EVALUATION OF CYTOTOXICITY OF ORTHODONTIC MINI IMPLANTS IN TWO DIFFERENT pH CONCENTRATIONS -AN *IN-VITRO* STUDY

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled **"EVALUATION OF CYTOTOXICITY OF ORTHODONTIC MINI IMPLANTS IN TWO DIFFERENT pH CONCENTRATIONS -AN *IN-VITRO* STUDY"**is a bonafide and genuine research work carried out by me under the guidance of **Dr. SHAHUL HAMEED FAIZEE, M.D.S.,** Professor, Department of Orthodontics and Dentofacial Orthopaedics, Ragas Dental College and Hospital, Chennai.

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CERTIFICATE

This is to certify that this dissertation titled **"EVALUATION OF CYTOTOXICITY OF ORTHODONTIC MINI IMPLANTS IN TWO DIFFERENT pH CONCENTRATIONS –AN *IN-VITRO* STUDY"**is a bonafide record of work done by **Dr. Peddada Revathi** under my guidance during her postgraduate study period **2013–2016.**

This dissertation is submitted to **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY**, in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY** in **BRANCH V** – Orthodontics and DentofacialOrthopaedics. It has not been submitted (partially or fully) for the award of any other degree or diploma.

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CONTENTS

Introduction

INTRODUCTION

Orthodontic devices such as brackets, bands, arch wires and temporary anchorage devices are constantly exposed to oral environment, which are inter related with dietary intake, temperature and plaque accumulation.[29]

Saliva is a hypotonic solution containing bioactonate, chloride, potassium, sodium, nitrogenous compounds, and proteins[4] which play an important role in maintaining the oral health. Although buffering action is an important function of saliva, food intake and microbial flora can induce a decrease in the physiologic pH of saliva from 7.8 to 5.3[2]. Even temperature variation, coping with cold of ice (0°C) to hot coffee also affects the pH of the saliva[4]. This transient variations in pH and temperature can affect corrosion resistance of metal devices and in addition to this, mechanical fatigue also add susceptibility of alloys to corrosion. Corrosion resistance refers to how well a substance; especially a metal can withstand damage caused by oxidization or other chemical reactions[2].

Thus, factors such as temperature, quantity and quality of saliva, plaque, pH, protein, and the physical and chemical properties of food and liquids as well as oral health conditions influence corrosion of the metallic orthodontic devices.

The elements of typical fixed appliance are mainly made of two types of alloys such as Stainless Steel and Nickel Titanium alloys. Metal ions such

as Copper, Chromium, Iron, and Nickel and Titanium are potentially released into the oral cavity from these alloys mainly because of the corossion.[29]

Corrosion is defined as a chemical or electrochemical process through which a metal is attacked by natural agents such as air and water resulting in partial or complete dissolution, deterioration or weakening of any solid substance. This corrosion can be of different types like Uniform, Pitting and Crevice corrosion. What so ever may be the type of corrosion, the metal ions released due to corrosion may results in the consequent adverse tissue reactions, which defines the biocompatibility of the metal alloy used.[7]

To concise about Biocompatibility, it is defined as the capacity of a material to perform its specific functions when applied to living tissues of certain hosts without causing any damage or harm.[33] Yet the different metal alloys used in the dentistry and the metal ions released by them lead to the variable cytotoxic effect on the tissues. Cytotoxicity is the degree to which an agent has specific destructive action on certain cells. According to the International Standard Organization (ISO 10993), in vitro cytotoxicity trials should be the first tests to evaluate the biocompatibility of any material to be included in biomedical devices. For the confirmation of their non-toxicity, investigation of the product's biocompatibility is necessary with trials using laboratory animals.[33]

Orthodontic temporary anchorage devices (TADs) are one of the metallic components successfully used in orthodontics to prevent many of the shortcomings of traditional anchorage methods. Literature in this regard has

reported a great number of clinical studies suggesting that TADs may provide stable anchorage during the orthodontic treatment without requiring patient cooperation.[2]

In an oral environment, mini screws are exposed to a number of potentially damaging physical and chemical agents contributing to corrosion of the metal components and number of studies has demonstrated that the oral cavity, owing to its peculiar physical, chemical, enzymatic, and microbial characteristics, plays a significant role in the biodegradation of metal which lead to corrosion.

This corrosion leads to the dissolution of the thin protective oxide layer of an implant. This layer is usually inactive with surrounding biological environment and quite compatible with living tissue. But the destruction of this protective oxide layer due to various oral environmental factors, induce the leaching of metallic ions into the surrounding tissues and organs. This might lead to the implant failure by aseptic loosening, due to inflammatory reactions like peri-implantitis or osteolysis. These free metal ions can either remain in the intercellular spaces near the site where they were released or it could migrate systematically and can be taken up by macrophages leading to mitochondrial dehydrogenase activity and decrease in the DNA synthesis which can be termed as cytotoxicity.[10]

Consequently, so far only limited studies exist in the literature with reference to the varying degree of cytotoxic effect of mini implants used in orthodontic and its effects on cytotoxicity when the pH concentrations were

altered. Hence aim of this study was to evaluate the cytotoxicity of different mini implants in two different pH concentrations.

Review of Literature

REVIEW OF LITERATURE

Biocompatibility of different materials used in the orthodontics is related to different factors such as pH of the oral fluid, duration of the material in the oral cavity, corrosion resistance and amount of metal ions released from the materials.

Hence, the review of literature for this study is categorized into two groups

I. Metal ions released from the different materials and their cytotoxicity.

II. Factors influencing the corrosion and Metal ion release from different materials.

I. Metal Ions Released From the Different Materials and Their Cytotoxicity

Wataha J.C et al (1991)[59] investigated ten dental casting alloys for alloy-element release into cell-culture medium. Results showed that Au, In, and Pd generally did not dissolve into the medium, but elements like Ag, Cd, Cu, Ga, Ni, and Zn frequently dissolved. Comparison of EMA ratios for Ag, Cu, and Zn showed that each element retained a behavioral identity in diverse metallurgical environments. The commercial alloys used in this study exhibited more complex and less predictable release than did the simpler alloy systems. With excellent corrosion properties, these materials can be applied as coatings on the dental alloys to improve corrosion resistance of substrates.

Frisken K W et al (2002)[19] investigated the levels of dissemination of titanium from threaded screw type implants following placement of single implants in sheep mandibles. Twelve sheep were implanted with a single 10x3.75mm self-tapping implant for time intervals of one, four and eight to 12 weeks. Regional lymph nodes, lungs, spleens and livers were dissected, frozen and subsequently analyzed. Results associated with successful implants showed some minor elevations in titanium levels within the lungs and regional lymph nodes. Two implants failed to integrate and these showed higher levels of titanium in the lungs and regional lymph nodes. Debris from a single implant insertion is at such a low level that it is unlikely to pose a health problem. Even though the number of failed implants was low, multiple failed implants may result in considerably more titanium release which can track through the regional lymph nodes.

Oh, Keun Taek et al (2005)[42] evaluated the Ion release and cytotoxicity of stainless steel wires in four types of SS wires with a cross sectional area of 0.41 × 0.56 mm. These wires were heat-treated in a vacuum, air, or argon environment, and were cooled in either a furnace or a water bath. The concentration of dissolved nickel ions in artificial saliva was measured for a period of up to 12 weeks. In all groups, the concentration of dissolved nickel ions in artificial saliva was lowest for the vacuum heat treatment-furnace cooling group. The concentration of dissolved nickel ions in artificial saliva was highest in the groups heat-treated in air while the amount of nickel

released was highest in the Remanium and Colboloy. The cytotoxicity was mild in all the experimental groups but the response index of the air groups was slightly higher than in the other groups. According to these results, SS wires retain their high corrosion resistance and low ion release rate when heat-treated in a vacuum and cooled in a furnace.

Jay Albretsen et al (2006)[2] reviewed the toxicity of iron and postulated that the iron localized in the mitochondria of cells and damages several cell organelles. Eventually, hypoglycemia, hyper-ammonemia, coagulation defects, and hepatic encephalopathy occur. Free iron inhibits the thrombin-induced conversion of fibrinogen to fibrin. Histopathologic evidence of iron-induced hepatic damage includes cloudy and swollen hepatocytes, portal iron deposition, fatty metamorphosis, and massive periportal necrosis.

Chia-Tze Kao et al (2007)[28] postulated the cytotoxic effects of four different orthodontic metal bracket with immersion media on primary human oral gingival fibroblasts and one permanent human osteogenic sarcoma cell line. The results showed microscopically no morphological changes in the HGF or U2OS cells exposed to the metal bracket immersion media. At pH 4, the survival rates of the U2OS cells and the HGFs differed statistically for the Unitek and Ormco groups. At pH 7, the survival rate for the HGFs and the U2OS cells differed statistically for the Dentaurum and Unitek groups. Results demonstrated that differing cells exhibit various cellular reactions on exposure

to metal bracket immersion media, although the four types of brackets appear to be biocompatible with HGF and U2OS cells.

Ke Zheng et al (2007)[61] evaluated the low effective concentrations of Al for short times and postulated that at higher concentrations of Al or longer exposure times, Al induces cell death and growth inhibition. Several apoptotic features appear during Al treatment, including cell shrinkage, vacuolation, chromatin marginalization, nuclear fragmentation, DNA degradation, and DNA strand breaks, as well as concomitant cell aggregation.

Fariborz Amini et al (2008)[3] studied the metal ion concentrations in the saliva of subjects with and without fixed orthodontic appliances. A total of 56 subjects were included in this study with twenty-eight healthy orthodontic patients with fixed appliances in both arches for a period of 12–18 months. He concluded that fixed orthodontic appliance therapy for an average period of 16 months can lead to increased levels of Ni and Cr ions in the saliva of patients. While the low levels of these metal ions can be of concern to patients with allergies, they do not lead to problems in the majority of orthodontic patients as toxic levels are never attained.

Monika Huber et al (2008)[40], investigated the peri prosthetic tissue containing solid corrosion products after aseptic loosening of second-generation metal-on-metal total hip replacements made of low-carbon cobalt–chromium–molybdenum alloy for the presence of immunologically

determined tissue changes. Peri prosthetic tissue of 11 cases containing uncommon solid deposits was investigated by light microscopy. In order to confirm the presence of corrosion products, additional methods including scanning electron microscopy (SEM) investigation, energy dispersive X-ray (EDX) and Fourier transform infrared microspectroscopy (FTIR) analysis were used. Various intense tissue reactions characteristic of immune response were observed in all cases. The simultaneous presence of corrosion products and hypersensitivity-associated tissue reaction indicates that a relationship between corrosion development and implant-related hypersensitivity may exist.

Dalia H. El-Rouby et al (2010)[16] evaluated the subcutaneous connective tissue reaction to the newly-developed nano-restorative materials such as Filtek Supreme XT, Ceram X and Ketac N100. The presence of inflammation, type and location of inflammatory cells, calcification and fibrous tissue formation were recorded. They found that the implanted materials induced different and time dependent inflammatory reactions, mast cells and microphages migration, in addition to distinct fibrosis development. Ketac N 100 revealed a less biocompatible tissue reaction compared to Filtek supreme NT and Ceram X.

Hideki Kamata et al (2010)[27] evaluated the electro deposition of collagen to a titanium surface under various conditions like pH of the collagen solution and electro deposition time. This was performed to understand the

optimal electro deposition conditions for the immobilization of collagen. Results showed that except with the pH 9, collagen fibrils were more attracted to a Ti cathode, and the durability of the immobilized layer was largest at pH 5. This is because collagen was more positively charged at pH 5 than pH 6. On the other hand, the alternating potential generated the thickest collagen layer with the fibrous network and the largest durability in water. Therefore, they concluded that the electro deposition with an alternating current at pH 5 is much more appropriate technique than the conventional immersion technique.

Matheus Melo Pithon et al (2010)[51] assessed the cytotoxic effect of orthodontic mini-implant on L929 fibroblast cells with eighteen orthodontic mini-implants made of Ti-6Al-4V alloy. Implants were divided into 6 groups. Cytotoxicity was evaluated in four different periods of time: 24, 48, 72, and 168 hours. After this period of time, they were fixed and a spectrophotometer was used for counting the viable cells and concluded that although mini-implants are made of the same alloy, there are differences in their cytotoxicity because of the different concentrations of chemical elements used for manufacturing them.

Marcin Mikulewicz et al (2011)[37] evaluated the new orthodontic appliance like wires, brackets, bands, and metal ligatures which are made of stainless steel. Demonstrated that elevated levels of metals in saliva are thought to occur by corrosion of the chemical elements in the alloys or welding material. The use of fixed orthodontic appliances made of stainless

steel can be a source of risk exposure to nickel. The concentration of Ni transferred from the appliances to the artificial saliva in this experiment was 11 times that of maximum levels set as acceptable for drinking water in Europe. The concentration of Mn was 1.4 times and the concentration of Fe is 12 times the acceptable levels.

Miceli Guimaraes Blaya et al (2011)[8] examined and compared 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 mini screw. Saliva of patients was collected at four time points: before mini screw placement, 10 minutes, 7 days and 30 days after mini screw placement. The release of nine different metal ions was observed: titanium, zinc, chromium, nickel, iron, copper, aluminum, Vanadium and cobalt. 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.

Muddugangadhar B C et al (2011)[5] postulated that phenomenal interest and improved application and improvement in the dental material and advocated that implantology provides many new and exciting ways to help dental patients to achieve the function and social wellbeing and provide a certain amount of personal and professional satisfaction to the dentist. Dental implantology will become a highly acceptable and predictable treatment modality for the restoration of human dental and oral apparatus. Also said that Implants are now being targeted in predoctorial and postdoctorial training programs nationally and internationally.

Amini et al (2012)[3] found that the level of Ni increased in oral mucosa cells in orthodontic patients from 12.23 ng/ml to 21.74 ng/ml in the experimental group in 16 months. The metallic ion release in the oral implant with super structure of different metals and alloys used in the clinical dentistry is determined. The measurement of the ion released was carried out by means of inductively coupled plasma mass spectrometry technique. The genotoxic effect on oral mucosa cells at the debonding phase of the orthodontic fixed appliance was found. Increased levels of Ni and Cr 30 days after debonding were not confirmed. Immediately after debonding, mean Cr and Ni contents were slightly higher in the test group.

Baricevic, Marinka et al (2012)[32] examined the genotoxicity of two dental casting alloys commonly used in fixed and removable prosthodontic appliances that are in contact with the oral epithelium for 5 years or more. For that purpose, 55 age-matched subjects were included in the study. Buccal cells of oral mucosa were collected and processed for further analysis. The cell viability has been assessed by trypan blue exclusion test, while genotoxic effect of metal ions on DNA in oral mucosa cells. It has been confirmed that metal ions released by the two base metal dental casting alloys examined in this study, might be responsible for DNA damage of oral mucosa cells. Therefore, the results of this study emphasize the importance of the in vivo evaluation of dental materials with respect to their genotoxicity, which is of major importance to ensure long-term biocompatibility.

Necati Menek et al (2012)[35] evaluated the nickel ion release from stainless steel crowns in artificial saliva at different days and pH's which is used in pediatric dentistry. Totally 120 stainless steel crowns for primary teeth were immersed to artificial saliva in this study. Nickel in aqueous solutions was determined by square wave voltammetry, using dimethylglyoxime as a complexion agent on mercury electrode. The study revealed that nickel ion release was decreased with increasing pH. Results showed that metal ions released in this experimental condition were well below the critical value to induce allergy and below daily dietary intake level.

Ajith Rajasekharan Pilla et al (2013)[49] investigated the cytotoxic activity of the media with MTT and comet assay. The results of the study show that the amount of nickel leached is capable of bringing damage to the fibroblast. Nickel solution at minimal concentration of 1.18 μg could damage human gingival fibroblast and the nickel released from the different brands of the brackets is not uniform.

