"COMPARATIVE EVALUATION OF OSTEOBLASTIC RESPONSE ON TITANIUM DISCS COATED WITH L-PRF (LEUCOCYTE PLATELET RICH FIBRIN) AND A-PRF+ (ADVANCED PLATELET RICH FIBRIN PLUS) - AN INVITRO STUDY."

By

REG. NO- IM0221004

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Head of Department

Dr. ANANDKUMAR G. PATIL M.D.S
Professor& Head
Department of Prosthodontics
and Crown & Bridge,
KAHER KLE Vishwanath Katti Institute
of Dental Sciences, Belagavi-590010.

Date: 4/4/24
Place: Belagavi

Principal

PRINCIPAL
KLE V.K. Institute of Dental Sciences
Nehru Nagar BELAGAVI-590010

Dr. ALKA D. KALE M.D.S.
Principal,
KAHER KLE Vishwanath Katti
Institute of Dental Sciences,
Belagavi-590010.

Date: 15/4/24
Place: Belagavi

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Nehru Nagar, Belagavi - 590 010, Karnataka State

Accredited 'A' Grade by NAAC (2nd Cycle) Placed in Category 'A' by MHRD (GoI)

☎: 0831-2470362 Web: http://www.kledental-bgm.edu.in
FAX: 0831-2470640 E-mail:principal@kledental-bgm.edu.in

Date : 4.04.2024 Serial No. : 168

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UG / PG / Ph.D / Staff : POST GRADUATE
Batch & Year : 2021 - 2024
Department : PROSTHODONTICS AND CROWN AND BRIDGE

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KLE V.K. Institute of Dental Sciences
(A Constituent unit of KLE Academy of Higher Education & Research
Deemed-to-be-University u/s 3 of the UGC Act, 1956)
Nehru Nagar, Belagavi-590 010 INDIA
Re-Accredited 'A' grade by NAAC (2nd Cycle) & Placed in Category 'A' by MHRD (GoI)
: 0831-2470362
FAX: 0831-2470640
Web: http://www.kledental-bgm.edu.in
E-mail: principal@kledental-bgm.edu.in

Biostatistics Clearance Certificate

This is to certify that the Biostatistics aspect of the Dissertation / Research work of **REG. NO- IM0221004** **Post Graduate Student**, under the guidance of **Professor, Department of PROSTHODONTICS AND CROWN AND BRIDGE,** entitled **"To evaluate and compare osteoblastic response on titanium discs coated with L-PRF (Leucocyte platelet rich fibrin) and A-PRF+ (Advanced platelet rich fibrin plus)- An in vitro study"** has been done under my guidance and considered satisfactory.

Place: Belagavi
Date: 24/02/2024

Name & Signature of Biostatistician
Dr. S. B. JAVALI Ph.D,
Sr. Associate Professor in Statistics
Department of Community Medicine
USM KLE International Medical Programme
BELAGAVI-590010.

LIST OF ABBREVIATIONS USED IN THE STUDY

ABBREVIATIONS	FULL FORMS
CONTROL GROUP	Untreated titanium discs
STUDY GROUP 1	Titanium discs treated with leucocyte platelet rich fibrin
STUDY GROUP 2	Titanium discs treated with advanced platelet rich fibrin plus
APRF	Advanced platelet rich fibrin
APRF+	Advanced platelet rich fibrin plus
ATSM	American society for testing and materials
ANOVA	Analysis of variance
ALP	Alkaline phosphatase activity
BIC	Bone Implant Contact
BMP	Bone morphogenetic protein
C.V.	Coefficient of Variation
CO_2	Carbon dioxide
°C	Degree Celsius
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EGF	Epidermal growth factor
HA	Hydroxyapatite
IL	Interleukin
IGF	Insulin like growth factor

i-PRF	Injectable platelet rich fibrin
LPRF	Leucocyte platelet rich fibrin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NTR	Natural tissue regeneration
OD	Optical density
PBS	Phosphate Buffer Saline
PRF	Platelet rich fibrin
PRP	Platelet rich plasma
PDGF	Platelet - derived growth factor
rpm	Revolutions per minute
Ra	Average surface roughness
S.D.	Standard Deviation
S.E.	Standard of error
SEM	Scanning electron microscope
Ti6Al4V	Titanium-6 aluminum-4 vanadium
TGF	Transforming growth factor
TNF	Tumor Necrosis Factor
TGF-β1	Transforming growth factor beta one
VEGF	Vascular endothelial growth factor

