EVALUATION OF HUMAN FETAL OSTEOBLAST PROLIFERATION AND MORPHOLOGY ON TITANIUM INFRAZYGOMATIC MINI-IMPLANTS

- AN IN VITRO STUDY

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled "**EVALUATION OF HUMAN FETAL OSTEOBLAST PROLIFERATION AND MORPHOLOGY ON TITANIUM INFRAZYGOMATIC MINI-IMPLANTS - AN IN VITRO STUDY**" is a bonafide and genuine research work carried out by me under the guidance of **Prof. Dr. V. K. SHAKEEL AHMED, M.D.S., M. Ortho RCPS (Glasgow), M. Ortho RCS (England), Diplomate of Indian Board of Orthodontics**, Department of Orthodontics and Dentofacial Orthopedics, Ragas Dental College and Hospital, Chennai.

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This dissertation is submitted to **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY,** in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY** in **BRANCH V - ORTHODONTICS AND DENTOFACIAL ORTHOPEDICS**. It has not been submitted (partially or fully) for the award of any other degree or diploma.

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Abstract

ABSTRACT

AIM:

The aim of this in vitro study was to:

1. Evaluating the proliferation and morphology of human fetal osteoblast (hFOB) cells seeded on the surface of two different brands of titanium infrazygomatic (IZC) mini-implants (Bioray & Dentos) in three different time periods (24, 48 & 72 hours).
2. Assess the chemical composition on the surface of two different brands of titanium IZC mini-implants in three different region (head, body & tip) and detailing the chemical component profile.

MATERIALS AND METHOD:

Six experimental units of two brands of titanium IZC mini-implants Dentos, (korea) (n=6) and Bioray (n=6) were used as a test material and polystyrene disk (n=3) was used as a control. hFOB cells seeded on the surface of sterilized titanium IZC mini-implants and polystyrene disk in three different time period (24, 48 and 72 hours). hFOB cells adhesion, proliferation was assessed quantitatively and morphology was assessed qualitatively in three time periods. As-received one titanium IZC mini-implant from each brands were subjected to surface composition analysis (head, body and tip) using Energy Dispersive X-ray analysis.

RESULTS:

1. Quantitative evaluation showed there was a significant increase in number of proliferated hFOB cells in both the titanium IZC mini-implants in three different time periods. One way ANOVA showed there was a statistical significant difference in the proliferated hFOB cells between the two titanium IZC mini-implants (p=0.000). Post hoc and Tukey tests also revealed there was a statistically significant difference between two titanium IZC mini-implants, Bioray IZC mini-implant had more number of proliferated hFOB cells in three different time period than Dentos IZC mini-implant (p=0.001, 0.028 & 0.000). Comparing both IZC mini-implants with polystyrene, polystyrene had more number of proliferated hFOB cells (p=0.000). Qualitative evaluation showed cell morphological change was observed on the surface (body, (thread & pitch) and tip) of titanium IZC mini-implants and surface of polystyrene disk in three different time periods (24, 48 & 72 hours).
2. The significant and most frequent element present on the surface of both the brands of titanium IZC mini-implants were titanium, aluminium and vanadium. Maximum amount of titanium was present on head portion of Bioray IZC mini-implant (90.10%)

followed by body and tip (89.31%, 88.12%) compared with Dentos IZC mini-implant.

CONCLUSION:

Time influenced hFOB cell proliferation was observed on all the surfaces of titanium IZC mini-implant (Dentos and Bioray) with lowest cell count values was at 24 hours, intermediate at 48 hours and highest at 72 hours which was statistical significant. More proliferation of hFOB cells was observed on the surface of polystyrene disk compared to IZC mini-implants (Bioray & Dentos) due to its high affinity. Comparing the hFOB cell proliferation between the titanium IZC mini-implant, Bioray titanium IZC mini-implant showed maximum number of proliferated hFOB cells in all three time periods. The increased cell count is attributed to the increased elemental composition of titanium on all its surfaces (head, body and tip) of the Bioray IZC mini-implant.

KEY WORDS: Human fetal osteoblast cell (hFOB), Titanium infrazygomatic mini-implants, cell culture, scanning electron microscopy (SEM), Energy Dispersive X-ray analysis (EDAX)

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Introduction

INTRODUCTION

Anchorage is one of the most important and fundamental part in orthodontic treatment, which is conventionally provided by various intra-oral and extra-oral devices.[36] In the recent years, an alternative method of anchorage is provided by miniscrew implants (MSI) which is considered as a sources of absolute osseous anchorage.[70, 28] The most common and successful area for the placement of MSI is the interdental area between the roots of the adjacent teeth, both in the maxilla and mandible. The other possible MSI insertion sites in the maxilla are 1) Anterior nasal spine, 2) Hard palate 3) Maxillary tuberosity 4) alveolar process, whereas in the mandible are 1) Mandibular symphysis 2) External oblique ridge 3) alveolar process. However, the interradicular insertion of MSI carries with it the risk of iatrogenic damage to the adjacent roots.[1, 69]

Due to the porous nature of the maxillary bone, the anchorage demands are very high in maxilla compared to mandible during orthodontic treatment. In order to overcome these undesirable side effects of avoiding inadvertent root contact and anchorage loss while placing MSI interdentally has led to the development of a new form of skeletal anchorage which is known as mini-implant, which is placed in infrazygomatic crestal region (IZC) in maxilla and buccal shelf area in mandible. These anatomical sites are distant away from the dentoalveolar region, which allows unobstructed

tooth movement and minimizing the chances of inadvertent root contact.[31,55, 7, 22]

IZC mini-implants are used as a skeletal anchorage for en-masse distalization, maxillary anterior retraction, and intrusion of maxillary posterior teeth. [69, 46]

To obtain good stability various sites for the placement of the IZC mini-implants has been reported in the literature. A cone beam computed tomography (CBCT) study by **Liou et al** [47] suggested placing IZC mini-implants more anterior and closer to the mesiobuccal root of the maxillary first molar (IZC 6). However **Lin et al** [45, 46] modified the site of placement of IZC mini-implant above the roots of first and second molar region (IZC 7). He claimed, the IZC 7 site to be more superior than IZC 6 site because of thicker buccal cortical plate in the maxillary second molar region as compared to the maxillary first molar region.

Liou et al [47, 46] claimed 97% of success rate using titanium IZC mini-implants as an adjunct in various orthodontic procedure. He also reported a lesser failure rates of about 3% using IZC mini-implants.

However, a recent study by **Uribe et al** [68] reported high failure rate of about 21.8% using titanium IZC mini-implants. Many factors have been reported in the literature regarding the success and failure rate of IZC mini-implant, which have been divided into different categories such as patient

related factors, IZC mini-implant related factors, host bone related factors and operator related factor.

Literature reports the IZC width has varying thickness of approximately 5 to 8mm.[47] Research evidence reports that the IZC area width varies in different skeletal malocclusion (class I, II & III) ranging from 5 to 7 mm. [44, 20] In general from a mechanical point of view the whole threaded part of mini-implant should be ideally inserted into the bone to achieve adequate stability.[10, 28] Anatomically IZC region being a smaller area in the maxilla, the whole width of the IZC region ranging between 5 to 8 mm will be partly engaged by the threaded portion of the longer sized IZC mini-implants (10 to 14mm) which might leads to inadequate retention.[30]

A more recent study by **Chang et al** [19] compared the failure rates between the stainless steel and titanium alloy IZC mini-implant placed in attached gingiva (AG) and movable mucosa (MM) of IZC region. He claimed that the factor responsible for the IZC mini-implant failures, which resulted in loosening were due to stainless steel IZC mini-implant placed in AG and right side has more failures (7.4%, 7.8%) than titanium IZC mini-implant placed in AG and in right side (5.1%, 5.2%). In comparison titanium IZC mini-implant offered a slightly lower failure rate than stainless steel IZC mini-implant.

IZC mini-implants are generally made of either titanium or stainless steel alloy. Therefore, the success or failure of an IZC mini-implant

largely depends on the degree to which it integrates with the host bone. In general stainless steel mini-implants attains the primary stability by mechanical retention, whereas the titanium mini-implants allows direct bone contact and attains stability by combination of mechanical retention and osseointegration.[13] Moreover, there is paucity of information whether commonly used titanium IZC mini-implant composition has biological property which will aid in stability and minimize its failure rates.

The search of best biomaterial for mini-implant is not complete with the material selection. It is found that cell interactions are highly dependent on surface topography and compositions. It would be advantageous to learn such characteristics of composition and surfaces, which would help in developing osteoblast friendly biomaterials.

Extensive research work is ongoing to understand osteoblastic cell interaction with the composition and surface of titanium dental implants and miniscrew implants (MSI). However, there are no studies in literature which has reported that human osteoblast is influenced by the composition of titanium IZC mini-implants, which would be an important material factor for biological retention aiding in its stability. Moreover the role of osteoblast cell during the initial stages of adhesion, proliferation and morphology on the surface of the titanium IZC mini-implants is less understood.

Hence this in vitro study aims to,

1. Evaluate the proliferation and morphology of human fetal osteoblast (hFOB) cells seeded on the surface of two different brands of self-drilling titanium IZC mini-implants (Bioray & Dentos) and polystyrene disk (control) in three different time periods 24, 48 and 72 hours.

2. To assess the chemical constitution on surface of two different brands of as received titanium IZC mini-implants in four different areas (head, body & tip) and detailing the chemical component profile.

Therefore, the null hypothesis presumed that there is no difference in proliferation, differentiation and maturation of hFOB cells on the surface of two different brands of titanium IZC mini-implants (Bioray and Dentos) when compared with Polystyrene disk (Control).

Aims and Objectives

AIMS AND OBJECTIVES

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The purpose of this in vitro study aims:

1. To evaluate the proliferation and morphology of human fetal osteoblast (hFOB) cells seeded on the surface of two different brands (Bioray & Dentos) of self-drilling titanium IZC mini-implants and polystyrene disk (control) in three different time periods (24, 48 and 72 hours).
2. To assess the chemical composition on the surface of two different brands of as received titanium IZC mini-implants in three different areas (head, body & tip) and detailing the chemical component profile.

Review of Literature

REVIEW OF LITERATURE

Anchorage, which is defined as resistance to unwanted tooth movement caused by the reacting force of orthodontic loading, plays a very important role in orthodontic treatment. **kanomi et** al[63] used miniscrew implants (MSI) to anchor orthodontic movements. MSI are also called as "Temporary Anchorage Device" (TADs) because they are designed to be used and removed at the end of orthodontic treatment. In order to eliminate the undesirable side effects of placing MSI interdentally has led to the development of a new skeletal anchorage device, infrazygomatic crest (IZC) mini-implant in maxilla. Which is an, anatomical sites away from the dentoalveolar region in maxilla.

SUCCESS AND FAILURE OF IZC MINI-IMPLANT

Lin et al[45] (2014) compared the six-month failure rates for IZC bone screws inserted into movable mucosa (MM) or attached gingiva (AG). Suggested that the alveolar bone thickness on buccal side of maxillary second molar is thicker than the maxillary first molar region. He has suggested the placement of the MSI in the IZC region between first and second molars at an angulation of 55-70°. If there is root interference, remove the IZC screw and replace it with a shorter screw in another location, as indicated.

Liou et al[46] (2007) evaluated the IZC thickness of 16 adult patients in CT. Coronal slice of the CT image containing the IZC and mesiobuccal root of the maxillary first molar were measured. The IZC thickness 13 to 17 mm above the maxillary first molar, measured at 40° to 75° ranged from 5.2±1.1 mm to 8.8 ±2.3 mm. He concluded that the clinical implication for MSI insertion in the IZC for adults is 14 to 16 mm above the maxillary occlusal plane and at an angle of 55° to 70° to the maxillary occlusal plane.

Liou et al[47] (2004) evaluated 16 adult patients with MSI of 2 mm in diameter and 17 mm in length inserted in the maxillary zygomatic buttress region as a direct anchorage for en masse anterior retraction. To prevent MSI from hitting any vital organs because of its displacement, it is recommended that the MSI has to be placed in a non–tooth-bearing area that has no foramen, major nerves, or blood vessel pathways, or in a tooth-bearing area allowing 2 mm of safety clearance between the MSI and dental root.

Eric Hsu et al[36] (2017) Compared the six-month failure rates for IZC bone screws inserted into movable mucosa (MM) or attached gingiva (AG). IZC mini-implants were highly successful (93.65%), and there was no significant difference between MM and AG, or any other variable tested, i.e. age, side, asymmetry or initial applied load.

Uribe et al[68] (2015) evaluated the failure rate of IZC mini-implants. The failure rates by characteristics of the patients listed in this study are age: ≥18yrs , gender: male(38.4%), presence of any related medical condition, less diameter of MSI:1.5-1.8mm, length of the MSI:6-8mm, force magnitude: >150gms, type of movement except intrusion, poor oral hygiene, inexperienced operator, no use of pilot hole, side of the patient. He concluded that there was a 21.8 % failure rate of mini-implants placed in the IZC region was observed. The differing results in success rates in this study may be attributed to the size, length of the MSI and the amount of the time period required for the treatment.

Chris Chang et al[19] (2018) placed TAD in IZC region has an overall success rate of 93.7%, of which only 6.3% of stainless steel and 5.7% of titanium alloy implants failed. Maxillary bone is less dense than in the mandible, so the strength of SS may not be necessary for the posterior maxilla. It was hypothesized that TiA has adequate strength for the IZC, and the absence of nickel would result in a lower failure rate compared with SS, particularly for patients predisposed to failure.

Lee et al[44] (2013) evaluated the IZC thickness in skeletal Class III children in CT. The bone thickness of the IZC area was measured at 35 locations on the right and left sides, perpendicular to the bone surface. There might be differences in the bone thicknesses at the IZC area among skeletal Class I, II, and III patients. According to his finding, the bone

thickness in the class III growing patients tended to be thicker at the superior and lateral areas of the zygomatic process of the maxilla. He states that this variation might be related to the development of the maxillary sinus in growing patients.

Chen et al[20] (2010) assessed 20 skeletal class II adult female, subdivided into three groups by FMA with cephalometric analyses: high FMA group: FMA.37.5 ± 2, 7 cases. Average FMA group: 28.8 ± 1.8, 8 cases and low FMA group: FMA.20.6 ± 2.5, 5 cases, observed that the cortical bone thickness was not significantly different in the five measured areas. The upper posterior area and the IZC area showed no significant difference among different FMA groups.

Chugh et al[22] (2013) summarized the results of studies relating to bone density and implant stability. They concluded that knowledge of low density sites prior to implant placement allows clinician to use longer implant in these areas to improve retention. In areas of high bone density, use of pre-drilling method avoids the breakage of implant. Sufficient irrigation should be done to prevent overheating of bone in that area. Immediate loading of mini-implants is possible because of higher bone density in all the areas of cortical bone. In areas of low bone density, it is necessary to augment the anchorage as per requirement.

Kravitz et al[41] (2007) assessed the potential risks and complications of orthodontic MSI in regard to insertion, orthodontic

loading, peri-implant soft-tissue health, and removal. Complications can arise during MSI placement and after orthodontic loading that affect stability and patient safety. A thorough understanding of proper placement technique, bone density and landscape, peri-implant soft tissue, regional anatomic structures, and patient home care are imperative for optimal patient safety and miniscrew success.

Ahmed et al[1] (2012) evaluated the reparative potential of cementum histologically after intentional root contact with a TAD. Root contact with the temporary skeletal anchorage device was confirmed by using a stereomicroscope. Cementum repair was assessed histo morphometrically. They concluded that 70% of all the teeth exhibited good repair by the end of week 12. Healing of cementum takes place after an injury with a temporary skeletal anchorage device, and it is a time-dependent phenomenon.

Asscherickx et al[7] (2005) inserted 20 MSI into the mandible of five beagle dogs. Each dog received two bracket screw bone anchors in each lower quadrant, between the roots of the second and third, and third and fourth premolars. Sequential point labelling was performed every 6 weeks with vital stains, and apical X-rays were taken every 6 weeks. Radiographic examination demonstrated damage at three roots because of insertion of the BSBAs. Histological examination at these three roots

demonstrated an almost complete repair of the periodontal structure (e.g. cementum, periodontal ligament and bone) in a period of 12 weeks.

Chen et al[21] (2008) evaluated the stability of MSI placed with intentional root contact. The root repair was evaluated after screw removal. During placement of MSI in the alveolar process, increased failure rates were noticed among those contacting adjacent roots. Failed MSI appeared to be surrounded with a greater volume of soft tissue. When more inflammation was present, the adjacent roots seemed to experience more resorption.

Poggio et al[57] (2006) determined the safe locations for MSI placement between the dental roots of the posterior teeth. CT images of 25 maxilla and 25 mandibles were taken. In the maxilla, the greatest amount of mesiodistal bone was on the palatal side between the second premolar and the first molar. The least amount of bone was in the tuberosity. The greatest thickness of bone in the buccopalatal dimension was between the first and second molars, whereas the least was found in the tuberosity. In the mandible, the greatest amount of mesiodistal dimension was between first and second premolar. The least amount of bone was between the first premolar and the canine. In the buccolingual dimension, the greatest thickness was between first and second molars. The least amount of bone was between first premolar and the canine.

Snehal Pathak et al[55] (2019) analysed the mandibular buccal shelf region and IZC region and provide anatomical map to clinician for bone screw placement. Thickness of cortical bone, bone density and soft tissue health directly affects implant stability. Mandibular buccal shelf and IZC offers enough bone quality and quantity for mini-implant insertion.

Krishnakumaran et al[42] (2021) measured the bone thickness of IZC region and to correlate the bone thickness with cervical vertebrae maturation index using CBCT. The superior and the lateral regions of the zygomatic process of maxilla have the maximum bone thickness and are the most appropriate site for placement of MSI or mini-implants. There was no statistically significant difference between the male and female groups. Bone thickness increased with cervical vertebrae maturation age.

Zhang et al[73] (2010) evaluated the healing time of self-drilling titanium MSI by histomorphologic and histomorphometric evaluations of osseointegration after immediate and early loading for evaluating the osseointegration property. Fifty-four MSI were bilaterally placed in the maxillary premolar regions of nine beagles. Then the MSI with various healing time of 0 day, 2 weeks, and 4 weeks were loaded with an orthodontic force (100 g) for 8 weeks. The three groups had significantly different degrees of osseointegration, and the 4week group showed the highest degree of osseointegration so 4 week should be considered before orthodontic loading to improve stationary anchorage.

Chaddad et al[17] (2008) evaluated the survival rate of two mini-implant systems with different surface characteristics under immediate orthodontic loading. Seventeen machined titanium (MT) mini-implants and 15 sandblasted, large grit, acid-etched (SLA) mini-implants were placed in 10 patients. The mini-implants were immediately loaded and the patients seen at 7, 14, 30, 60, and 150 days. They concluded that surface characteristics did not appear to influence survival rates of immediately loaded mini-implant.

Nguyen et al[52] (2017) investigated the influence of cortical bone thickness on the amount of micro damage caused immediately after insertion of orthodontic mini screw implants. They concluded that influence of cortical bone thickness on the amount of micro damage formed immediately after insertion in porcine bone. An increase in cortical bone thickness was statistically associated with an increase in micro damage. Inserted of MSI into 1.0 or 1.5 mm cortical bone thickness should have a 3.5-mm safety zone from important anatomic structures or from the initial entry site if reinsertion is required during treatment

PROLIFERATION OF HUMAN OSTEOBLAST, ANIMAL OSTEOBLAST CELLS

Ricardo et al[16] (2014) evaluated the proliferation and morphology of human osteoblasts cultured on two brands of mini-implants after 24, 48, and 72 hours, in addition to the chemical composition found on their surface. Two brands of mini-implant (Morelli and Neodent) were evaluated; polystyrene was used as a control group. Osteoblasts were cultured on the surface of sterilized mini-implants in different time periods. Osteoblast proliferation was increased over time. No difference was seen between the mini-implants evaluated in terms of chemical composition. Cell adhesion after 72 hours suggests that areas of bone remodeling can be achieved, thus initiating the process of mini-implant anchorage.

Schmidt et al[62] (2001) tested the behavior of osteoblasts on cpTi, Ti-6Al-7Nb, and stainless steel with surface designs similar to clinical implants. After surface characterization by SEM. Cell proliferation and the differentiation parameters of alkaline phosphatase activity and osteocalcin were measured. CpTi provides the best biocompatibility with regard to proliferation, in addition to more reliable early and late differentiation markers of human osteoblasts in vitro.

Subramaniam et al[65] (2002) hFOB cell line has minimal chromosome abnormalities, exhibit the matrix synthetic properties of differentiated osteoblasts, and are immortalized but non-transformed cell line. These hFOB cells thus appear to be an excellent model system for the study of osteoblast biology in vitro.

Alves et al[2] 2009 evaluated the biocompatibility of a dental implant surface, observing adhesion, cell morphology and proliferation of osteoblast-like cells cultivated on a commercially available titanium dental implant. After seeding the cells, the samples were prepared for analyses through SEM. Based on the surface analysis, the osteoblastic cells adhered to the machined surface after 24 hours in culture. In 48 hours, the cells spread over the implant surface, and after 72 hours a proliferation of cells with large and flat bodies was observed over the machined implant surface. These results demonstrate that the machined titanium surface studied is biocompatible and it preserved the integrity of the cultivated osteoblast-like cells for a period of 24 to 72 hours, allowing their adhesion and proliferation, and maintaining their morphologic characteristics.

Harris et al[34] (1995) reported the establishment of a hFOB cell line derived from biopsies obtained from a spontaneous miscarriage. Treatment of hFOB cells with 1-34 parathyroid hormone (PTH) resulted in an increase in CAMP levels. They concluded that clonal cell line hFOB

provides a homogeneous, rapidly proliferating model system to study certain stages of human osteoblast differentiation.

Ryan JA[59] (2008) primary cell cultures, had difficulty in attaching to glass. By the 1960s, plastic flasks, dishes, and 96 well plates were all available commercially. Most of these vessels were manufactured from polystyrene. Polystyrene was chosen because it has excellent optical clarity, is easy to mold, and can be sterilized by irradiation. It also has one significant drawback: it is a very hydrophobic (nonwettable) polymer to which cells have difficulty attaching. For good cell attachment the hydrophobic polystyrene surface must be modified to a more hydrophilic surface. This allows cell attachment proteins (vitronectin and fibronectin) found in the serum containing culture medium to adhere and spread on the vessel bottom, thus providing a better surface for cells to attach. The freshly molded polystyrene surface is treated using either electrode discharge under atmospheric conditions or gas-plasma under vacuum. So that the surface becomes hydrophilic and negatively charged once medium is added. By the mid-1970s most researchers were growing their cell cultures in treated polystyrene vessels rather than glass.

Hendrich et al[35] (2002) evaluated the **e**ffect of standard implant materials on osteoblast proliferation and differentiation was investigated using a human osteoblast cell culture system. hFOB were cultured on stainless steel, cobalt-chrome molybdenum, and commercially pure

titanium for 12 days. Tissue culture polystyrene was used as a control. Electronic cell counting and proliferation assays showed lower cell numbers and delayed proliferation on stainless steel and cobalt-chrome-molybdenum compared with titanium and polystyrene. They concluded that the hFOB cell provides a rapidly proliferating and differentiating system for testing biomaterials in which differences in osteoblast proliferation and differentiation on implant materials.

Vande vannet et al[69] (2007) partial osseointegration represents a distinct advantage in orthodontic applications, allowing effective anchorage to be combined with easy insertion and removal. This article reports the histomorphometric findings of the osseointegration of bracket screw bone anchors. This is an experimental animal study, four BSBAs were inserted in the alveolar process of the lower jaw in each of five male beagle dogs. After 6 months, histological evaluation of the eight remaining screws was performed to evaluate the extent of osseointegration. They concluded that miniscrew, used for temporary anchorage in orthodontics, partially Osseointegrate.

Passeri et al[54] (2009) analyzed the morphology and proliferation of human osteoblastic cells in vitro on five commercially available titanium surfaces. Human primary cells of the osteoblastic lineage were obtained from bone explants. Cell morphology was studied after 6, 24, 72 h, 7 and 14 days of culture by SEM. Commercially pure, grade 4 Polished and

machined titanium disk were used. They evaluated the morphology of osteoblast cells after 6, 24, 72 h, 7 and 14 days of culture. After 6 h of culture, cells attached on all the different surfaces; more spread and flattened with large lamellipodia. At 24 h, most of the cells appeared flat, with some interspersed round and loosely attached cells and cellular edge formed large lamellipodia ending with filopodia. At 72 h, cell-to-cell contacts were frequent and had an elongated shape and a parallel orientation. At day 7, cells reached confluence and they were flat, parallel and arranged in a polarized monolayer. At day 14, cells were confluent and no difference was detectable among the surfaces.

Leonardo et al[26] (2015) evaluated the behavior of osteoblast-like MG-63(human osteosarcoma cell line) on the surfaces of titanium, which is coated with polytetrafluorethylene (PTFE) and titanium nitride (TiN). The sample were constituted of titanium disk. Cell viability or proliferation was determined using MTT reagents. PTFE coating could be considered as a possible choice for a surface treatment of temporary skeletal anchorage devices in orthodontics.

Denizard et al[58] (2008) evaluated the in vitro cell attachment and bone tissue formation of human fetal osteoblast cells cultured on γTiAl with different surface roughness, and hence determine its biocompatibility using SEM. hFOB appeared to spread and anchor on both metal surfaces,

independent of surface roughness (rough and smooth surfaces). Titanium does not appear to affect the biological activity**.**

Khoo et al[39] (2020) evaluated the surface chemistry and topography of commercially available laser-modified titanium implants, together with evaluating the cell morphology and cell adhesion of hFOB seeded onto the same implants. Laser-modified titanium implants have the potential for increasing the chances of successful osseointegration. The hFOB with filopodia infer the possibility of successful osseointegration. Cell adhesion is possible after 48 hours in laser-modified titanium.

Anselme et al[5] (1999) evaluated and compared the adhesion of human osteoblasts cultured on smooth titanium alloy (Ti6Al4V), stainless steel, glass and standard tissue culture polystyrene (Thermanox1) substrates.. They concluded that the chemical composition of substrates with comparable surface topographies did not significantly influence adhesion protein expression by primary human osteoblastic cells. Adhesion of human osteoblasts was comparable on smooth Ti6Al4V and stainless steel surfaces and higher than on glass and tissue culture polystyrene.

Berger et al[11] (2018) compared either human male or female osteoblasts exhibit sex-specific differences in response to E2 and 1α, 25(OH) 2D3 when cultured on micro structured Ti surfaces. This study

revealed that male and female osteoblasts respond to Ti surface micro/meso topography in a comparable manner and are regulated by 1α25 (OH) 2D3 in a comparable way.

Babuskaet al[9] (2015) compared the nanostructured titanium with different grain size with respect to biocompatibility using human fibroblast cell line (HFL1) as well hFOB cells. There were significant differences related to the initial phase of attachment, but not in proliferation. Also indicate that osteoblasts grow best on material with grain size of 160 nm with a longitudinal section in comparison with other examined materials.