Laura-cristina Usu et al (2014)[57] evaluated the cytotoxic potential of the most used dental alloys, the Ni–Cr alloy and the Co–Cr alloy. The tests were made on cell culture of pure cell line dermal fibroblasts and of those obtained from skin biopsies, for both, dental alloys and their eluates. The results were compared with control samples. At seven days after inoculation, they observed the relative similarity between the Ni–Cr alloy and the Co–Cr alloy, where the cells did not detach from the plate and they grow to the edge

of the material. In case of the eluates, there were no fragments detached, the cells having a relatively high confluence. Concluded that the cytotoxicity of the alloys tested was similar and had minimal in vitro effects on fibroblasts from cell culture. They believe that the tested alloys can be successfully used in the dental practice despite of the tendency to give up metal in this medical field.

Marcin Mikulewicza et al (2014)[38] investigated the release of metal ions from an orthodontic appliance with animal test conducted on 24 pigs divided equally into an experimental and a control group. Noninvasive matrices (hair 0, 3, and 6 months) and invasive matrices (kidneys, liver, lungs, aorta, and oral mucosa) were collected for multi-elemental analysis from the experimental and control groups. The greatest differences in the content of toxic metals were found in the aorta, in the cheek and in the hair sampled after 3 months. Metal ions were released from the appliances in low doses, in particular at the beginning of the experiment. The doses of toxic metal ions did not reach toxic levels. Hair was found to be a noninvasive biomarker of exposure to metal ions released from orthodontic appliances.

Ozturk Firat et al (2014)[56] evaluated the cytotoxicity of four different orthodontic cement materials using the real-time xCELLigence system. Four orthodontic glass ionomer cement were selected for this study, namely: GC Fuji, Ultra Band Lok, Multi Cure, and Meron. Ten test cylinders measuring 5×2mm of each material were fabricated, making a total of 40 cylinders.

Human gingival fibroblasts were taken for the study. A real-time cell analyzer was used to evaluate cell survival. GHF cells were seeded with bioactive components released from cement materials. When the data were evaluated at 24 and 48hrs, all tested materials showed significant decreases in HGF cell index compared to the control group**.** According to the results of this study, all tested cements were found to have cytotoxic effects to the HGFs.

Sahmali et al (2014)[38] postulated the effects of dental alloys containing Ni on the level of this element in serum, liver, kidney, and oral mucosa of guinea pigs. The test was conducted for 15 days and found that guinea pigs sensitized to Ni had higher levels of Ni in the serum, oral mucosa, liver, and, slightly, in kidney as compared with the control group. Significant differences were found between liver and oral mucosa Ni content in the experimental and control groups.

Carvalho Bueno et al (2015)[9] evaluated the human osteoblast proliferation and morphology on orthodontic mini-implants by culturing the Osteoblasts on the surface of sterilized mini-implants in a CO2 incubator at different time periods (24, 48, and 72 hours). They found that Osteoblast proliferation was successful on the mini implant surface, which increased over time without a significant difference between commercial brands. The most frequently observed elements present in the alloys were Ti, Al, V, and Fe, a characteristic that did not differ significantly between brands.

Veronica Mercut et al (2015)[59] evaluated the in vivo effect of nickel and copper compounds on the oral mucosa cells, including their ability to induce cell death, by analyzing the cytochrome Immune histochemical expression. Gingival mucosa fragments obtained from the subjects with dentures manufactured by nickel or copper casting alloys were processed through the histological technique of paraffin inclusion. The sections obtained were stained to highlight the histopathological lesions and analyzed using the immune histochemical technique in order to study the cyst expression. The papillomatosis lesions were observed in the gingival mucosa fragments obtained from the subjects with nickel-based alloy dentures and the condyloma acuminata lesions were observed in those obtained from the subjects with copper-based alloy dentures. The cyst immune histochemical expression was different in the epithelial layer of two types of mucosal fragments, but it was the same in their lamina propria connective tissue. They conclude that the two types of metal alloys have different effects on the adjacent gingival mucosa.

II. Factors Influencing the Corrosion and Metal Ion release from different materials.

Hamoon Zohdi et al (2000)[63] reviewed the comprehensive studies of galvanic corrosion behavior of dental alloys and reported that failures of some implants are due to the galvanic-type corrosion. The galvanic current passes through the metal/metal junctions, which may finally cause pain owing to release of metal ions. The oral environment is particularly favorable for corrosion. The corrosive process is mainly of an electrochemical nature and natural saliva present acts as a good electrolyte. Fluctuations in temperature, changes in pH because of diet, and decomposition of food, all contribute to this process. It is also mentioned that the parameters like, pH and the presence of fluoride could severely affect galvanic corrosion. Besides, in this review, it is shown that new types of prosthesis, implants like metallic glasses could be applied as new generation of implants.

Athanasios E. Athanasiou et al (2002)[15] reviewed the critical issues of corrosion potential and nickel leaching from alloys by investigating the effect of intraoral conditions on the surface reactivity of the materials. After an overview of fundamentals of metallurgical structure of orthodontic alloys, they provided an analysis of corrosion processes occurring in vivo. They also presented recent evidence suggesting the formation of a proteinaceous biofilm on retrieved orthodontic materials that later undergoes calcification. They illustrated the vastly irrelevant surface structure of aged alloys and discussed

the potential implications of this pattern in the reactivity of the materials. Finally, they present a comprehensive review of the issue of nickel release, based on three perspectives: its biologic effects, the methods used for studying its release, and nickel-induced hypersensitivity in orthodontic patients.

Denizoglu S et al (2004)[12] evaluated the influence of salivary pH on the corrosion of two base-metal alloys. Cobalt – chromium and nickel – chromium alloy samples were taken and submerged in artificial saliva of different pH values (pH 4, pH 5 and pH 7). The amount of each ion present in solution after 1 month was measured by flame atomic absorption spectrophotometry. Results showed that pH significantly affected total Cobalt ion release, but not Ni or Cr ion release. The alloy type did not affect total ion release, but was significant for Cr ion release. Alloy – pH interaction significantly affected Cr and total ion release. To prevent metal particle release, the alloys used for dental restoration should, whenever possible, be made from noble metals and corrosion-resistant alloys

Karthega et al (2006)[30] evaluated the corrosion behavior of Ti-6Al-4V in naturally aerated artificial saliva in the presence of 1% sodium fluoride at different pH namely 7.0, 5.0 and 3.5 using open circuit potential, potentiodynamic polarization. Results showed that Fluoride ions at 3.5 and5.0 pH can attack the titanium oxide formation which causes changes in the protective passive layer on the metal, leading to the porosity of the oxide layer which reduces its protection. Potentiodynamic polarization studies show that

for pH 7.0, the potential shifts to more noble direction, indicating the formation of passive layer. The EIS studies indicate that, resistance increases with increase in pH. Hence, Ti-6Al-4V shows a much better corrosion resistance in artificial saliva containing 1% NaF at pH 7.0.

Douglas C. Hansen et al (2008)[23] advocated that Corrosion is one of the major issues resulting in the failure of biomedical implant devices. The nature of the passive oxide films formed, and the mechanical properties of the materials form some of the essential criteria for selection of alternative or development of new materials. That is, the coating of the alloy with hydroxyapatite plays a dual role: minimizing the release of metal ions by making it more corrosion resistant, as well as making the surface more bioactive and stimulating bone growth. Other surface modification techniques, such as hard coatings, laser nitriding, bio ceramics, ion-implantation, and biomimetic coatings have great potential to improve the performance of biomedical implants and improving the lives of their recipients.

Yoshimitsu Okazaki et al (2008)[43] evaluated the Metal release from stainless steel, Co–Cr–Mo–Ni–Fe and Ni–Ti alloys which are used for stents and stent grafts. The quantities of Fe and Ni released from stainless steel gradually decreased with increasing pH of the solution from 2–7.5. For Co–Cr–Mo– Ni–Fe alloy, the quantity of Cr released steadily increased as pH decreased to 6 and reached nearly zero at pH higher than 6 (pH 6–7.5). Although the rapid increases were observed at approximately pH 2, the

quantities were even higher than that of Co released from the Co–Cr–Mo and Co–Cr–Mo–Ni–Fe alloys.

Daniel G. Olmedo et al (2009)[44] illustrated the interface between the implant and its peri implant tissues *in situ* by means of failed Dental Implant for the investigation. It is concluded from the analysis of failed human dental implants that in traumatized tissues significant drops in pH values have been found, reaching values as low as pH 4 during the healing process. These values greatly increase the aggressiveness of tissues towards metallic materials. They have found that the reduction of pH in the electrolytic medium as a consequence of local inflammatory processes and it may also act as a corrosion-stimulating agent.

Belma Muhamedagić et al (2010)[11] overviewed the existing dental metals and alloys. Based on the existing dental metals and alloys in contexts with their anticorrosive characteristics, anticorrosive protective films they postulated that the metal alloys in a mouth are exposed to the influence of chemical, biological, mechanical, thermal and electrical forces which can have a negative impact on therapeutic work or surrounding tissue. Electrochemical corrosion is the most important damaging factor of dental works. The corrosive resistance of metal is its important characteristic during implantation into a mouth and concluded by saying that precious alloys are the most suitable for dental use.

Marcin Mikulewicz et al (2010)[36] conducted a systematic literature review on release of metal ions from orthodontic appliances under in vitro conditions and he identified 40 studies, among which eight met the selection criteria and gave conclusion that the presence and activity of microflora to a large extent is responsible for the process of corrosion, in particular, bio deterioration. The general conclusions from the papers discussed in the review were that the less biocompatible material was SS, which released the highest quantity of nickel and chromium. Acidic environment significantly increased the degree of metal ions release.

Nilo A. S. Sampaioet al (2010)[52] illustrated the influence of Ni and Cr Content on Corrosion Resistance of Ni-Cr-Mo alloys by using the three Ni-Cr-Mo alloys. Corrosion resistance has been determined in naturally aerated 0.05% NaF solution, pH 6.0 at 37°C using electrochemical techniques. The open circuit potential curves for the three different alloys in 0.05% NaF showed that the Fluoride ion presence in solution can alter the oxide layer structure making it more porous. This leads to a decrease in corrosion resistance in the considered medium. These observations seem to be consistent with the differences in the alloys compositions, since alloy A, by having a smaller chromium amount in its composition, becomes more susceptible to corrosion in medium containing fluoride because Chromium is responsible for corrosion resistance and for an oxide film formation which is commonly called passive film.

Rahul Bhola et al (2010)[7] reviewed on the Corrosion in Titanium Dental Implant and concluded that the metallic titanium dental implants used in dentistry today derive their biocompatibility from the alloying elements responsible for the formation of a continuous stable TiO2 passive film on its surface. There is a significantly small release of alloying ions even under the ideal conditions of passivity and with no damage to the implant surface. Corrosion of these implants may occur when the oral conditions are unfavorable as under mechanical trauma to the implant surface during placement, subject induced, and trauma to assault.

Hoffman B et al (2011)[24] interpreted the coating on the mini implant to evaluate the ion release of the implant material, which can cause inflammation or allergy reactions in the body. Ti6Al4V and Co28Cr6Mo discs were used as substrates. The results show, that a TiO2-coating has a cover function by reducing the ion release of the bulk material. Most probably the ion release is an essential factor, which influences the biocompatibility.

Lamia S. Kheiralla et al (2011)[4] studied the corrosion behavior of Ti and Ti6Al4V implants coupled either with metal-ceramic or all-ceramic superstructures. Results showed cpTi couples showed significantly superior corrosion resistance properties than Ti 6Al 4V couples. The use of fluoride therapy with concentrations higher than 0.1 M might cause a significant decrease in the corrosion resistance. Ti 6Al 4V couples were more affected by fluoride than commercially pure Ti couples. They also said that the biological

characteristics of each individual represent a variable that cannot be reproduced easily in vitro, in terms of composition, acidity of saliva, dental hygiene, eating habits, administration of medicine, and caries.

Keren Shemtov-Yona et al (2012)[53] evaluated the influence of fluid environment mimicking intra-oral conditions. 3.75-mm diameter implants fatigue performance has been essentially studied in room air, based on the premise that the implant material is relatively resistant to corrosion in the intra-oral environment. Results showed that artificial saliva acts as an aggressive environment for dental implant fatigue. Transgranular fracture and secondary parallel micro cracks are the main fracture micro-mechanisms. For this reason it is recommended to include testing in artificial saliva into the existing requirements for implants in order to evaluate the fatigue performance of dental implants in conditions that mimic better the oral environment.

Srinivas kumarkarnam et al (2012)[29] determined the amount of nickel, chromium, copper, cobalt and iron ions released from simulated orthodontic appliance made of new arch wires and brackets. These appliances were immersed in 50 ml of artificial saliva solution and stored in polypropylene bottles in the incubator to simulate oral conditions. After 90 days the solution were tested for nickel, chromium, copper, cobalt and iron ions using atomic absorption spectrophotometer. Results showed that high levels of nickel ions were released from all four groups, compared to all other ions, followed by release of iron ion levels.

Giban and Peter et al (2013)[47] investigated the Cobalt based alloy by modifying the composition by adding titanium which can increase the corrosion resistance and at the same time it can also improve the alloy biocompatibility. They found that by using a higher amount of Ti, the hardness will be further improved, but at the same time a negative effect has been observed on the workability. The simultaneous addition of Ti and Zirconium leads to the formation of extended Zirconium covered areas which interrupt the homogeneity of the structure. As corrosion resistance in a simulated oral cavity environment concerns, no significant release of metal ions was observed.

Ivana D. Dimić et al (2013)[13] investigated ion release from grade 2 cpTi in artificial saliva with different pH values. Concentrations of titanium ions released from cpTi grade 2 in the artificial saliva with different pH values 4.0, 5.5 and 7.5 after 1, 3 and 6 weeks was quantified. Results showed that the ion release from cpTi grade 2 in artificial saliva of different pH values indicate that the concentrations of released titanium ions increased with increasing immersion time. Also, the decrease of the pH value led to the increase of the concentrations of released titanium ions. Mechanical resistance, chemical inertness, absence of toxicity, extraordinary specific strength, low Young's modulus, and outstanding biocompatibility of titanium, combined with low ion-release tendency, confirmed in this study, recommends the usage of cpTi as a biomaterial for dental implants.

Anuja Agarwal et al (2014)[1] reviewed corrosion aspect of dental implant and postulated that the good biocompatibility of Ti is related to the thin oxide layer formed on Ti surface. TiO2 is inactive with the surrounding biological environment and quite compatible with living tissues. But the localized destruction causes corrosion of the implant, thus, weakening it and can induce the leak of small metallic particles or ions into living tissues. He also reviewed various aspects of corrosion and biocompatibility of dental titanium implants as well as supra structures, and the methods to prevent it. Said that the Implant failure in the form of aseptic loosening, or osteolysis, may result from metal release in the form of wear debris or electrochemical products generated during corrosion events. Metal ions such as Ti4+, Co2+, and Al3+ have been shown to decrease DNA synthesis, mitochondrial dehydrogenase activity, mineralization, and mRNA expression of alkaline phosphatase.

Luís Gustavo Costa de Castro et al (2014)[22] assessed the electrochemical behavior of a Ni-Cr-Mo commercial dental alloy, in a physiological environment that simulates the aggressiveness of oral cavity (0.9% NaCl solution) with its pH varying from 2.0 to 6.0. The corrosion behavior was assessed by electrochemical measurements which are commonly applied in metals corrosion study, with quantitative parameters to estimate corrosion resistance. On the potentiodynamic curves it was observed that the re-passivation potential decreased with diminishing pH, suggesting that the re-

passivation does not occur at pH 2.0. With decreasing pH, a higher difficulty of the passive film to regenerate was observed.

O. Petka et al (2014)[48] evaluated the influence of alloying additions iron or aluminium on corrosion resistance in artificial saliva solution of a dental cobalt alloy. Pitting corrosion resistance was evaluated on the basis of anodic polarization curves obtained during electrochemical potentiodynamic polarization tests, which was also performed for samples after one and twelve hours of passivation. Corrosion potential, repassivation potential and corrosion current were picked as an assessment criteria. Results obtained in the test showed that both alloying additions have a slight influence on the pitting corrosion resistance of the tested alloy. Nevertheless, comparing iron and aluminium additions, corrosion parameters values were better for samples admixed with aluminium. On the basis of microscopic observations it was stated, that tested alloys have the structure with noticeable dendritic segregation.