ABSTRACT

STATEMENT OF PROBLEM

Success of a dental implant relies on its osseointegration process. Ever since osseointegration concept was introduced, there have been number of techniques and materials that have been developed to increase the bone implant contact and decrease the time required for osseointegration. PRF is an autologous fibrin-based (membrane, matrix or scaffold), living biomaterial, derived chairside from centrifugation of blood without any external additives or anti-coagulant, contains growth factor rich platelets , fibrinogen (acting as a supportive matrix), and cells (mostly the leucocytes, and stem cells for their antibacterial, neo-vascularization and regenerative properties), thus making it the most autogenic ideal source for regeneration and healing .The research related to the osteogenic potential of PRF coating on titanium surfaces are at sparse. Hence it becomes necessary to evaluate the effect of PRF and its osteogenic potential for early osseointegration of dental implants. Therefore, this invitro study was undertaken with an intent to evaluate and compare osteoblastic response on titanium discs coated with A-PRF+ (Advanced platelet rich fibrin plus) and L-PRF (Leucocyte platelet rich fibrin) coated titanium discs.

AIM: The aim of the present study was to evaluate and compare the osteoblastic response of Advanced platelet-rich fibrin plus (A-PRF+) and Leucocyte platelet rich fibrin (L-PRF) coated titanium discs.

MATERIALS AND METHODOLOGY

A total of the 243 identical commercially available Titanium Grade V, 10 mm x 2 mm (ASTM B348) disc shaped specimens were used. 81 titanium discs were coated with

Leucocyte platelet rich fibrin (L-PRF) which made up the study group 1, 81 titanium discs coated with Advanced platelet rich fibrin plus (A-PRF+) which made the study group 2, while the remaining 81 titanium discs without any PRF coating acted as a control. Out of the total titanium discs (n=243), 81 titanium discs were used to assess the cell attachment, 81 titanium discs were used to assess the cell proliferation and the remaining 81 titanium discs were used to assess the cell mineralization by Alizarin red S staining. All the specimens from each group were subjected to quantitative evaluation of surface roughnessusing profilometer and one specimen from each group was subjected to Scanning Electron microscopy for qualitative assessment of surface roughness. A mean roughness profile was evaluated for each specimen to describe the overall roughness of the surface. Both the types of PRF (LPRF and APRF+) were prepared and the discs in the study groups 1 and 2 were coated by dipping method simultaneously.

The osteogenic potential was evaluated by assessing the cell attachment, cell proliferation and cell mineralization on PRF coated and noncoated discs. Cell attachment and cell proliferation were evaluated at 24 hours, 48 hours and 72 hours. Cell mineralization was evaluated at day 7, day 14 and day 21 using alizarin red-S staining of MG-63 cells. The cell Attachment was evaluated using hemocytometer while cell proliferation was assessed using MTT Assay. The resultant data was tabulated and then subjected to statistical analysis to draw conclusion from experimental data. ($p<0.05$).

RESULTS

The results showed that the cell attachment was higher for the PRF coated discs, with the highest seen in the APRF+ group at all three-time intervals as compared

to noncoated titanium discs. There was a statistically significant difference in the cell attachment between the time intervals for the study groups (LPRF and APRF +). The cell proliferation was higher in the study groups (LPRF and APRF+) and with statistically significant results at all three-time intervals. The APRF+ coated titanium discs showed highest mineralization when compared to LPRF coated titanium discs. Hence, within the limitations of the study, we can conclude that PRF could be used as a surface coating rendering increased osteogenic properties and APRF + proved to have enhanced cell proliferation, cell attachment and cell mineralization, aiding in early osseointegration.