Yeniyol et al[72] (2014) evaluated the effect of surface roughnesses and crystalline structures of modified surfaces on hFOB cells morphological behavior and adhesion after 24 hours. hFOB cells culture system permits a reproducible examination for investigating the biocompatibility of dental implant materials in vitro. There is inconclusive evidence about effect of surface roughness and composition on the morphology and adhesion of hFOB cells cultured on surfaces.

Le Guehennec et al[43] (2008) compared the osteoblastic cell (Mouse calvaria) was used. Behavior on various titanium implant surfaces. Four groups were investigated: mirror-polished (Smooth-Ti), alumina-blasted and acid-etched (Alumina–Ti), SLA (sandblasted, large-grit, acid-

etched. Titanium discs were used. MC3T3-E1 cells attached, spread and proliferated on the substrates. Different rough titanium surfaces were correlated to osteoblastic cell adhesion, viability and differentiation in comparison with plastic and smooth titanium.

K. Anselme et al[4] (2000) they compared the behaviour of a mouse osteoblastic cell line and of primary human osteoblastic cells on the Ti6Al4V samples with various surface roughnesses. They observed a lower proliferation and adhesion on rough surfaces than on smooth ones. Their findings show the significance of chemical surface analysis after any surface treatment of titanium-based implants before any biological use.

Schmidt et al[63] (2002) investigated the morphology of human osteoblasts on stainless steel, cobalt chromium alloy, commercially pure titanium, Ti-6Al-4V, and Ti-6Al-7Nb with surface designs similar to those used as clinical implants. Morphology was investigated at three different time period after 12 hours, 72 hours and 7 days. The materials were examined by scanning electron microscopy at different points of time. On the surface of titanium disk after 12 hours, the cells were well spread and formed many filopodia extending from the base of the cell mass and also possessed many thin, capillary-like extensions are seen. After 72 hours, the polygonally spread osteoblast-like cells started to fuse their cell borders or the cells overgrew each other and the beginning of multilayer

growth the cells were very flattened to the material surface. Cells grew very dense, they were not as flattened but formed multilayers and nodules.

Wu et al[71] (2011) evaluated and compare the effects of surface topography on Staphylococcus epidermidis and hFOB behavior like cell adhesion, viability and differentiation on four clinically relevant titanium surfaces: mirror-polished, satin, grit blasted and plasma-sprayed titanium. Ti alloy, disks were used. The initial adhesion and subsequent proliferation of S. epidermidis and osteoblasts were visualized both by SEM and osteoblast proliferation also evaluated. The disk were exposed to hFOB osteoblasts and cultured for 1, 4, 8 and 16 days. After 16 days of culture the cells were flattened and spread with numerous cytoplasmic extensions and lamellipodia on the polished, grit-blasted and plasma-sprayed surfaces

Aybar et al[8] (2009) evaluated the behavior of neonatal rat calvarial osteoblast-like cells cultured on different titanium discs with different surface roughness (Sandblasted acid etched (SLA)) surfaces of 2 different companies with different alloy properties were used properties with different composition. Cell morphologies were evaluated by SEM, for 24 hours and 7 days. SEM images of both the SLA surfaces expressed osteoblasts with stretched appearance also shortening of cytoplasmic extensions is observed. SLA surface implants are being widely used today for their mechanical increased stability in bone.

Cui et al[24] (2012) examined the surface properties and osteblastic responses to a titanium alloy were investigated in vitro. Group I was an untreated machined smooth titanium surface (Ti S), Group II was a titanium-6 aluminum-4 vanadium and Group III was a titanium-8. Tantalum-3 neobium discs. The surface morphology was examined by scanning electron microscopy. SEM images of cell attachment and spreading on all samples. Which were polygonal in shape and had many cytoplasmic extensions indicative of cell spreading. Some cells presented triangular or round in shape with no significant difference in proliferation between the groups. They concluded that improved surface characteristics and osteoblastic response to the Ti–8Ta–3Nb alloy compared to Ti S and Ti–6Al–4V alloy. These properties can make a Ti–8Ta–3Nb alloy a new implant material for medical and dental implants.

Subramani et al[64] (2016) evaluated the surface roughness and carboxyl functionalization of multi-walled carbon nanotubes mixed with collagen coated onto titanium (Ti) substrates on MC3T3-E1 osteoblasts. Smooth-surfaced titanium discs were used. They concluded that coatings containing MWCNT-COOH (increased hydrophilic surface chemistry) influence osteoblast proliferation, differentiation, and matrix mineralization and should be further studied for applications in orthodontic MSIs.

Galli et al[30] (2005) determined the tissue response to implants and therefore their clinical outcome. The aim of the present study was to compare two commercially available titanium disk surfaces: plasma sprayed (TPS) and sand-blasted, acid-etched surface (SLA) were used. Human osteoblasts were obtained from jaw bone specimens resulting from a surgical intervention on a 5-year-old patient. The effect of the surfaces on human mandibular osteoblasts was then studied in terms of cell morphology, adhesion, proliferation, and differentiation. Human osteoblasts from the mandible were cultured on these two surfaces and evaluated at 3, 6, 24, and 48 hours to determine cell attachment and morphology. Osteoblasts on SLA appeared more elongated and spindle shaped than those on TPS, and their adhesion at 3 and 6 hours was weaker, but reached that of cells on TPS at hour 24. Cell proliferation was greater on SLA surfaces.

Conserva et al[23] (2012) they investigated the in vitro comparison of osteoblast-like cell (osteosarcoma,SaOS-2) adhesion, proliferation and differentiation response to six titanium dental implants (length 13 mm and diameter was 5 mm) with four different surface treatments: turned, sandblasted, acid-etched, anodized and to determine the interaction between cells and implant is influenced by surface structure and chemical composition. Thirteen specimens were used cell morphology, adhesion, and proliferation qualitative evaluation by SEM and one for cell counting

at various magnifications. SaOS-2 cell morphology appeared to be influenced by the type of surface treatment at 6, 24, and 72 h of growth. SaOS-2 cells spread more rapidly on sandblasted surfaces.

EVALUATING THE CHEMICAL CONSTITUTION

Saldana et al[60] (2006) evaluated the thermal oxidation of this alloy at 500 and 700°C for 1 h results in lower Titanium and Aluminium ion release, compared with the same alloy in the as-received state. Also evaluated the lower release of these metal ions from the thermally oxidized alloy may result in improved cell response. To test it, human primary osteoblasts were exposed to different concentrations of Ti, Al, or both the metal ions, and cell proliferation, metabolic activity, differentiation, and mineralization were evaluated. Ti and Al ions affected primary human osteoblast proliferation, metabolic activity, and differentiation in a dose-dependent manner. Treatments with individual Ti or Al metal ions in similar concentration ranges than released from the surfaces did not alter osteoblast response, which also remained unaffected after treatments with combinations of Ti plus Al applied in the proportional relations than detected in ion-release experiments. They concluded that thermal oxidation treatments of Ti6Al4V alloy may improve the biocompatibility of the alloy by reducing both Ti and Al release, and thus attenuating ion-mediated interference with osteoblast differentiation.

Barros et al[10] (2021) tested the null hypothesis that there is no difference in the mechanical strength of SS-MIs and TA-MIs, and to analyze, by SEM, the SS-MI, and TA-MI threads resistance to morphological damage after insertion. The null hypothesis was rejected. SS-MIs were 13.2% and 20.2% more resistant to torsional fracture and deflection, respectively. The threads of the SS-MIs and TA-MIs were not damaged during the insertion and removal process. Thus, the use of SS-MI can reduce the fracture risk without increasing the MI diameter.

Bollero et al[13] (2018) compared the in vivo Titanium Alloy with Stainless Steel miniscrews TAD using removal torque and SEM analysis. For each patient, a TiA TAD and a SS TAD with same length and width were implanted following a randomized split mouth study design. TiA and SS miniscrews had comparable removal torque values. SEM photomicrographs showed no evidence of osteointegration with both TADs having similar biological responses.

Daniele Francioli et al[28] (2010) analyzed the mechanical performance of a self-tapping surgical stainless steel mini-screw system. Surgical stainless steel mini-screw system withstood greater force magnitude compared to other commercially available mini-screw systems (titanium and titanium alloy); therefore the examined mini-screw system is provided with greater mechanical properties.

Chin-Yun Pan et al[51] (2012) evaluated the influence of different implant materials on the primary stability of orthodontic mini-implants by measuring the resonance frequency. Twenty-five orthodontic mini-implants with a diameter of 2 mm were used. The first group contained SS mini-implants with two different lengths (10 and 12 mm). The second group included TiA mini-implants with two different lengths (10 and 12 mm) and stainless steel mini-implants 10 mm in length. The resonance frequency of the mini-implants in the artificial bone was detected with the Implomates device. Resonance frequency was not influenced by the implant materials TiA or SS. Therefore, the primary stability of a mini-implant is influenced by insertion depth and not by implant material. Insertion depth is extremely important for primary implant stability and is critical for treatment success.

Brown et al[15] (2014) evaluated the detailed mechanical and histologic properties of stainless steel MSI and titanium alloy MSI used for temporary orthodontic anchorage with identically sized MSI. Forty-eight stainless steel and 48 titanium alloy miniscrew implants were inserted into the tibias of 12 rabbits. Insertion torque and primary stability were recorded. All implants were stable at insertion and after 6 weeks. The only significant difference was the higher (9%) insertion torque for stainless steel. Stainless steel and titanium alloy miniscrew implants

provide the same mechanical stability and similar histologic responses, suggesting that both are suitable for immediate orthodontic clinical loads.

Morais et al[49] (2009) evaluated the concentration of Ti, aluminum, and vanadium, as a function of time, in the kidneys, livers, and lungs of rabbits that had Ti-6Al-4V alloy orthodontic mini-implants placed in their tibia. Low amounts of Ti, Al, and V were detectable in the 1-week, 4-weeks, and 12-weeks groups, confirming that release of these metals from the mini-implants occurs, with diffusion and accumulation in remote organs. The tendency of ion release when using the Ti alloy as orthodontic mini-implants, the amounts of metals detected were significantly below the average intake of these elements through food and drink and did not reach toxic concentrations.

Ananthanarayanan et al[3] (2016) assessed the composition, surface characterization and corrosion resistance of five commercially available mini-implants by assaying ion release in artificial saliva. Ten mini-implants each from five companies were used. Group 1 – AbsoAnchor, Group 2 – Micro-implant Anchorage System, Group 3 – The Orthodontic Mini Anchorage System, Group 4 – mini-implants (Denticon, Group 5 – orthodontic mini-implants J.J.Orthodontics .One mini-implant from each group was subjected to characterization and surface microstructure analysis using EDAX and SEM. Ten mini-implants were immersed for 30 days in Fusayama-Meyer artificial saliva solution and the release of

titanium, aluminium and vanadium ions was detected. The composition of all the implants was comparable, there was a statistically significant difference in the Ti, Al and V release between Group 4 – the group with maximum release – and Group 2, the group with least release.

Blaya et al[12] (2011) examined and compare the levels of several metal ions released in the saliva of patients with orthodontic appliances, at different time points before and after insertion of a miniscrew. Saliva of patients was collected at four time points: before miniscrew placement (T1), 10 minutes (T2), 7 days (T3) and 30 days after miniscrew placement (T4). Salivary metal concentrations from different time points of miniscrew treatment were compared using Wilcoxon paired tests (α=5%). At time point T4, there was a quantitative increase in the salivary concentration of Cu, Ti, V, Zn, as well as a quantitative decrease in the salivary concentration of Al, Co, Cr, Fe, Ni, when compared with T1.The placement of fixed orthodontic appliances associated with miniscrews does not lead to an increase of salivary metal ion concentrations.

Hanawa[33] (2004) discussed the mechanism of metal ion release form metallic implant materials and the behavior of released metal ion in vivo. Metal ion release from metallic implant is inhibited by the surface oxide as a passive film where partial dissolution and reprecipitation are repeated in aqueous solutions. Released ion, as well as its amount and toxicity must be considered to discuss safety of metallic biomaterials.

Woodman et al[70] (1984) quantify the amount of titanium, aluminum, and vanadium release from titanium-based prosthetic segmental replacements in the long bones of baboons. Forty-five baboons that had received titanium-based fiber metal composite segmental bone replacements were studied along with 13 controls without implants. Thirty-eight baboons with implants were sacrificed, and titanium, aluminum, and vanadium levels were assayed in homogenized lung, kidney, spleen, liver, adjacent muscle, and regional lymph nodes. The blood and urine samples were obtained for trace metal analysis as well as for biochemical and hematological profiles. An increased titanium levels were noted in the lungs, spleen, adjacent muscle and regional lymph nodes in comparison to those of six sacrificed controls without implants. In addition, vanadium was significantly elevated in the lungs of some animals, while aluminum increases were noted in adjacent muscle, lung, and regional lymphnodes.

Lukaszewska et al[47] (2018) evaluated the surface topography and chemistry of various modified titanium surfaces. All examined surfaces were found to be biocompatible. Favourable cell reactions were observed for Al2O3 and HA blasted surfaces. The surface roughness patterns influenced the growth orientation while the surface topography influenced osteoblast morphology.

Gongadze et al[32] (2011) evaluated the adhesion of cells to a nanorough titanium implant surface with sharp edges. The basic assumption was that the attraction between the negatively charged titanium surface and a negatively charged osteoblast is mediated by charged proteins with a distinctive quadrupolar internal charge distribution. Similarly, cation-mediated attraction between fibronectin molecules and the titanium surface is expected to be more efficient for a high surface charge density, resulting in facilitated integrin mediated osteoblast adhesion. They suggest that osteoblasts are most strongly bound along the sharp convex edges or spikes of nanorough titanium surfaces where the magnitude of the negative surface charge density is the highest. It is therefore plausible that nanorough regions of titanium surfaces with sharp edges and spikes promote the adhesion of osteoblasts.

Morais et al[49] (2007) analyzed the immediately loaded mini-implant fixation and to gauge the vanadium ion release during the healing process. Titanium alloy mini-implants were inserted in the tibiae of rabbits. After 1, 4 and 12 weeks, they were submitted to removal torque testing. There was no increase in the removal torque value between 1 and 4 weeks of healing, regardless of the load. Nevertheless, after 12 weeks, a significant improvement was observed in both groups, with the highest removal torque value for the unloaded group. The kidney, liver and lung were also extracted evaluated the content of vanadium, increased slightly

after 1 week, significantly increased after 4 weeks and decreased slightly after 12 weeks, without reaching the 1 week values. They concluded that titanium alloy mini-implants can be loaded immediately with no compromise in their stability. The detected concentration of vanadium did not reach toxic levels in the animal model.

Galli et al[30] (2005) determined the tissue response to implants and therefore their clinical outcome by comparing two commercially available titanium disk surfaces: plasma sprayed (TPS) and sand-blasted, acid-etched surface (SLA) were used. Human osteoblasts were obtained from jaw bone specimens resulting from a surgical intervention on a 5-year-old patient. The effect of the surfaces on human mandibular osteoblasts was then studied in terms of cell morphology, adhesion, proliferation, and differentiation. Human osteoblasts from the mandible were cultured on these two surfaces and evaluated at 3, 6, 24, and 48 hours to determine cell attachment and morphology. Osteoblasts on SLA appeared more elongated and spindle shaped than those on TPS, and their adhesion at 3 and 6 hours was weaker, but reached that of cells on TPS at hour 24. Cell proliferation was greater on SLA surfaces.

Jayaraman et al[38] (2004) investigated surface efficacies of two different titanium dental implants, a sandblasted and acid-etched surface and an experimental grooved surface were compared through in vitro systems. The titanium dental implants were seeded with osteoblast-like

primary cells and maintained for a period of 1–7 days. Expressions of fibronectin and osteonectin were assessed through immunogold labelling by scanning electron microscopy. The grooved surface, supported better osteoblastic cell adhesion and proliferation than the rough surfaces. In conclusion, grooved surfaces offered better cell attachment and proliferation than the other rough surfaces studied.

Morais et al[50] (2007) measured the concentration of titanium, aluminum, and vanadium in rabbits' tissues (kidney, liver and lungs) after the insertion of Ti-6Al-4V alloy orthodontic mini-implants (2mm diameter 6 mm length). Eighteen New Zealand rabbits had four mini-implants inserted in their left tibia and five rabbits were used as control. After 1, 4, and 12 weeks selected tissues were extracted from rabbit, and prepared to analysis by graphite furnace atomic absorption spectrometry. Varied amounts of Ti, Al, and V were detected in the tested groups proving that diffusion of these metals from Ti-6Al-4V orthodontic mini-implants exists. The tendency of ion release when using the titanium alloy, the amount of metals detected were very low. Then, Ti-6Al-4V orthodontic mini-implants are safe auxiliary orthodontic anchorage devices.

Patil et al[54] (2015) evaluated the surface and elemental analyses of orthodontic retrieved self-drilling titanium MSI (8mm long 1.5 mm in diameter, dentos) and as-received MSI. In order to investigate the behavior of MSIs while in contact with bone and soft tissues, oral fluids,

and food. All MSIs were subjected to EDAX microanalysis to investigate the changes in surface elemental composition and to SEM to analyze their surface topography were done at 4 zones of each MSI: head, neck, body, and tip. Retrieved MSIs exhibit morphologic surface change in the form of dullness, blunting of threads and tips, corrosion, craters, and occasional tearing of thin threads.

Natarajan et al[50] (2017) analysed the surface topography, elemental adsorption, and type of bone tissue adherent to the retrieved Ti and SS orthodontic mini-implant, which served as orthodontic anchorage. Sixteen self-drilling mini-implants with a diameter of 1.5mm and a thread length of 9 mm were obtained (S.K. Surgicals) of which 12 implants were placed for the purpose of orthodontic anchorage, after 12 months, all 12 mini-implants were retrieved and examined for bone tissue on surface. The other four mini-implants served as a control. The experimental and control group specimens were subjected to surface analysis and alteration in composition with SEM and EDAX and the nature of bone tissue was assessed with histological examination. Blunting (of the tips and threads) of all retrieved mini-implants were evident on SEM images and craters were observed in body region of retrieved stainless steel mini-implant. The retrieved titanium implants showed oxygen, carbon, calcium, and nitrogen on the surface, whereas only carbon and oxygen elements were found on retrieved stainless steel implants.

Brezulier et al[14] (2020) validate a model of 3D culture of human fetal osteoblasts cells to study mechanobiology. Proliferation was evaluated by measuring diameters, monitoring glucose levels, and conducting Hoechst/propidium iodide staining. The 3D model shows good cell viability. 3D osteoblastic cultures validate this model system for exploring biomolecule release and analysing gene transcription.

Tseng et al[67] (2017) investigated the correlation between the mechanical strengths and gripping volume (GV) of mini-implants. Thirty mini-implants of three types (Type A: 2 mm x 10 mm, cylindrical, titanium alloy; Type B: 2 mm x 10 mm, tapered, stainless steel; and Type C: 2 mm x 11 mm, cylindrical, titanium alloy) were inserted 7 mm into artificial bones. In the GV measurement, Type C (14.4 mm3) was significantly larger than Type B (11.4 mm3) and Type A (9.2 mm3). They concluded, that a trend that larger GVs indicate greater mechanical strengths of mini-implants. Also they suggested that a higher value of GV is an important factor for the design of orthodontic mini-implants.

Ting et al[66] (2020) evaluated the mechanical properties of three types of micro-implants. All micro-implants were manually driven into artificial bones at an 8-mm depth. The insertion torque (IT), pullout strength (PS), and gripping volume (GV) of each type were measured. In conclusion, the design of thread and its GV were the important factors on the mechanical strengths of micro-implant.

Materials & Methods

MATERIALS AND METHODS

The present study was carried out in Department of Orthodontics and Dentofacial Orthopedics, Ragas Dental College and Hospital, Chennai.

This in vitro study was done to:

1. Evaluate the proliferation and morphology of human fetal osteoblast cells seeded on the surface of two different brands (Bioray & Dentos) of titanium infrazygomatic (IZC) mini-implants in three different time periods (24, 48 & 72 hours).
2. Assess the chemical composition on the surface of two different brands of titanium IZC mini-implants in three different region (head, body & tip) and detailing the chemical component profile.

TITANIUM INFRAZYGOMATIC MINI-IMPLANTS AND POLYSTYRENE DISK

The studied factors are two different brands of self-drilling titanium IZC mini-implants (Test material) and polystyrene disk (Control) for evaluating cell adhesion, proliferation and morphology of hFOB cells. Bioray titanium IZC mini-implant (Korea) of 11mm in length and 1.8mm in diameter, and Dentos titanium IZC mini-implant (Korea) with 12mm length and 2mm in diameter were chosen for this study. Both the IZC mini-implants had machined surface (smooth surface), whereas polystyrene disk was 13mm in diameter and 1mm in thickness with smooth surface was used as control (Nest biotech, China). (**Figure. 18, 19 & 20**).

Sample size consisted of twelve (n=6: Bioray and n=6: Dentos) self-drilling titanium IZC mini-implants (test material) and three polystyrene disks (control). Two IZC mini-implant from each test material (Bioray and Dentos) and one polystyrene disk were used in each time period (24, 48 & 72 hours) for evaluating the cell adhesion, proliferation and morphology of hFOB cells. The specification of the titanium IZC mini-implant and polystyrene disk are described in **Table 1**.

PREPARATION OF BASAL MEDIUM FOR HUMAN FETAL OSTEOBLAST (hFOB) CELL CULTURE

The basal culture medium is the most important component of the cell culture environment, because it provides the necessary nourishments, growth factors, hormones for the cell growth, regulates pH and osmotic pressure of the cell culture. Basal medium for hFOB cell proliferation (cell line) contains 1:1 mixture of 50ml Dulbecco's modified eagle's medium and 50ml Ham's F12 medium (DMEM/F-12) (Gibco by life technologies, India). This basal medium is widely used for supporting the growth of hFOB cells, which contains high concentration of glucose, amino acids and vitamins for cell growth (**Figure. 4 & 5**). The basal medium was freshly prepared each time by mixing 50ml of Dulbecco's modified eagles medium and 50 ml of Ham's F12 medium. In addition to make the growth medium complete, 10 ml of 10% of fetal bovine serum was added, which contains protein, lipid and growth factor which is not present in DMEM/F-12 (**Figure. 6**). 1 ml of gentamycin was

added to the basal medium to prevent bacterial and fungal growth (**Figure. 7**). This completely prepared medium (recommended basal medium) was then transfered into a sterillized tissue cultured flask (T flask) (**Figure. 8**).

HUMAN FETAL OSTEOBLAST CELL (hFOB 1.19)

Human fetal osteoblast cell (hFOB 1.19), (CRL-11372) were obtained from American Type Culture Collection (ATCC), Manassas, Va, USA, Patent number 5,681,701. The hFOB cells were cultured in accordance with ATCC recommendations. The frozen hFOB cells was delivered in dry ice packing under temperature of -78.9°C. After unwrapping the pack, the frozen hFOB cells were stored in a cryowell, which contained about 1ml of hFOB cells (**Figure. 1, 2 & 3**). All the procedures involving the live cell culture was performed in a laminar flow cabinet in order to prevent contamination.

HANDLING OF HUMAN FETAL OSTEOBLAST BEFORE & AFTER CULTURE

PROCEDURE:

To insure highest level of viability of hFOB cells, thawing of the cryowell content was initiated as soon as possible. Thawing the cryowell containing hFOB cells was done by gentle agitation at 37°C water bath for approximately two minutes (**Figure. 9**). During thawing, to minimize the possibility of contamination, the cryowell was held with a paper strip around

its neck (O-ring and cap) to stabilize and prevent it from getting submerged into the water bath. Thawing was done to convert the temperature of cells from the freezing temperature to normal room temperature (37°C) for its optimal hFOB cell growth. After thawing, the cryowell containing hFOB cells were transferred into a centrifuge tube containing 9 ml of the freshly prepared basal medium (**Figure. 10 & 11**). The centrifuge tube containing hFOB cells were spun at approximately 3000 to 5000 rpm for 5 to 7 minute in a centrifuge machine (Doctor Centrifuge Machine 8 Tube × 15 ML, India) (**Figure. 12**). This agitation (centrifuging) was carried out to eliminate the glycerol (cryosolution) present in the hFOB cells. After centrifuging, the sedimented hFOB cells were collected at the bottom of the centrifuge tube. The supernatant basal medium after centrifuging was gently discarded (which contains glycerol, which will prevent cell growth) by pipetting out without disturbing the sedimented cell pellet collected at the bottom of centrifuge tube (**Figure. 13**). 2ml of freshly prepared basal medium was gently added using micropipette to the sedemented hFOB (resuspended cell pellet). Then gently add 9 ml of prepared basal medium into the centrifuge tube to reduce the concentration of sedimented cells, so that hFOB cells can grow appropriately. 2ml of resuspended cell culture was transferred into five different tissue cultured flask (T flask 25 cm^2) each containing 15 ml of freshly prepared basal medium (**Figure. 8 & 14**). All the five T flask containing prepared basal medium and hFOB cells were placed into an incubator at 34 °C for hFOB cell proliferation and its growth (**Figure. 15, 16 & 17**).

SUB-CULTURING THE HUMAN FETAL OSTEOBLAST CELLS

The cultured hFOB cells were kept viable throughout the experiment by sub-culturing the cells. Repeated sub-culturing was done by adding and removing the basal medium repeatedly for every 2 to 3 days.

PROCEDURE:

To maintain the viability of the hFOB cells throughout the experiment sub-culturing was done periodically.

1) Remove and discard the old supernatant basal medium from the T- flask containing the cultured hFOB cells, which were stored in the incubator

2) Briefly rinse the cell layer with 2 to 3 ml of trypsin-EDTA (ethylene diamine tetra acetic acid) solution to detach the hFOB cells which were adhering to the T-flask (the function of trypsin is to break down the proteins, which enable the cells to adhere to the vessel) and 1ml of Dulbecco phosphate buffered saline was added to avoid osmotic shock to the hFOB cells. Then T flask containing hFOB cells was observed under an invert phase contrast microscope at various magnification (10x, 40x and 100x) (Jayagen biologics private limited, India) until hFOB cell layer were dispersed. In order to avoid clumping of hFOB cells T flasks were not agitated, which allows the hFOB cells to detach from the T flask. (**Figure. 37, 38 & 39**)

3) Again add 15 ml of freshly prepared basal medium into the T flask, so that the serum content in the medium will inactivate the remaining trypsin activity, which is toxic to the hFOB cell growth (trypsin should be neutralized with

serum that contained in the medium because prolonged exposure of trypsin could damage the cell surface receptor)

4) Finally all the T flask were placed in the incubator at 34°C.

hFOB cells obtained through the subculturing was ready for experiment to evaluate the cell adhesion, proliferation and its morphology.

STERILIZATION OF TITANIUM IZC MINI-IMPLANTS AND POLYSTYRENE DISK

Prior to seeding of the hFOB cells on the surface of titanium IZC mini-implants and polystyrene disk, they were completely immersed in 10 ml of 70% ethanol for 30 minutes to achieve sterilization. (**Figure. 21**)

SEEDING OF CULTURED hFOB CELLS ON THE SURFACE OF TITANIUM IZC MINI-IMPLANT AND POLYSTYRENE DISK

Cultured hFOB cells were seeded on the surface of the titanium IZC mini-implants and polystyrene disk in three different time periods (24, 48 and 72 hours), in order to evaluate the cell adhesion by means of proliferation and morphology.