Gabriella MP Juanito et al (2015)[45] summarized the current data regarding the influence of fluoride and bleaching agents on the degradation of titanium and Ti6Al4V alloy surfaces. Thirty eight studies from an initial yield of 180 studies were selected. Results indicated that therapeutic substances used in dental practice such as fluoride, hydrogen and carbamide peroxides are related to corrosion and wear processes of titanium-based structures. Consequently, corrosive processes occurring on titanium leading to the release

of ions and wear particles to surrounding peri-implant tissues and organs. The relation between ion release and inflammatory reactions into human tissues is not clear yet.

Tbrah Nuoh et al (2015)[41] evaluated the influence of temperature and pH on corrosion resistance of Ni-Cr and Co-Cr dental alloys, in order to characterize the physical and mechanical properties of corrosion resistance property of the Ni-Cr and Co-Cr. The corrosion behavior of three different dental alloy, were tested in Ringer's solution, artificial saliva at different pH values and temperature. On the basis of the results obtained it has been shown that the corrosion resistance of the alloys was decreased when the pH of the solution was lowered to pH 2.5 as well as the temperatures raised to 40°C. The metal and different alloys have thermal, microbiological and enzymatic properties. A metal in aqueous solution will be thermodynamically unstable and there is a tendency to pass from a solid state to anionic form which is associated with a decrease in energy. The direction of energy changes is influenced by factors such as surface morphology, salivary composition, pH and temperature. An unstable metal may corrode, releasing metal ions into solution and may have adverse biological, aesthetic and functional effects.

Materials and Methods

MATERIALS AND METHODS

Cytotoxicity and the amount of metal ions released were evaluated using three different Companies of mini implants that were popularly used in India in the field of orthodontics, such as:

1) Dentos, Anchor Dentons Inc., Korea,

2) Denticon, Dental Instrument Co, Mumbai, India,

3) SK Mini Implants, SK Surgicals, Om Labs, Pune, India,

(Figure no. 1)

Composition of selected Titanium implants (wt. %)

Implant	Fe	Al	V	Ti
DENTICON	0.35	2.75	0.64	96.36
DENTOS	0.30	6	4	89.7
SK Surgicals	0.30	5.62	4.7	89.3

Twelve mini implants of each selected company were evaluated for cytotoxicity by immersing them in a Sodium Chloride solution (NaCl) at two different pH concentrations. For this purpose, Sodium Chloride solution of pH 4 and pH 7 concentrations was prepared.

Preparation of pH Solution:

45ml of Sodium Chloride solution at two different pH concentrations which are pH 4 and pH 7 were prepared in two separate beakers. Two different pH concentrations were obtained by adding Sodium Hydroxide (NaOH) and Hydrochloric acid (HCL) and distilled water. (Figure no. 2). Both the pH concentrations were verified by using digital pH meter (Figure no. 3).

Study Methodology

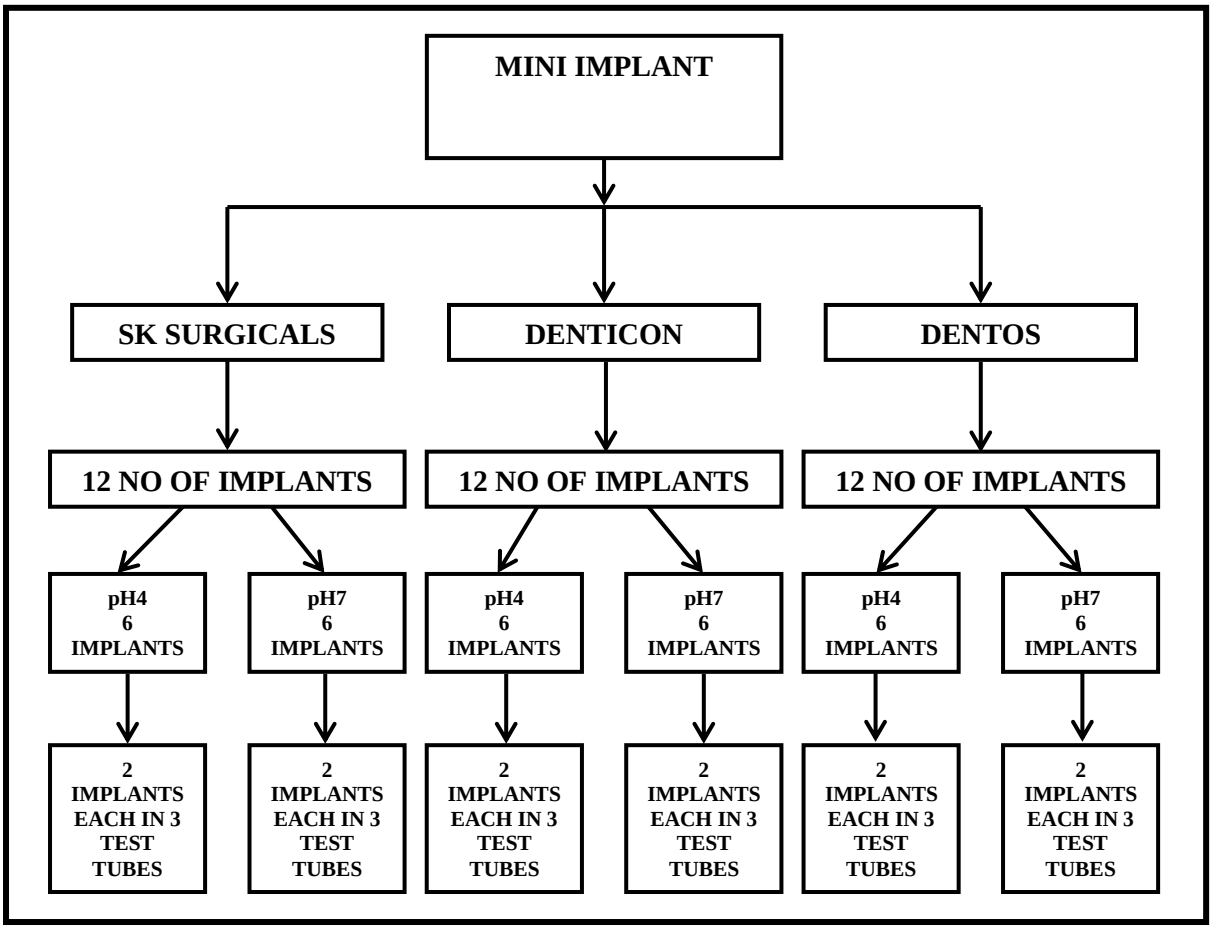
All the mini implants were sterilized using autoclave before starting the study. Among 12 implants of each company, six implants were immersed in the solution of pH 4 and other six implants were immersed in the solution of pH 7 (Figure no. 4). These 6 implants were segregated in 3 test tubes with 2 implants per each test tube. This accounts to a total of 6 test tubes for each company (3 test tubes for pH 4 and 3 test tubes for pH 7). Similar methodology was followed for all the companies of mini implants selected in our study. (Thus there were 6 test tubes for each company, a total up to 18 test tubes for all the companies). Out of 18 test tubes, 9 test tubes pertain to pH 4 and other 9 test tubes to pH 7. NaCl solution of pH 4 and pH 7 without adding implants were considered as control.

The samples were stored under stationary condition at room temperature. The solution containing the mini implant is termed as the eluate. An eluate can be described as the solution of solvent and dissolved matter

resulting from elution. (Elution is the process of extracting one material from another by washing with a solvent, as in washing of loaded ion-exchange resins to remove captured ions).

1ml of eluate was collected from each test tube at Day 15, Day 30 and Day 60. The collected eluates were used for the evaluation of cytotoxicity and amount of metal ions leached from the mini implants (Figure no. 5, 6).

Schematic representation of Study Methodology



Cell Culture

To evaluate the cytotoxicity of the mini implants, Primary Human Gingival Fibroblast (HGF) and Human Osteogenic Sarcoma (U2OS) cell lines were used in this study. Both the cell lines were obtained from The National Centre for Cell Sciences, An Autonomous Institute of Dept. of Biotechnology, Pune, India.

Human Gingival Fibroblasts (HGF)

Human Gingival Fibroblasts Cell Vial is thawed by gentle agitation in a 37°C water bath. This thawing is done to activate the cells. As soon as the contents are thawed, they are decontaminated by spraying with 70% ethanol. All of the operations from this point were carried out under strict aseptic conditions. The decontaminated cell vials are added to 9 ml of the growth medium. The growth medium used to culture these Human Gingival Fibroblast cell vials is Dulbecco's Modified Eagle's Medium. In order to mix the cell vial completely with the growth medium, the mixture is transferred to centrifuge test tube and centrifuged at approximately 125 rpm for 5 to 7 minutes. The mixture of these cells and the growth medium after the centrifugation is termed as cell pellet. The obtained cell pellet is dispensed into 96 well culture plate and cell culture is incubated at 37°C in 5% CO_2 until the cells get matured (Figure no. 7).

Human Osteogenic Sarcoma cells (U2OS)

Human Osteogenic Sarcoma cell lines were also cultured in the similar condition as mentioned above for the Human Gingival Fibroblast cell culture.

Evaluation of cytotoxicity:

Cell viability is evaluated using the MTT assay. This is done by adding the 40 µl of the collected eluates and the control solutions to the matured cell culture of both the Human Gingival Fibroblast (HGF) and Human Osteogenic Sarcoma Cells (U2OS) (Figure no. 8).

After adding the eluate to the cell cultures, they are incubated for 48 hours. This is done for cells to absorb and react to the eluates. After 48 hours of incubation, cell cultures are now treated with MTT dye and incubated at 37°C for 4 hours and then with DMSO (Dimethyl Sulfoxide) at room temperature for 1 hour (Figure no. 9).

Principle of the MTT Assay

MTT-[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide] assay. MTT is a membrane permeable dye which is metabolized to dark-blue crystals of formazan by mitochondrial succinate dehydrogenase of living cells. These crystals are impermeable to cell membranes and get accumulated in the cells. After lysis of the cell using DMSO and solubilization of the formazan crystals, the optical density (OD) of the dye is quantified using a multiwell-

spectrophotometer at 550nm. The number of living cells is directly proportional to the development of dark-blue color, i.e. higher the viability cells the darker the blue color (Figure no. 9).

Since the reduction of MTT to formazan can occur in only metabolically active cells, the measure of activity is the measure of viability. MTT assay is done for all the eluates collected at day 15, day 30 and day 60 by placing the well plate in the ELISA reader. The values were tabulated for statistical analysis (Figure no. 10).

Ion Coupled Plasma – Atomic Emission Spectrometer (ICPMS)

Ion Coupled Plasma – Atomic Emission Spectrometer is advocated to find out the amount of metal ions released from the mini implant eluates at day15, day 30 and day 60.

Principle of ICP-MS

The fundamental principle is the use of high temperature plasma discharge to generate positively charged ions; the sample typically in the liquid form is pumped into the sample introduction system, which is made up of spray chamber and nebulizer. It emerges as an aerosol and eventually finds its way through sample injector into the base of plasma. As it travels through the different heating zones of the plasma torch, it is dried, vaporized, atomized and ionized. During this time the sample is transformed from a liquid aerosol to solid particle, then into a gas. When it finally arrives at the analytical zone

of the plasma it exists as exited atoms and ions representing the elemental composition of the sample (Figure no. 11).

Statistical analysis:

All statistical analysis were performed using Statistical Package for Social Science (SPSS, version 17) for Microsoft windows. The data were normally distributed and non-parametric tests were performed. Descriptive statistics were presented as numbers and percentages. The data were expressed as Mean and SD. A one way analysis of variance with a post hoc Tukey's HSD test was used. Independent sample student t test were used to compare continuous variables between two groups. A two sided p value < 0.05 was considered statistically significant.

