) of Ti culture was at the equivalent intensity as that of the control culture. Hence Ti did not promote the osteoblast formation and differentiation. But on other hand, GC plates showed higher DNA content and ALP activity and the osteoblastic formation on HA and Ti revealed more squashed shapes with some dorsal disturbance.

Our results shows consistence with previous study of Knabe et al, 2002 which clearly illustrated that all surfaces enhanced the production of RBM, which in turn support osteoblast formation verified by alkaline phosphatase activity, except

HA cells during the first phase at day 5 and day 8. Conversely, the method of elevated ALP activity on HA and GC at day 15 is unknown. An increased level of ALP activity on HA or GC could encourage formation of mineralized tissue. The hidden phenomenon under ALP activity appears to be that alkaline phosphatase supplied phosphate ions from the organophosphate substrate culture medium for the formation of apatite and then for the purpose of Ca transport binds to Ca^{+2} ion in the medium.

Starting from day 1 to day 8, increased Ca production on Co, Ti, GC and HA was examined (Figure 6). The reason behind this increased activity might be due to physicochemical aspects occurring within the culture medium.

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Starting from day 1 to day 8, increased Ca production on Co, Ti, GC and HA was examined (Figure 6). The reason behind this increased activity might be due to physicochemical aspects occurring within the culture medium. At day 8 GC revealed highest Ca content in comparison with HA, this might be due to the fact that as soon as GC submerged in a culture medium, the phenomenon of interaction between culture medium and ceramics would occur resulting in release of the vital constituents of GC.

There is no significant difference was observed between Ti surface and the control (Figure 8). As soon as the formation of mineralized nodule would appear on the culture plates, it was regarded as the end result of osteoblastic differentiation.

CONCLUSION

For the determination of physicochemical reactions and various properties of surfaces like Ti, GC and HA there should be direct contact of the culture cells with these materials. We concluded that our study of rabbit bone marrow cell culture allows consistent growth of osteoblastic cells on multiple implant materials like Ti, GC and HA. For appropriate contemplation about the implant materials results must be clarified and justified accurately. Additional studies including more assays for the purpose of considering communications between cultured cells and implant material should be performed.

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