PROCEDURE:

Two brands of twelve titanium IZC mini-implants and three polystyrene disk (control) were horizontally placed in a well plate containing 2 ml of freshly prepared basal medium (**Figure. 8**). 1 ml of cultured hFOB cells were added to

each well (**Figure. 22, 23 & 24**). The response variable was cell adhesion, which was evaluated both quantitatively and qualitatively according to proliferation (evaluate the cell count using hemocytometer) and cell morphology of hFOB (SEM) respectively.

Human fetal osteoblast cells adhesion was evaluated using trypan blue vital dye (exclusion method) in the cultured basal medium containing titanium IZC mini-implants in three different time periods (24, 48 and 72 hours) (**Figure. 25**). To get uniform distribution of hFOB cells, the suspension was thoroughly stirred using a micropipette. In addition 10 microliters (µL) of the cell suspension was diluted in 10µL of trypan blue dye. From 20 microliters (µL), 10 microliters (µL) of mixed suspension was transferred into a hemocytometer (neubauer chamber; weber scientific USA) to evaluate cell proliferation (**Figure. 26 & 27**). In order to eliminate the bias in evaluating the cell proliferation counting was done at three times in three different time periods. The hFOB cells were counted under an invert phase contrast microscope at 10x, 40x and 100x magnification (**Figure. 28**). It is based on the principle that live cells possess intact cell membrane that excludes certain dyes, such as trypan blue, eosin, or propidium iodide, whereas dead cells will imbibe the dyes (**Figure. 29**). The total number of proliferated hFOB cells present were counted on each square grids of the neubauer chamber at different time periods (24, 48 and 72 hours) obtained are as follows,

HUMAN FETAL OSTEOBLAST (hFOB) CELLS COUNT

Total number of cells = Number of cell counted ×initial volume ×dilution× 10^4 Square considered for counting[29, 16]

HUMAN FETAL OSTEOBLAST CELL MORPHOLOGY

Human fetal osteoblast cell morphology was analyzed using a scanning electron microscope (SEM, Hitachi S-3400N, Singapore). Three different time periods were chosen to evaluate the morphology of hFOB cells on the surface of Dentos and Bioary titanium IZC mini-implants (body (thread, pitch) and tip) and polystyrene disk (24, 48 and 72 hours). Before morphological examination the hFOB cells on the titanium IZC mini-implants surface were fixed using karnovsky solution (**Figure. 30**). Titanium IZC mini-implants and the polystyrene disk were transfered into the karnovsky solution for 1 hour at room temperature (37^{o} C) (**Figure. 31**).

Titanium IZC mini-implants and the polystyrene disk were positioned and secured on a metal stubs which contained carbon tab for fixation (**Figure. 32**). After fixation the samples were placed in a sputter coating unit (Hitachi E-1010) for 30 seconds (**Figure. 33**). Gold sputter coating was done for 30 seconds (**Figure. 34**). After gold sputter coating the samples were studied under scanning electron microscope for morphology of hFOB in three time period at various magnifications (10x, 30x, 50x up to 500x) (**Figure. 35, 36, 40-48**).

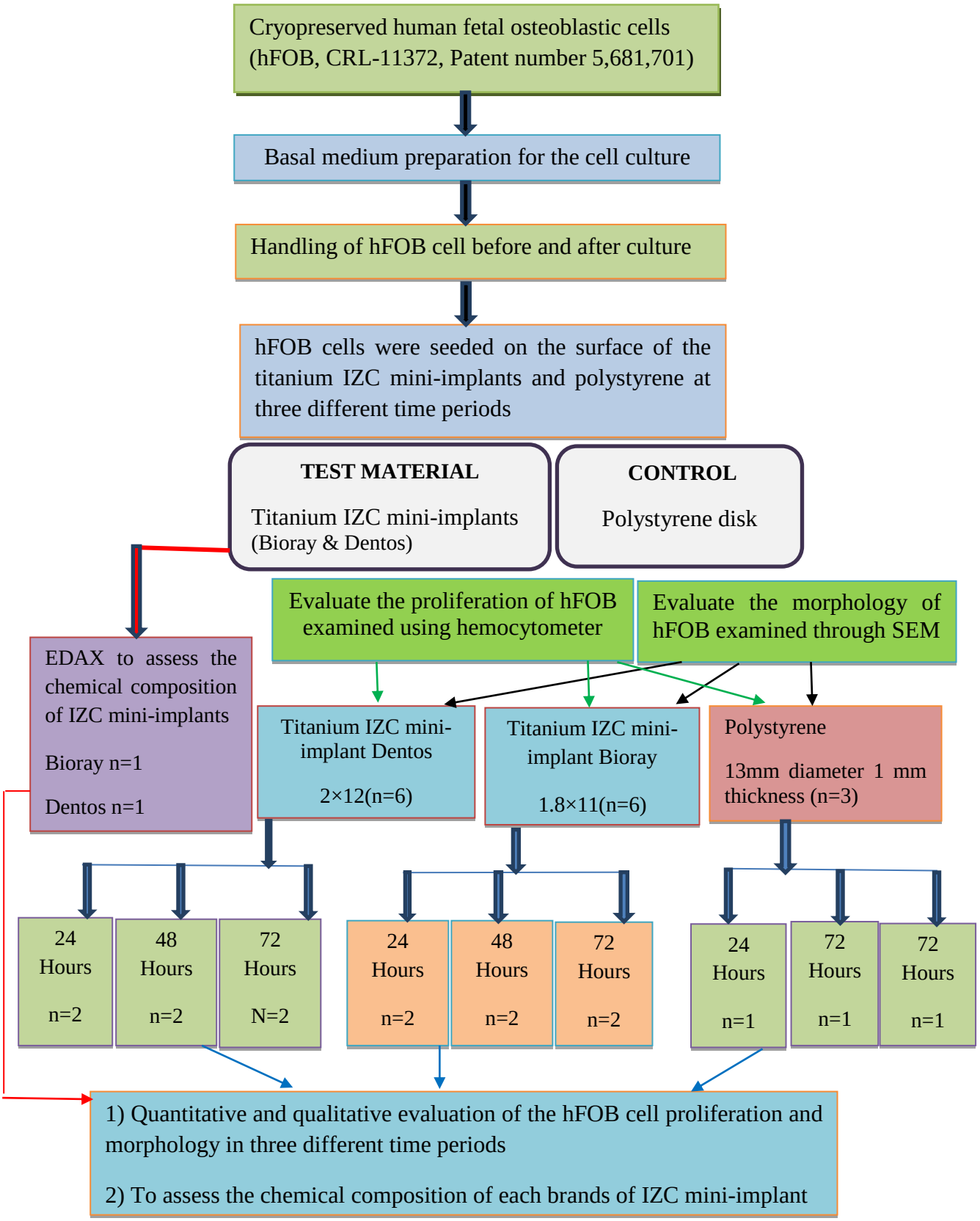
CHEMICAL COMPOSITION OF TWO BRANDS OF AS RECEIVED TITANIUM IZC MINI-IMPLANTS WAS ASSESSED USING EDAX ANALYSIS

One sample of as-received titanium IZC mini-implant from each brands was subjected to composition analysis using an Energy Dispersive X-ray analysis (EDAX, Horiba Instruments japan TMP/RP based vacuum system operated at 15kv). Head, body (thread & pitch) and tip regions of the Dentos and Bioray IZC mini-implant were assessed for surface composition.

STATISTICAL ANALYSIS

Data entry and statistical analysis was performed with using the SPSS v.17 (SPSS Inc., Chicago, Illinois, USA). The hFOB cell proliferated on the surface of two brands of IZC mini-implant and polystyrene disk data were assessed for normality by using Kolmogorov-Smirnov and Shapiro-Wilk test. Intra group comparison of hFOB cell proliferation were done using one way ANOVA with 95% confidence interval was performed. For multiple comparisons within the parameters Tukey and Post hoc test was done. hFOB cell morphology was described in terms of cell type and its shape of hFOB cells. Descriptive statistics were done to find the composition of Bioray and Dentos IZC mini-implants. A p value of less than or equal to 0.05 was taken as significant.

SCHEMATIC REPRESENTATION OF THE PRESENT IN VITRO STUDY



Figures

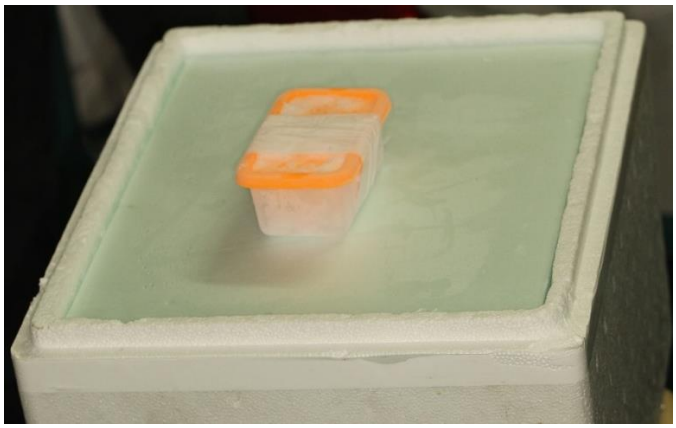


Figure 1. Human fetal osteoblast cells (hFOB) cells are stored in dry ice pack (-78.9^{o}c)

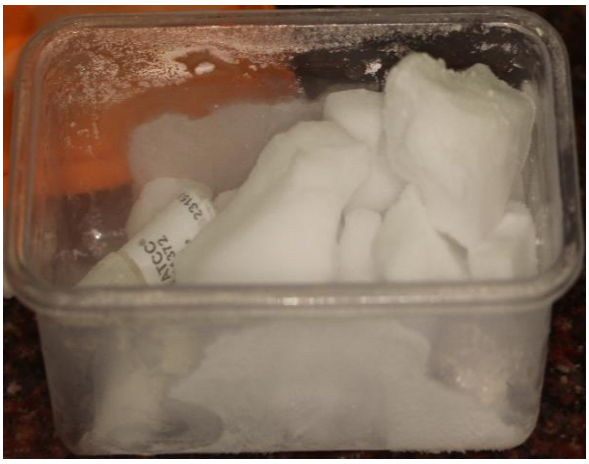


Figure 2. hFOB cells unwrapped from the dry ice packing

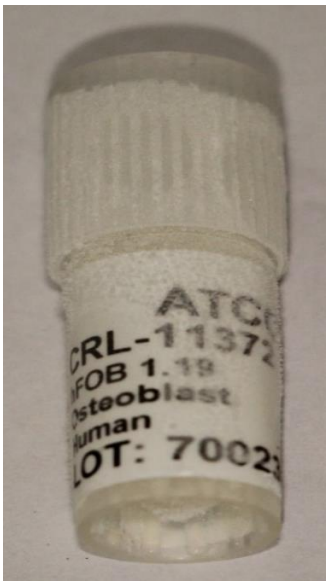


Figure 3. Cryowell containing 1ml of hFOB cells (CRL-11372)

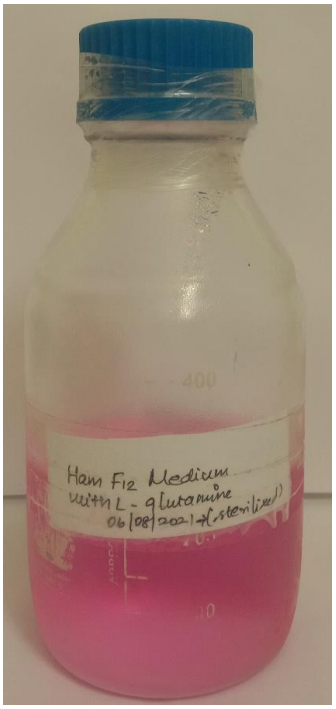


Figure 4. Ham's F12 medium

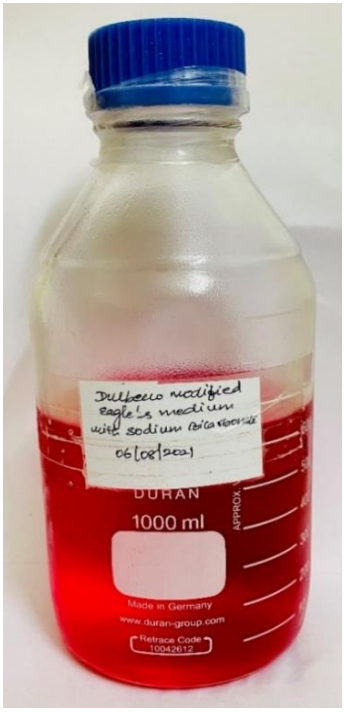


Figure 5. Dulbecco's modified eagle's medium

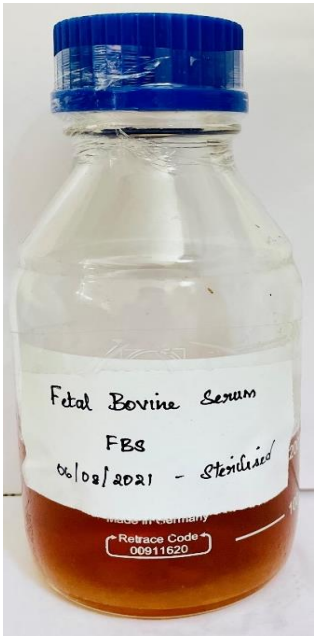


Figure 6. 10% of fetal bovine serum medium

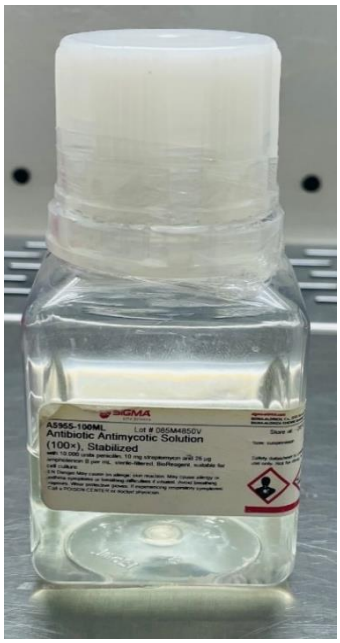


Figure 7. Antibiotic solution 1 ml of gentamycin

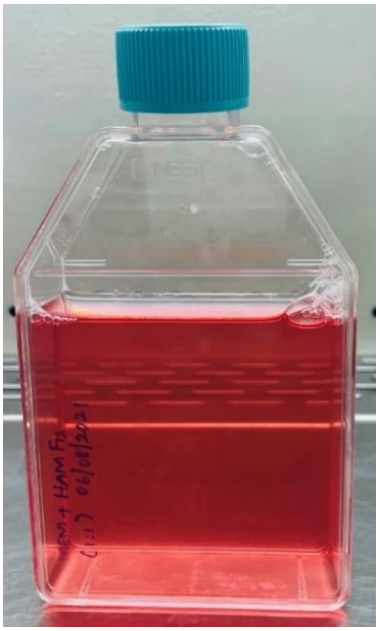


Figure 8. Completely prepared basal medium in T flask

Figure 9. Thawing of hFOB cells at 37 ^{o} water bath

Figure 10. Transferring 9 ml of freshly prepared basal medium using micropipette in a centrifuge tube

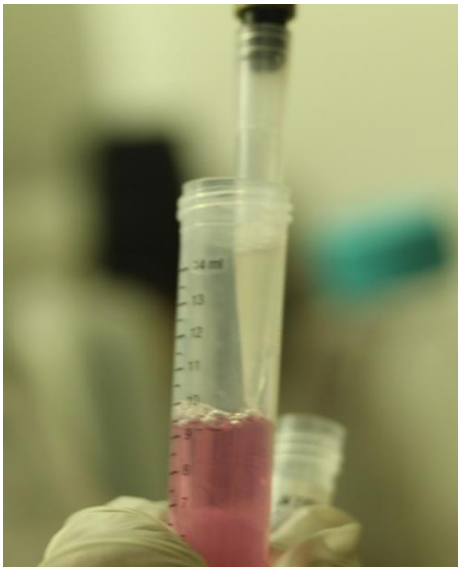


Figure 11. Tranfering 1ml of hFOB cells to centrifube tube using micropipette contained 9 ml of freshly prepared basal medium

Figure 12. Centrifuge machine

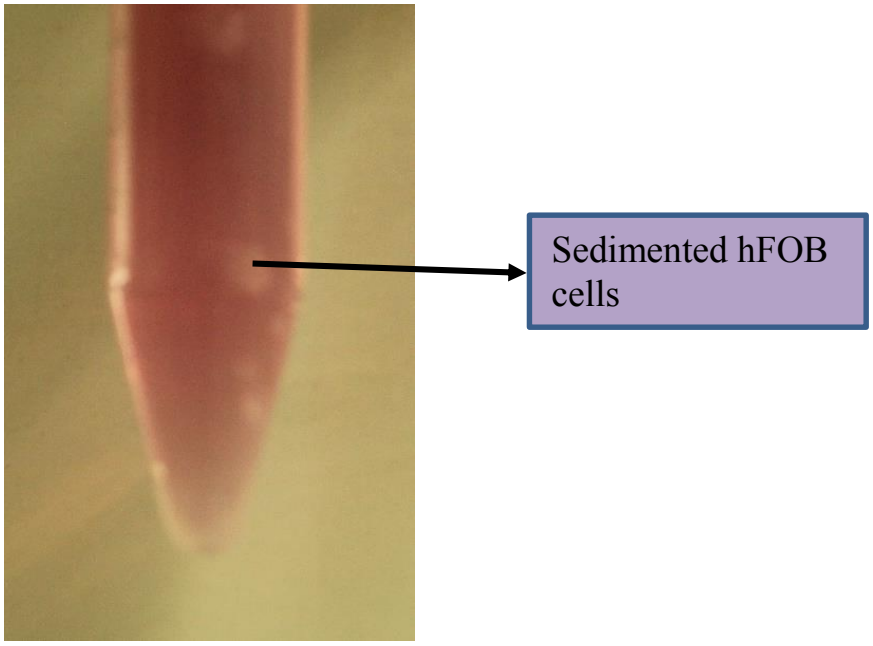


Figure 13. hFOB cells sedimentation (resuspended cell) at the bottom of centrifuge tube after centrifugation

Figure 14. Empty tissue culture flask

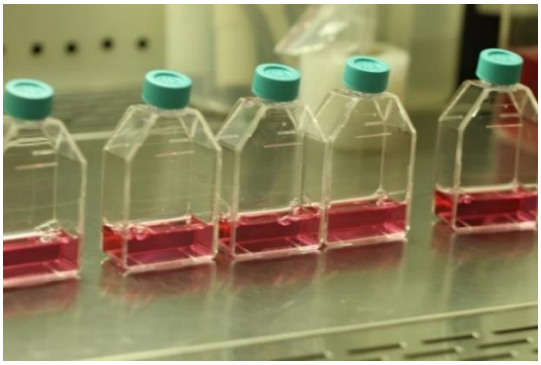


Figure 15. 15 ml of prepared basal medium and 2ml of cultured hFOB cells in a T flask

Figure 16. Incubator set with a temperature at 34°C

Figure 17. T flask with prepared basal medium and hFOB cells placed inside the Incubator

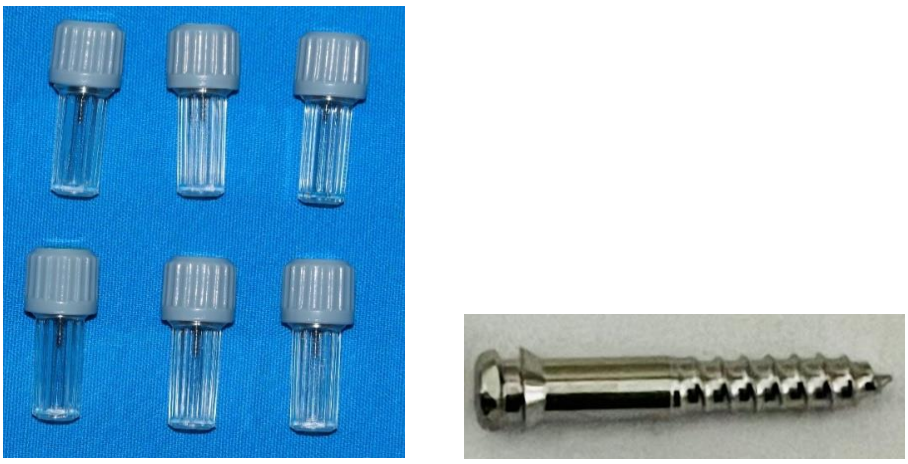


Figure 18. Bioray (korea) titanium IZC mini- implant 1.8x11(diameter x length)

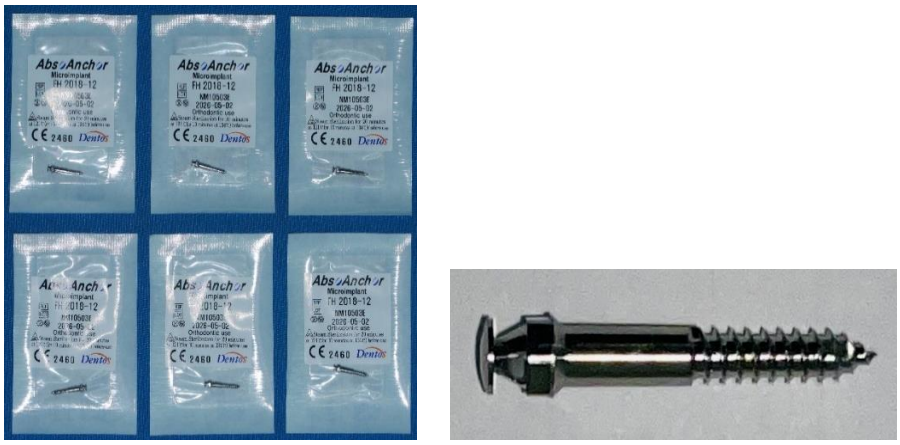


Figure 19. Dentos (korea) titanium IZC mini- implant 2x12 (diameter x length)

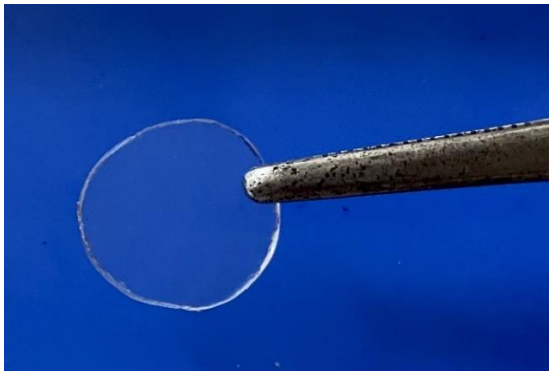


Figure 20. Polystyrene disk 13m diameter with 1mm thickness

Figure 21.Titanium IZC mini-implants and polystyrene were immersed in 10 ml

Figure 22. Empty well plate

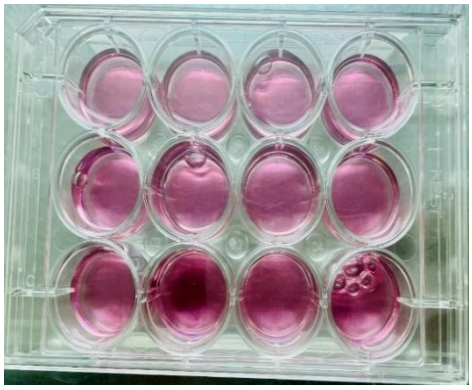


Figure23. Well plate that contain 2 ml of freshly prepared basal medium and 1 ml of hFOB cells

Figure 24.Titanium IZC mini-implants and polystyrene placed in well plate containing 2ml of basal medium and 1ml of cultured hFOB cells

Figure 25. Trypan blue vital dye

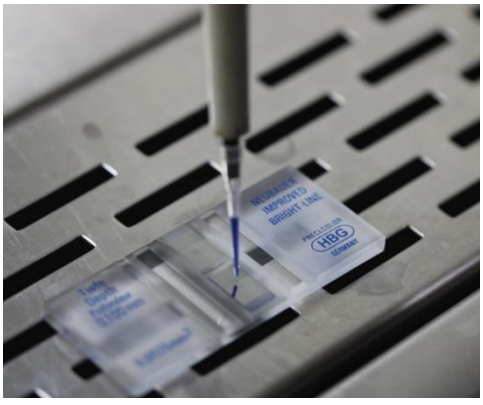


Figure 26. 10µL of mixed suspension placed in hemocytometer using micropippete

Figure 27. Hemocytometer with mixed suspension

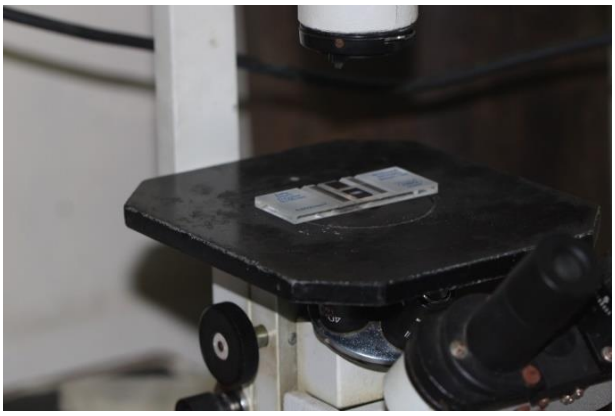


Figure 28. Hemocytometer is examined under an invert phase contrast microscope for counting the proliferated hFOB cells

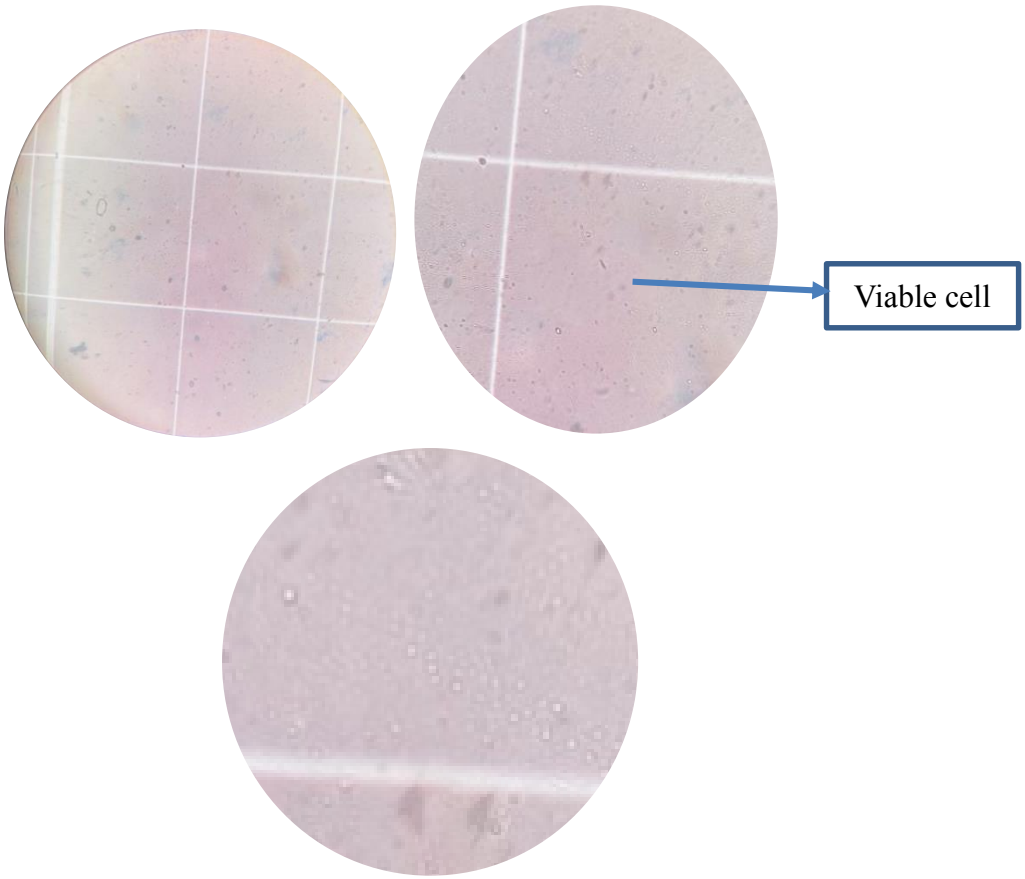


Figure 29. Viable hFOB cells examined under invert phase contrast microscope at various magnification (10x, 40x, and 100x)

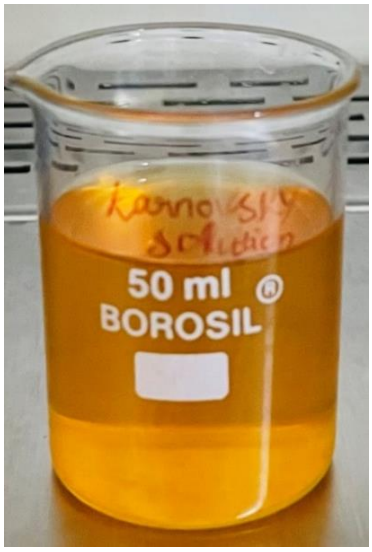


Figure 30. Karnovsky solution for fixing the hFOB cells on the surface of titanium IZC mini-implants and the polystyrene disk before morphology examination

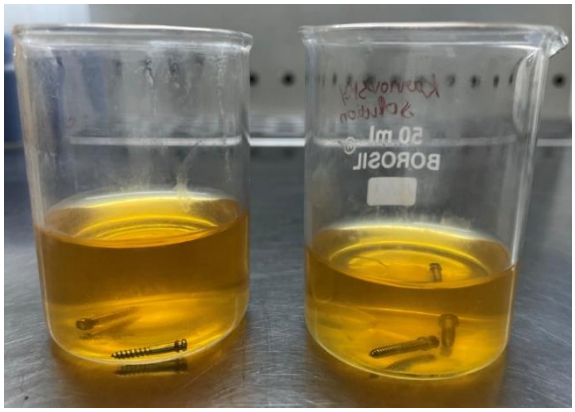


Figure 31. IZC mini-implant and polystyrene disk placed in the solution.