Figures

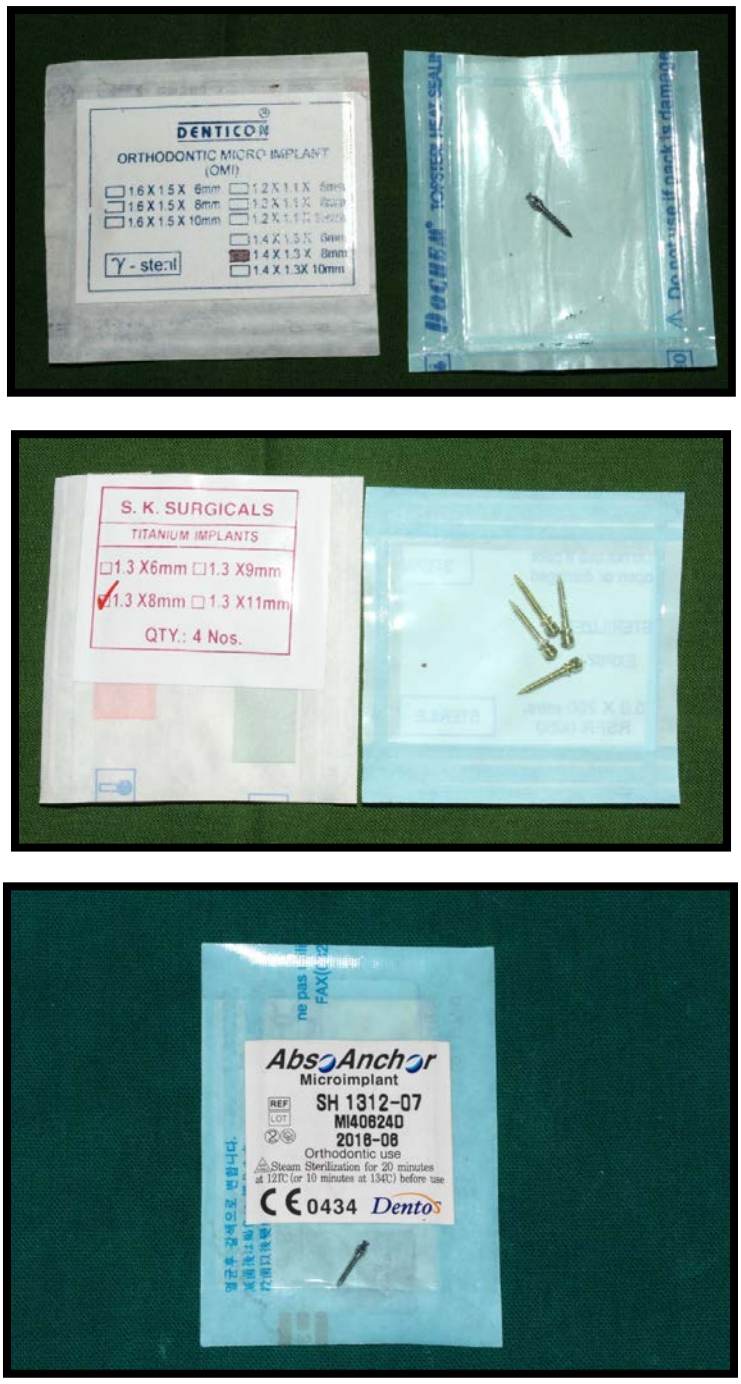


Figure 1: Mini Implants used in the study to evaluate the cytotoxicity

Figure 2: Materials used for pH solution

Figure 3: Prepared solution is checked for exact pH with pH meter

Figure 4: Implants placed in pH solution

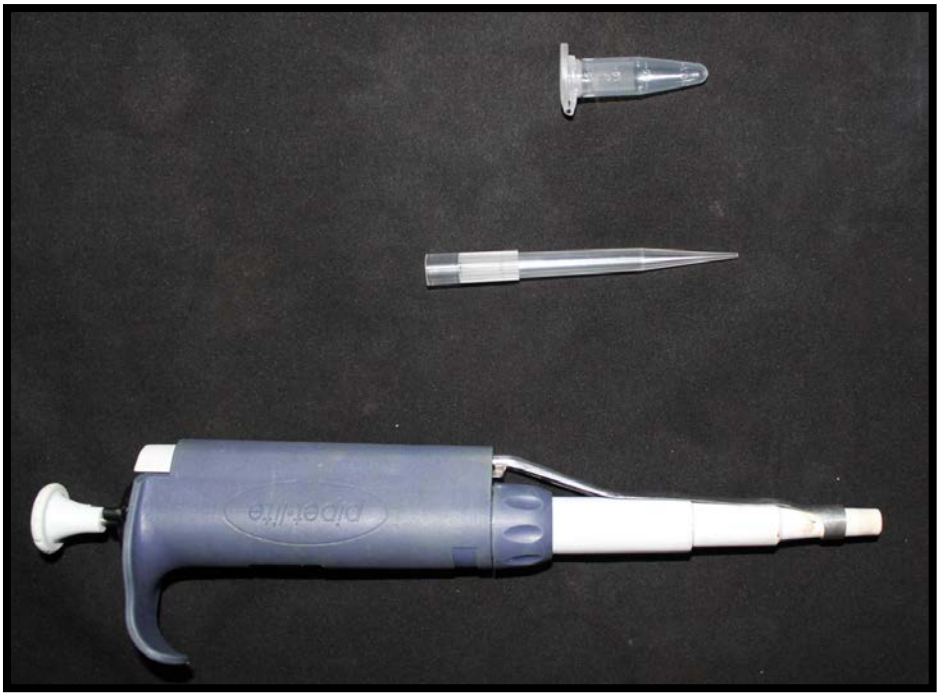
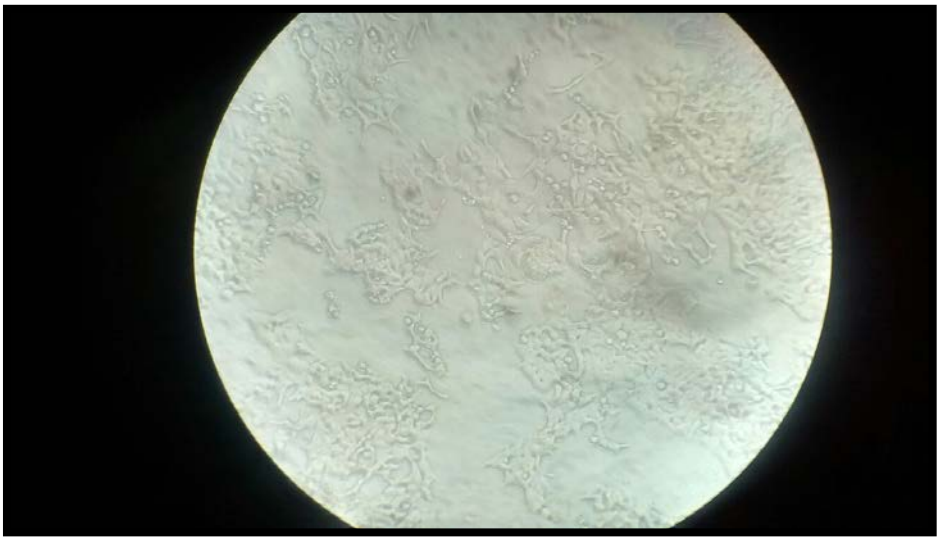


Figure 5: Micro pipette used to collect eluates

Figure 6: Collected eluates at day 15, day 30, day 60

Figure 7: CO_2 incubator with cell lines and growth media



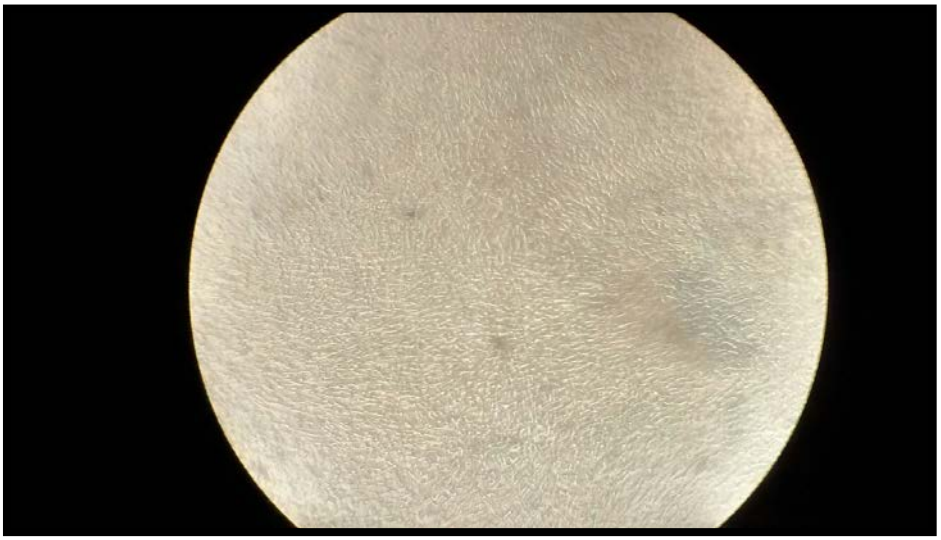
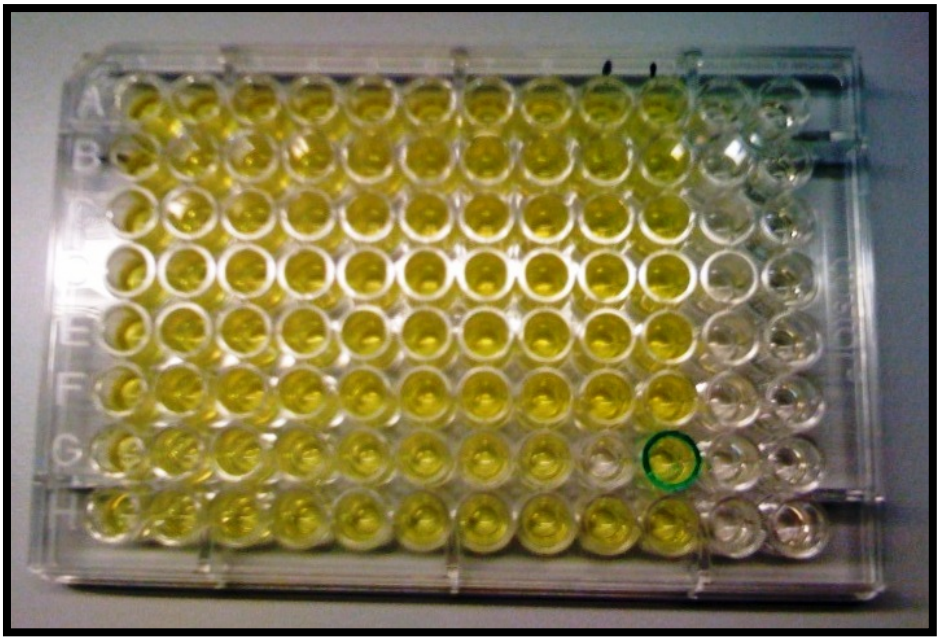


Figure 8: Matured U2OS and HGF Cells



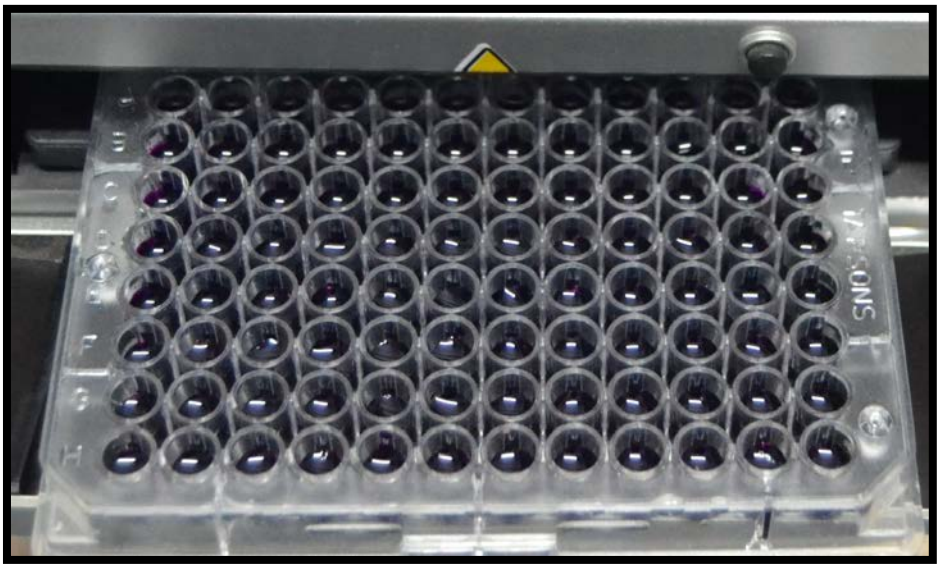


Figure 9: Well plates with MTT dye and DMSO

Figure 10: Well plates placed in Elisa Reader

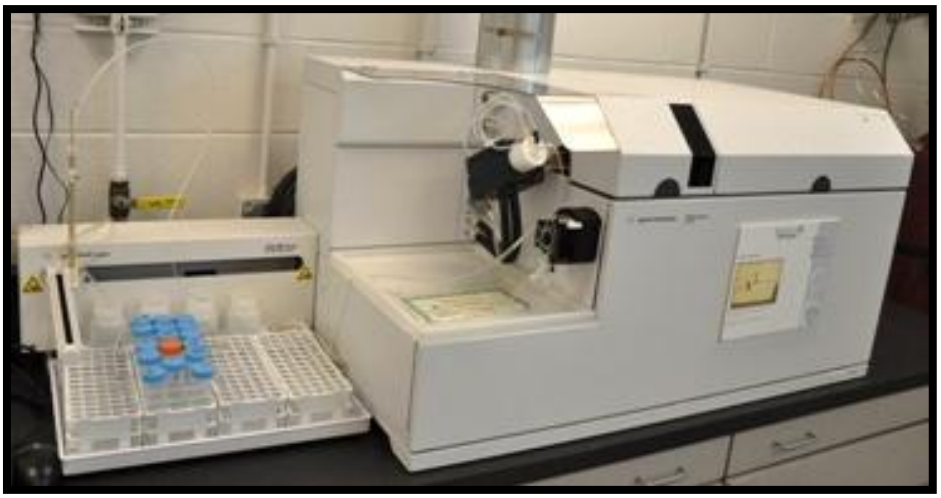


Figure 11: Inductive Coupled Plasma – Mass Spectrometry

(Meter Agilent 7700, ICP-MS System Mass Hunter 4.1)

Results

RESULTS

Effect of pH on Cytotoxicity of SK Mini Implants with Human Osteogenic Sarcoma Cell (U2OS)

Non parametric independent t test was done to compare the mean values. On day 15, When comparing the cell death between the pH 4 and pH 7 in SK Implants with U2OS, it was found that there was more cell death in pH 4 (**8.95%)** than in pH 7 (**7.74%)**. However the difference was not statistically significant for the day 15, (p value >0.05). When comparing the cell death between pH 4 and pH 7 on day 30, it was found that there was more cell death in pH 4 (**19.86%)** than in pH 7 (**12.75%)**. The results were statistically significant (p valve <0.05). On day 60, when comparing cell death between pH 4 and pH 7, it was also found that there was more cell death in pH 4 (**45.32%**) than in pH 7 (**23.77%)** and the difference was statistically significant (p valve <0.05) (Table no.1) (Graph no.1).

Further analysis was done with one way post hoc and Tukey's HSD for multiple comparisons. When comparing the cell death in SK Implant with control in pH 4 there was significantly more cytotoxic effect with SK implants with a mean difference of **4.35%** on day 15, **15.56%** on day 30 and **40.72%** on day 60. (Table no.2).

When comparing the cell death in SK Implant with control in pH 7 there was statistically significant cytotoxic effect with a mean difference of **5.29%** on day 15, **10.30%** on day 30 and **21.32%** on day 60. (Table no.3)

Effect of pH on Cytotoxicity of SK Mini Implants with Human Gingival Fibroblast (HGF)

Non parametric independent t test was done to compare the mean values. On day 15, When comparing the cell death between the pH 4 and pH 7 with SK Implant on HGF, it was found that there was more cell death in pH 4 (**10.56%**) than in pH 7 (**11.94%**). However the differences were not statistically significant (p value >0.05). On day 30, when comparing the cell death between pH 4 and pH 7, it was found that there was more cell death in pH 4 (**24.82%**) than in pH 7 (**14.73%**). The difference were statistically significant (p valve <0.05). On day 60, when comparing cell death between pH 4 and pH 7, it was found that there was more cell death in pH 4 (**34.68%**) than in pH 7 (**22.81%**). The difference was statistically significant (p valve <0.05) (Table no. 4) (Graph no. 1).

Further analysis was done with one way post hoc and Tukey's HSD for multiple comparisons. When comparing the SK Implant with control in pH 4 there was statistically significant Cytotoxic effect with a mean difference of **4.33%** on day 15, **18.59%** on day 30 and **28.45%** on day 60 (Table no. 5). When comparing the SK Implant with control in pH 7 there was statistically

significant Cytotoxic effect with a mean difference of **4.10%** on day 15, **10.61%** on day 30 and **18.69%** on day 60. (Table no.6)

Effect of pH on Cytotoxicity of Denticon Mini Implants with Human Osteogenic Sarcoma Cell (U2OS)

Non parametric independent t test was done to compare the mean values. On day 15, When comparing the cell death between the pH 4 and pH 7 for Denticon implant with U2OS, it was found that there was more cell death in pH 4 (**12.36%**) than in pH 7 (**13.53%**). However the difference was not statistically significant (p value >0.05). On day 30, when comparing the cell death between pH 4 and pH 7, it was found that there was more cell death in pH 4 (**26.10%**) than in pH 7 (**13.91%**). The difference was statistically significant (p valve<0.05). On day 60, when comparing cell death between pH 4 and pH 7, it was found that there was more cell death in pH 4 (**29.81%**) than in pH 7 (**19.31%**). The difference was statistically significant (p valve <0.05) (Table no. 7) (Graph no.1).

Further analysis was done with, one way post hoc and Tukey's HSD for multiple comparisons .When comparing the Denticon Implant with control in pH 4 there was statistically significant Cytotoxic effect with a mean difference of **8.96%** on day 15, **21.50%** on day 30 and **25.21%** on day 60. (Table 8)

When comparing the Denticon Implant with control in pH 7 there was statistically significant Cytotoxic effect with a mean difference of **10.15%** on day 15, **11.46%** on day 30 and **16.86%** on day 60. (Table no.9)

Effect of pH on Cytotoxicity of Denticon Mini Implants with Human Gingival Fibroblast (HGF)

Non parametric independent t test was done to compare the mean values. On day 15, When comparing the cell death between the pH 4 and pH 7 for Denticon implant with HGF, it was found that there was more cell death in pH 4 (**12.08%**) than in pH 7 (**11.94%**). The difference was not statistically significant (p valve>0.05). On day 30, when comparing the cell death between pH 4 and pH 7, it was found that there was more cell death in pH 4 (**25.29%**) than in pH 7 (**14.37%**). The difference was statistically significant (p valve <0.05). On day 60, when comparing cell death between pH 4 and pH 7, it was found that there was more cell death in pH 4 (**26.15%**) than in pH 7 (**17.14%**). The difference was statistically significant (p valve <0.05) (Table no.10) (Graph no.1).