CONCLUSION

On comparing the osteogenic potential, the cell attachment was higher on titanium surfaces which were treated with APRF + followed by LPRF and noncoated titanium discs at different time intervals. APRF+ coated titanium discs showed increase in proliferation of MG63 cell lines from 24 hours to 72 hours than compared to LPRF, thus showing that APRF+ helps in accelerating proliferation of MG63 cell lines. The APRF+ coated titanium discs showed highest calcium rich deposits than compared to LPRF coated titanium discs, at all the three-time intervals i.e. day 7, day 14 and day 21. Thus, indicating APRF + coated titanium discs showed increased cell attachment, cell proliferation and mineralization.

KEYWORDS: Leucocyte Platelet Rich Fibrin, Advanced Platelet Rich Fibrin Plus, Coated Materials, Osseointegration, Titanium.

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INTRODUCTION

Natural teeth are frequently lost due to caries, periodontal diseases and trauma that affect the patient's natural appearance, speech, and masticatory system. Rehabilitation using dental implants becomes the most approachable option for restoring missing teeth, masticatory function, and esthetics, thereby improving patient's quality of life.[1] Restoring the patient to their best possible speech, appearance and overall health, is the goal of contemporary dental care, irrespective of any kind of impairment to the stomatognathic system.

Dental implants, also referred to as a replacement for tooth roots, are inert metallic anchors which are placed by surgery, beneath the gum line in the jaw bone to support a prosthetic crown in the absence of natural teeth .[2]

The dental implants are biocompatible and made of titanium and its alloys. Ti-6 aluminum-4 vanadium (Ti6Al4V) remains the most commonly used titanium alloy and is preferred due to its superior mechanical properties and excellent biocompatibility. [3] They are left undisturbed in the bone, where healing takes place around the implant , termed as osseointegration and is critical for long term prognosis.

Osseointegration can be affected by a number of factors including the material properties, surface topography, and geometrical features of dental implants.[4] Osseointegration may also be affected in cases of low bone density and quantity, due to systemic disorders like osteoporosis or be encountered as a side effect of radiotherapy. These conditions point towards a difficult bone remodelling scenario and the implant survival rates could drop to 55% in certain circumstances, which

would require additional surface modifications that would stimulate new bone formation and promote early osseointegration. [5]

As the implant surface forms an intimate contact with the living tissues, the surface modification of the implant enhances the materials biological response and osseointegration without affecting the bulk properties.[4] Therefore, improvement of the osteoconductive nature of the dental implant is always desirable to achieve clinical success.

There are various techniques pertaining to surface modifications that have been researched and applied to improve biological surface properties, that support the mechanism of osseointegration, these include subtractive methods such as sandblasting, acid etching or a combination of both. The additive methods include plasma spraying, hydroxyapatite coating and also coating with biomimetic agents. [6]

Most of these implant surface treatments aim at enhancing the osteoblastic activity and the action of their mediators to increase the new bone formation and promote early osseointegration and higher secondary implant stability. [6]

One of the surface chemical modifications to further stimulate osseointegration is the coating of implant surfaces with biological components, which include coatings with peptides, growth factors, calcium phosphates and lipids. Different organic coatings, biopolymers and biomimetic agents have been explored as biomaterials used for dental implants which can potentially influence cellular activity during peri-implant healing, and promote an intimate bone-implant contact, promoting osseointegration by the proliferation of osteoblasts.[7]

Numerous surface alterations have been suggested in order to change the characteristics, maximize bone to implant interaction, improve osteogenesis, and ultimately to achieve greater and faster osseointegration. However, search for an ideal implant surface coating is still continued. One of the most important initial steps of bone implant healing is the generation of a solid clot of fibrin in contact with the implant surface to provide a provisional scaffold for the migration of differentiating osteogenic cells towards the implant surface.[9]

Clinicians are studying and utilizing platelet rich fibrin (PRF), an autologous bioinert material produced from patient's blood to promote bone, soft tissue healing and regeneration. The optimal approach for in vivo regrowth of tissues necessitates a combination of leucocytes, stem cells, platelets, growth factors, and scaffolds (fibronectin matrix).[10] When appropriately integrated and processed, these essential components of PRF play a crucial role in tissue repair and regrowth processes such as cell proliferation and differentiation, extracellular matrix formation, chemotaxis, and angiogenesis (neo-vascularization).