Figure 32. Samples secured in a stub which contains carbon tab for fixation

Figure 33. Samples were placed in a sputtering unit for 30 seconds for gold sputtering.

Figure 34. Gold sputter coated IZC titanium IZC mini-implants

Figure 35. Scanning electron microscope to assess hFOB cells morphology.

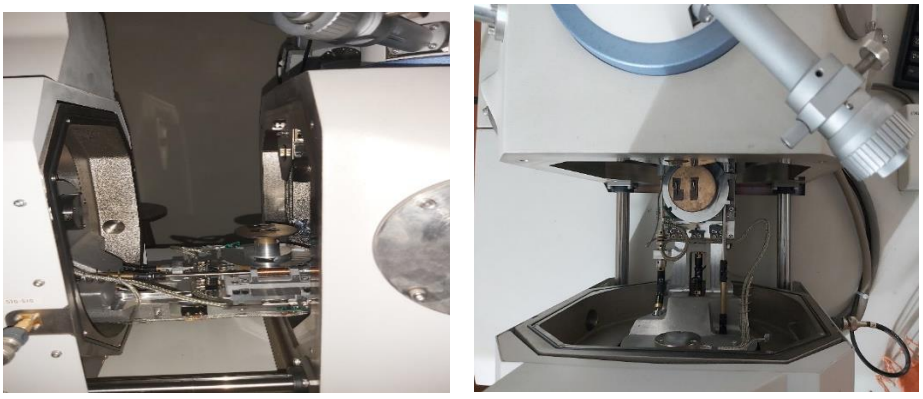


Figure 36. Samples are placed under the Scanning electron microscope for evaluating hFOB cell morphology.

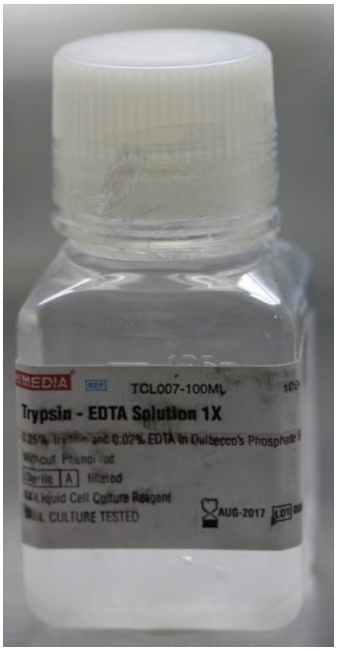


Figure 37. Trypsin EDTA solution for detaching hFOB cells from the T Flask

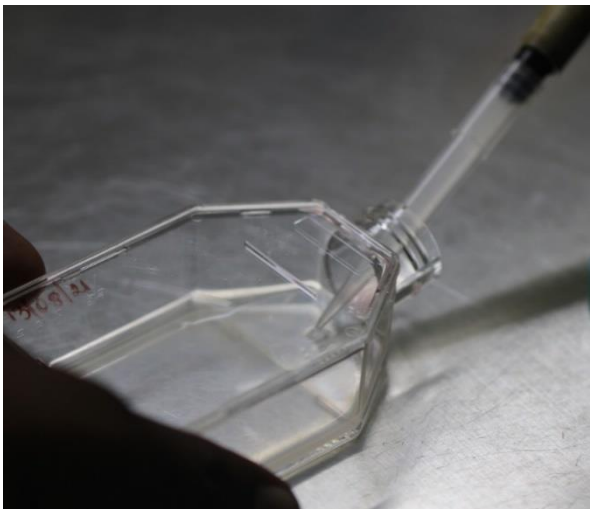


Figure 38. Rinse the cell layer with 2 to 3 ml of trypsin EDTA solution in the T flask

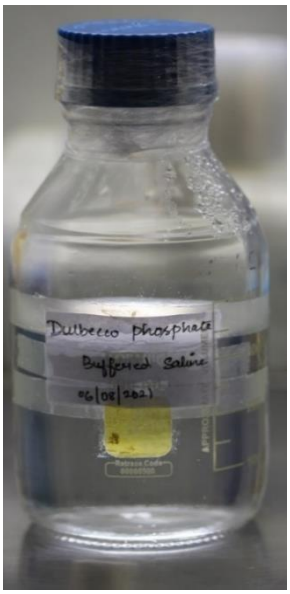


Figure 39. 1ml of Dulbecco phosphate buffered saline to avoid osmotic shock to the hFOB cells

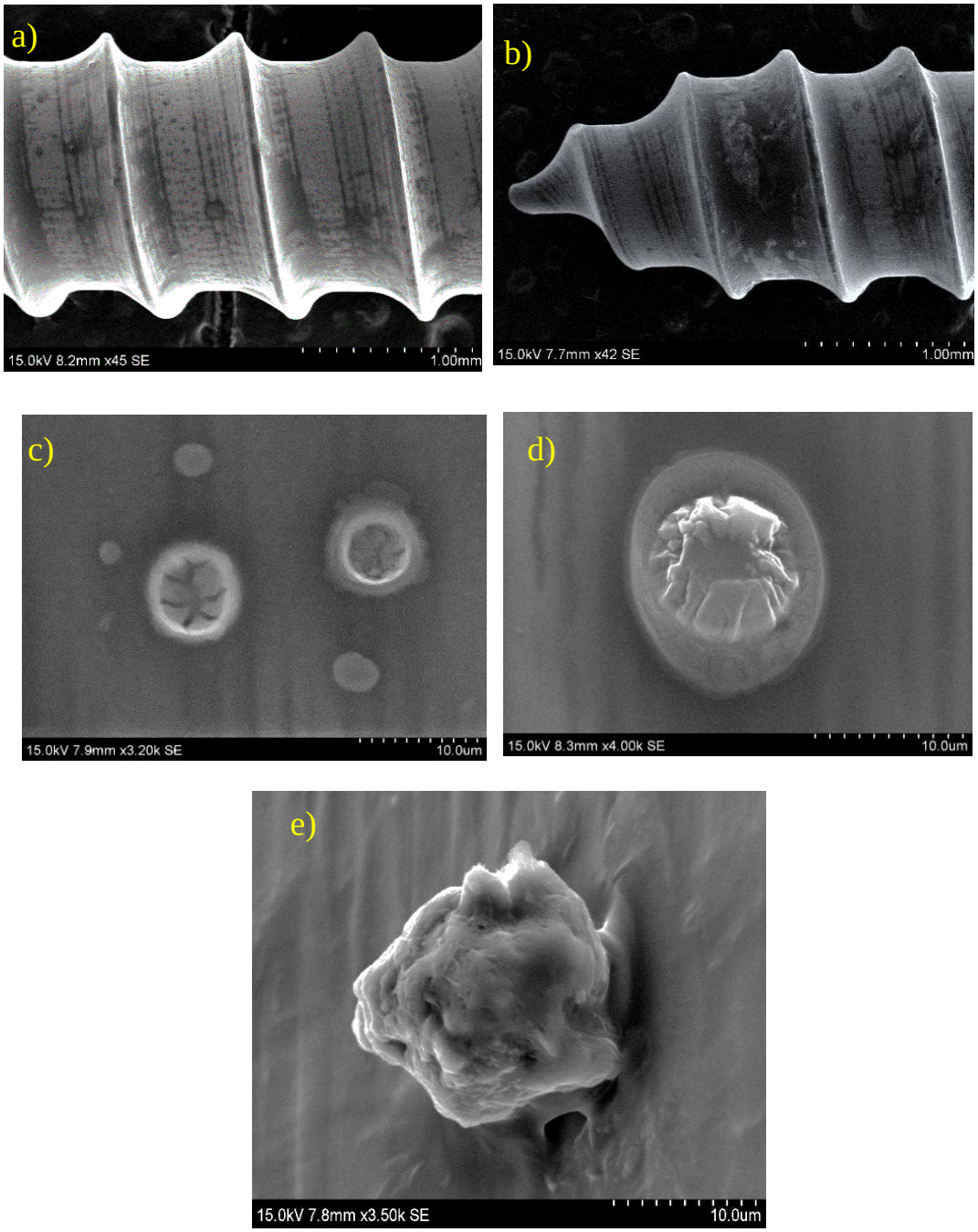


Figure 40 a, b. 24 hours surface analysis showed hFOB cell adhered more on the body (pitch & thread) of the Bioray IZC mini-implant than in the tip portion (SEM 500x, 10x) , c, d, e. round shaped cells at 24 hours.

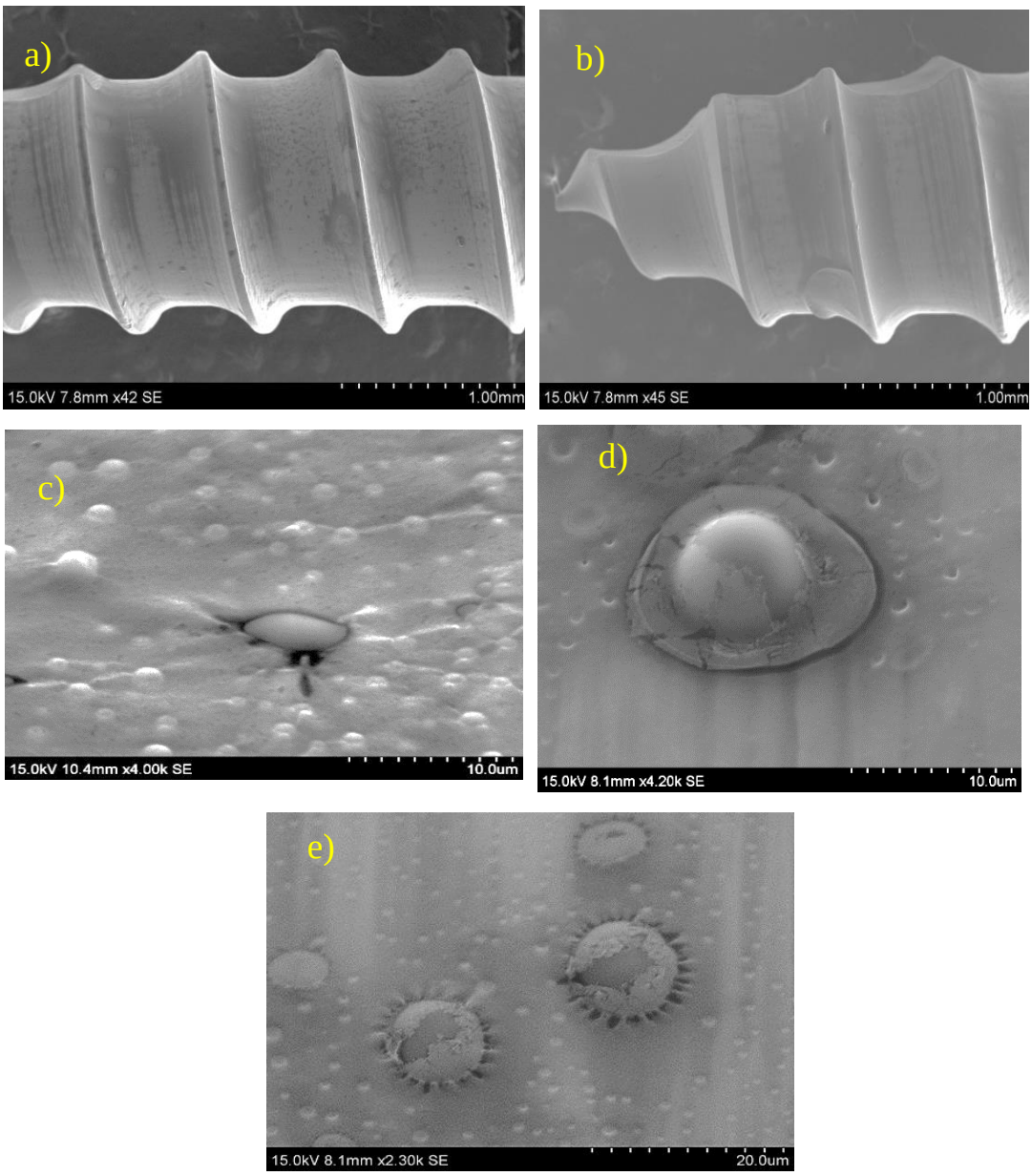


Figure 41 a, b. 48 hours surface analysis showed hFOB cells spreaded over the body (pitch & thread) of the Bioray IZC mini-implant compared to the tip portion, which is comparatively less (SEM 500x, 10x, 20x). c, d,e elongated shaped cells at 48 hours.

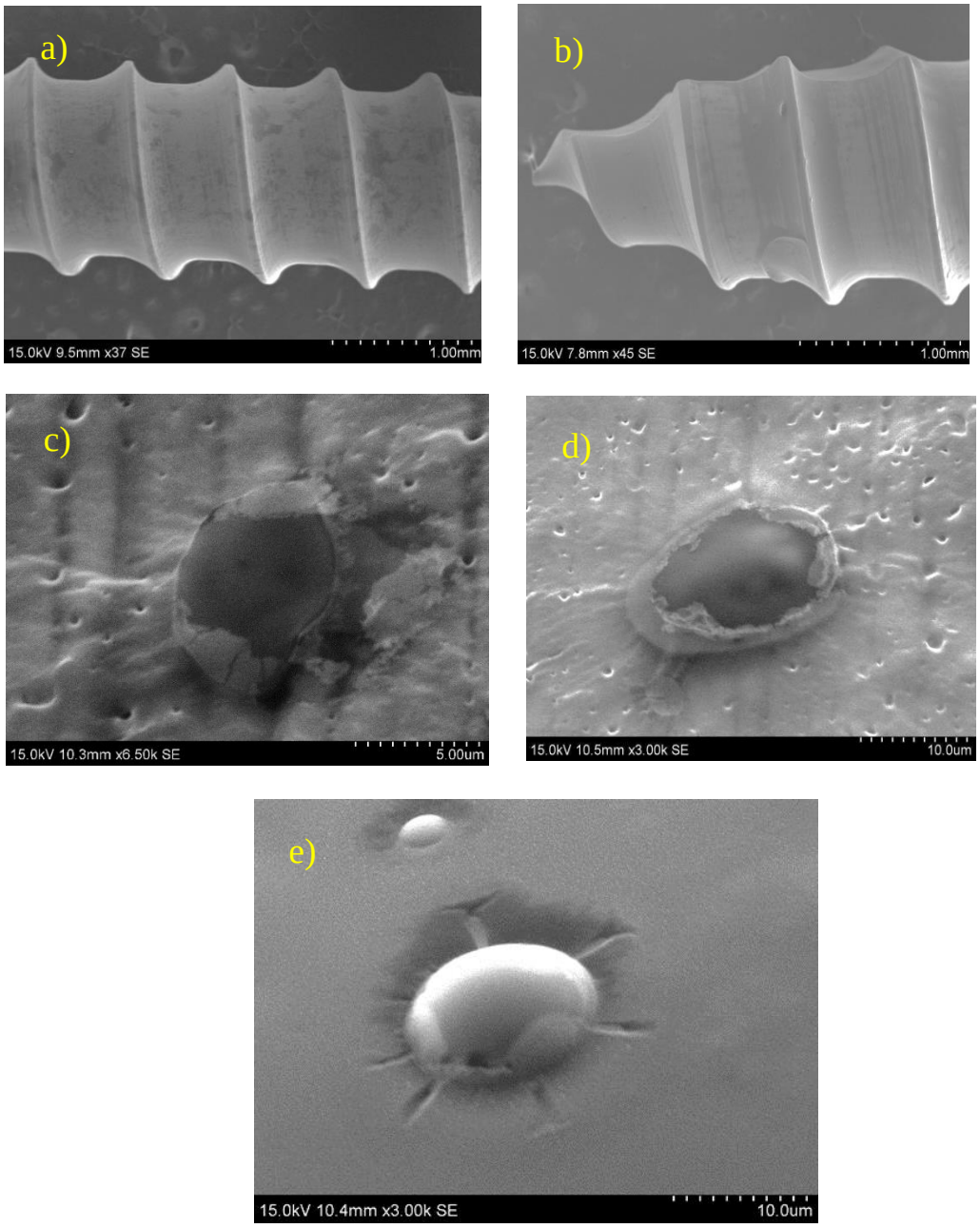


Figure 42. a, b. 72 hours surface analysis showed proliferating hFOB cells. Spreading and proliferation of cells over the body and tip of the Bioray IZC mini-implant (SEM 500x, 10x), c,d,e. stellate shaped hFOB cells with elongation was observed.

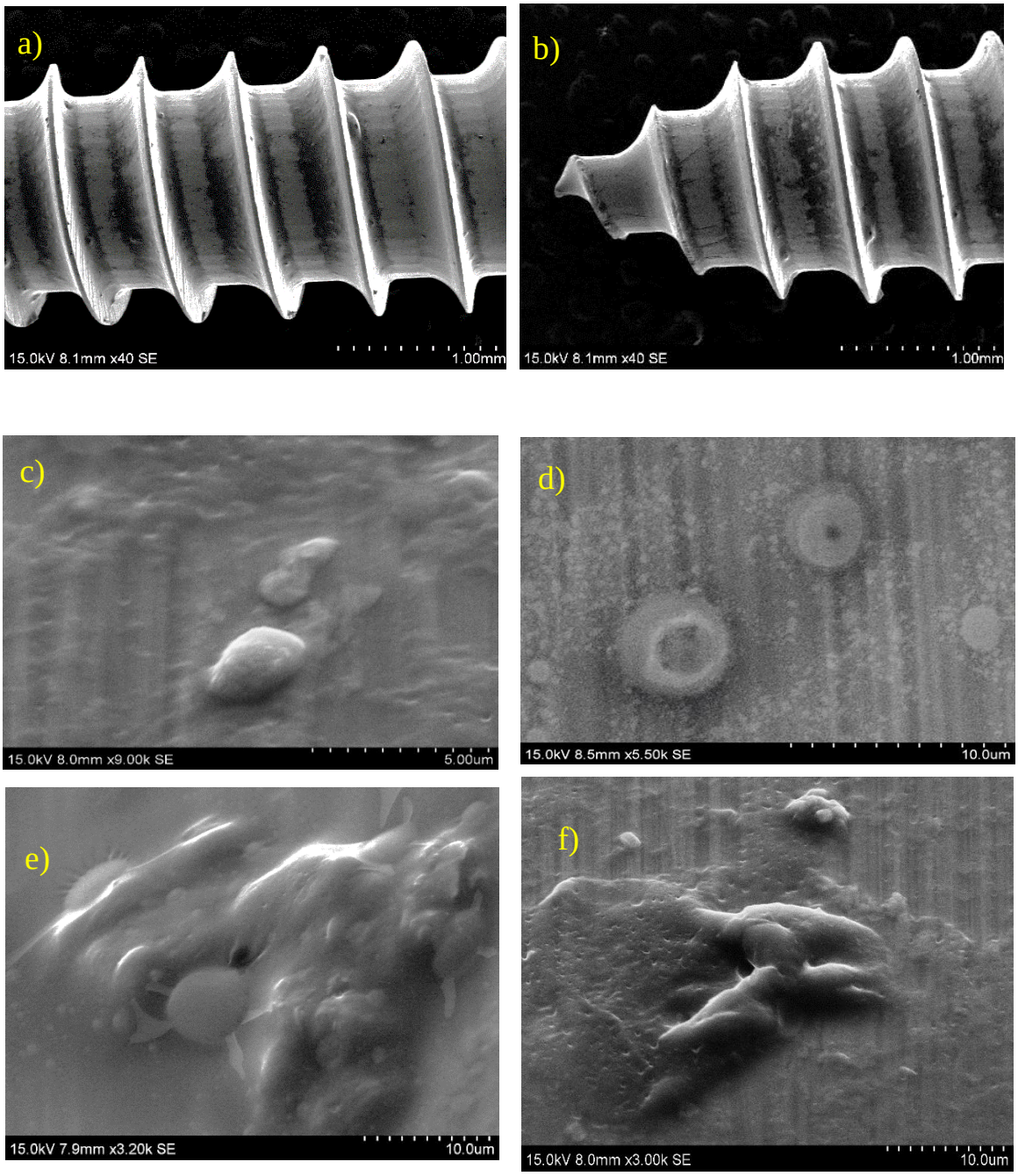


Figure 43 a, b. 24 hours surface analysis showed hFOB cell adhered more on the body (pitch & thread) of the Dentos IZC mini-implant than in the tip portion.(SEM 500x, 10x) c, d, e, f round shaped cells at 24 hours.

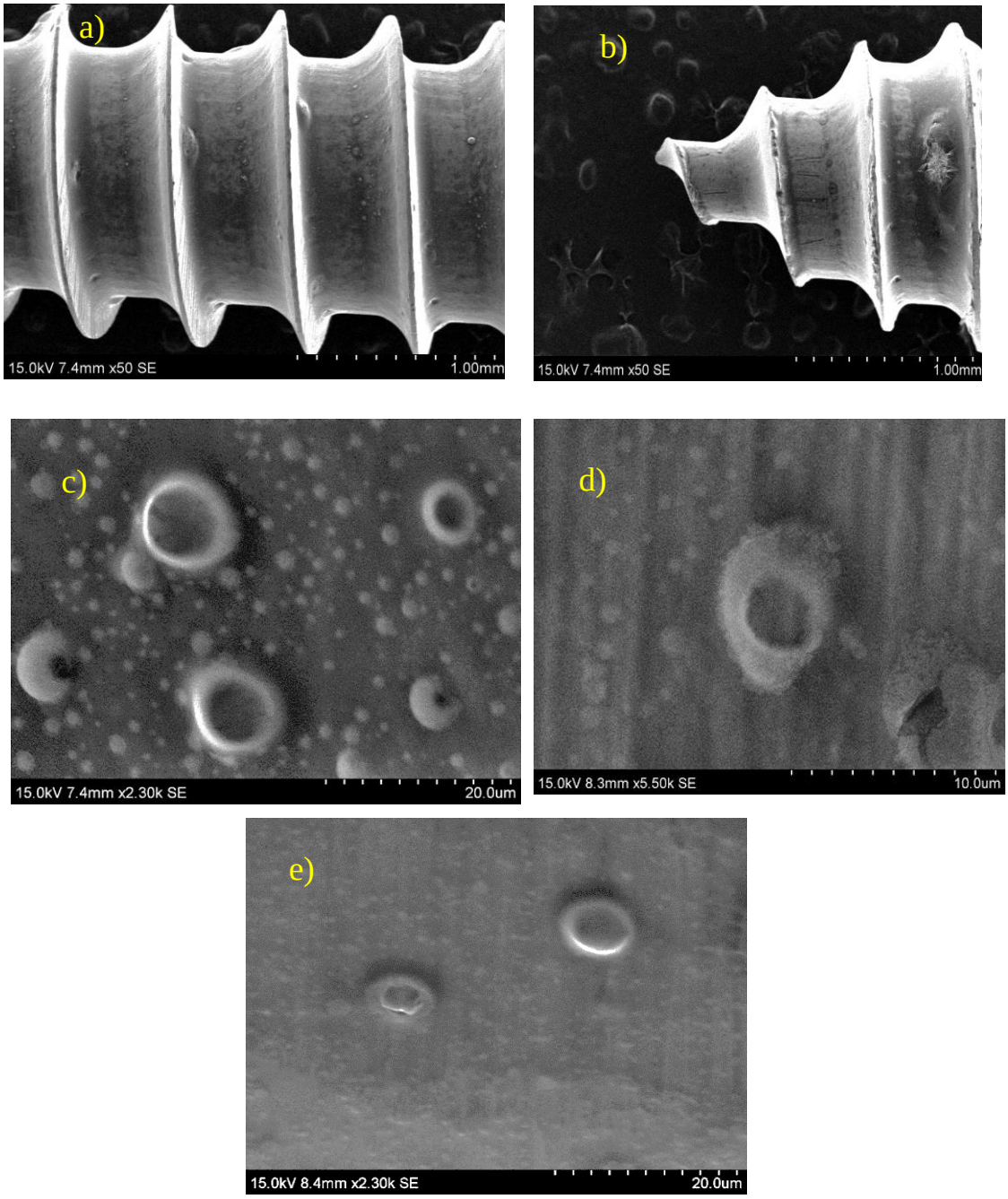


Figure 44 a, b. 48 hours surface analysis showed hFOB cells spreads over the body (pitch & thread) of the Dentos IZC mini-implant compared to the tip portion, which is comparatively less (SEM 500x, 10x, 20x). c,d,e elongated shaped cells at 48 hours.