Further analysis was done with, one way post hoc and Tukey's HSD for multiple comparisons. When comparing the Denticon Implant with control in pH 4 there was statistically significant Cytotoxicity effect with a mean difference of **5.85%** on day 15, **19.06%** on day 30 and **19.92%** on day 60. (Table no.11)**.** When comparing the Denticon Implant with control in pH 7

there was statistically significant Cytotoxicity effect with a mean difference of **7.83%** on day 15, **10.25%** on day 30 and **13.02%** on day 60. (Table no.12)

Effect of pH on Cytotoxicity of Dentos Mini Implants with Human Osteogenic Sarcoma Cell (U2OS)

Non parametric independent t test was done to compare the mean values. On day 15, When comparing the cell death between the pH 4 and pH 7 for Dentos implant with U2OS, it was found that there was more cell death in pH 4 (**8.12%**) than in pH 7 (**7.13%**). However the difference was not statistically significant (p value >0.05). On day 30, when comparing the cell death between pH 4 and pH 7, it was found that there was more cell death in pH 4 (**14.66%**) than in pH 7 (**11.39%**).The difference was statistically significant (p valve <0.05). On day 60, when comparing cell death between pH 4 and pH 7, it was found that there was more cell death in pH 4 (**46.87%**) than in pH 7 (**36.78%**). The difference was statistically significant (p valve <0.05)

Further analysis was done with, one way post hoc and Tukey's HSD for multiple comparisons. When comparing the Dentos Implant with control in pH 4 there was statistically significant Cytotoxicity effect with a mean difference of **3.52%** on day 15, **10.06%** on day 30 and **42.27%** on day 60. (Table no.14). When comparing the Dentos Implant with control in pH 7 there was statistically significant cytotoxicity effect with a mean difference of **4.68%** on day 15**, 8.91%** on day 30 and **34.33%** on day 60. (Table no. 15)

Effect of pH on Cytotoxicity of Dentos Mini Implants with Human Gingival Fibroblast (HGF)

Non parametric independent t test was done to compare the mean values. On day 15, When comparing the cell death between the pH 4 and pH 7 for Dentos implant with HGF, it was found that there was more cell death in pH 4 (**7.56%**) than in pH 7 (**7.98%**). However the difference was not statistically significant (p value >0.05). On day 30, when comparing the cell death between pH 4 and pH 7, it was found that there was more cell death in pH 4 (**19.85%**) than in pH 7 (**12.48%**). The difference was statistically significant (p valve <0.05). On day 60, when comparing cell death between pH 4 and pH 7, it was found that there was more cell death in pH 4 (**47.19%**) than in pH 7 (**23.54%**). The difference was statistically significant (p valve <0.05) (Table no.16) (Graph no.1)

Further analysis was done with, one way post hoc and Tukey's HSD for multiple comparisons. When comparing the Dentos Implant with control in pH 4 there was statistically significant Cytotoxicity effect with a mean difference of **1.33%** on day 15, **13.62%** on day 30 and **40.96%** on day 60. (Table no.17). When comparing the Dentos Implant with control in pH 7 there was statistically significant Cytotoxicity effect with a mean difference of **3.86%** on day 15, **8.36%** on day 30 and **19.42%** on day 60. (Table no.18) (Graph no. 1).

II. Comparison of cell death between SK, Denticon and Dentos at day 15, day 30 and day 60.

II. A) Human Osteogenic Sarcoma Cell at pH 4

One Way ANOVA was done to compare the mean values. When comparing the results between the three groups SK, Denticon and Dentos implants with U20S at pH 4 with the control, On day 15, Denticon Implants revealed highest cell death (**13.53%**) followed by Dentos Implant (**8.95%**) and the least cell death was recorded in SK Implants (**8.12%**). On day 30, Denticon Implants revealed a highest cell death (**26.10%**), followed by SK Implants (**19.86%**) and the least cell death was recorded in Dentos Implants (**14.66%**). On Day 60, Dentos Implants revealed a highest cell death (**46.87%**), followed by SK Implants (**45.32%**) and the least cell death was recorded in Denticon Implants (**29.81%**). All the above results were statistically significant (Table no.19, 21) (Graph no. 2)

II.B) Human Osteogenic Sarcoma Cell at pH 7

When comparing the results between the three groups SK, Denticon and Dentos Implants with U20S cells at pH 7 it was found that, On day 15, Denticon Implant revealed highest cell death (**12.56%**), followed by SK Implants (**7.74%**) and the least cell death was recorded in Dentos Implant (**7.13%**). On day 30, Denticon Implant revealed a highest cell death (**13.91%**),

followed by SK Implant (**12.75%**) and the least cell death was recorded in Dentos Implants (**11.39%**). However on Day 60, Dentos Implant revealed a highest cell death (**36.78%**), followed by SK Implants (**23.77%**) and the least cell death was recorded in Denticon Implant (**19.31%**) and all the above results were statistically significant (Table no.20, 21) (Graph no.2).

II. C) Human Gingival Fibroblast at pH 4

When comparing the results between the three groups SK, Denticon and Dentos Implants with HGF at pH 4 with the control, on day 15, Denticon Implants revealed highest cell death (**12.08%**), followed by SK Implants (**10.56%**) and the least cell death was recorded in Dentos Implants (**7.56%**). On day 30, Denticon Implants revealed highest cell death (**25.29%**), followed by SK Implants (**24.82%**) and the least cell death recorded in Dentos Implants (**19.85%**). On Day 60, Dentos Implants revealed a highest cell death (**47.19 %**), followed by SK Implants (**34.68%**) and the least cell death recorded in Denticon Implants (**26.15%**). All the above results were statistically significant (Table no. 22, 24) (Graph no 3).

II. D) Human Gingival Fibroblast at pH 7

When comparing the results between the three groups SK, Denticon and Dentos Implants with HGF at pH 7 with the control, on day 15, Denticon revealed highest cell death (**11.94%**), followed by SK Implants (**8.22%**) and the least cell death was recorded in Dentos Implants (**7.98%**). On day 30, SK

Implants revealed a highest cell death (**14.73%**), followed by Denticon Implants (**14.37%**) and the least cell death was recorded in Dentos Implants (**12.48%**). On Day 60, Dentos Implants revealed a highest cell death (**23.54%**), followed by SK Implants (**22.81%**) and the least cell death was recorded in Denticon Implants (**17.14%**). All the above results were statistically significant (Table no.23, 24) (Graph no. 3)

ICP-MS Results for metal ion release at pH 4:

Non parametric one way ANOVA was done to compare the mean values of metal ions released. It was observed that Aluminum release on day 15 was higher in Denticon Implants **(0.81ppm)** followed by SK Implants **(0.38 ppm)** and least aluminum release was in Dentos **(0.26 ppm**). Similarly, Iron release was recorded highest in Denticon **(2.50 ppm)**, followed by SK Implants **(0.67 ppm)** and least Iron release was in Dentos **(0.30 ppm).** Also, when comparing the total amount of metal ion release, Denticon showed highest metal ion release **(3.37 ppm)** followed by SK Implant **(1.51 ppm)** and least amount of metal ion release was observed in Dentos **(0.82 ppm).** On day 30 Aluminum release was highest in Denticon Implants **(1.04 ppm)** followed by SK Implants **(0.89 ppm)** and least aluminum release in Dentos **(0.44 ppm).** Similarly, Iron release was recorded highest in Denticon **(3.32 ppm),** followed by SK Implants **(0.98 ppm)** and least Iron release was observed in Dentos **(0.44 ppm).** Also, when comparing the total amount of metal ion release, highest metal ion release was observed in Denticon **(4.45 ppm)** followed by

SK Implant **(2.59 ppm)** and least amount of metal ion release was observed in Dentos **(1.96 ppm)**

On day 60, Aluminum release was highest in Dentos Implants **(4.90ppm)** followed by SK Implants **(4.73 ppm)** and least aluminum release was in Denticon **(4.20 ppm**). Similarly, Iron release was recorded highest in Dentos **(3.80 ppm**), followed by SK Implants **(3.40 ppm)** and least Iron release were observed in Denticon Implants **(3.36 ppm).** Also, when comparing the total amount of metal ion release, highest metal ion release was observed in Dentos **(9.30 ppm)** followed by SK Implant **(8.52 ppm)** and least amount of metal ion release were observed in Denticon Implants **(7.88 ppm).** However, the results were not statistically significant (Table no. 25) (Graph no 4).

ICP-MS Results in pH7:

Non parametric one way ANOVA was done to compare the mean values of metal ions. It was observed that, on day 15, Aluminum release was highest in Denticon Implants **(0.31)** followed by Dentos Implants **(0.09 ppm)** and least aluminum release was in SK Implants **(0.08 ppm).** Similarly, Iron release was recorded highest in Denticon Implants **(0.31 ppm),** followed by Dentos Implants **(0.24 ppm)** and least Iron release were in SK Implants **(0.19 ppm).** Also, when comparing the total amount of metal ion release, highest was observed in Denticon **(0.78 ppm)** followed by SK Implant

(0.72 ppm) and least amount of metal ion release were observed in Dentos **(0.69 ppm).**

On day 30, Aluminum release was highest in Denticon Implants **(0.26 ppm)** followed by SK Implants **(0.16 ppm)** and least aluminum release was in Dentos Implants **(0.10 ppm**). Similarly, Iron release was recorded highest in Dentos Implants **(0.68 ppm),** followed by Denticon Implants **(0.40 ppm**) and least Iron release were observed in SK Implants **(0.28 ppm).** But when comparing the total amount of metal ion release, highest metal ion release was observed in SK Implants **(1.13 ppm)** followed by Denticon Implant **(1.08 ppm)** and least amount of metal ion release were observed in Dentos implants **(0.82 ppm).**

It was observed, on day 60, Aluminum release was highest in SK Implants **(2.33 ppm)** followed by Denticon Implants **(1.14 ppm)** and least aluminum release was observed in Dentos Implants **(1.02 ppm).** Similarly, Iron release was recorded highest in Dentos **(5.47 ppm**), followed by SK Implants **(2.74 ppm)** and least Iron release were observed in Denticon **(1.61 ppm).** Also, when comparing the total amount of metal ion release, highest metal ion release was observed in Dentos Implants **(7.06 ppm),** followed by SK Implant **(5.95 ppm)** and least amount of metal ion release were observed in Denticon **(3.58 ppm).** However, the results were not statistically significant. (Table no.26) (Graph no. 5)

Tables and Graphs

TABLE 1: Cytotoxicity of SK Implants with U2OS on day 15, 30 and 60 at pH4 and pH7

Day	pH	Mean	Std. Deviation	P vale
15	pH4	8.95	0.40	0.421
	pH7	7.74	0.60	0.414
30	pH4	19.86	0.79	0.000
	pH7	12.75	0.62	0.000
60	pH4	.45.32	4.81	0.005
	pH7	23.77	4.17	0.004
CONTROL	pH4	4.60	0.00	0.000
	pH7	2.45	0.00	0.000

Table 2: Comparison of SK Implants Cytotoxicity with control on U2OS at pH4

Day	GROUP	Mean	Mean diff	Std. deviation	P value
15	SK	8.95	4.35	0.40	0.421
	CONTROL	4.60		0.00	0.000
30	SK	19.86	15.26	0.79	0.000
	CONTROL	4.60		0.00	0.000
60	SK	45.32	40.72	4.81	0.005
	CONTROL	4.60		0.00	0.000

TABLE 3: Comparison of SK Implants Cytotoxicity with Control on U2OS at pH7

Day	GROUP	Mean	Mean Diff	Std. Deviation	P Value
15	SK	7.74	5.29	0.60	0.414
	CONTROL	2.45		0.00	0.000
30	SK	12.75	10.30	0.62	0.000
	CONTROL	2.45		0.00	0.000
60	SK	23.77	21.32	4.17	0.004
	CONTROL	2.45		0.00	0.000

TABLE 4: Cytotoxicity of SK Implants with HGF on day 15, 30 and 60 at pH4 and pH7

Day	Ph	Mean	Std. Deviation	P Value
15	pH4	10.56	1.21	0.912
	pH7	11.94	1.68	0.911
30	pH4	24.82	1.99	0.003
	pH7	14.73	1.41	0.002
60	pH4	34.68	2.48	0.004
	pH7	22.81	1.81	0.003
Control	pH4	6.23	0.00	0.000
	pH7	4.12	0.00	0.000

TABLE 5: Comparison of SK Implants Cytotoxicity with control on HGF at pH4

Day	GROUP	Mean	Mean diff	Std. deviation	P value
15	SK	10.56	4.33	1.21	0.912
	CONTROL	6.23		0.00	0.000
30	SK	24.82	18.59	1.99	0.003
	CONTROL	6.23		0.00	0.000
60	SK	34.68	28.45	2.48	0.004
	CONTROL	6.23		0.00	0.000

TABLE 6: Comparison of SK Implants Cytotoxicity with control on HGF at pH7

Day	GROUP	Mean	Mean diff	Std. deviation	P value
15	SK	8.22	4.10	1.68	0.911
	CONTROL	4.12		0.00	0.000
30	SK	14.73	10.61	1.41	0.002
	CONTROL	4.12		0.00	0.000
60	SK	22.81	18.69	1.81	0.003
	CONTROL	4.12		0.00	0.000

TABLE 7: Cytotoxicity of Denticon Implants with U2OS on day 15, 30 and 60 at pH4 and pH7

Day	pH	Mean	Std. Deviation	P Vale
15	pH4	12.36	1.12	0.214
	pH7	13.53	0.69	0.200
30	pH4	26.10	0.24	0.012
	pH7	13.91	2.36	0.0001
60	pH4	29.81	2.72	0.016
	pH7	19.31	0.79	0.003
CONTROL	pH4	4.60	0.00	0.000
	pH7	2.45	0.00	0.000

TABLE 8: Comparison of Denticon Implants Cytotoxicity with control on U2OS at pH4

Day	GROUP	Mean	Mean diff	Std. deviation	P value
15	DENTICON	13.56	8.96	1.12	0.214
	CONTROL	4.60		0.00	0.000
30	DENTICON	26.10	21.50	0.24	0.012
	CONTROL	4.60		0.00	0.000
60	DENTICON	29.81	25.21	2.72	0.016
	CONTROL	4.60		0.00	0.000

TABLE 9: Comparison of Denticon Implants Cytotoxicity with control on U2OS at pH7

Day	GROUP	Mean	Mean diff	Std. deviation	P value
15	DENTICON	12.56	10.15	0.69	0.200
	CONTROL	2.45		0.00	0.000
30	DENTICON	13.91	11.46	2.36	0.000
	CONTROL	2.45		0.00	0.000
60	DENTICON	19.31	16.86	0.79	0.003
	CONTROL	2.45		0.00	0.000

TABLE 10: Cytotoxicity of Denticon Implants with HGF on day 15, 30 and 60 at pH4 and pH7

Day	pH	Mean	Std. Deviation	P Vale
15	pH4	12.08	0.97	0.43
	pH7	11.94	0.99	0.36
30	pH4	25.29	0.49	0.008
	pH7	14.37	2.05	0.001
60	pH4	26.15	1.38	0.014
	pH7	17.14	2.69	0.007
CONTROL	pH4	6.23	0.00	0.000
	pH7	4.12	0.00	0.000

TABLE 11: Comparison of Denticon Implants Cytotoxicity with control on HGF at pH4

Day	GROUP	Mean	Mean diff	Std. deviation	P value
15	DENTICON	12.08	5.85	0.97	0.43
	CONTROL	6.23		0.00	0.000
30	DENTICON	25.29	19.06	0.49	0.008
	CONTROL	6.23		0.00	0.000
60	DENTICON	26.15	19.92	1.38	0.014
	CONTROL	6.23		0.00	0.000

TABLE 12: Comparison of Denticon Implants Cytotoxicity with control on HGF at pH7

Day	GROUP	Mean	Mean diff	Std. deviation	P value
15	DENTICON	11.95	7.83	0.99	0.36
	CONTROL	4.12		0.00	0.000
30	DENTICON	14.37	10.25	2.05	0.001
	CONTROL	4.12		0.00	0.000
60	DENTICON	17.14	13.02	2.69	0.007
	CONTROL	4.12		0.00	0.000

TABLE 13: Cytotoxicity of Dentos Implants with U2OS on day 15, 30 and 60 at pH4 and pH7

Day	pH	Mean	Std. Deviation	P vale
15	pH4	8.12	0.57	0.353
	pH7	7.13	2.64	0.306
30	pH4	14.66	1.08	0.012
	pH7	11.39	1.33	0.025
60	pH4	46.87	0.98	0.045
	pH7	36.78	4.11	0.014
CONTROL	pH4	4.60	0.00	0.000
	pH7	2.45	0.00	0.000