Platelet-rich fibrin is obtained by centrifugation of blood without any external additives or anti-coagulant. It concentrates the blood platelets and has found application in several areas of dentistry. [11] These days, platelet concentrates come in a variety of biologic forms, such as platelet-rich plasma (PRP) and platelet-rich fibrin (PRF), which are utilized to aid in wound healing. Clinicians are interested in PRF technology because it is readily prepared chairside from individual's own blood, is easy to use; and widely applicable in dentistry, while being financially realistic for the patient and the clinician, and with virtually no risk of a rejection reaction (foreign body response).[11]

This three dimensional scaffold allows a continuous release of cytokines and growth factors enhancing mainly the first phase of tissue repairs, such as TGF (transforming growth factor), PDGF (platelet derived growth factor), VEGF (vascular endothelial growth factors) among many others, enhancing the healing process up to 10 days.[12] The PRF can be applied in injectable form (iPRF) or liquid form, as a plug, as a barrier (L-PRF, A-PRF, or A-PRF +), or as a fragmented membrane in standalone therapies (i.e., plug, filler, or protective barrier); additive therapies (i.e., added or mixed to bone substitutes); or used in combination therapies with other biomaterials.[12]

According to the study conducted by Mara Simoes-Pedro et al in the year 2022, for invitro testing of mechanical and structural properties like tensile strength of APRF +, APRF and LPRF, it was concluded that notably greater tensile strength was with the A-PRF+ protocol, making this type of membrane the most appropriate for suturing and handling.[13]

Another study conducted by Marco Lollobrigida et al in the year 2018, concluded that Liquid L-PRF products contributes to the development of a thick fibrin clot in vitro on surfaces of implants that are micro or nano-roughened. [9]

According to study conducted by Rucha Shah et al in the year 2021 to evaluate the osteoblastic response to the effect of injectable platelet-rich fibrin coating on titanium discs, it was concluded that i-PRF improves mineralization at days 1, 7, 14, and 21 of MG-63 osteoblast-like cells and promotes differentiation and alkaline phosphatase activity.[14] However , no studies have been conducted to evaluate the osteoblastic efficacy of surface modified dental implants using the more

stable APRF +, so the present comparative study was conducted to evaluate and compare the osteoblastic efficacy comparing L-PRF and APRF +.

An improved understanding of the biological and physiological properties and characteristics of the APRF+, for its use in hard and soft tissue repair and remodelling is necessary, which will lead to successful therapeutic applications in the field of implant dentistry.

NEED FOR STUDY

Implant dentistry is unique because it has the ability to accomplish the goals of normal function, contour, aesthetics, phonetics, speech and general oral health inspite of atrophy, disease or injury to stomatognathic system[15].

Titanium-6 aluminum-4 vanadium (Ti6Al4V), these alloys are the most commonly used for implants due to their excellent mechanical properties and superior biocompatibility in comparison to conventional materials such as stainless steel 316L and cobalt–chromium alloys. However, titanium in its use as implant materials have some limitations. Titanium as an alloy is bioinert lacking the ability to form any chemical bond with surrounding tissues, it only forms physical bond with the bone tissue and the stability of this physical bond is less than chemical osseous bonding leading to a higher risk of failure or implant loosening. [16]

With the current implants present in the market using titanium alloys, it takes atleast 4 – 6 months for complete osseointegration with the surrounding bone. Moreover, in cases with impaired quality of bone, systemic diseases longer waiting periods are required and the osseointegration is not complete. The bone-to-implant contact area averages between 70% and 80%, with a minimum percentage of 60%, even with successful implants that have survived for up to 17 years, according to study of titanium implants that have been recovered.[91]