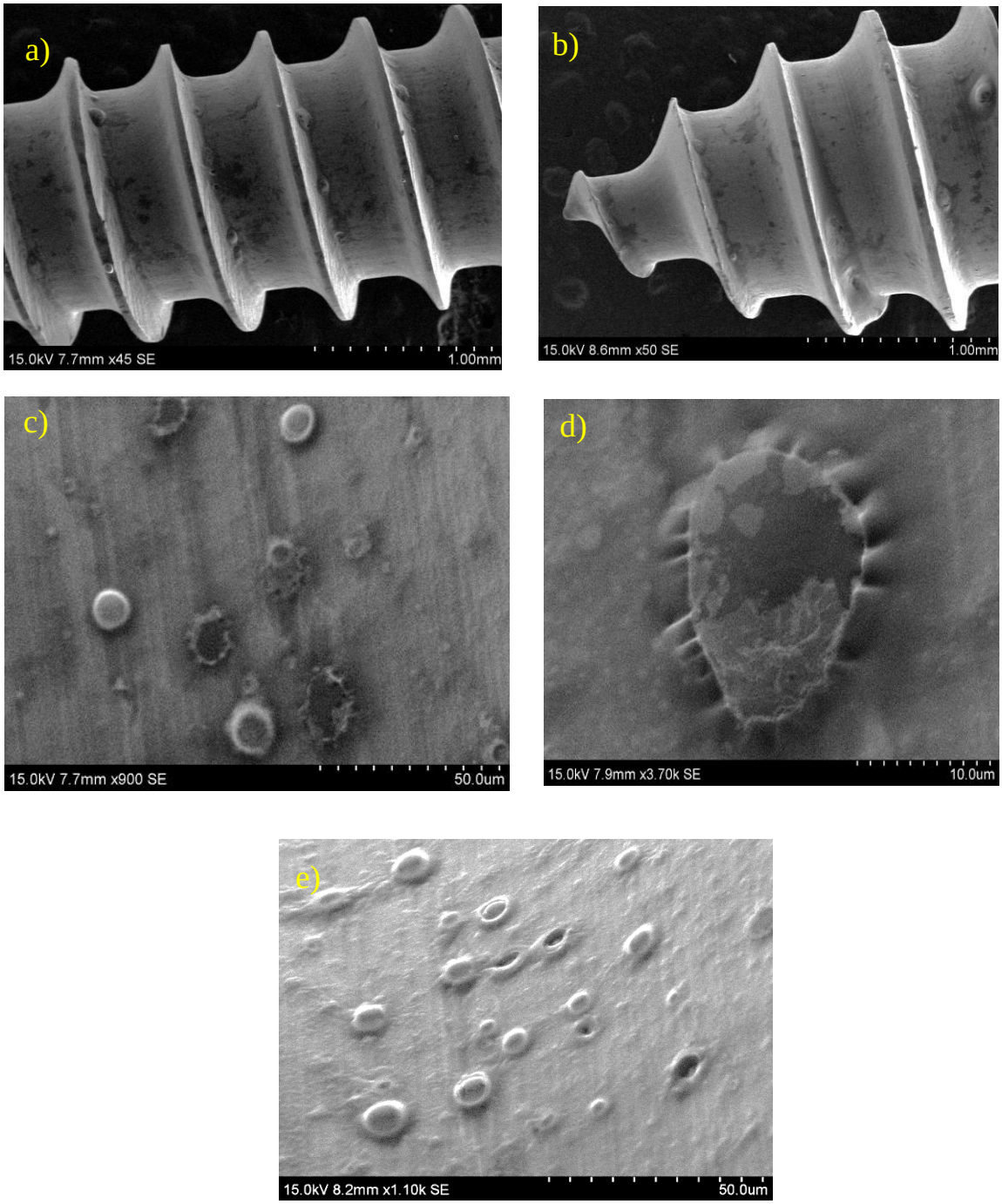


Figure 45 a, b. 72 hours surface analysis showed proliferating hFOB cells spreading and proliferation of cells over the body and tip of the Dentos IZC mini-implant (SEM 500x, 50x, 10x). c, d, e stellate shaped cells with elongated proliferation at 72 hours

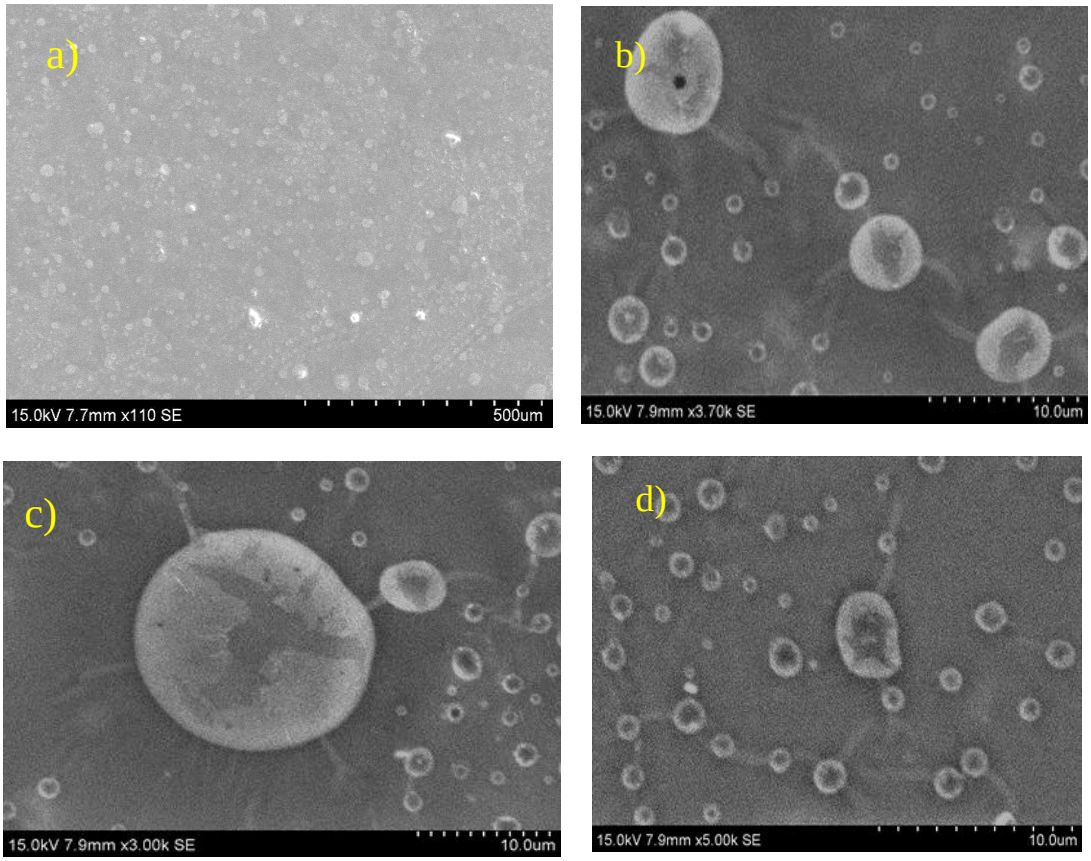


Figure 46 a. 24 hours surface analysis showed hFOB cell adhered on the surface of polystyrene disk (SEM 500x, 10x) b, c, d. round shaped cells at 24 hours)

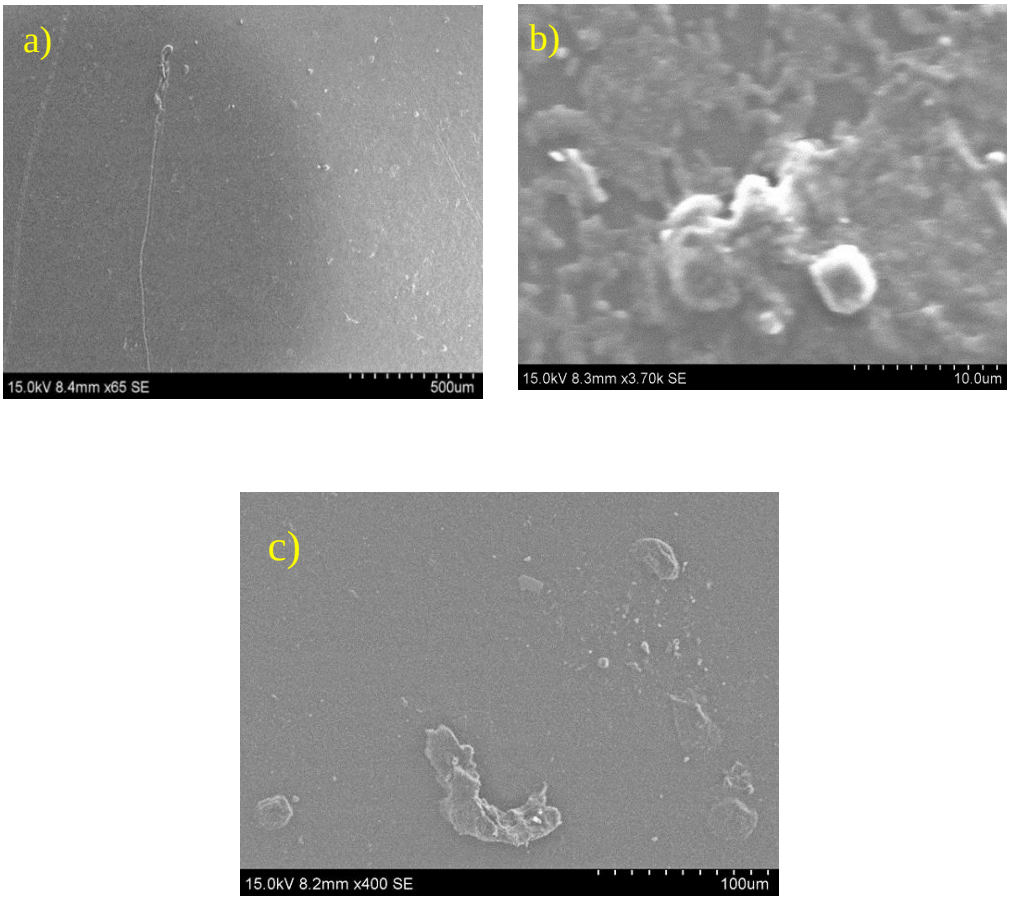


Figure 47 a. 48 hours surface analysis showed hFOB cells spreads over the surface of polystyrene disk. (SEM 500x). b, c. elongated shaped cells at 48 hours

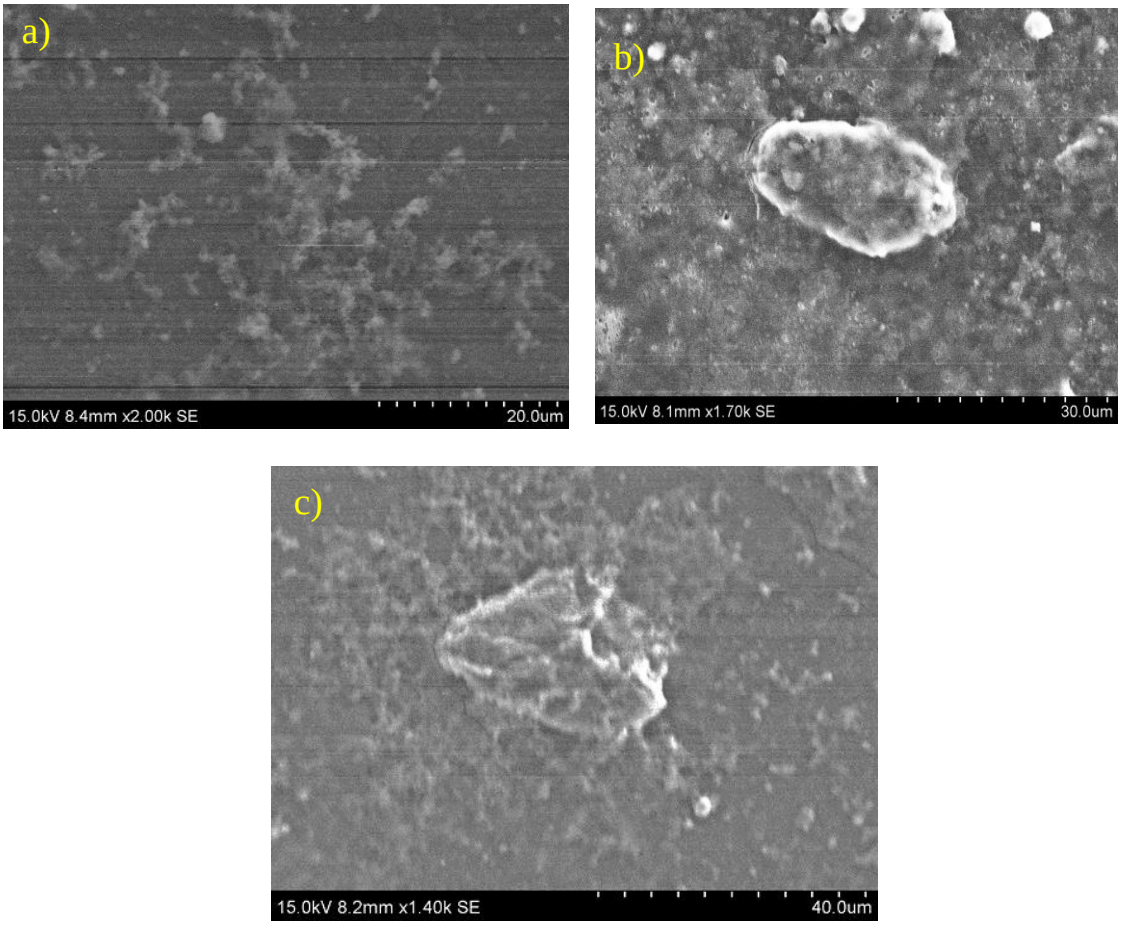


Figure 48 a. 72 hours surface analysis showed proliferating hFOB cells. Spreading and proliferation of cells over the surface of Polystyrene disk (SEM 20x, 40x). b, c. stellate shaped cells with elongated proliferation at 72 hours.

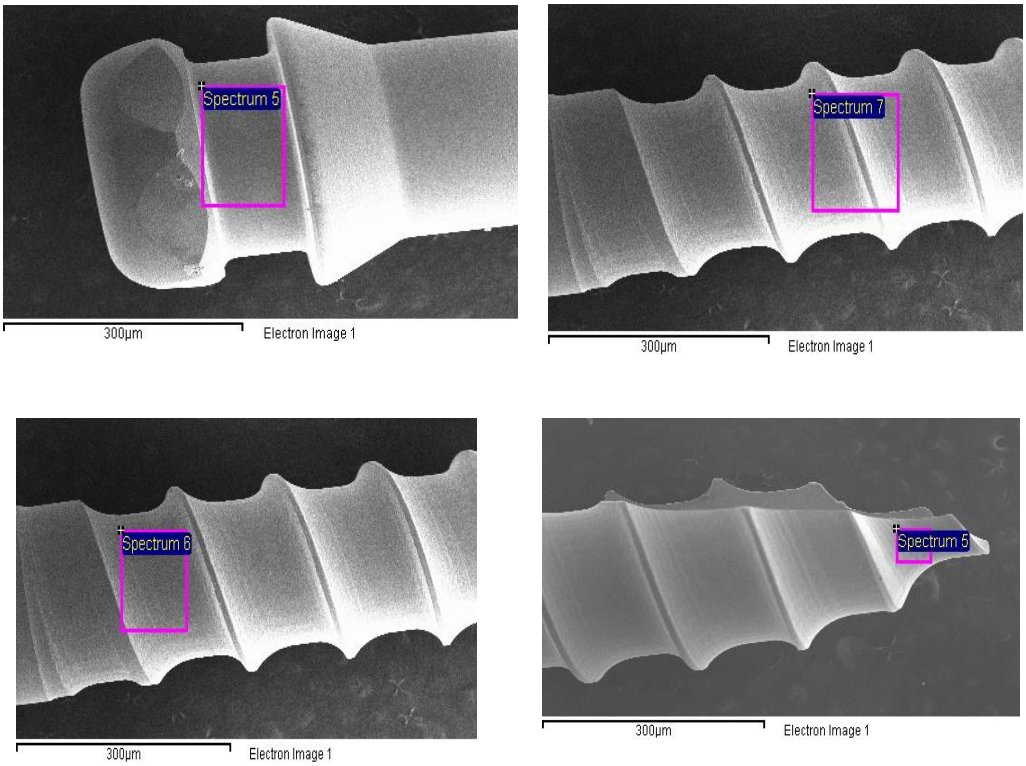


Figure 49. As-received elemental weight percentage of assessed on the surface of head, body and tip region Bioray IZC mini- implant using EDAX.

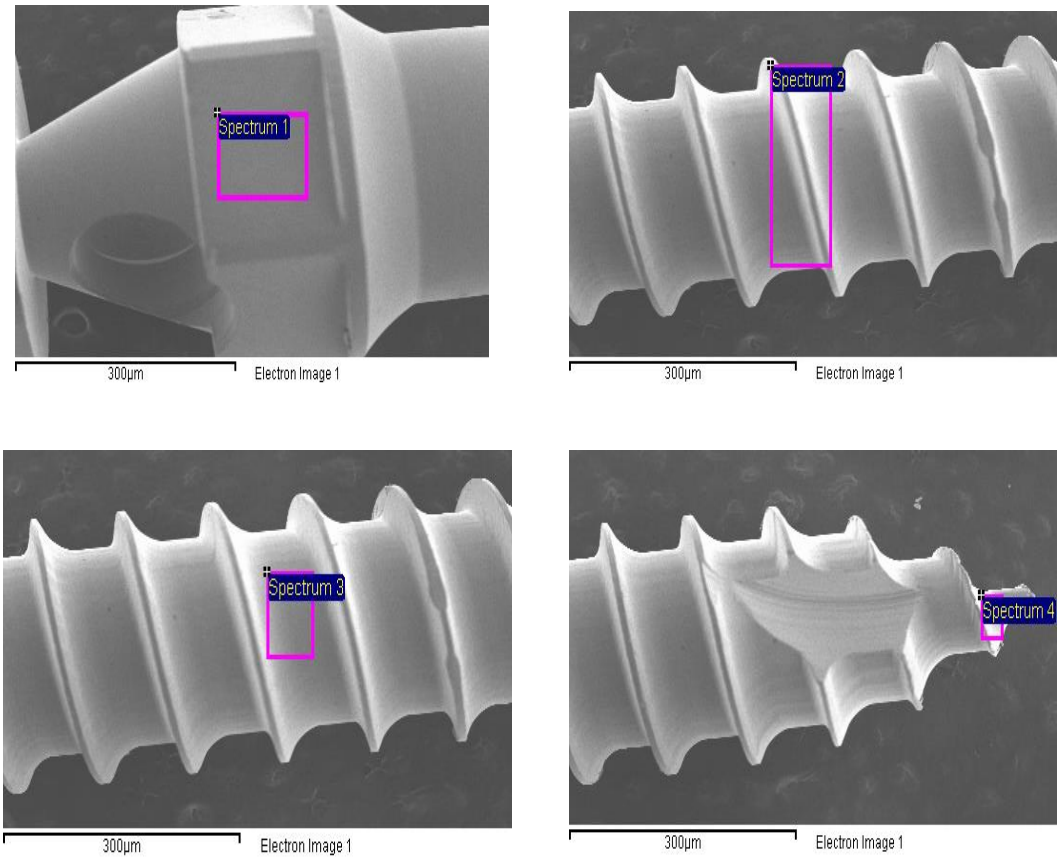


Figure 50. As-received elemental WG% of assessed on the surface of head, body and tip region Dentos IZC mini-implant using EDAX

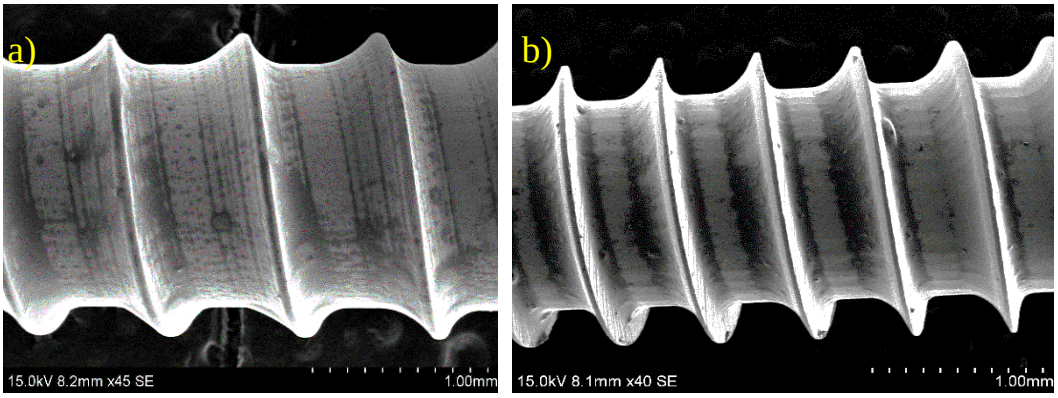


Figure 51 a, b. hFOB cells adhesion on the surface of Bioray and Dentos IZC mini-implant. Since the elemental composition varied between the IZC mini-implants, a higher adhesion, spreading and proliferation was seen over the surface of Bioray IZC mini-implant than the Dentos IZC mini-implant.

Results

RESULTS

This in vitro study was done in two parts,

1. Evaluation of proliferation and morphology of human fetal osteoblast (hFOB) seeded on the surface of two different brands (Bioray & Dentos) of self-drilling titanium IZC mini-implants (Test material) and polystyrene disk (Control) at three different time periods (24, 48 &72 hours).
2. To assess the chemical constitution on the surface of two different brands of as received titanium IZC mini-implants in three different areas (head, body & tip) and detailing the chemical component profile. (**Table. 1**)

For hFOB cell attachment and proliferation efficacies, hFOB cells were counted in the cultured basal medium containing IZC mini-implant in three different time period (quantitatively). Whereas the morphology of hFOB cells were evaluated on the smooth surface of titanium IZC mini-implants (qualitatively).

EVALUATION OF HUMAN FETAL OSTEOBLAST CELL PROLIFERATION IN THE CULTURED BASAL MEDIUM CONTAINING TWO DIFFERENT BRANDS OF TITANIUM IZC MINI-IMPLANT

QUANTITATIVE ANALYSIS

Proliferated human fetal osteoblast cells were assessed and quantified in three different time periods (24, 48 and 72 hours). The cells were examined under an invert phase contrast microscope at various magnifications (10x, 40x and 100x) in a neubauer chamber.

Proliferated hFOB cells counted in the medium containing Dentos IZC titanium mini-implant (Test material)

The mean proliferated hFOB cells counted in the medium containing Dentos titanium IZC mini-implant at 24 hours was $3.27 \pm 0.15x10^6$, whereas at 48 hours it was $5.27 \pm 0.10x10^6$ and at 72 hours it was $7.27 \pm 0.08x10^6$. From 24 hours to 72 hours there was a significant increase in number of proliferated hFOB cells. (**Table.2**, **graph.1**)

Proliferated hFOB cells counted in the medium containing Bioray IZC titanium mini-implant (Test material)

The mean proliferated hFOB cells counted in the medium containing Bioray titanium IZC mini-implant at 24 hours was $5.54 \pm 0.23x10^6$, whereas

at 48 hours it was 5.71 ± 0.20x10^6 and at 72 hours it was 8.45 ± 0.10x10^6. From 24 hours to 72 hours there was highly significant increase in number of proliferated hFOB cells. (**Table. 2**, **Graph. 1**)

Proliferated hFOB cells counted in the medium containing Polystyrene disk (Control) (**Table. 2**, **Graph. 1**)

The mean proliferated hFOB cells counted in the medium containing polystyrene disk at 24 hours was 6.54 ± 0.30x10^6, whereas at 48 hours it was 8.23 ± 0.15x10^6 and at 72 hours it was 9.59 ± 0.06x10^6. From 24 hours to 72 hours there was a highly significant increase in the number of proliferated hFOB cells.

Intragroup comparison among two brands of titanium IZC mini-implant (Dentos and Bioray) and polystyrene disk (control) in three different time periods (24, 48 and 72 hours) was done using one way ANOVA analysis.

The mean proliferated hFOB cells counted in the medium containing Dentos titanium IZC mini-implant at three different time period was 3.26x10^6 ± 0.15, 5.27x10^6 ± 0.09 & 7.26x10^6 ± 0.07 respectively with a p value of 0.000*, which was statistically highly significant at 24, 48 and 72 hours and there was progressive increase in the number of proliferated hFOB cells. **(Table. 3, Graph. 2)**

The mean proliferated hFOB cells counted in the medium containing Bioray titanium IZC mini-implant in three different time period was $4.53x10^6 \pm 0.22$, $5.68x10^6 \pm 0.16$ & $8.45x10^6 \pm 0.09$ respectively with a p value of 0.000*, which was statistically highly significant at 24, 48 and 72 hours and there was progressive increase in the number of proliferated hFOB cells. **(Table. 3, Graph. 2)**

At 24, 48 and 72 hours the mean proliferated hFOB cells counted in the medium containing polystyrene disk was $6.56\times10^6 \pm 0.29$, $8.23\times10^6 \pm 0.15$ & $9.59\times10^6 \pm 0.05$ respectively with a p value of 0.000*, which was statistically highly significant at 24, 48 and 72 hours and there was progressive increase in the number of proliferated hFOB cells. **(Table. 3, Graph. 2)**

The one way ANOVA analysis revealed a statistical highly significant difference between the titanium IZC mini-implant and polystyrene disk (p=0.000). There was a significant and progressive increase in number of proliferated cells observed between 24hours to 72 hours, and in each time period. Comparatively highly proliferated hFOB cells were observed in polystyrene disk followed by Bioray and Dentos titanium IZC mini-implant.

Intergroup comparison of hFOB cell proliferation in the medium containing test material and the control at three different time periods were done using Post hoc and Tukey tests.