TABLE 14: Comparison of Dentos Implants with control on U2OS at pH4

Day	GROUP	Mean	Mean diff	Std. deviation	P value
15	DENTOS	8.12	3.52	0.57	0.353
	CONTROL	4.60		0.00	0.000
30	DENTOS	14.66	10.06	1.08	0.012
	CONTROL	4.60		0.00	0.000
60	DENTOS	46.87	42.27	0.98	0.045
	CONTROL	4.60		0.00	0.000

TABLE 15: Comparison of Dentos Implants Cytotoxicity with control on U2OS at pH7

Day	GROUP	Mean	Mean diff	Std. deviation	P value
15	DENTOS	7.13	4.68	2.64	0.306
	CONTROL	2.45		0.00	0.000
30	DENTOS	11.39	8.91	1.33	0.025
	CONTROL	2.45		0.00	0.000
60	DENTOS	36.78	34.33	4.11	0.014
	CONTROL	2.45		0.00	0.000

TABLE 16: Cytotoxicity of Dentos Implants with HGF on day 15, 30 and 60 at pH4 and pH7

Day	pH	Mean	Std. Deviation	P vale
15	pH4	7.56	1.42	0.778
	pH7	7.98	1.26	0.778
30	pH4	19.85	1.93	0.009
	pH7	12.48	1.14	0.005
60	pH4	47.19	1.94	0.000
	pH7	23.54	1.19	0.000
CONTROL	pH4	6.23	0.00	0.000
	pH7	4.12	0.00	0.000

TABLE 17: Comparison of Dentos with control on HGF at pH4

Day	Group	Mean	Mean Diff	Std. Deviation	P Value
15	DENTOS	7.56	1.33	1.42	0.778
	CONTROL	6.23		0.00	0.000
30	DENTOS	19.85	13.62	1.93	0.009
	CONTROL	6.23		0.00	0.000
60	DENTOS	47.19	40.96	1.94	0.000
	CONTROL	6.23		0.00	0.000

TABLE 18: Comparison of Dentos Cytotoxicity with control on HGF at pH7

Day	Group	Mean	Mean Diff	Std. Deviation	P Value
15	DENTOS	7.98	3.86	1.26	0.778
	CONTROL	4.12		0.00	0.000
30	DENTOS	12.48	8.36	1.14	0.005
	CONTROL	4.12		0.00	0.000
60	DENTOS	23.54	19.42	1.19	0.000
	CONTROL	4.12		0.00	0.000

Table 19: Cell Cytotoxicity on U2OS in pH4 at day 15, 30 and 60

Day		Mean	Std. Deviation	Mean Diff	P Value
15	SK	8.95	0.40	3.52	0.00
	DENTICON	13.53	0.69	7.76	0.00
	DENTOS	8.12	0.57	4.35	0.00
30	SK	19.86	0.79	15.25	0.00
	DENTICON	26.10	0.24	21.50	0.00
	DENTOS	14.66	1.08	10.60	0.00
60	SK	45.32	4.81	40.71	0.00
	DENTICON	29.81	2.72	25.21	0.00
	DENTOS	46.87	0.98	42.27	0.00

Table 20: Cell Cytotoxicity on U2OS in pH7 at day 15, 30 and 60

Day	GROUP	Mean	Std. Deviation	Mean Diff	P Value
15	SK	7.74	0.60	-5.28	0.01
	DENTICON	12.56	1.12	-11.08	0.00
	DENTOS	7.13	2.64	-4.67	0.01
30	SK	12.75	0.62	-10.29	0.00
	DENTICON	13.91	2.36	-11.46	0.00
	DENTOS	11.39	1.33	8.14	0.00
60	SK	23.77	4.17	-22.32	0.00
	DENTICON	19.31	0.79	-16.86	0.01
	DENTOS	36.78	4.11	-34.33	0.00

Table 21: Cytotoxicity of Implants in descending order for U2OS at pH4 and pH7

Day	pH	1st in Order	2nd in Order	3rd in Order
15	pH4	DENTICON (13.53%)	SK (8.95%)	DENTOS (8.12%)
	pH7	DENTICON (12.56%)	SK (7.74%)	DENTOS (7.13%)
30	pH4	DENTICON (26.10%)	SK (19.86%)	DENTOS (14.66%)
	pH7	DENTICON (13.91%)	SK (12.75%)	DENTOS (11.39%)
60	pH4	DENTOS (46.87%)	SK (45.32%)	DENTICON (29.81%)
	pH7	DENTOS (36.75%)	SK (23.77%)	DENTICON (19.31%)

Table 22: Cell Cytotoxicity on HGF at pH4 at day 15, 30 and 60

Day		Mean	Std Deviation	Mean Diff	P Value
15	SK	10.56	1.21	5.84	0.00
	DENTICON	12.08	0.97	4.33	0.03
	DENTOS	7.56	1.26	1.4	0.36
30	SK	24.82	1.99	18.59	0.00
	DENTICON	25.29	0.49	19.56	0.00
	DENTOS	19.85	1.93	13.61	0.00
60	SK	34.68	2.48	28.44	0.00
	DENTICON	26.15	1.38	19.92	0.00
	DENTOS	47.19	1.94	40.95	0.00

Table 23: Cell cytotoxicity in HGF at pH7 at day 15, 30 and 60

Day		Mean	Std. Deviation	Mean Diff	P Value
15	SK	8.22	1.68	4.09	0.00
	DENTICON	11.94	0.99	7.81	0.01
	DENTOS	7.98	1.42	3.86	0.01
30	SK	14.73	1.41	10.61	0.00
	DENTICON	14.37	2.05	10.25	0.00
	DENTOS	12.48	1.14	8.36	0.00
60	SK	22.81	1.81	18.69	0.00
	DENTICON	17.14	2.69	13.01	0.00
	DENTOS	23.54	1.19	19.42	0.00

TABLE 24: Cytotoxicity of Implants in descending order for HGF at pH4 and pH7

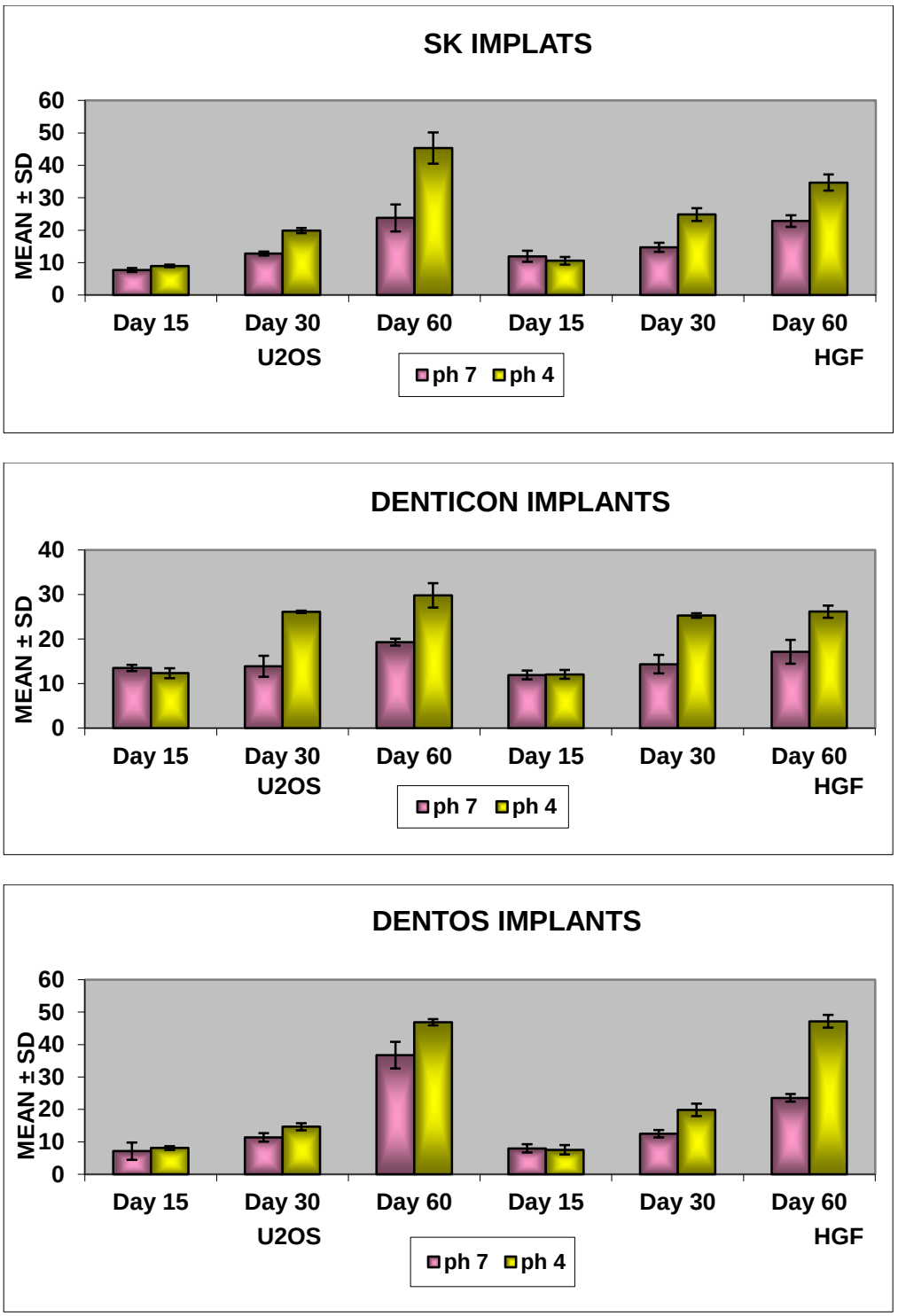
Day	pH	1st in Order	2nd in Order	3rd in Order
15	pH4	DENTICON (12.08%)	SK (10.56%)	DENTOS (7.56%)
	pH7	DENTICON (11.94%)	SK (8.22%)	DENTOS (7.98%)
30	pH4	DENTICON (25.29%)	SK (24.82%)	DENTOS (19.85%)
	pH7	SK (14.73%)	DENTICON (14.37%)	DENTOS (12.48%)
60	pH4	DENTOS (47.19%)	SK (34.68%)	DENTICON (26.18%)
	pH7	DENTOS (23.54%)	SK (22.81%)	DENTICON (17.14%)

Table 25: Table representing the ICP-MS values at pH4

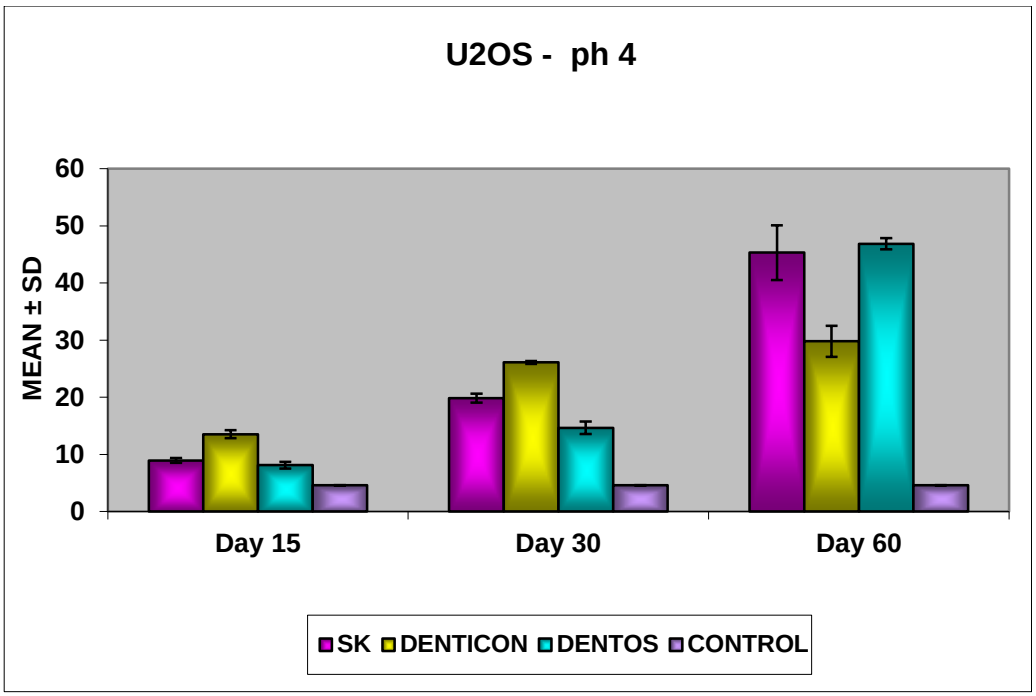
DAY	IMPLANT	Al(ppm)	Fe(ppm)	V(ppm)	Ti(ppm)	Total	P value
15	SK	0.38	0.67	0.01	0.45	1.51	0.28
	DENTICON	0.81	2.50	0.02	0.04	3.37	
	DENTOS	0.26	0.30	0.01	0.25	0.82	
30	SK	0.89	0.98	0.16	0.56	2.59	0.11
	DENTICON	1.04	3.32	0.04	0.05	4.45	
	DENTOS	0.44	0.35	0.13	1.04	1.96	
60	SK	4.73	3.40	0.10	0.29	8.52	0.12
	DENTICON	4.20	3.36	0.07	0.25	7.88	
	DENTOS	4.90	3.80	0.20	0.40	9.30	

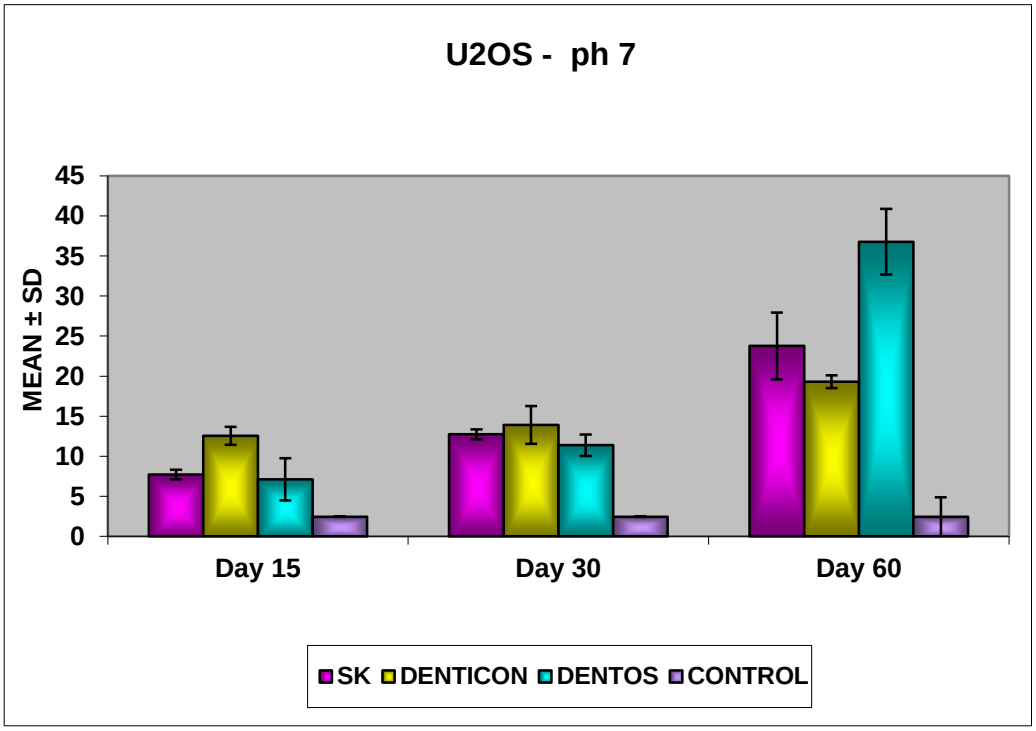
Table 26: Table representing the ICP-MS values at pH7

DAY	IMPLANT	Al(ppm)	Fe(ppm)	V(ppm)	Ti(ppm)	Total	P value
15	SK	0.08	0.19	0.10	0.44	0.72	0.25
	DENTICON	0.31	0.31	0.13	0.03	0.78	
	DENTOS	0.09	0.24	0.01	0.35	0.69	
30	SK	0.16	0.28	0.14	0.55	1.13	0.23
	DENTICON	0.26	0.40	0.13	0.29	1.08	
	DENTOS	0.10	0.68	0.01	0.03	0.82	
60	SK	2.33	2.74	0.64	0.24	5.95	0.21
	DENTICON	1.14	1.61	0.70	0.13	3.58	
	DENTOS	1.02	5.47	0.35	0.22	7.06	