So, the real challenge lies in reducing the time of osseointegration and increase the bone to implant contact ratio. Therefore, majority of the implant surface treatments aim at increasing the activity of osteoprogenitor cells and their mediators

to increase the new bone formation, promote earlier osseointegration and higher secondary implant stability.[17]

Growth factors-based coatings are a category of substances that play a role in tissue growth and cell division. The combination of each growth factor's recognition of a particular membrane receptor either promotes or prevents cell division. Vascular endothelial growth factor (VEGF) and bone morphogenetic proteins (BMPs) are the two main growth factors used in implant coatings. However, the osteoblastic efficacy of these growth factors to act as a implant surface coating needs to be investigated. [8] One of the universal factors in the implant bone healing process is the development of a stable fibrin clot in contact with the implant surface to provide a provisional scaffold for the migration of differentiating osteogenic cells in the direction of the implant area. In order to achieve this, numerous modifications have been developed till date that promote adhesion of fibrin to improve osseointegration.[6]

PRF is an autologous fibrin-based (membrane, matrix or scaffold), living biomaterial, derived chairside from centrifugation of blood without any external additives or anti-coagulant, contains growth factor rich platelets , fibrinogen (acting as a supportive matrix), and cells (mostly the leucocytes, and stem cells for their antibacterial, neo-vascularization and regenerative properties), thus making it the most ideal autogenic source for regeneration and healing.[10]

Bone implant healing and remodelling occurs in several stages; however cell adhesion remains crucial for subsequent proliferation and differentiation of osteoblastic cells producing bony tissue and extracellular matrix, which will ensure a high bone-implant contact. So, it is imperative that implant titanium surface forms an

adequate bond with the osteoblasts and much room remains for improving the surface quality in terms of the rate and strength osseointegration.[11]

The research related to the osteogenic potential of PRF coating on titanium surfaces are at sparse. Hence it becomes necessary to evaluate the effect of PRF and its osteogenic potential for early osseointegration of dental implants. Therefore, this invitro study was undertaken with an intent to evaluate and compare osteoblastic response on titanium discs coated with A-PRF+ (Advanced platelet rich fibrin plus) and L-PRF (Leucocyte platelet rich fibrin) coated titanium discs.

HYPOTHESIS

NULL HYPOTHESIS:

There is no difference in osteoblastic response between A-PRF+ and L-PRF coated titanium discs.

RESEARCH HYPOTHESIS:

There is a difference in osteoblastic response between A-PRF+ and L-PRF coated titanium discs.

AIM AND OBJECTIVES

AIM OF THE STUDY:

The aim of the present study was to evaluate and compare the osteoblastic response of Advanced platelet-rich fibrin plus (A-PRF+) and Leucocyte platelet rich fibrin (L-PRF) coated titanium discs.

OBJECTIVES:

1. To evaluate cell adhesion, cell proliferation and cell mineralization of MG-63 osteoblast like cells on L-PRF coated titanium discs.
2. To evaluate cell adhesion, cell proliferation and cell mineralization of MG-63 osteoblast like cells on A-PRF+ coated titanium discs.
3. To evaluate and compare cell adhesion, cell proliferation and cell mineralization of MG-63 osteoblast like cells on L-PRF and A-PRF+ coated titanium discs.

REVIEW OF LITERATURE

1. **Gregory R. Parr et al in 1985** highlighted the important dental materials aspects of titanium and its alloys. Titanium and its alloys are important in dental and surgical implants because of their high degree of biocompatibility, their strength, and their corrosion resistance. The alloys most commonly used for dental implants are of the alpha-beta variety. Of these, the most common contains 6% aluminum and 4% vanadium (Ti-6Al-4V). After heat treatment these alloys possess many favorable physical and mechanical properties that make them excellent implant materials. [19]

2. **Sennerby et al in 1991** conducted a study to examine key implant factors that determine the bone-metal interface reactions that occur around a titanium screw. Tissue reactions to titanium versus Titanium-6aluminum-4Vanadium were examined, relevant surface characteristics and surface structure for achieving reliable osseointegration, as well as probable bonding processes over the bone-to-titanium interface, were outlined. This article indicates that elements linked to the implant alone do not dictate the bone-metallic interfacial responses, but that other factors such as surgical technique and loading circumstances are equally significant for establishing a reliable osseointegration.[20]