A) Comparison between the groups at 24 hours

Comparing between the Bioray and Dentos IZC titanium mini-implant at 24 hours, the mean difference in proliferated hFOB cells was 1.270 with a p value of 0.001**, which was statistically significant. The Bioray IZC mini-implant had more number of proliferated hFOB cells compared with Dentos IZC mini-implants at 24 hours. **(Table. 5, Graph. 3)**

Comparing Polystyrene and Dentos IZC titanium mini-implant at 24 hours the mean difference in proliferated hFOB cells was 3.296 with a p value of 0.000**, which was statistically highly significant. Polystyrene had more number of proliferated hFOB cells compared with Dentos IZC mini-implants at 24 hours. **(Table. 5, Graph. 3)**

Comparing Polystyrene and Bioray IZC mini-implants at 24 hours the mean difference in proliferated hFOB cells was 2.026 with a p value of 0.000**, which was statistically highly significant. Polystyrene had more number of proliferated hFOB cells compared with Bioray IZC mini-implants at 24 hours. **(Table. 5, Graph. 3)**

B) Comparison between the groups at 48 hours

Comparing Bioray and Dentos IZC mini-implants at 48 hours the mean difference in number of proliferated hFOB cells was 0.410 with a p value of 0.028*, which was statistically significant. Bioray IZC mini-implant had more number of proliferated hFOB cells compared with Dentos IZC mini-implant at 48 hours. **(Table. 5, Graph. 4)**

Comparing polystyrene and Dentos IZC mini-implant at 48 hours the mean difference in number of proliferated hFOB cells was 2.960 with a p value of 0.000**, which was statistically highly significant. Polystyrene had more number of proliferated hFOB cells compared with Dentos IZC mini-implant at 48 hours. **(Table. 5, Graph. 4)**

Comparing polystyrene and Bioray IZC mini-implant at 48 hours the mean difference in number of proliferated hFOB cells was 2.550 with a p value of 0.000**, which was statistically highly significant. Polystyrene had more number of proliferated hFOB cells compared with Bioray IZC mini-implant at 48 hours. **(Table. 5, Graph. 4)**

C) Comparison between the groups at 72 hours

Comparing Bioray and Dentos IZC mini-implant at 72 hours the mean difference in number of proliferated hFOB cells was 1.183 with a p value of 0.000**, which was statistically highly significant. Bioray IZC mini-implant

had more number of proliferated hFOB cell compared with Dentos IZC mini-implant at 72 hours. **(Table. 5, Graph. 5)**

Comparing polystyrene and Dentos IZC mini-implant at 72 hours the mean difference in number of proliferated hFOB cells was 2.326 with a p value of 0.000**, which was statistically highly significant. Polystyrene had more number of proliferated hFOB cells compared with Dentos IZC mini-implant at 72 hours. **(Table. 5, Graph. 5)**

Comparing polystyrene and Bioray IZC mini-implant at 72 hours the mean difference in number of proliferated hFOB cells was 1.143 with a p value of 0.000**, which was statistically highly significant. Polystyrene had more number of proliferated hFOB cell compared with Bioray IZC mini-implant at 72 hours. **(Table. 5, Graph. 5)**

Time influenced increase in number of proliferated hFOB cell was observed in the medium containing both the titanium IZC mini-implants and Polystyrene disk in all three time periods. The lowest proliferated hFOB cell count was at 24 hours, intermediate value at 48 hours and highest value was at 72 hours. (**Table. 3, Graph. 1, 2**)

EVALUATION OF MORPHOLOGY OF HUMAN FETAL OSTEOBLAST CELLS ON THE SURFACE OF TWO DIFFERENT BRANDS OF TITANIUM IZC MINI-IMPLANTS

QUALITATIVELY ANALYSIS

Morphology of the hFOB cells on the smooth surface of both the titanium IZC mini-implants (Bioray & Dentos) and polystyrene disk (control) was assessed using scanning electron microscopy (SEM) in three different time periods (24, 48 &72 hours). **Figure. 40 to 48** shows the morphological changes observed in hFOB cells on the smooth surface of two different brands of titanium IZC mini-implants (Bioray and Dentos) and polystyrene disk (control) at 24 ,48 and 72 hours.

Morphology of hFOB cells observed on the surface of Bioray titanium IZC mini-implant

hFOB cells followed the underlying topographical structure of smooth surfaces of the IZC mini-implant by the formation of an oriented adhesion along the ups and downs on these surfaces. The number of adhered hFOB cells were shown to be denser in the body than the tip of the IZC mini-implant. In 24 hours there was a significant increase in adherence of hFOB cells was observed in the body (pitch & thread) of the IZC mini-implant than the tip of the Bioray IZC mini-implant**.** The morphology of the adhered hFOB

cells at the body and tip of the Bioray IZC mini-implant were typically round in shape. **(Figure. 40a, b, c, d, e)**.

At 48 hours hFOB cells spreaded over the body surface (pitch & thread) of the Bioray IZC mini-implant was more when compared with its tip. But the number of spreaded cells were dense and uniform in the body and tip. The morphology of spreaded hFOB cells on the Bioray IZC mini-implant were more elongated in shape than 24 hour. Cell to cell contact were present. The elongated hFOB cells showed the presence of fewer lamellipodia and filopodia (both are cytoskeletal and cytoplasmic extension from the edges of the cells that will helps in cell migration). **(Figure. 41a, b, c, d, e)**

At 72 hours hFOB cells completely spreaded and proliferated over the body and tip of the Bioray IZC mini-implant. Proliferated hFOB Cells were denser both in the body and tip. Proliferated hFOB cells were predominantly stellate shaped and elongated. Fewer lamellipodia and filopodia were present at the borders of the cells. **(Figure. 42a, b, c, d, e)**

Morphology of hFOB cells observed on the surface of Dentos titanium IZC mini-implant

hFOB cells followed the underlying topographical structure of smooth surfaces by the formation of an oriented adhesion along the ups and downs on these surfaces. The number of cells adhered were shown to be less dense in the body and tip of the IZC mini-implant. In 24 hours there was lesser

adherence of hFOB cells in the body (pitch & thread) and the tip of the Dentos IZC mini-implant**.** The morphology of adhered hFOB cells at the body and tip of the Dentos IZC mini-implant were typically round in shape. **(Figure.43 a, b, c, d)**.

At 48 hours hFOB cells spreaded over the body surface (pitch & thread) of the Dentos IZC mini-implant were more than its tip. The number of spreaded hFOB cells were dense and uniform in the body and tip. The morphology of spreaded hFOB cells on the Dentos IZC mini-implant were more elongated than round shaped. Cell to cell contact was present. The elongated hFOB cells showed the presence of fewer lamellipodia and filopodia, which was lesser compared to Bioray IZC mini-implant. **(Figure.44a, b, c, d, e)**

Whereas at 72 hours hFOB cells completely spreaded and proliferated over the body and tip of the Dentos IZC mini-implant. Proliferated hFOB cells were predominantly stellate shaped and elongated. Fewer lamellipodia and filopodia were present at the borders of the cells. Proliferated hFOB Cells were less dense both in the body and tip as compared to Bioray IZC mini-implant. **(Figure. 45a, b, c, d, e)**

Morphology of hFOB cells observed on the surface of polystyrene disk (control)

hFOB cells followed the underlying topographical structure of smooth surfaces by the formation of an oriented adhesion along the flat surface of polystyrene disk The number of cells adhered were shown to be denser on all the surface. In 24 hours there was high adherence of hFOB was observed on the surface of polystyrene disk. The morphology of the hFOB cells were typically round in shape. Cell to cell contact were present **(Figure. 46a, b, c, d)**

At 48 hours, hFOB cells showed more number of cells spread over the surface of the polystyrene disk. The spreaded cells were dense and uniform. The morphology of proliferated hFOB cells were more elongated shaped than round**. (Figure. 47a, b, c)**

At 72 hours hFOB cells spreaded and proliferated cells over the surface of disk. The morphology of proliferated hFOB cells were denser on all the surface of polystyrene disk. Predominantly stellate shaped hFOB cells with elongation. **(Figure. 48a, b, c)**

2. CHEMICAL COMPOSITION OF TWO BRANDS OF AS RECEIVED TITANIUM IZC MINI-IMPLANTS ASSESSED USING EDAX ANALYSIS

Dimension (diameter x length), composition and surface characteristics of the tested materials (Dentos and Bioray) and control (Polystyrene disk) are shown in **Table 1**.The weight percentage (wg %) composition of two different brands of titanium IZC mini-implants was studied using Energy Dispersive X-ray analysis (EDAX). This technique measures the composition or amount of particles near and at the surface and their respective weight percentage (wg %). The wg% percentage values are tabulated for comparison. (**Table. 7, Graph. 6**). The elements most abundant in both the as-received IZC mini-implants (Bioray and Dentos) were titanium, aluminium and vanadium.

ELEMENT WEIGHT PERCENTAGE OF AS-RECEIVED BIORAY IZC MINI-IMPLANT (head, body & tip)

As-received Bioray IZC mini-implant had maximum WG% of titanium in their head portion (90.10%) followed by body (89.31%) and tip (88.12%) respectively. Whereas maximum amount of aluminum was present in the body portion (8.82%) followed by tip and head (8.22% & 6.24%) respectively. The composition of vanadium was uniform in the head, tip and body (3.66%, 3.65% & 3.62%) respectively (**Table. 7, Graph. 6 a, b & c)**

ELEMENT WEIGHT PERCENTAGE OF AS-RECEIVED DENTOS IZC MINI-IMPLANT (head, body & tip)

Whereas, as-received Dentos IZC mini-implant had maximum titanium content in the head portion (89.47%), followed by tip (87.32%) and body (74.42%) respectively. The maximum amount of aluminum was present in the head (10.53%) followed by tip and body (9.16%, & 6.72 %) respectively. Whereas maximum amount of vanadium was present in tip (3.52%) and minimal amount of vanadium was present in body & head (2.07% & 0%) respectively (**Table. 7, Graph. 6a, b & c)**

COMPARISON ELEMENT WEIGHT PERCENTAGE BETWEEN AS-RECEIVED BIORAY & DENTOS IZC MINI-IMPLANT (head, body & tip)

Comparing as-received Bioray and Dentos IZC mini-implant maximum amount of titanium was present in head portion of Bioray IZC mini-implant (90.10%) and minimum amount of titanium was present in the body portion of Dentos IZC mini-implant (74.4%).

Comparing the aluminium content between as-received Dentos and Bioray IZC mini-implant maximum amount of aluminium was present in head portion of Dentos IZC mini-implant (10.53%) and minimum amount was present in head portion of Bioray IZC mini-implant (6.24%).

Comparing the vanadium between as received Dentos and Bioray IZC mini-implant maximum amount of vanadium was present in the head of Bioray IZC mini-implant (3.66%) and minimum amount was present in head portion of Dentos IZC mini-implant (0%).

3. CHEMICAL COMPOSITION OF TWO BRANDS OF TITANIUM IZC MINI-IMPLANTS SUBJECTED TO VARIOUS GROWTH MEDIUM ASSESSED USING EDAX ANALYSIS

After evaluating the hFOB cell proliferation and morphology, one sample of IZC mini-implant from each tested material (Dentos & Bioray) which were earlier subjected to various growth medium during this in vitro cell culture were again re-investigated for their chemical composition using EDAX in two different time periods (24 and 72 hours). The compositional weight percentage values are tabulated for comparison between the as-received IZC mini-implant and IZC mini-implant which were subjected to various growth medium in two time periods (24& 72 hours) **Table. 8 & 9**. The most abundant elements in both Bioray and Dentos IZC mini-implant after subjected to basal medium were titanium, aluminium and vanadium. Compared with as-received IZC mini-implant, there was a significant variation in the elemental composition of the IZC mini-implants which were subjected to various growth medium.

There was a significant variation in the elemental composition of titanium in the body and tip of the IZC mini-implant which was subjected

to various medium compared to as received Bioray and Dentos IZC mini-implant. A minimal increase in the titanium was found in the head region in contrast there was a significant decrease in titanium in the body and tip region of Bioray and Dentos IZC mini-implants. Elemental weight percentage of aluminium and vanadium significantly reduced in the body and tip of both the IZC mini-implants. (**Table. 8 & 9, Graph. 7-11**)

Tables and Graphs

Table 1. Specification of titanium IZC mini-implants (Test material) and polystyrene disk (Control)

Titanium IZC Mini-implants & polystyrene disk	Size(Diameter x Length)	Composition and surface characteristics
Bioray (Korea)	1.8x11	Titanium(TiAl4V), smooth surface
Dentos (Korea)	2x12	Titanium(TiAl4V), smooth surface
Polystyrene (Nest biotech, China)	Disk shape measuring 13 mm in diameter by 1 mm in thickness	Homopolymer, (plastic), smooth surface

Table 2. Total mean proliferated hFOB cells counted in the medium containing titanium IZC mini-implants (Bioray & Dentos) and polystyrene disk (control) in three different time periods.

Material		**TIME**	
	24 HOURS	**48 HOURS**	**72 HOURS**
	Mean ± SD	Mean ± SD	Mean ± SD
Dentos IZC mini-implant	3.27 ± 0.15	5.27 ± 0.10	7.27 ± 0.08
Bioray IZC mini-implant	4.54 ± 0.23	5.71 ± 0.20	8.45 ± 0.10
Polystyrene disk	6.54 ± 0.30	8.23 ± 0.15	9.59 ± 0.06

Values are means and standard deviation of (10^6) cell count

Table 3. Intragroup comparison among two brands of titanium IZC mini-implant (Dentos and Bioray) and polystyrene disk (control) in 3 different time periods

MATERIAL	TIME PERIOD	MEAN ± S.D	P VALUE
DENTOS	24 HOURS	3.26 ± 0.15	**0.000***
	48 HOURS	5.27 ± 0.09	
	72 HOURS	7.26 ± 0.07	
BIORAY	24 HOURS	4.53 ± 0.22	**0.000***
	48 HOURS	5.68 ± 0.16	
	72 HOURS	8.45 ± 0.09	
POLYSTYRENE	24 HOURS	6.56 ± 0.29	**0.000***
	48 HOURS	8.23 ± 0.15	
	72 HOURS	9.59 ± 0.05	

P ≤0.05 statistically significant difference

Mean and standard deviation ($\times 10^6$) of cell count.

Table 4. Mean, standard deviation and 95% confidence interval of hFOB cell proliferation in different time periods two IZC mini-implant using one way ANOVA analysis.

TIME PERIOD	MATERIAL	MEAN±S.D	MINIMUM-MAXIMUM	95%CI
24 HOURS	DENTOS	3.26 ± 0.15	3.10-3.40	2.88-3.64
	BIORAY	4.53 ± 0.22	4.28-4.71	3.973-5.10
	POLYSTYRENE	6.56 ± 0.29	6.28-6.87	5.82-7.29
48 HOURS	DENTOS	5.27 ± 0.09	5.18-5.37	5.03-5.50
	BIORAY	5.68 ± 0.16	5.49-5.78	5.26-6.09
	POLYSTYRENE	8.23 ± 0.15	8.10-8.40	7.85-8.61
72 HOURS	DENTOS	7.26 ± 0.07	7.18-7.33	7.07-7.45
	BIORAY	8.45 ± 0.09	8.37-8.56	8.20-8.69
	POLYSTYRENE	9.59 ± 0.05	9.53-9.63	9.45-9.73

Mean and standard deviation ($\times 10^6$) of cell count.

Table 5. Intergroup comparison of hFOB cells counted in the medium containing test material (Bioray & Dentos) and the control (Polystyrene disk) using one way ANOVA- analysis and Post hoc and Tukey tests

DEPENDENT VARIABLE	MATERIALS		MEAN DIFFERENCE	P - VALUE
24 HOURS	**DENTOS**	BIORAY	-1.270	**0.001***
		POLYSTYRENE	-3.296	**0.000****
	BIORAY	DENTOS	1.270	**0.001****
		POLYSTYRENE	-2.026	**0.000****
	POLYSTYRENE	DENTOS	3.296	**0.000****
		BIORAY	2.026	**0.000****
48 HOURS	**DENTOS**	BIORAY	-0.410	**0.028***
		POLYSTYRENE	-2.960	**0.000****
	BIORAY	DENTOS	0.410	**0.028***
		POLYSTYRENE	-2.550	**0.000****
	POLYSTYRENE	DENTOS	2.960	**0.000****
		BIORAY	2.550	**0.000****
72 HOURS	**DENTOS**	BIORAY	-1.183	**0.000****
		POLYSTYRENE	-2.326	**0.000****
	BIORAY	DENTOS	1.183	**0.000****
		POLYSTYRENE	-1.143	**0.000****
	POLYSTYRENE	DENTOS	2.326	**0.000****
		BIORAY	1.143	**0.000****

Table 6. One way ANOVA test done for intergroup comparison in three different time periods (24, 48 & 72 hours)

TIME PERIODS	VARIANCE	SUM OF SQUARES	MEAN SQUARE	F VALUE (ANOVA)	P VALUE
24 HOURS	BETWEEN GROUPS	16.588	8.294	153.406	**0.000***
48 HOURS	BETWEEN GROUPS	15.432	7.716	383.250	**0.000***
72 HOURS	BETWEEN GROUPS	8.121	4.060	649.092	**0.000***

Table 7. Weight percentage composition of as received Bioray and Dentos IZC mini-implant at three different regions (head, body & tip)

SITE	ELEMENTS					
	Titanium WG%		Aluminium WG%		Vanadium WG%	
	Bioray IZC mini-implant	Dentos IZC mini-implant	Bioray IZC mini-implant	Dentos IZC mini-implant	Bioray IZC mini-implant	Dentos IZC mini-implant
Head	90.10	89.47	6.24	10.53	3.66	0
Body (thread , pitch)	89.31	74.4	8.82	6.72	3.62	2.07
Tip	88.12	87.32	8.22	9.16	3.65	3.52

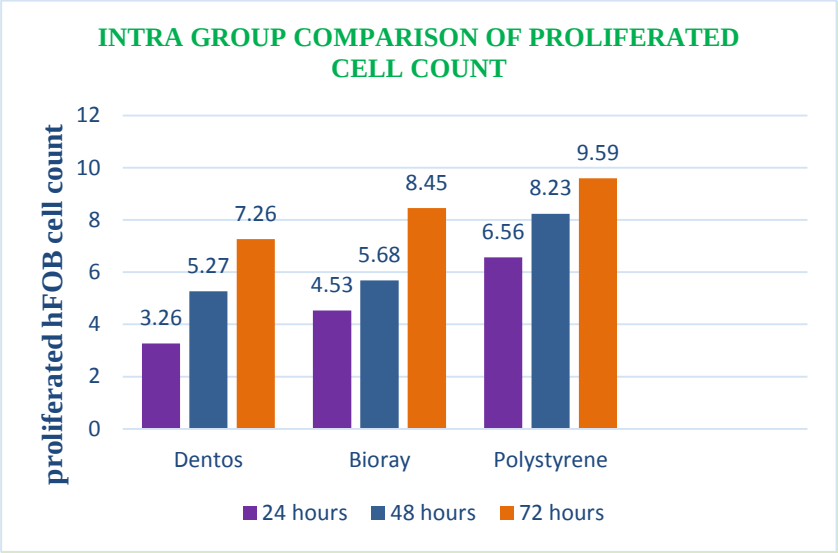
Table 8. Weight percentage composition of Bioray IZC mini-implant as received and after subjected to examination at 24 and 72 hours at three different regions (head, body & tip)

BIORAY IZC MINI-IMPLANT SITE	ELEMENT								
	TITANIUM (Wg%)			ALUMINIUM (Wg%)			VANADIUM (Wg%)		
	As-received	After study at 24 hrs.	After study at 72 hrs.	As-received	After study at 24 hrs.	After study at 72 hrs.	As-received	After study at 24 hrs.	After study at 72 hrs.
Head	90.10	94.77	95.87	6.24	10.06	9.62	3.66	0.80	1.07
Body (Thread& pitch)	89.31	31	19.1	8.82	1.14	1.72	3.62	0.00	0.00
Tip	88.12	0.82	-1.27	8.22	1.24	0.72	3.65	0.00	0.00

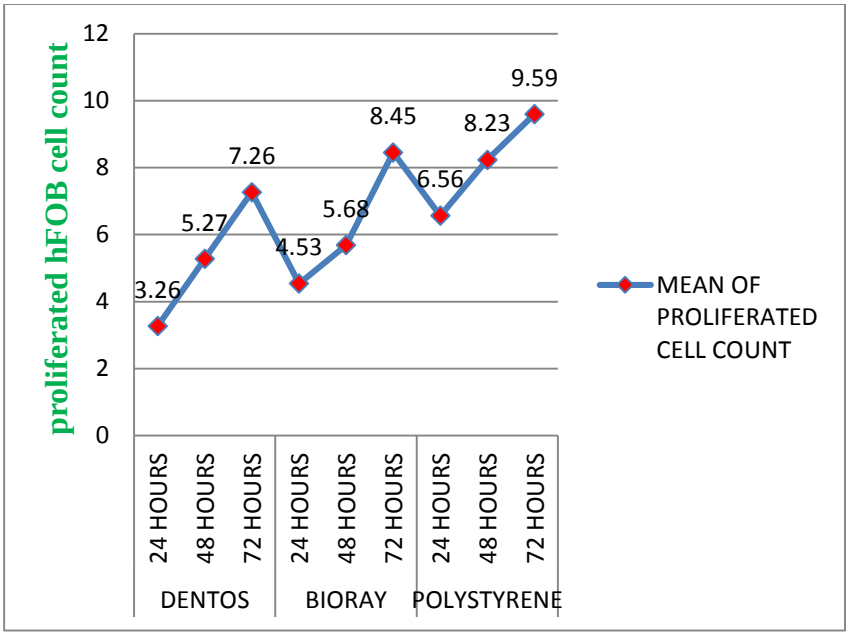
Table 9. Weight percentage composition of Dentos IZC mini-implant as received and after subjected to examination at 24 and 72 hours at three different region (head, body & tip)

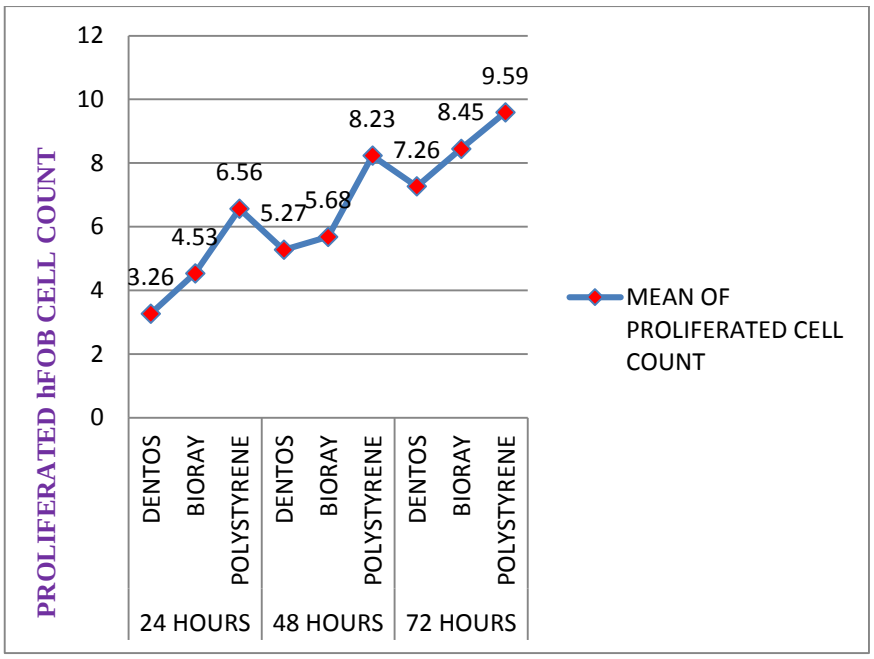
DENTOS IZC MINI-IMPLANT SITE	ELEMENT								
	TITANIUM (Wg%)			ALUMINIUM (Wg%)			VANADIUM (Wg%)		
	As-received	After study at 24 hrs.	After study at 72 hrs.	As-received	After study at 24 hrs.	After study at 72 hrs.	As-received	After study at 24 hrs.	After study at 72 hrs.
Head	89.47	90.39	89.28	10.5	10.45	9.54	0.00	6.23	3.24
Body (Thread & Pitch	74.42	18.14	9.46	6.72	0.94	2.22	2.07	0.00	0.00
Tip	87.32	0.11	1.08	9.16	0.00	0.51	3.52	0.00	0.00

Graph 1. Intra group comparison of proliferated hFOB cells count

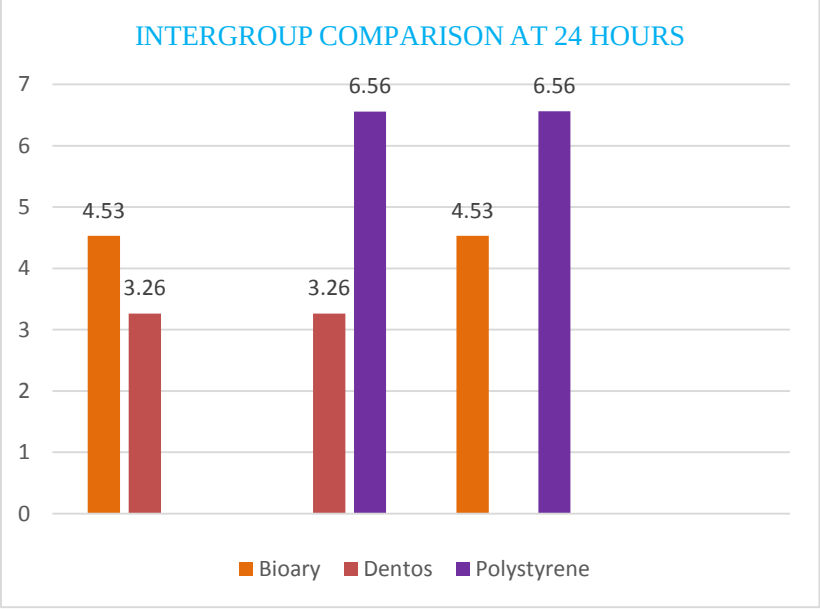


Graph 2. Mean proliferated hFOB cells counted at three different time periods between test material and control.