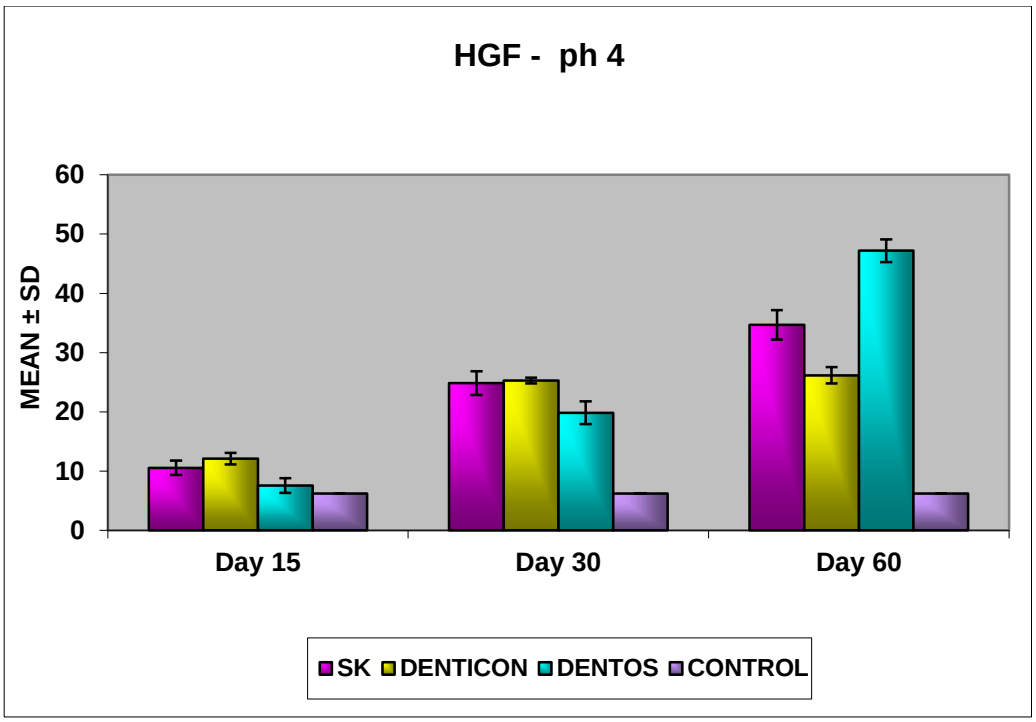


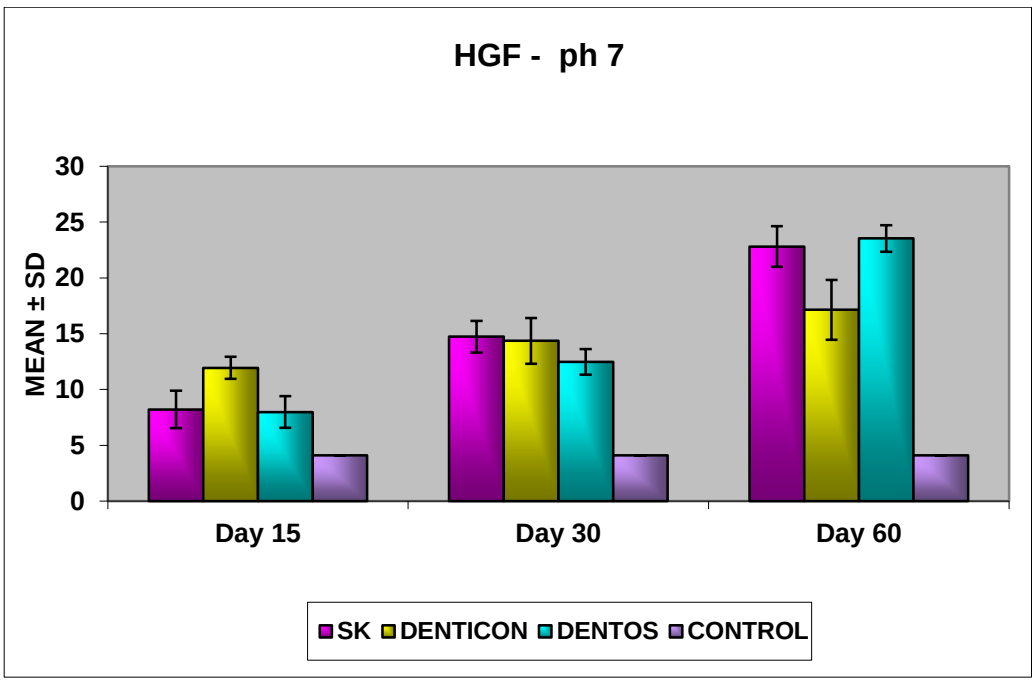
Graph 1: Bar diagram depicting cytotoxicity of different company of implants





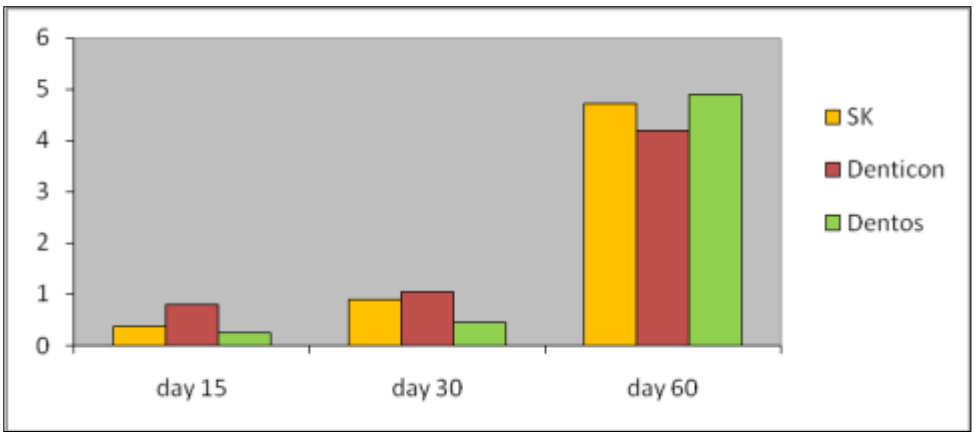
Graph 2: Citotoxicity of selected implants at pH4 and pH7 on U2OS



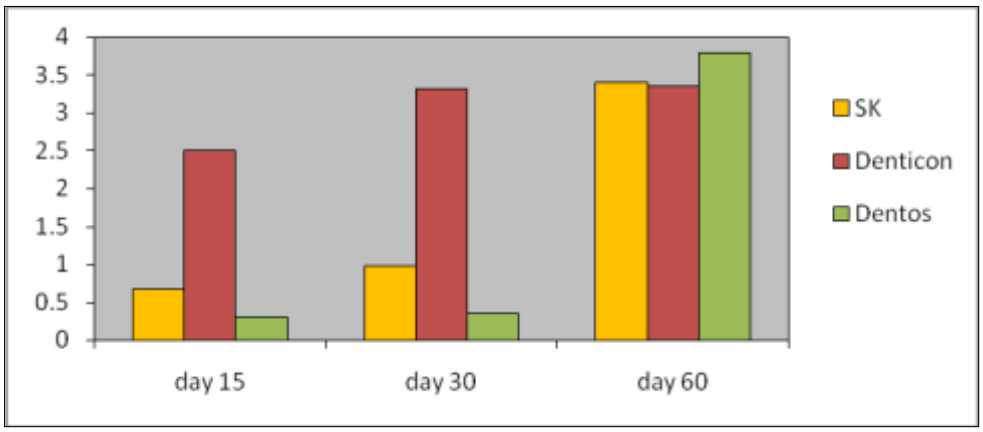


Graph 3: Citotoxicity of selected implants at pH4 and pH7 on HGF

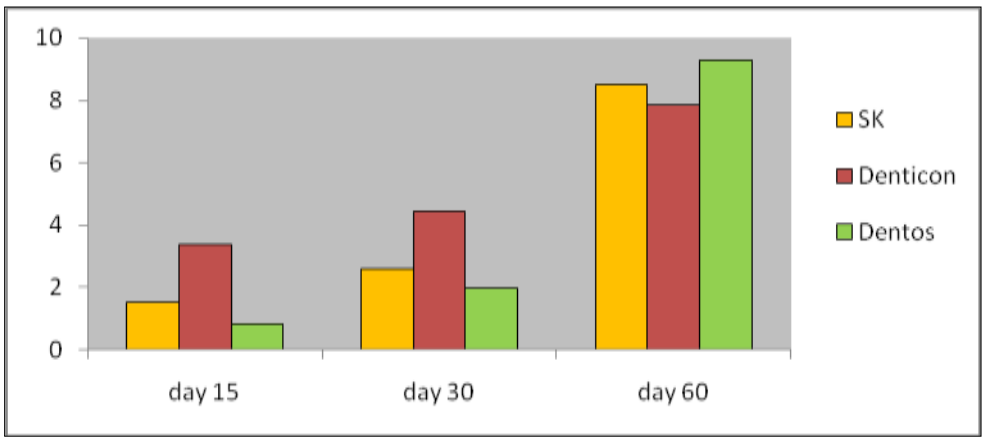
Aluminium ion release in pH 4



Iron ion release in pH 4

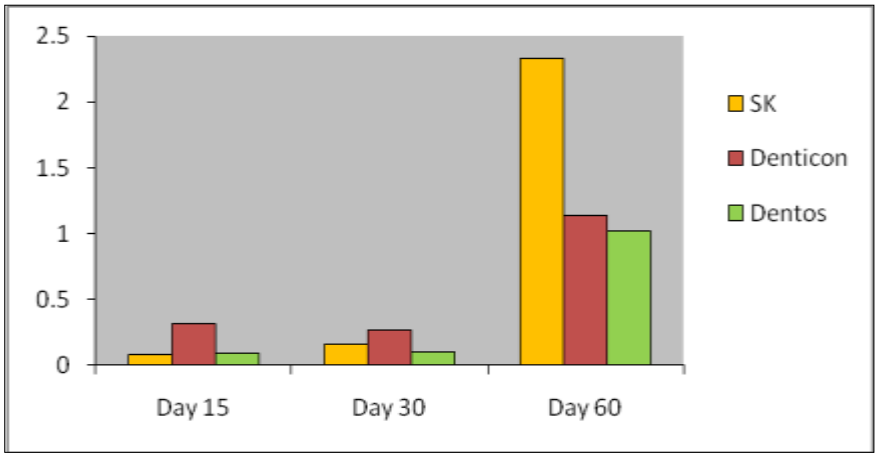


Total ions release in pH 4

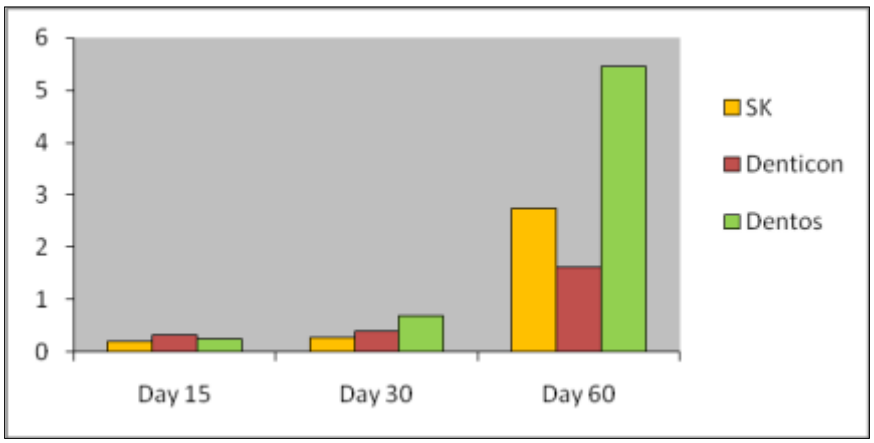


Graph 4: Bar diagram depicting Metal ions released in pH 4

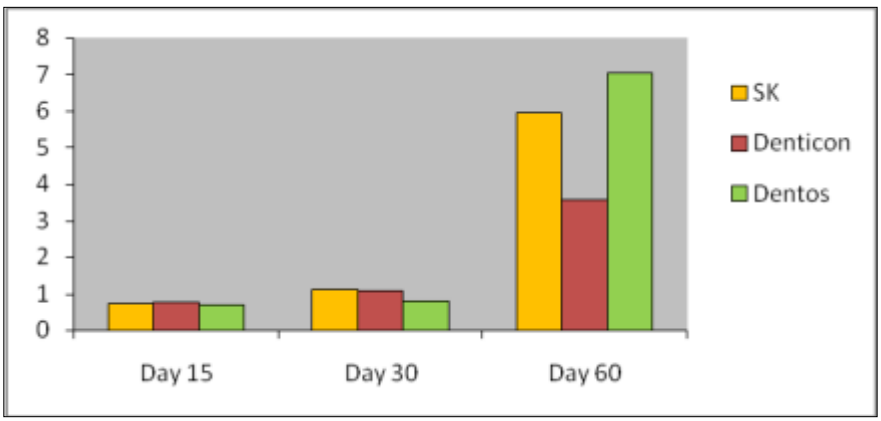
Aluminium ion release in pH 7



Iron ion release in pH 7



Total ions release in pH 7



Graph 5: Bar diagram depicting Metal ions released in pH 7

Discussion

DISCUSSION

The selection and production of materials that are biocompatible have been one of the greatest challenges in the area of healthcare. Toxic, inflammatory, allergic or mutagenic reactions are the possible biological responses to these materials and cytotoxicity is one of the main parameters for evaluation of biocompatibility.[16]

The performance of any material in the human body is controlled by two sets of characteristics, they are biofunctionality and biocompatibility. Now a day with the advent of wide range of material alloys, it is relatively easy to satisfy the requirements for mechanical and physical functionality of implantable devices. Never the less, the selection of materials for medical applications is usually based on considerations of biocompatibility.[22]

In orthodontics, materials like brackets, bands, wires and implants are made up of different alloys of metal, plastic and ceramic which are directly in contact with oral soft tissues and oral fluid. Moreover, they should be biocompatible so as it should not cause any damage to the living tissues. These Orthodontic materials in the different oral environmental conditions like pH of the saliva, temperature, oral flora, plaque and the duration of the materials in the oral cavity may lead to the series of effects like corrosion and leaching of metal ions.[64] This release of metal ions into the oral cavity may affect the oral

tissue viability or allergic reactions in the surrounding tissue and systemic migration of these metal ions may lead to adverse reactions.

Temporary anchorage devices are made up of different materials and metal alloys so as to improve their efficiency, strength and biocompatibility. However, these metals were not adequately tested for cytotoxicity in recent past. Therefore, aim of the present study was to evaluate the cytotoxicity and correlate this cytotoxicity with the amount of metal ions leached from the mini implants. For this purpose, three mini implant such as Dentos, Denticon and SK Surgicals were selected which are popularly used for orthodontic purpose in our country.

All of these three mini implants were immersed in two pH concentration solutions such as pH 4 and pH 7. pH solutions were prepared with Sodium Chloride Solution (NaCl) as a base and Sodium Hydroxide (NaOH) and Hydrochloric Acid (HCl) were used to bring about exact desired pH concentration. Sodium Chloride (NaCl) was preferred over the artificial saliva because its use has been validated in previous studies as a standard ISO (International Organization for Standardization) protocol. Moreover, it has been established that In-vitro corrosion studies with physiologic saline solutions produced more reliable results which are similar to the studies advocated in other extra cellular fluids and in the natural saliva.[20] Mini implants of different companies named 1. Dentos implants 2. Denticon and 3. SK Mini Implants; were used in the present study to evaluate cytotoxicity. The

implants were immersed in the Sodium Chloride (NaCl) solutions of two different pH and eluates were collected at day 15, day 30 and day 60. The solution in which the implants were immersed is called as an eluate. Eluate is commonly defined as the solution of adsorbed material in the eluent obtained during the process of elution.