3. **Zhao G et al in the year 2005**, performed a study where discs were prepared and coarse grit-blasted with 0.25– 0.50 mm carborundum disc. Modified sandblasted surfaces were produced with same sandblasting and acid-etching procedure and named as SLA surfaces. Surface topography, cell culture with

MG63 cells was performed. Water contact angles were determined by dynamic contact angle analysis. It was concluded, that this novel process to protect the titanium surfaces from the surrounding environment during production diminishes hydrocarbon contamination and enhances surface energy. Osteoblast-cells cultured on these chemically hydrophilic surfaces produced more differentiation.[48]

4. **Joseph Choukroun et al in the year 2006**, did a review of the literature to assess the platelet-associated features of platelet rich fibrin. They did a comparative study to quantify PDGF-BB, TGFβ-1, and IGF-I within PPP (platelet-poor plasma) supernatant and PRF clot exudate serum. These initial analyses revealed that slow fibrin polymerization during PRF processing leads to the intrinsic incorporation of platelet cytokines. This result implied that PRF, unlike the other platelet concentrates, would be able to progressively release cytokines during fibrin matrix remodelling, such a mechanism might explain the clinically observed healing properties of PRF.[28]

5. **David M. Dohan in the year 2006**, performed a review of the literature to describe the conceptual and technical evolution from fibrin glues to platelet concentrates. This retrospective analysis was necessary and evaluated the biochemical properties of 3 generations of surgical additives, respectively fibrin adhesives, concentrated platelet-rich plasma (cPRP) and PRF. The 3-dimensional fibrin architecture is dependent on artificial clinical polymerization processes, such as massive bovine thrombin addition. Recently, the slow polymerization during PRF preparation seemed to generate a fibrin network very similar to the natural one.[30]

6. **Zhe Qu et al in the year 2007**, aimed to investigate the influence of hydrophobic acid-etched (A) and coarse-blasted large-grit and acid-etched (SLA) surfaces as well as hydrophilic modified acid-etched (modA) and modified coarseblasted large-grit and acid-etched (modSLA) surfaces on the behavior of MG63 cells grown on these surfaces through determination of cell attachment and cell proliferation, timelapse microscopy of fluorescence-labeled cells, and analysis of gene expression by reverse transcription-polymerase chain reaction (RT-PCR). No significant difference of cell attachment on various titanium surfaces was found. Also, the modSLA surface resulted in an enhanced cluster formation of osteoblasts grown on this surface and in an increased expression of key osteogenic regulatory genes in osteoblasts.[36]

7. **Simonpieri A et al in the year 2009**, described a new technique of total maxillary preimplant bone grafting using allograft, Choukroun's PRF membranes and metronidazole. This focused on the preimplant reconstructive treatment using allogeneic bone granules. They concluded that PRF membranes are particularly helpful to protect the surgical site and foster soft tissue healing. This fibrin biomaterial represents a new opportunity to improve both the maturation of bone grafts and the final esthetic result of the peri-implant soft tissue.[42]

8. **Wennerberg A et al in 2010** in their study put forth that the roughness of an implant surface is the key factor in osseointegration and in the long-term success of the implant. The study concluded that the optimum roughness value is in the range of 1–2 μm, as this provides an optimum degree of roughness to

promote osseointegration, compared to the smoother or rougher surfaces. The best clinical results have been obtained with moderately rough surfaces (Sa between 1.0–2.0 μm).[21]

9. **Ehrenfest DM et al in the year 2010**, examined in vitro effects of PRF on human bone mesenchymal stem cells (BMSC), harvested in the oral cavity after preimplant endosteal stimulation. Cell counts, cytotoxicity tests, alkaline phosphatase (ALP) activity quantification, Von Kossa staining and mineralization nodules counts were performed at 3, 7, 14, 21 and 28 days. It was concluded that PRF generated a significant stimulation of the BMSC proliferation and differentiation. The combination of oral BMSC and PRF might offer many potential clinical and biotechnological applications, and deserves new studies.[44]