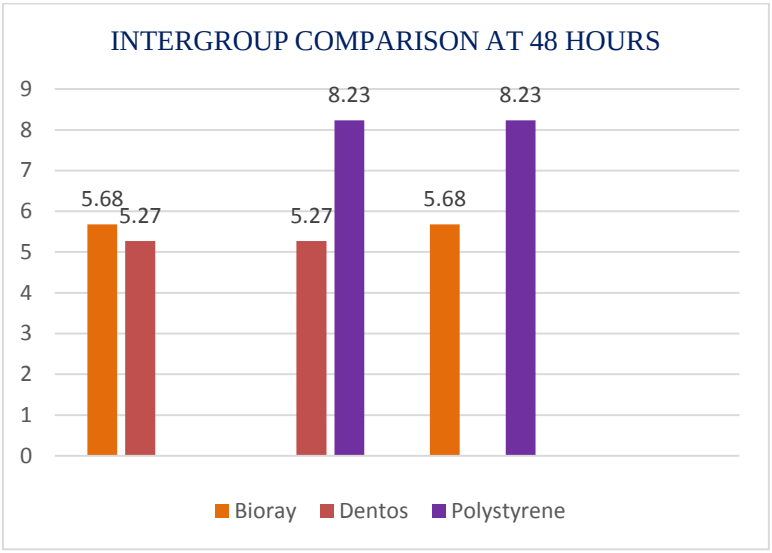




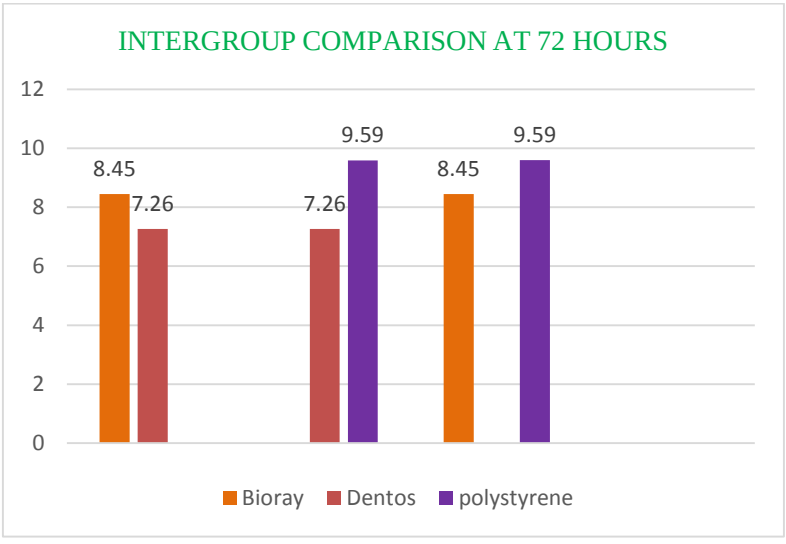
Graph 3. Intergroup comparison of mean proliferated hFOB cells counted at 24 hours



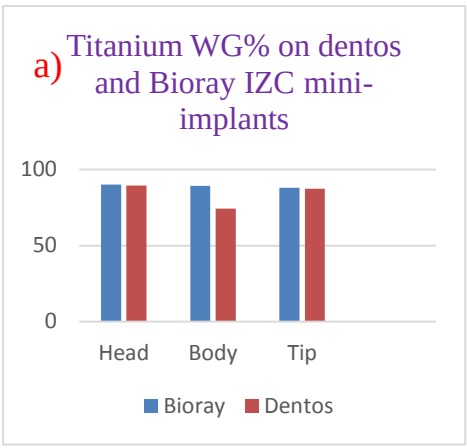
Graph 4. Intergroup comparison of mean proliferated hFOB cells counted at 48 hours

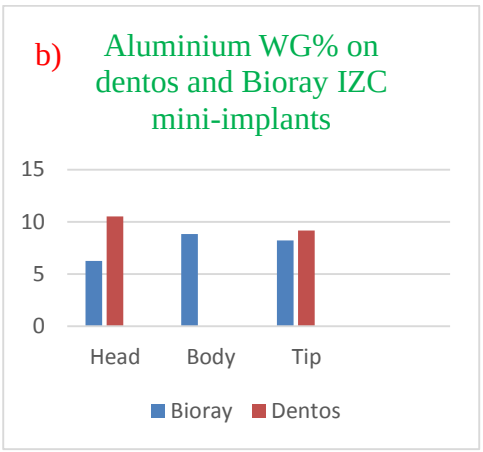


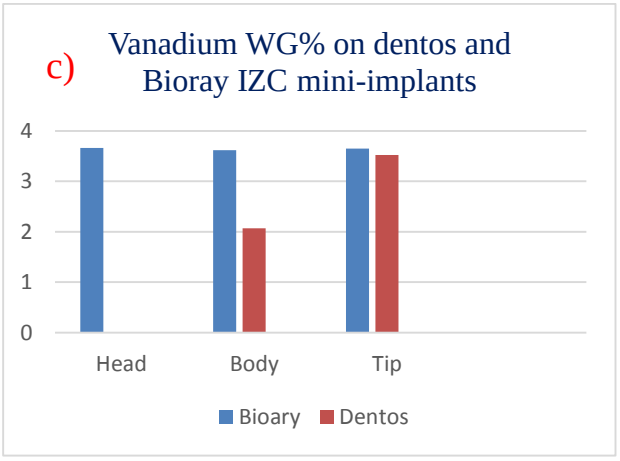
Graph 5. Intergroup comparison of mean proliferated hFOB cells counted at 72 hours



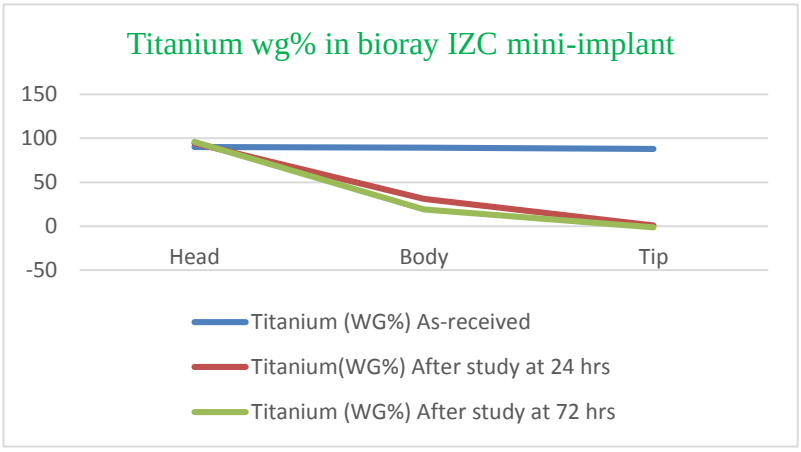
Graph 6. Weight percentage composition of as received Bioray and Dentos IZC mini-implant at three different regions (head, body & tip)



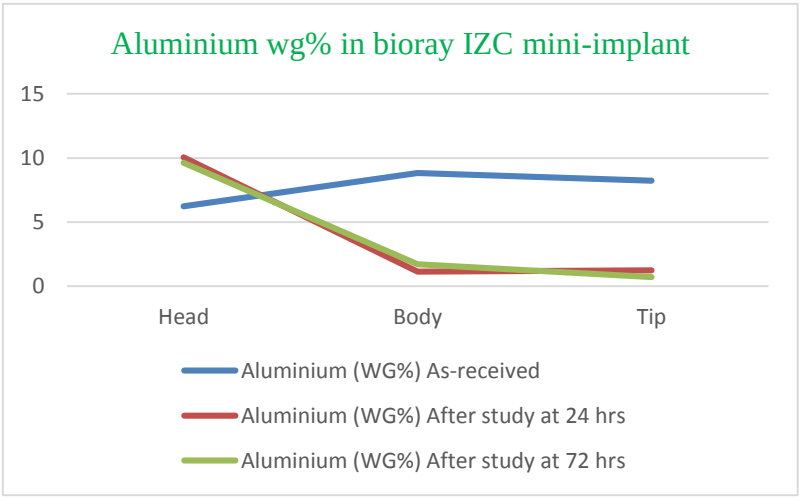




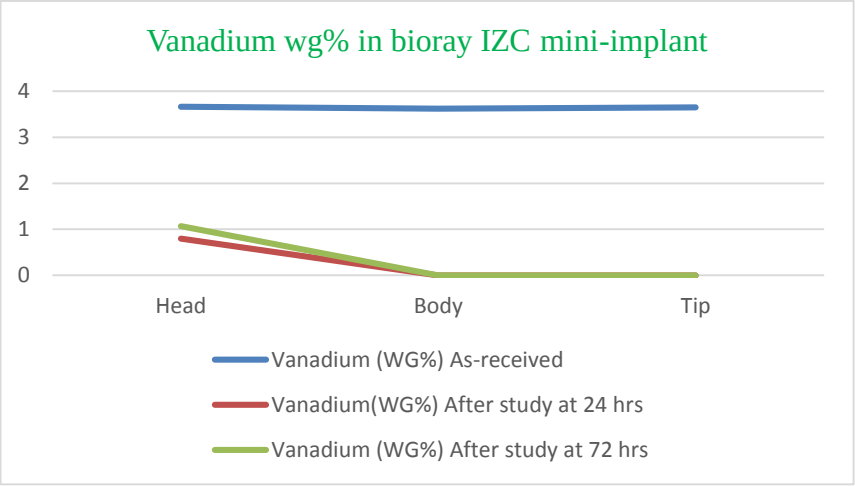
Graph 7: Weight percentage of titanium in Bioray IZC mini-implant: as-received, after study at 24 and 72 hour



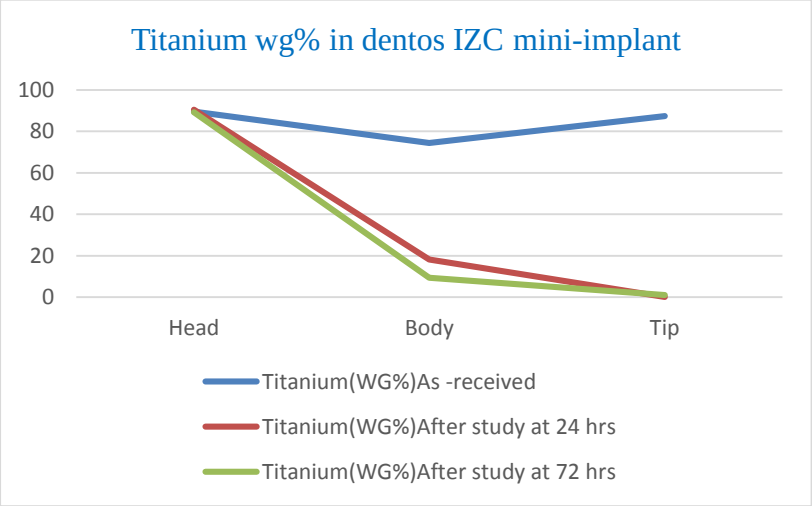
Graph 8: Weight percentage of aluminium in Bioray IZC mini-implant: as-received, after study in 24 and 72 hours



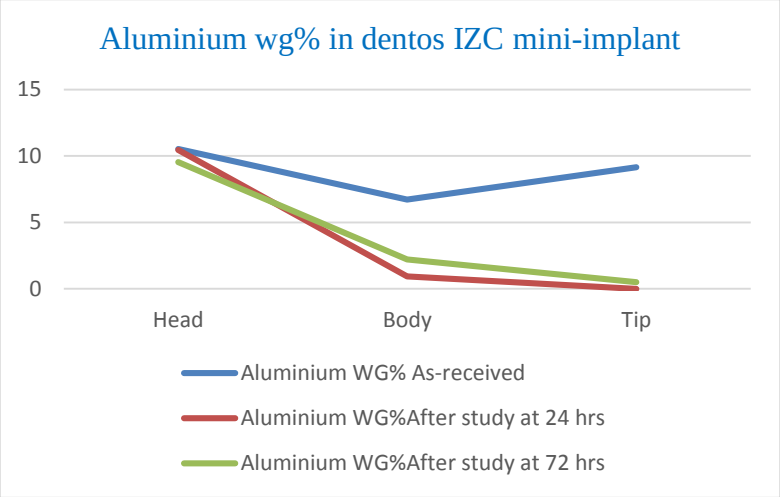
Graph 9: Weight percentage of vanadium in Bioray IZC mini-implant: as-received, after study in 24 and 72 hours



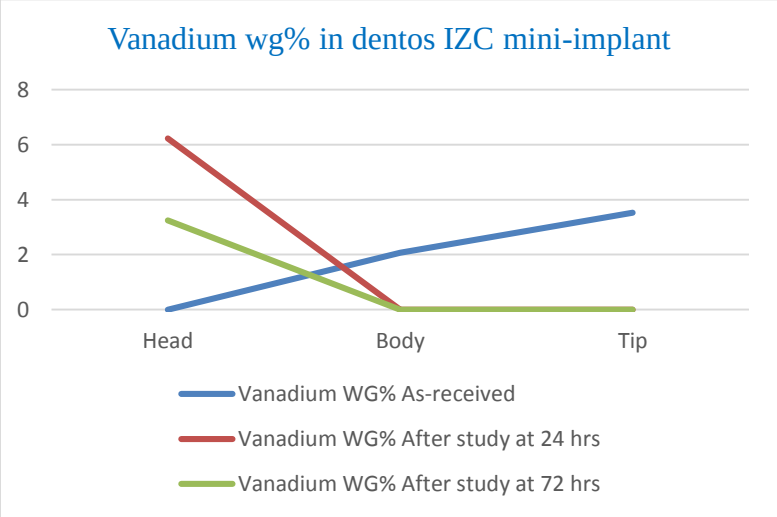
Graph 10: Weight percentage of titanium in Dentos IZC mini-implant: as-received, after study in 24 and 72 hours



Graph 11: Weight percentage of aluminium in Dentos IZC mini-implant: as-received, after study in 24 and 72 hours



Graph 12: Weight percentage of vanadium in Dentos IZC mini-implant: as-received, after study in 24 and 72 hours



Discussion

DISCUSSION

In the last few decades numerous advances in material science has facilitated the fabrication of implants which would result in enhancing their stability. Various metals have been tested and used to manufacture dental and miniscrew implants (MSI) such as stainless steel, cobalt chromium molybdenum, titanium, and a multitude of titanium alloys.[63]

In 1997 **Wehrbein et al** used short titanium MSI in the maxilla for orthodontic anchorage and in the same time period **Kanomi et** al used MSI to anchor orthodontic tooth movement.[62] Unlike dental implants, MSI are designed to be used and removed at the end of orthodontic treatment hence are called "Temporary Anchorage Devices" (TADs).[13] Orthodontic MSI offer various advantages to both the orthodontist and patient: simple insertion and removal, immediate loading, requires less patient cooperation and favourable cost-benefit ratio. There are few disadvantages in using MSI like MSI loosening, slippage, nerve involvement, MSI bending or fracture. But the proximity of roots while placing MSI interdentally is considered as one of the major factor for MSI failure, whereas a direct root contact with MSI will results in either blunting of its tips or fracture during its insertion and removal.[37,1, 39, 7, 21]

In order to eliminate these undesirable side effects of placing MSI interdentally has led to the development of a new form of skeletal

anchorage device known as infrazygomatic crest (IZC) mini-implant in maxilla and buccal shelf mini-implant in mandible, which are anatomical sites away from the dentoalveolar region of maxilla and mandible allowing unobstructed tooth movement and minimize the chances of root contact.[31]

Many factors have been reported in the literature regarding the success and failure rate of IZC mini-implant, which have been divided into different categories such as patient related factors (age, gender, oral hygiene, medical condition), IZC mini-implant related factors (size, length, diameter, composition and design), host bone related factors (quality and quantity of bone) and operator related factor.[53]

Liou et al [47, 48] claimed 97% of success rate using titanium IZC mini-implants as an adjunct in various orthodontic procedure. He also reported a lesser failure rates of about 3% using titanium IZC mini-implants.

A study by **Uribe et al** [68] reported high failure rates of 21.8% using titanium IZC mini-implant placed in the IZC region. Most of these studies reported in the literature have focused on either patient related factors, clinician related factors or host related factors.[69]

A more recent study by **Chang et al** [19] reported failure rates comparing the stainless steel and titanium IZC mini-implant placed in the attached gingiva (AG) and movable mucosa (MM) near the IZC region. He claimed that the factors responsible for the IZC mini-implant failures, which resulted in loosening were due to stainless steel IZC mini-implant placed in

AG and right side has more failures (7.4%, 7.8%) than titanium IZC mini-implant placed in AG and in right side (5.1%, 5.2%). Titanium IZC mini-implant offered a slightly lower failure rate compared with stainless steel IZC mini-implant.

Misch et al [40, 18, 22] claimed that anatomically the maxillary bone is less dense as compared to mandible. Which indicates that the density of the posterior maxilla is minimal (D3 refers to density within 350-850 HU) as compared to anterior region of maxilla (D1 refers to values greater than 1250 HU, D2 has 850-1250 HU). According to **Liou et al** [46] the mean thickness of the IZC region varied approximately between 5 and 8 mm. Moreover there are studies in the literature reporting variations in the thickness and bone density of the IZC region in various skeletal malocclusions (class I, II and III) which ranged between 5 to 7mm.[20, 44]

Anatomically IZC region being a smaller area in the maxilla, the whole width of the IZC region ranging between 5 to 8 mm will be partly engaged by the threaded portion of the longer sized IZC mini-implants (10 to 14mm) which might leads to inadequate retention.[31]

Moreover the materials used to manufacture IZC mini-implant must be nontoxic, biocompatible, possess excellent mechanical properties, provide resistance to stress/strain and resistance to corrosion.[16] The choice of the materials used for manufacturing IZC mini-implant are generally made of

either titanium alloy (TiA) or stainless steel (SS), but the mechanical and physical properties of these materials differ significantly.

Stainless steel tends to develop a fibrous tissue interface between the IZC mini-implant and bone (mechanical retention).[62] whereas, titanium has the property that, it allows direct bone contact between IZC mini-implant and the host bone interface (biological retention). Titanium IZC mini-implant are highly biocompatible with human tissues, high corrosion resistance in body fluids, lack allergenicity, high strength, and low elastic modulus when compared with other metallic biomaterials. Despite the differences between Ti and SS, both materials provide relatively predictable clinical outcomes.[13]

There is paucity of information, whether the commonly used titanium IZC mini-implant and its chemical composition has a distinct advantage which aids in favourable biological bonding along with mechanical retention.

Osteoblast adhesion, migration, spreading, proliferation, and differentiation on the surface of titanium alloy are important factors to achieve biological integration during the initial phase [23, 15]

Therefore complete understanding the cell adhesion and proliferation particularly, employing human fetal osteoblast (hFOB) cells on the surface of implant materials is now essential to optimize the bone and IZC mini-implant interface. Moreover the quality of this adhesion will influence the

osteoblast morphology and their capacity for proliferation and differentiation.[4]

Hence this in vitro study was done,

1. To evaluate the proliferation and morphology of human fetal osteoblast (hFOB) cells seeded on the surface of two different brands (Bioray &Dentos) of self-drilling titanium IZC mini-implants (test material) and polystyrene disk (control) in three different time periods (24, 48 and 72 hours).

2. To assess the chemical composition on the surface of two different brands of as received titanium IZC mini-implants in three different areas (head, body & tip).

Previous in vitro studies have either used animal or adult human osteoblast cells to study biological integration with titanium alloy. The main drawback of using animal osteoblast cells is the interspecies variations, biology and structure of bone which cannot be extrapolated in humans.[25]

In contrast the main advantage of using human adult osteoblast cells is their clinical applicability and the reduced need for accounting for interspecies variation, as in the case when other animal cell sources or cell lines are used. The primary human osteoblasts tend to retain their differentiated phenotype in vitro, but many factors influence the human

adult osteoblast cell proliferation and differentiation such as donor age, site of isolation and gender difference has been reported in the literature. [25, 63, 53]

A study by **Voegele et al** using human adult osteoblast cells reported that donors age younger than 65 years old were shown to have shorter population doubling times than adult osteoblast cells from older donors. **Fedarko et al** in his study reported decreased collagen levels around 65% in human adult osteoblast cells donors above the age of 20 years. Moreover **Davis et al** [27, 65] reported age-related changes occurring in human adult bone composition which occurs mostly in the lumbar and hip regions in postmenopausal women due to the progressive decrease in systemic estrogen levels. Whereas, osteosarcoma cells grown in laboratory culture, their properties and tumor origin render them questionable as representative of normal human osteoblast cells and do not demonstrate a complete pattern of in vitro differentiation.

To overcome the drawbacks of using adult human fetal osteoblast cells, **Harris et al** [34] recommended the use of human fetal osteoblastic cell lines (hFOB 1.19) by stably transfecting fetal bone-derived osteoblast cells, which is an osteoprogenitors from fetal long bones. Which can used for an investigation of cell adhesion, proliferation, differentiation, metabolic activity and viability.[72, 35, 57]

When compared with human adult osteoblast cells, human fetal osteoblast (hFOB) cells have distinct advantageous in research purposes

because, they provides a homogenous rapid proliferating and differentiating system and respond well to environmental stimuli.[34,62] This line is closer to the healthy cell than the tumoural lines and allows suitable assays and comparison to others studies. An in vitro study by **Yeniyol et al** [72] has used hFOB cells for evaluating the adhesion of hFOB cells on commercially pure titanium surface and surface-modified Ti (polishing, sand blasting) in the same period of 24 hours. In a recent study **Brezulier et al** [14] has used three-dimensional (3D) of hFOB cells in an innovative culture to study cell proliferations and mechanobiology application of various intensities of orthodontic forces.

Hence in the present in vitro study human fetal osteoblast cells (hFOB 1.19) were used, which provided a better representation of the in vivo situation than using commercially available cell lines. The hFOB cell line provides a rapidly proliferating and differentiating system for testing biomaterials in which differences in osteoblast proliferation and differentiation on mini-implant materials could be revealed, suggesting that the chemistry of biomaterials has a dynamic effect on proliferation and differentiation of human osteoblasts.[35]

The present in-vitro study using hFOB cells concurs with the above mentioned studies reported that hFOB cells have distinct advantage of relatively well-controlled variables and allow the investigation of parameters that are difficult to study in vivo.

Hence this in vitro study done to assess the biological property between two different brands of commercially available titanium IZC mini-implants (Bioray & Dentos) as a test material and polystyrene disk were used as a control. Since the osteoblast adhesion, proliferation, differentiation and morphology of the hFOB cells is influenced by composition of the titanium. The direct relation between the hFOB cells and titanium IZC mini-implant surface in three different time period (24, 48 and 72 hours) was evaluated.

Cell growth, attachment and proliferation are major events that need to occur in order to maintain a tissue or repair. Since osteoblast cells are sensitive to the physical properties of the materials with which they interact, the effects of the material on the surrounding tissue will determine its ability to be used as an implant in the human body.[59]

In general 24 hours is regarded as a key time point for analysing the short-term cellular interactions to biomaterials. By this time, cell adhesion gets firmly established on both polished and machined surfaces of the implant material.[54]

Hence in the present study hFOB cells proliferation in the initial stages was assessed quantitatively and qualitatively in three different time periods (24, 48 and 72 hours).

EVALUATION OF HUMAN FETAL OSTEOBLAST CELL PROLIFERATION IN THE CULTURED BASAL MEDIUM CONTAINING TWO DIFFERENT BRANDS OF TITANIUM IZC MINI-IMPLANT

Quantitative analysis

Human fetal osteoblast cell proliferation was assessed and quantified in three different time periods (24, 48 and 72 hours). The hFOB cells were examined under an invert phase contrast microscope at various magnifications (10x, 40x and 100x) in a neubauers chamber. Total mean proliferated hFOB cells counted on the surface of two different brands of titanium IZC mini-implants (Bioray & Dentos) and polystyrene disk in three different time period was evaluated (24, 48 and 72 hours) are given below.

Proliferated hFOB cells counted in the medium containing Dentos IZC titanium mini-implant (Test material)

The mean proliferated hFOB cells counted in the medium containing Dentos titanium IZC mini-implant at 24 hours was $3.27 \pm 0.15x10^6$, whereas at 48 hours it was $5.27 \pm 0.10x10^6$ and at 72 hours it was $7.27 \pm 0.08x10^6$. From 24 hours to 72 hours there was a significant increase in number of proliferated hFOB cells. (**Table. 2**, **Graph. 1**)

Proliferated hFOB cells counted in the medium containing Bioray IZC titanium mini-implant (Test material)

The mean proliferated hFOB cells counted in the medium containing Bioray titanium IZC mini-implant at 24 hours was 5.54 ± 0.23x10^6, whereas at 48 hours it was 5.71 ± 0.20x10^6 and at 72 hours it was 8.45 ± 0.10x10^6. From 24 hours to 72 hours there was highly significant increase in number of proliferated hFOB cells. (**Table. 2**, **Graph. 1**)

Proliferated hFOB cells counted in the medium containing Polystyrene disk (Control) (**Table. 2**, **Graph. 1**) (**Table.2**, **Graph. 1**)

The mean proliferated hFOB cells counted in the medium containing polystyrene disk at 24 hours was 6.54 ± 0.30x10^6, whereas at 48 hours it was 8.23 ± 0.15x10^6 and at 72 hours it was 9.59 ± 0.06x10^6. From 24 hours to 72 hours there was a highly significant increase in the number of proliferated hFOB cells

Ricardo et al[16] conducted a similar study on the proliferation of osteoblast cells seeded on two different brands of titanium MSI (Morelli & Neodent) in three different time periods. Osteoblast proliferation on the Moreli MSI at 24 hours was 3.18 ± 0.56×10^4, at 48 hours 4.51 ± 0.34×10^4 and at 72 hours 8.88 ± 0.80×10^4 and Neodent MSI was at 24 hours 3.99 ± 0.97×10^4, at 48 hours 4.51 ± 0.78×10^4 and at 72 hours 9.99 ± 0.59×10^4

respectively. He concluded that there was no significant difference in human osteoblast proliferation on both the titanium MSI.

The results of the present study concurs with the findings and results of above mentioned study regarding the proliferation of osteoblast.

In contrast the present study we observed a significant increase in proliferation of hFOB cells seeded on the surface of different brands titanium IZC mini-implants (Dentos & Bioray), which is statistically significant. One way ANOVA analysis and Tukey tests revealed there was a statistical significant difference in hFOB cell proliferation between Dentos and Bioray IZC titanium mini-implant and polystyrene disk also ($p< 0.05$).

This increase in proliferation of hFOB cells may probably be due to the increased surface composition of titanium, larger surface area of both the titanium IZC mini-implants compared to titanium MSI.

Alves et al [2] conducted a similar study by seeding the osteoblast like cells on the surface of titanium dental implants (Neodent). They evaluated the adhesion, proliferation and morphology of osteoblast like cells cultivated on the surface of titanium dental implant at 24, 48 and 72 hours using SEM. Based on SEM surface analysis at 24 hours the osteoblastic cells adhered well on the surface of dental implant, at 48 hours the cells spreads over the dental implant whereas at 72 hours proliferation of cell on the surface of dental implant.

The present study concurs with the findings of above mentioned study of similar adhesion, proliferation and morphology of hFOB seeded on both IZC titanium mini-implants(Dentos & Bioray) in three different time period (24, 48 & 72hours). The hFOB cells proliferated on all the surfaces of titanium IZC mini-implant studied with lowest value was at 24 hours, intermediate at 48 hours and highest number of cells proliferated at 72 hours.

An invitro study **Yeniyol et al** [72] evaluated the adhesion of hFOB cells on titanium disk (10 mm diameter and 2 mm thickness) which were surface modified by polishing and sand blasting at 24 hours using SEM analysis. At the end of 24 hours cell adhesion and spread were quantified on all the surface of the Titanium disk using hemocytometer. He concluded that hFOB cells adhered well on the smooth and polished surface of titanium disk in 24 hours. In addition hFOB cell culture system permits a reproducible examination for investigating the biocompatibility of dental implant materials in vitro.

Hendrich et al [35] evaluated the proliferation and differentiation of hFOB 1.19 cells on the disk (diameter of 15.6 mm and 1mm in height) surface of different materials such as, stainless steel, cobalt-chromium-molybdenum (CoCrMo), and commercially pure Titanium for 12 days and polystyrene was used as a control. The results of their study showed that

lesser cell number and delayed proliferation of hFOB cells on stainless steel and CoCrMo compared with titanium and polystyrene.

They concluded that surface composition of having titanium influences increased proliferation and differentiation of hFOB cells and may play a major role in the process of osseointegration.

Schmidt et al [63] in an in vitro assessed the proliferation of human adult osteoblasts on the disk (20 mm in diameter) surface of commercially pure titanium (cpTi) Ti-6Al-7Nb, and stainless steel similar to clinical implants. They determined cell proliferation in four time periods (3, 7, 11, 15days). They found that higher cell proliferation on titanium and the titanium alloy than other material. Titanium provides the best biocompatibility with regard to proliferation, in addition to more reliable early and late differentiation markers of human osteoblasts in vitro.