This time protocol was under taken in order to validate the amount of metal ions leaching and its effect on cytotoxicity in different periods of times as it has been postulated by Miceli guimarars Blaya[8] et al that there is a quantitative increase in the salivary concentration of nine different metal ions suck as Titanium, Zinc, Chromium, Nickel, Iron, Copper, Aluminium, Vanadium and Cobalt as the duration increases. When the implants surfaces are exposed to oral fluid and the oral flora, its protective oxide coating wears off with increase in the duration. Because of this, the corrosion resistance property of the material is lost, and the amount of metal ions leaching will be also increased.

In order to evaluate the cytotoxicity of the mini implants from the collected eluates, two different cell cultures such as Human Osteogenic Sarcoma cells (U2OS) and Human Gingival Fibroblast cell lines (HGF) were selected.

Human Osteogenic Sarcoma cells are considered as one of the first generated cell lines and it has the ability to grow fast because of their high transfection efficiency. Moreover it does not have susceptibility to adenoviral

infections. Because of these advantages, Human Osteogenic Sarcoma cell lines (U2OS) were widely used in various biomedical researches.

Likewise Human Gingival Fibroblast (HGF) is also considered as one of the most easily accessible mammalian cell types to grow in culture. Moreover it would be appropriate to evaluate the cytotoxicity on Human Gingival Fibroblast (HGF) cells because they are the major constituents of gingival tissue and plays a key role in its maintenance, which is adjacent to mini implants after their insertion.

40µl of eluates are added to the matured cell culture of both Human Gingival Fibroblast (HGF) and Human Osteogenic Sarcoma cells and incubated for 48 hours and then evaluated for cell viability with MTT assay. [3-(4, 5-dimethythiazole-2-yl)-2, 5-diphenyle tetrazolium bromide]

After 48 hours of incubation in CO_2 incubator, MTT dye is added to the cell culture in well plates and well plates are inserted in the ELISA reader to get the cell viability readings. This indicates the level of cytotoxicity of the selected implants. Subsequently, the collected eluates were also assessed for metal ions leached from the implants. For this purpose Inductive Coupled Plasma – Mass Spectroscopy (ICP-MS) is used. With this technique, elements can be detected at or below the parts of trillion, which is considered to be one of the most precise method for evaluating the amount of metal ions leached. The principle composition of mini implants that were used in this study are

Aluminum (Al), Titanium (Ti), Vanadium (V) and Iron (Fe). Therefore traces of these elements were examined with ICP-MS.[8]

All the collected readings were subjected to statistical analysis. It has been revealed that the cytotoxicity is significantly more for pH 4 than pH 7 for all the mini implants and also there was an increase in cell death percentage from day 15 to day 30 and day 30 to day 60 in both Human Osteogenic Sarcoma Cells (U2OS) and Human Gingival Fibroblast (HGF). (Table 1, 4, 7, 10, 13, 16). However results revealed that survival rates for Human Osteogenic Sarcoma Cells (U2OS) and Human Gingival Fibroblast (HGF) cells are different. This difference shows that cells reactions differ on exposure to metals ions. This was in accordance to study done by Chia Tze kao et al[28] who also had similar findings and concluded, that at pH 4 and pH 7 the survival rates of U2OS and HGF cells differed.

The reason for increased cell death in pH 4 than pH 7 might be attributed to the fact that reduction of pH may act as a corrosion stimulating agent[11,3] and consequently amount of metal ions leached from these metal due to corrosion also increases[11]. This high concentration of metal ions leached in pH 4 when compared to pH 7 can be further validated from our results obtained from the ICP-MS (Table 25, 26) values in which sum of metal ions leached was observed high in pH 4 than in pH 7 at day 15, day 30 and day 60. This phenomenon of more metal ions leaching as the pH decreases was also observed by the study conducted by Tbrah Nuoh et al [41] and

Hamoon Zohdi et al[24] concluded that the corrosion resistance of the alloy was decreased when the pH of the solution was lowered to pH 2.5. He reasoned this as the metal in aqueous solution will be thermodynamically unstable and there is a tendency to pass from a solid state to anionic stage which is in association with the decrease in the energy and the direction of energy is influenced by factors such as surface morphology, salivary composition, pH and the temperature. An unstable metal in different influencing factors will corrode, releasing metal ions into the solution and may have adverse biological and functional effect (Table 1, 4, 7, 10, 13, 16).

Subsequently in pH 4 concentration on day 15 and day 30 results of this study revealed that the amount of cytotoxicity recorded highest in Denticon followed by SK mini implants and the least cell death was noted in Dentos with U2OS cell culture. Same order of the cell death was noted for HGF as well.

However the percentage of cell death is not same between U2OS and HGF, i.e. both cells reacted differently. This was in accordance with the study done by Chia Tze Kao[28] who demonstrated that the HGF and U2OS cells survival rates differ and exhibit various cellular reactions. The Rationale behind the increased cell death in Denticon when compared to SK and Dentos Implants (Table 21, 24) may be because of the total amount of metal ions released was higher for Denticon Implants for both day 15 and day 30 followed by SK Implants and the least amount of metal ions were leached in

Dentos Implants for both day 15 and day 30 (Table 25, 26). Moreover it can be appreciated that the amount of Aluminium and Iron ion released for Denticon Implants is also higher than that of SK Implants and Dentos Implants on day 15 and day 30. This may be because of the loss of protective titanium oxide layer as these Denticon implants are made of cp titanium and has a thin protective layer compared to the Ti5Al4V alloys (Table 25). This can be accounted as a reason behind the increased cell death observed in Denticon Implants.

The cytotoxic effect of Aluminium and Iron was also studied by Ke Zheng et al[62] and they concluded that Aluminium induces typical apoptotic characteristics like cell shrinkage, nuclear fragmentation, vacuolation, chromatin marginalization and also DNA degeneration. Similarly Amuja Agarwal et al[1] stated that the metal ions such as Titanium (Ti), Cobalt, Aluminium (Al) are responsible to decrease the DNA synthesis and mitochondrial dehydrogenase activity which is responsible for cell death. Furthermore Ke Zheng et al[62] also stated that cell growth inhibitory effects were correlated with Aluminium concentration.

When evaluating the cytotoxic effect of all the mini implants in day 60, the proportions of cell death were highest in Dentos Implants followed by SK Mini implants and least amount of cell death was noted in Denticon Implants for both U2OS and HGF cells. It is interesting to note that the Denticon

Implants which had highest percentage of cytotoxicity in day 15 and day 30, now exhibiting the least amount of cytotoxicity in day 60 (Table 21, 22, 24).

Conversely the Dentos Implant which exhibited lowest cytotoxicity in day 15 and day 30 was now exhibiting high cell death in day 60. This can be correlated to the values of ICP-MS, that the amount of metal ions leached was more in Dentos Implant followed by SK Implant and the least metal ions released was in Denticon Implant at day 60 (Table 25). Moreover amount of Aluminium (Al) and Iron (Fe) released was also highest in Dentos Implant followed by SK Implant and the least metal ion release was observed in Denticon Implan on day 60.

In the study conducted by Jay Albretsen et al[2] reviewed the effect of Iron ions on cell toxicity and concluded that the free Iron ions increases lipid peroxidation with resulting membrane damage to mitochondria, microsomes and other cellular organ release.

Likewise, for the pH 7 results of day 15 revealed that the highest cell death was recorded with Denticon Implant followed by SK Implants and the least cell death was noted in Dentos Implants. However the percentage of cytotoxicity is not same between U2OS and HGF cells, i.e. both the cells reacted differently. Reason behind this increased cell death in Denticon Implant when compared to SK and Dentos Implants has been explained earlier in discussion with pH 4.[18] The cell death for day 30 at pH 7, varied in the order of cell death from pH 4 as the highest cell death in S K implant followed

by Denticon Implants and the least in Dentos Implants. This difference in order can be either because of the increased amount of Titanium (Ti) leached (Table 26) or might be a reading error in methodology.

Finally When evaluating the cytotoxicity for day 60, similar to the results of pH 4, the amount of cytotoxicity was highest in Dentos Implants followed by SK Implants and the least amount of cytotoxicity in Denticon Implants for both U2OS and HGF, Dentos Implants which had the least percentage of cytotoxicity in day 15 and day 30 was now exhibiting the highest amount of cytotoxicity in day 60 which can be attributed to the ICP-MS values.

Generally Titanium implants are also classified as Alpha, Beta, Alpha+ Beta and neutral phased according to the type of the stabilizer present in it. Stabilizer plays a vital role in corrosion resistance.[52] Alpha stabilizers constitutes Aluminium (Al), Cobalt (Co) and Beta stabilizers constitute Molybdenum, Vanadium and neutral stabilizers are Zerconium+alpha+beta. Implants with both alpha and beta stabilizers such as Dentos Implants due to presence of both alpha and beta stabilizer there exists a double layer of Titanium oxide (TiO_2) and they have an advantage of high corrosion resistance. This double layer of Titanium oxide prevents the corrosion initially; hence there was less metal ion leaching in day 15 and day 30. However in day 60 because of prolonged exposure to corrosion elements there was initiation and propagation of pits. Moreover acidity of oral environment

accelerates the effect of corrosion and dissolves the Titanium oxide protective layer with which in turn causes localized destruction and leaching of metal elements. Denizog lu et al[12] evaluated the influence of salivary pH on the corrosion of two base metal alloys and concluded that the pH significantly affect total cobalt ion release. Alloy pH interaction affects the titanium oxide layer and total amount of metal ion release increases subsequently.

Results were in accordance with Lima C.N. Elias et al, who stated that the dental implants made with Ti-6Al-4V can release ions into tissues locally and systemically due to the effect of corrosion. In accordance to this study, Ti6Al4V Implants like Dentos had increased cytotoxic effect than other implants selected in this study.

From this study it is evident that though double Titanium oxide layer which claims to have high corrosion resistance in implants, in prolonged duration they exhibit higher cytotoxic effect. This can be because of presence of high concentration of Aluminium and Venidium. However these elements were added in order to increase the strength, modulus of elasticity and formability of the implant.[7] Thus, it is prudent to conclude that as far as the cytotoxic effects are concerned, the Denticon Implants are superior to the other implants used in our study. It is because Denticon Implant contains comparatively lesser concentration of Aluminium, Iron and also the total amount of metal ions leached is also significantly less. However, its strength,

and fracture resistance during insertion should be validated in the further studies.

Although all experimental steps of this study were conducted in a judicious manner and strictly according to the protocol, in vitro studies have limitations. Consequently this in-vitro study cannot be directly correlated with in vivo situation. The presence and activity of micro flora to a large extent is responsible for the process of corrosion and deterioration and this cannot be replicated in the in-vitro study[20] as the pH of the oral cavity does not remain constant, it changes between pH 4 to pH 9.5. Moreover the biological considerations in terms of composition and acidity of saliva, dental hygiene, eating habits, oral temperature, administration of medicine and susceptibility to caries also influence the corrosion.[4]

Therefore it is recommended that further studies should be conducted with larger sample size, longer duration and with different pH concentrations. We also recommend the manufacturers for the production of mini implants with lesser amounts of Aluminium but without compromising on strength of the implant. May be the use of Zirconium and noble alloys for manufacturing of mini implants can be beneficial.[47]

Moreover other surface modification techniques such as hard coatings, laser nitriding, bio ceramics, ion implantation and biomimetic coating can be advocated in the implant as they have great potential to improve the performance of biomedical implants and improves the life of their recipients.[23]

Summary and Conclusion

SUMMARY AND CONCLUSION

The purpose of this in-vitro study was to evaluate the cytotoxicity of orthodontic mini implants in two different pH concentrations. For this purpose three different companies of mini implants named 1. Dentos implants, Anchor Dentos Inc, Korea 2. Denticon, Dental Instrument Co, Mumbai, India and 3. SK Mini Implants, SK surgical, Om lab Services, Pune, India were selected and the cytotoxicity is evaluated at two different pH concentrations such as pH 4 and pH 7 at day 15, day 30 and day 60.

Twelve implants of each company were analyzed for evaluation of their cytotoxicity by immersing them in a NaCl solution at pH 4 and pH 7. The eluates are collected after the day 15, day 30, and day 60. The cytotoxicity was evaluated on two different cell lines such as Human Osteogenic Sarcoma Cell Lines and Human Gingival Fibroblast cell lines by exposing them to the eluates collected at day 15, day 30 and day 60.

The evaluation of cytotoxicity is done by examining the percentage of viability of the cell culture after the exposure to the eluates and this is advocated by means of MTT Assay. This cell death after the exposure of the cells to eluates is correlated with the amount of the metal ions released from the mini implants after day15, day 30 and day 60.

The evaluation of the amount of metal ions released such as Aluminium, Titanium, Iron and Vanadium which are principle metal

composition of the mini implants is assessed by Inductive Coupled Plasma Mass Spectroscopy (Agilent 7700, ICP-MS system Mass Hunter 4.1). After the evaluation of the metal ion release, these results are correlated with the cytotoxicity.

The results of the study indicate that:

1. When comparing the cell death at pH 4 and pH 7, cell death at pH 4 is always greater than the cell death at pH 7 in both U2OS and HGF cell in all the three companies of mini implants tested in the present study.

2. When comparing the cell death at three different time periods, such as day 15, day 30 and day 60, there is a gradual increase in the cell death from day 15 to day 30 and from day 30 to day 60 in all the mini implants tested.

3. When comparing the cytotoxicity at day 60 in both U2OS and HGF; Dentos revealed a highest cell death followed by SK Implant and least cell death was observed in Denticon mini implant.

4. When comparing the total amount of metal ion released at day 60, Dentos showed a highest amount of metal ion release followed by SK Implant and least amount of metal ion release was observed in Denticon implant.

5. Cytotoxicity at day 60, can be correlated to the ICP-MS values in day 60, that the amount of total metal ions leached was more in Dentos Implants followed by SK Implants and least was in Denticon Implants.

CONCLUSIONS:

The following conclusions were made from the results of the study:

From this study it is evident that mini implants with double Titanium oxide layer which thought to have high corrosion resistance might have higher cytotoxic effect when exposed for longer duration. This can be because of presence of high concentration of Aluminium and Venidium. However these elements are added in order to increase the strength, modulus of elasticity and formability of the implant.

Thus, it is prudent to conclude that as far as the cytotoxic effects are concerned, the Denticon Implants are superior to the other implants used in our study. It is because Denticon Implant contains less concentration of Aluminium, Iron and also the total amount of metal ions leached was significantly less. However its strength, and fracture resistance during insertion should be validated in the further studies.

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Annexure

ANNEXURE I

RAGAS DENTAL COLLEGE & HOSPITAL
(Unit of Ragas Educational Society)
Recognized by the Dental Council of India, New Delhi
Affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai
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TO WHOM SO EVER IT MAY CONCERN

Date: 05-01-2016
Place: Chennai

From
The Institutional Review Board,
Ragas Dental College & Hospital,
Uthandi,
Chennai – 600119.

The thesis topic **'EVALUATION OF CYTOTOXICITY OF ORTHODONTIC MINI IMPLANTS IN TWO DIFFERENT pH CONCENTRATIONS -AN IN-VITRO STUDY'**, submitted by **Dr. PEDDADA REVATHI**, has been approved by the institutional review board of Ragas Dental College & Hospital on 5th May, 2014.

(Dr. S. RAMACHANDRAN M.D.S.)
Secretary, Institutional Review Board,
Head of the Institution,
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ANNEXURE II

DECLARATION OF PLAGIARISM CHECK

From,

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To,

The Head of the Department

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SUB: Declaration of plagiarism check of my dissertation to be submitted to The Tamilnadu Dr. M G R Medical University - April 2006.

I, hereby declare that I have checked my dissertation for plagiarism using smallseotools plagiarism checker software on 7-1-2016 for this dissertation. The unique content was 85 % and plagiarism content was 15%. The plagiarism content corresponds to definitions and terminologies that have to be quoted.

Yours sincerely

Revathi Peddada.