10. **Naik B et al in the year 2013**, critically reviewed the strategies available for use of platelet rich fibrin as healing aid in dentistry. According to this review they concluded that PRF can be used to promote wound healing, bone regeneration, graft stabilization, wound sealing, and hemostasis. Because the fibrin matrix is better organized, it is able to more efficiently direct stem cell migration and the healing program. It was also added that although PRF belongs to a new generation of platelet concentrates, the biologic activity of fibrin molecule is enough in itself to account for significant cicatricial capacity of the PRF. However, it is necessary to look further into platelet concentrates and inflammatory features of this biomaterial.[47]

11. **Shahram Ghanaati et al in the year 2014**, performed an invitro study where Choukroun's platelet-rich fibrin (PRF) is obtained from blood without adding

anticoagulants. In this study, protocols for standard platelet-rich fibrin (S-PRF) (2700 rpm, 12 minutes) and advanced platelet-rich fibrin (A-PRF) (1500 rpm, 14 minutes) were compared by histological cell detection. Platelets were detected throughout the clot in both groups, although in the A-PRF group, more platelets were found in the distal part. Decreasing the rpm while increasing the centrifugation time in the A-PRF group gave an enhanced presence of neutrophilic granulocytes in the distal part of the clot. Thus, A-PRF might influence bone and soft tissue regeneration, especially through the presence of monocytes/macrophages and their growth factors.[24]

12. **Jemat A et al in 2015** conducted a review which covered several basic methodologies of surface treatment and their effects on titanium implants. Significant surface roughness played an important role in providing effective surface for bone implant contact, cell proliferation, and removal torque, despite having good mechanical properties. An acid etched surface-modified and a coating application on commercial pure titanium implant was most preferable in producing the good surface roughness. Thus, a combination of a good surface roughness and mechanical properties of titanium could lead to successful dental implants.[22]

13. **Amparo Mendoza-Arnau et al in 2016** conducted a study to characterize the surface topography of dental implants. A rough implant surface improves cellular adhesion however, too rough surface hinders osseointegration and biological response. An ideal Ra roughness range (0.775µm± 0.058µm) and Rt range (5.258µm ± 0.554) had been proposed. All the implant systems analyzed in this study had lower scores. This study also found that a medium

Ra achieved the best cell adhesion, coming to the conclusion that high roughness is not necessary in order to achieve the best cell response.[23]

14. **Eizaburo Kobayashi et al in the year 2016**, compared growth factor release over time from platelet-rich plasma (PRP), platelet-rich fibrin (PRF), and a modernized protocol for PRF, the advanced-PRF (A-PRF). Up to 10 days, it was found that A-PRF released the highest growth factors. Also, A-PRF released higher total protein accumulated over a 10-day period when compared to PRP or PRF. The results indicated that the new formulation of PRF (A-PRF) released higher total quantities of growth factors when compared to traditional PRF.[29]

15. **Ralf Smeets et al in the year 2016**, aimed to review various surface modifications of dental implants and their effect on osseointegration. The new techniques like Discrete Crystalline Deposition, laser ablation, and surface coatings with proteins, drugs, or growth factors were reviewed. It was concluded that advancements have been made in developing novel surfaces of dental implants. These innovations set the stage for rehabilitating patients with high success and predictable survival rates even in challenging conditions.[37]

16. **Johan Hartshorne et al in the year 2016** performed a comprehensive clinical review of Platelet Rich Fibrin (PRF). According to him, the use of PRF enables local delivery of a fibrin matrix, cells, growth factors, and proteins that provide unique biological properties for promoting new blood vessel formation, and potentially accelerating wound healing. PRF has an outstanding potential cost-to-benefit ratio., which is readily available,

inexpensive biomaterial that is beneficial in implant dentistry, oral surgery, and periodontal procedures.[11]