The above mentioned studies concurs with findings of the present in vitro study that the osteoblast cells adhered and proliferated well on the surface of titanium surface and also showed titanium has good proliferating and differentiation ability compared with other implant materials.

In the present study, we compared the hFOB cells proliferation between Dentos and Bioray IZC titanium mini-implant. Bioray titanium IZC mini-implant showed maximum hFOB cell proliferation in all three time periods. (**Table. 2, Graph. 1**). The most probable reason could be attributed to the

elemental composition of titanium in Bioray titanium IZC mini-implant. When compared between polystyrene (control) and the tested titanium IZC mini-implants, polystyrene had more proliferated hFOB cells which is due to the high affinity of hFOB cells on the surface of polystyrene and its surface area of the disk.

The chemical and topographic features of mini-implant surfaces are regarded as important in terms of their interface with bone cells because of their high quality of osseointegration. Since osteoblast morphology can be influenced by composition, hence present study we evaluated the morphology of hFOB cells on the surface of two brands of IZC mini-implant in three time periods.

EVALUATION OF MORPHOLOGY OF HUMAN FETAL OSTEOBLAST ON THE SURFACE OF TWO DIFFERENT BRANDS OF TITANIUM IZC MINI-IMPLANTS

Qualitatively analysis:

Morphology of hFOB cells were observed on the surface of two different brands of titanium IZC mini-implants (Bioray and Dentos) and polystyrene disk (control) in three time periods (24, 48 and 72 hours) using a SEM. SEM analysis showed the hFOB cells adhered, spreaded and proliferated well on the all the surfaces of both titanium IZC mini-implants (body & tip,) and the polystyrene disk. **Figure 40 to 48** shows the

morphological changes observed in hFOB cells on the smooth surface of two different brands of titanium IZC mini-implants (Bioray and Dentos) and polystyrene disk (control) at 24 ,48 and 72 hours.

Body and tip region of titanium IZC mini-implant were assessed for morphology of the hFOB cells. This areas of titanium IZC mini-implant body (thread and pitch) and tip was considered unique because this region is exposed to the bone surface which contribute to the biological bonding, whereas the head portion of IZC mini-implant is exposed to saliva and food.

Morphology of hFOB on the surface of Bioray titanium IZC mini-implant in three time periods.

hFOB cells followed the underlying topographical structure of smooth surfaces of the IZC mini-implant by the formation of an oriented adhesion along the ups and downs on these surfaces. The number of adhered hFOB cells were shown to be denser in the body than the tip of the IZC mini-implant. In 24 hours there was a significant increase in adherence of hFOB cells was observed in the body (pitch & thread) of the IZC mini-implant than the tip of the Bioray IZC mini-implant**.** The morphology of the adhered hFOB cells at the body and tip of the Bioray IZC mini-implant were typically round in shape. **(Figure.40a, b, c, d, e)**.

At 48 hours spreaded hFOB cells over the body surface (pitch & thread) of the Bioray IZC mini-implant was more when compared with its tip. But the number of spreaded cells were dense and uniform in the body and tip. The morphology of spreaded hFOB cells on the Bioray IZC mini-implant were more elongated in shape than 24 hour. Cell to cell contact were present. The elongated hFOB cells showed the presence of fewer lamellipodia and filopodia (both are cytoskeletal and cytoplasmic extension from the edges of the cells that will helps in cell migration). **(Figure. 41a, b, c, d, e)**

At 72 hours hFOB cells completely spreaded and proliferated over the body and tip of the Bioray IZC mini-implant. Proliferated hFOB Cells were denser both in the body and tip. Proliferated hFOB cells were predominantly stellate shaped and elongated. Fewer lamellipodia and filopodia were present at the borders of the cells. **(Figure. 42a, b, c, d, e)**

Morphology of hFOB cells on the surface of Dentos titanium IZC mini-implant in three time periods

hFOB cells followed the underlying topographical structure of smooth surfaces by the formation of an oriented adhesion along the ups and downs on these surfaces. The number of cells adhered were shown to be less dense in the body and tip of the IZC mini-implant. In 24 hours there was lesser adherence of hFOB cells in the body (pitch & thread) and the tip of the Dentos IZC mini-implant**.** The morphology of adhered hFOB cells at the

body and tip of the Dentos IZC mini-implant were typically round in shape. **(Figure. 43 a, b, c, d)**.

At 48 hours hFOB cells spreaded over the body surface (pitch & thread) of the Dentos IZC mini-implant which were more than its tip. The number of spreaded hFOB cells were dense and uniform in the body and tip. The morphology of spreaded hFOB cells on the Dentos IZC mini-implant were more elongated than round shaped. Cell to cell contact was present. The elongated hFOB cells showed the presence of fewer lamellipodia and filopodia, which was lesser compared to Bioray IZC mini-implant. **(Figure. 44a, b, c, d, e)**

Whereas at 72 hours hFOB cells completely spreaded and proliferated over the body and tip of the Dentos IZC mini-implant. Proliferated hFOB cells were predominantly stellate shaped and elongated. Fewer lamellipodia and filopodia were present at the borders of the cells. Proliferated hFOB Cells were less dense both in the body and tip as compared to Bioray IZC mini-implant. **(Figure. 45a, b, c, d, e)**

Ricardo et al [16] evaluated the morphology of osteoblast on the surface of titanium MSI in three time periods. In 24 hours, the osteoblast cells demonstrated typical morphological features, which was round in shape with fewer elongations. At 48 hours, the cells varied from round to stellate, with more frequent elongations. At 72 hours predominantly cell shape were stellate in nature with numerous elongations.

Findings of our study in three time periods regarding the morphology were in accordance with the above mentioned study. At 24 hours cells were typically round shaped, at 48 hours elongated cells with lamellipodia and filopodia, whereas at 72 hours predominantly stellate shaped cells with elongated proliferation.

The observed morphology of the hFOB cells in all three time periods in the present study concurs with the findings of **Ricardo et al**, the only difference being titanium MSI was used in their study, whereas in the present study titanium IZC mini-implants was used had dimensional variation.

A study by **Passeri et al** [54] evaluated the behaviour and morphology of human adult osteoblastic cells on the surface of five commercially available titanium surfaces using SEM analysis. Commercially pure, grade 4 titanium were used which were polished and machined titanium disk (8mm diameter and 1mm thickness). Morphology of osteoblast cells were evaluated in five time periods (6, 24 & 72 hours, 7 and 14 days). At 24 hours, most of the cells appeared flat, with some interspersed round and loosely attached cells and cellular edge formed large lamellipodia ending with filopodia. At 72 hours, cell-to-cell contacts were frequent and had an elongated shape and a parallel orientation.

The results of present study on the morphology of the osteoblast in 24 and 72 hours concurs with the findings of the studies by **Passeri et al** and **Ricardo et al**.

All the above mentioned study are in concordance with the findings of the present study showing that human osteoblast have the ability to proliferate well on the titanium surface and has better biological bonding. [58]

CHEMICAL COMPOSITION OF TWO BRANDS OF AS RECEIVED TITANIUM IZC MINI-IMPLANTS ASSESSED USING EDAX ANALYSIS

Many brands of self-drilling IZC mini-implants are currently available. Though various factors might direct the clinician to his or her choice of IZC mini-implant selection.

Hence in the present in-vitro study, two brands of commonly used titanium IZC mini-implants (Bioray & Dentos) with different dimensions were used. Bioray had 11 mm length, 1.8mm diameter and 8 number of threads, whereas Dentos had 12 mm length, 2mm diameter and 10 number of threads were used to assess the surface composition.

Comparison between two titanium IZC mini-implants was done to assess whether titanium IZC mini-implant had an added advantage of biological bonding during the initial stages of mini-implant insertion which will aid in its stability and minimize its failure rates. The surface of titanium

IZC mini-implants tested for this study was machined (smooth surface), surface did not receive any treatment to alter the roughness. However in the clinical scenario such modifications on the surface of IZC mini-implant are not exercised during its placement. Hence in the present study, surface alteration were not done in the IZC mini-implants (Dentos and Bioray).

In the present study composition on the surfaces of as-received titanium IZC mini-implant (head, body and tip) was evaluated using Energy Dispersive X-ray analysis (EDAX). EDAX is a technique that uses X-rays as a source of excitation for analysing the elementary semi-quantitative composition of implant surfaces. This technique measures the composition or amount of particles near and at the surface and their respective weight percentage (wg %) also. This analysis showed, heavy metals were present on the surfaces of both brands of IZC mini-implant (Dentos and Bioray) with most abundant element being titanium, aluminium, and vanadium.

The head, thread, pitch and tip region were assessed for chemical composition. These areas were considered unique because each region is exposed to a different environment in the oral cavity. The head of the mini-implant is exposed to oral fluids and food, whereas the neck is in contact with oral mucosa and gingiva, both are points of force application. In contrast, the body and tip of the IZC mini-implant are in contact with bone surfaces and contribute to the stability of mini-implants.[51, 3]

Hence in the present study composition of the head, body and tip of the both brands of IZC mini-implant were assessed for the clinical biocompatibility, biological bonding, osteoblast adhesion, proliferation and morphology which are influenced by the composition and surface chemistry of IZC mini-implant.

ELEMENT WEIGHT PERCENTAGE OF AS-RECEIVED BIORAY IZC MINI-IMPLANT (head, body & tip)

As-received Bioray IZC mini-implant had maximum wg% of titanium was in the head portion (90.10%) followed by body (89.31%) and tip (88.12%) respectively. Whereas maximum amount of aluminum was present in the body portion (8.82%) followed by tip and head (8.22% & 6.24%) respectively. The composition of vanadium was uniform in the head, tip and body (3.66%, 3.65% & 3.62%) respectively (**Table. 7, Graph. 6 a, b & c)**

ELEMENT PERCENTAGE OF AS-RECEIVED DENTOS IZC MINI-IMPLANT (head, body & tip)

Whereas, as-received Dentos IZC mini-implant had maximum titanium content in the head portion (89.47%), followed by tip (87.32%) and body (74.42%) respectively. The maximum amount of aluminium was present in the head (10.53%) followed by tip and body (9.16%, & 6.72 %)

respectively. Whereas maximum amount of vanadium was present in tip (3.52%) and minimal amount of vanadium was present in body and no vanadium was found on the head of the Dentos IZC mini-implant (2.07% & 0%) respectively (**Table. 7 Graph. 6a, b & c)**

COMPARISON ELEMENT WEIGHT PERCENTAGE BETWEEN AS-RECEIVED BIORAY & DENTOS IZC MINI-IMPLANT (head, body & tip)

Comparing the as-received Bioray and Dentos IZC mini-implant maximum amount of titanium was present in head portion of Bioray IZC mini-implant (90.10%) and minimum amount of titanium was present in the body portion of Dentos IZC mini-implant (74.4%). When compared the aluminium content between as-received Dentos and Bioray IZC mini-implant maximum amount was present in head portion of Dentos IZC mini-implant (10.53%) and minimum amount in head portion of Bioray IZC mini-implant (6.24%). When comparing the vanadium content between as received Dentos and Bioray IZC mini-implant maximum amount was present in the head of Bioray IZC mini-implant (3.66%) and in contrast no vanadium was present in the head portion of Dentos IZC mini-implant (0%).

The most probable reason for differences in titanium composition in the head, body and tip could be attributed to fabrication process of thread size, thread shape, taper and varying diameter by subtractive method.

This was first in vitro study which has used the IZC titanium mini-implant for evaluating the proliferation and morphology of the hFOB cells on the surface of the mini-implant at three different time period (24, 48 & 72). As the time of contact between the cells and surface increased, suggesting a favourable biological relationship.

Even though the length and diameter of the Dentos (12x12) titanium IZC mini-implant compared with Bioray (11x1.8) titanium IZC mini-implant had minimal variations in dimension. Bioray titanium IZC mini-implant showed maximum hFOB cell proliferation in all three time periods. The most probable reason could be attributed to the increased elemental composition of titanium on all surfaces of Bioray titanium IZC mini-implant (head, body and tip).

A study by **Schmidt et al[63]** investigated the morphology of human adult osteoblast-like cells in five different disk (20 mm diameter) composed of stainless steel, cobalt chromium alloy (CoCrMo), commercially pure titanium (cpTi), Ti-6Al-7Nb, and Ti-6Al-4V. Morphology of osteoblast was investigated in three different time period after 12 hours, 72hours and 7days assessed under scanning electron microscopy. They found the titanium disks, had more cells attached to its surface in 12 hours and with well

spread filopodia extending from the base of the cell mass and also possessed many thin, capillary-like extensions. After 72 hours the cells were well oriented and formed monolayers which spreaded polygonally. Cells were very dense, matured into multilayers and nodules.

Another study by **Yeniyol et al** [72] evaluated the adhesion of hFOB cells on titanium disk. He concluded that hFOB cells adhered well on the surface of titanium disk as earlier as 24 hours.

Hendrich et al [35] in his study evaluated the proliferation and differentiation of hFOB cells on the disk on surface of three different materials such as, stainless steel, cobalt-chromium-molybdenum (CoCrMo), and commercially pure titanium for 12 days. The results of their study showed that lesser cell number and delayed proliferation of hFOB cells in stainless steel disk and CoCrMo disk compared with titanium and polystyrene.

Schmidt et al [63] in his in vitro study assessed the proliferation of human adult osteoblasts on the surface of pure titanium disk in four different time periods (3, 7, 11, 15days). Result of their study showed that higher cell proliferation on titanium surface. They concluded that titanium provides the best biocompatibility with regard to proliferation.

Denizard et al [58] conducted an in vitro study assessing the cell attachment and bone tissue formation of human fetal osteoblast cells cultured on gamma titanium aluminide (γTiAl) and Ti-6Al-4V disks using

SEM. hFOB cells were proliferated on the surface of both γTiAl and Ti-6Al-4V disks. Their observation of a mitosis-like structure, with cells in the process of separating, clearly indicated that hFOB cells can grow and proliferate on γTiAl, suggesting that titanium alloy has the potential for being used as implant material.

Ricardo et al [16] evaluated the composition of two different brands of titanium MSI (Neodent & Morelli). The most abundant element in both the MSI analysed were Ti, Al, V and Fe. MSI with highest amount of titanium composition was in the Morelli (88.37%) compared to Neodent (87.93%).

In contrast, the present study assessed the composition of IZC mini-implant which showed maximum weight percentage of titanium was present in the head, body and tip of the Bioray IZC mini-implant when compared with Dentos IZC mini-implant and MSI.

The probable reason for increased composition of titanium in both the IZC mini-implant when compared with MSI could be due to its dimension (length, diameter and surface area) and manufacturing of the IZC mini-implant.

There is a significant correlation between the compositions of titanium alloy which influences the adhesion, spread, proliferation and morphology of the human osteoblast.

Hence the finding of the present study concurs with the results of the above mentioned studies.

In contrast most of the studies assessing osteoblast proliferation had used pure titanium disk which are not used in clinical scenario. Except the study of **Ricardo et al** which explored the successful proliferation of osteoblast cell suggesting titanium MSI has better biological bonding (partial osseointegration). Similar findings were observed in the present study suggesting that IZC mini-implants with optimum level of titanium would be a better adjuvant to increase stability and minimize failure.

IZC mini-implants are made of titanium and stainless steel alloy. Mini-implants made of titanium alloy incorporates Aluminium (Al) and Vanadium (V) to enhance to withstand orthodontic loads, greater strength and fracture resistance.

Titanium alloys are highly biocompatible with human tissues, high corrosion resistance in body fluids, lack of allergenicity, high specific strength, and low elastic modulus when compared with other metallic biomaterials. In contrast, stainless steel mini-implants incorporates chromium, nickel, molybdenum and a maximum of carbon.[27,16] Stainless steel MSI demonstrated to have acceptable biocompatibility, mechanical properties, and cost-effective.[54, 63]

A recent study by **Chang et al**[19] claimed maxillary bone being less dense compared to mandible, suggested using titanium IZC mini-implant with adequate strength for placing in the IZC region than using high strength stainless steel IZC mini-implant which might not be necessary for the posterior maxilla.

Since less dense bone is found in the posterior maxilla (D_3), when heavy insertion torque forces are applied in a smaller width of infrazygomatic crest region using stainless steel IZC mini-implant might result in micro cracks and crack propagation in the bone around the mini-implant results in either loosening or failure of mini-implant. Moreover the lack of pilot drill protocol in IZC region may result in increased insertion torque. To decrease the amount of micro fracture of bone, the strain on the bone should be reduced using pilot drill.[22, 53]

However **Chang et al**[19] also revived placing self-drilling stainless steel bone screws mini-implant in the mandibular buccal shelf. Stainless steel was selected as the material rather than titanium because of its toughness (resistance to fracture) when placed in the dense cortical bone.

CHEMICAL COMPOSITION OF TWO BRANDS OF TITANIUM IZC MINI-IMPLANTS SUBJECTED TO VARIOUS GROWTH MEDIUM ASSESSED USING EDAX ANALYSIS

After evaluating the hFOB cell proliferation and morphology, one sample of IZC mini-implant from each tested material (Dentos & Bioray) which were earlier subjected to various growth medium during this in vitro cell culture were again re-investigated for their chemical composition using EDAX in two different time periods (24 and 72 hours). The compositional weight percentage values are tabulated for comparison between the as-received IZC mini-implant and IZC mini-implant which were subjected to

various growth medium in two time periods (24& 72 hours) **Table. 8 & 9**. The most abundant elements in both Bioray and Dentos IZC mini-implant after subjected to basal medium were titanium, aluminium and vanadium. Compared with as-received IZC mini-implant, there was a significant variation in the elemental composition of the IZC mini-implants which were subjected to various growth medium.

There was a significant variations in the elemental composition of titanium in the body and tip of the IZC mini-implant which was subjected to various medium compared to as received Bioray and Dentos IZC mini-implant. A minimal increase in the titanium was found in the head region in contrast there was a significant decrease in titanium in the body and tip region of Bioray and Dentos IZC mini-implants. Elemental weight percentage of aluminium and vanadium significantly reduced in the body and tip of both the IZC mini-implants. (**Table. 8 & 9, Graph. 7-11**)

According to **woodman et al [70]** in his long term clinical study of metal ion release has stated that, titanium has been regarded as a biologically inert to the bio fluid rather than tissue fluid. In the titanium implants, superficial oxide is composed of TiO2, with small amounts of Al2O3, hydroxylic groups and vanadium is not present in the superficial oxide layer. Aluminium and vanadium are added to the implants to stabilise the alpha and beta phase of titanium. The Alpha-Beta phase in the titanium alloy allows a considerable increase of fracture limits.[2]

Titanium and aluminium are the metal ions most likely to be released from the titanium implant surface. Both aluminium and vanadium in titanium implants are potentially toxic. Aluminium accumulation in the human body may leads to osteomalacia and pulmonary granulomatosis. The aluminium ions affect the proliferation, metabolic activity of osteoblast by hindering their proliferation and differentiation.[60]

Vanadium is an essential element for the functioning of our organism. When vanadium absorbed in high dose, brings about acute and toxic effects. Hence **Hanawa**[33] suggested that the most harmful components of titanium alloy implants is vanadium. The toxic effect of vanadium may elicit local, systemic reaction and inhibit cellular proliferation.

A study was done by **Ananthanarayanan et al**[3] assessed the composition of five commercially available MSI (Length 6.0 mm & diameter 1.5 mmm) (Dentos, Biomaterials, Dentaurum, Denticon and J.J. Orthodontics) by assessing ion release in artificial saliva. They also evaluated the surface characterization and corrosion resistance. Similar to our study one as-received MSI from each group was subjected to characterization using EDAX and surface microstructure was analysed with the help of a SEM in three different regions of each MSI (head, body and tip). And they concluded that the ions released from all of the groups were lower than their respective toxicity levels.

In contrast with present study **Ananthanarayanan et al** had not mentioned the region of the MSI which was evaluated for the elemental composition. In the present study the composition was evaluated in three regions (head, body and tip) of Bioray and Dentos IZC mini-implant.

Blaya et al[12] examine and compare the levels of several metal ions released in the saliva of patients with orthodontic appliances, at different time points before and after insertion using MSI. This is only clinical study in humans which evaluated the ion release from MSI placed during orthodontic treatment. Saliva of patients was collected at four time period: before MSI placement (T1), 10 minutes (T2), 7 days (T3) and 30 days after miniscrew placement (T4). At time point T4, there was a quantitative increase in the salivary concentration of titanium, vanadium as well as a quantitative decrease in the salivary concentration of aluminium when compared with T1.

A similar and interesting findings was observed in the present invitro study using IZC mini-implant. There was a significant variations in the elemental composition of titanium IZC mini-implant which were subjected to various growth medium.

In the present in vitro study though there was a significant variations in the elemental composition of titanium IZC mini-implant which were subjected to various growth mediums, the human fetal osteoblast cells did not have toxic effect that affect the proliferation, morphology and differentiation of cells.

To the best of our knowledge, this is the first study which evaluated the hFOB cell proliferation, morphology and differentiation on the surface of titanium IZC mini-implant. The promising results of hFOB cell adhesion, proliferation suggest that areas of bone remodelling during the initial stages can be achieved which initiates the process of biological osseointegration.

However, the outcome of present study could only be compared with in vivo studies which have assessed the cell proliferation, morphology and differentiation in human osteoblast cells using mini-screws, titanium dental implants and titanium disk. Future studies are needed to investigate the speed and time of biological osseointegration using titanium IZC mini-implants.

Hence, in our study the null hypothesis was rejected as there was a significant increase in the proliferation of hFOB cells in polystyrene disk (control) followed by Bioray and Dentos IZC mini-implant respectively.

Limitation of the study:

The limitation of this study pertains to the number of sample size. All the findings of our in vitro study cannot be extrapolated to clinical situation and hence further clinical research should be done in vivo to evaluate the results of this study.

Summary and conclusion

SUMMARY AND CONCLUSION

A new form of skeletal anchorage is IZC mini-implant placed in infrazygomatic crest region of maxilla. The factors responsible for failure rates of the IZC mini-implants have been extensively studied. Literature reports failure rates using IZC mini-implants ranging between 7.5 to 21.8%. Many factors which has been reported in the literature regarding the success and failure rates of IZC mini-implant, are host related factors, IZC mini-implant related factors, host bone related factors and operator related factors. IZC mini-implants are made up of either titanium or stainless steel alloys. Studies have reported a significant correlation between composition of titanium alloy and osteoblast adhesion with greater proliferation is regarded for higher quality of biological osseointegration. Hence, the composition and surface related advantages of using titanium MSI and titanium disk has been reported in the literature.

However, there is a paucity of information whether the commonly used titanium IZC mini-implant has biological property during the initial stages of IZC mini-implant insertion to increase the stability and minimize its failure rates.

Therefore this study was done 1. To evaluate the proliferation and morphology of human fetal osteoblast cell (hFOB) on the surface of titanium IZC mini-implants (Bioray & Dentos). 2. To assess the chemical

composition on the surface of two different brands of titanium IZC mini-implants in three different region (head, body & tip) using Energy Dispersive X-ray analysis (EDAX).

Conclusions drawn from this study are:

The human fetal osteoblast (hFOB) cells adhered, spreaded and proliferated well over all the surfaces of both titanium IZC mini-implant. However there was a significant difference between the two different brands of titanium IZC mini-implants which is attributed to elemental composition on its surface.

When compared between polystyrene with both titanium IZC mini-implant, polystyrene had more proliferated hFOB cells in different time periods which is due to its high affinity. When compared between the two brands of titanium IZC mini-implants, Bioray titanium IZC mini-implant showed maximum hFOB cell proliferation in all three time periods which is attributed to its increased surface composition of titanium.

The shape of hFOB cells on the surface of a material is an important parameter for determining the biocompatibility of the material because cell spreading is closely linked to cell survival and differentiation. Morphology of hFOB cells varied between titanium IZC mini-implants and polystyrene in different time periods. During the initial stages hFOB cells adhered, spreaded and proliferated well on the contacting surface of titanium IZC mini-implants. At 24 hours hFOB cells were typically round shaped, at 48 hours elongated

cells with lamellipodia and filopodia, whereas at 72 hours predominantly stellate shaped cells with elongated proliferation.

The extrinsic structural and variations in the composition between the two titanium IZC mini-implants led to some minor differences in the hFOB cell attachment and proliferation, whereas there was no significant variations in their cell morphology.

The head, body and tip region of both the IZC mini-implants were assessed for their chemical composition. These areas were considered unique because each region of the IZC mini-implant is exposed to different environment in the body. The head is exposed to oral fluids and food, whereas the neck is in contact with oral mucosa and gingiva, both these regions acts as a point of force application. In contrast, the body and tip of IZC mini-implant are in contact with bone and hence contributes to its stability. Bioray IZC mini-implant has increased titanium content this might be the probable reason for increased cell adhesion and proliferation on the surface of Bioray IZC mini-implant.

From a clinical perspective, maxillary bone being a less dense than mandible, when heavy insertion torque forces are applied using high strength stainless steel IZC mini-implant in the posterior maxilla (D_3) which might lead to micro crack propagation resulting in loosening of IZC mini-implants. Moreover stainless steel IZC mini-implants are dependent on mechanical retention for its stability.

However, in addition to mechanical retention a good biological bonding of IZC mini-implant is needed in the IZC region during the initial stages of mini-implant insertion due to the anatomical variations in the width of various skeletal malocclusion. Moreover, to decrease the micro crack propagation of bone and the strain in the IZC region, titanium IZC mini-implant with low modulus of elasticity having adequate strength will minimize the failure rates.

Based on the finding of this study it can be concluded that as the time of contact between titanium surface and hFOB cells increased, so did the cell proliferation in both the brands of titanium IZC mini-implants achieving added advantage of favorable biological integration during initial stages, thus initiating the process of skeletal anchorage.

It is the orthodontist's responsibility to understand the biological aspect, materials aspect and structural limitations of these devices and the principles of application to minimize performance failure. Longer cell culture experiments or clinical studies using titanium IZC mini-implants are to be performed in order to study formation of extra cellular matrix and bone mineralization. Further in vivo studies are needed to validate and/or amend these insights.

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Annexures

ANNEXURE I

RAGAS DENTAL COLLEGE & HOSPITAL

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TO WHOM SO EVER IT MAY CONCERN

24-01-2022
Chennai

From,
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The dissertation topic titled **"EVALUATION OF HUMAN FETAL OSTEOBLAST PROLIFERATION AND MORPHOLOGY ON TITANIUM INFRAZYGOMATIC MINI IMPLANTS - AN INVITRO STUDY"** Submitted by **Dr. Deepthi Chandran. B** has been approved by the Institutional Ethics Committee of Ragas Dental College and Hospital.

Dr.N.S.AZHAGARASAN, MDS
Member Secretary
The Institutional Ethics Committee
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ANNEXURE II

Curiginal

Document Information

Analyzed document	EVALUATION OF HUMAN FETAL OSTEOBLAST PROLIFERATION AND MORPHOLOGY ON TITANIUM INFRAZYGOMATIC MINI-IMPLANTS -.docx (D127641951)
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