17. **Johan Hartshorne in the year 2016,** performed a comprehensive clinical review of Platelet Rich Fibrin (PRF) and its role in promoting tissue healing and regeneration. According to him, purpose of PRF technology is to extract the key elements from a patients' blood sample and to prepare it in a clinically usable form such as, a membrane or plug (A-PRF, L-PRF or CGF) or injectable liquid (i-PRF). The function of PRF is to connect the various elements within the fibrin matrix with local tissues (bone and soft tissue) to accelerate neo-angiogenesis within the tissue and to enhance its healing and regeneration potential.[10]

18. **Bansal S et al in the year 2017**, reviewed the merits and demerits of Platelet-rich plasma (PRP) and Platelet-rich fibrin (PRF) which is natural fibrin-based biomaterial prepared from an anticoagulant-free blood harvest without any artificial biochemical modification enriched with platelets and growth factors. The slow polymerization during centrifugation, fibrin-based structure, ease of preparation, minimal expense makes PRF somewhat superior in some aspect to PRP. It was concluded that the results obtained from PRF for various treatments are encouraging but still further studies are necessary to support its common use in day today practice with its clinical efficacy and long-term stability. Most importantly, establishing a scientifically sound, evidence-based rationale is critical to have the ultimate success of PRF.[43]

19. **Richard Miron et al in the year 2018,** calculated centrifugal g-forces at the PRF clot (referred to as relative centrifugal force [RCF]-clot) as opposed to

the international standard method described at the bottom of centrifugation tubes (RCF-max). It was further highlighted that RCF-clot is not only a deviation from the standard international method used to report g-force values, but one subject to significant error owing to centrifugation time, patient hematocrit levels, initial volume of blood collected, and many factors.[33]

20. **Ratajczak J et al in the year 2018,** aimed to fully characterize the angiogenic potential of L-PRF. With an antibody array, the growth factors released by L-PRF were determined and high levels of CXC chemokine receptor 2 (CXCR-2) ligands and epidermal growth factor (EGF) were found. L-PRF induced in vitro key steps of the angiogenic process: endothelial proliferation, migration and tube formation. It was concluded that L-PRF is induces blood vessel formation in vivo, when chorioallantoic membrane assay was performed. It was also concluded that because of the angiogenic capacity of L-PRF both in vitro and in vivo, it can be used as a potential biomaterial.[46]

21. **Strauss FJ et al in the year 2018**, performed a systematic review to assess the impact of platelet-rich fibrin (PRF) on implant dentistry. A total of 12 randomized controlled trials (RCT) were included in the study. It was concluded that there is moderate evidence supporting the clinical benefit of PRF on ridge preservation and in the early phase of osseointegration. It remains unclear whether PRF can reduce pain and improve soft tissue healing. More research support is necessary to comment on the role of PRF to improve other implant therapy outcomes. [45]

22. **Richard J. Miron et al in the year 2019,** compared 3 different commercially available centrifuges at both high and low g-force protocols. Two separate

protocols were tested on each machine including the original leucocyte and platelet-rich fibrin (L-PRF) protocol (~ 700 RCF max (~ 400 RCF clot) for 12 min) and the advanced platelet-rich fibrin (A-PRF+) protocol (~ 200 g RCF max (~ 130 g RCF clot) for 8 min). Tested groups were compared for cell numbers, growth factor release, scanning electron microscopy (SEM) for morphological differences, and clot size (both weight and length/width). This study demonstrated the reproducibility of a scientific concept (reduction in RCF produces PRF clots with more evenly distributed cells and growth factors) utilizing different devices.[27]

23. **Dara Ghaznavi et al in the year 2019**, evaluated the effect of advanced-platelet-rich fibrin modified by gold nanoparticles on the osteoblastic differentiation of human mesenchymal stem cells. MTT assay revealed 0.0125 mM gold nanoparticles had no cytotoxic effects on stem cells after 7 days. By using Alizarin Red S staining, red-colored calcium deposits were observed in the group treated with advanced-platelet-rich fibrin. Advanced-platelet-rich fibrin conditioned medium was unable to promote calcium deposition compared to the combination of advanced-platelet-rich fibrin and gold nanoparticles.[25]

24. **Staehlke S et al in the year 2019**, aimed of to present a systematic characterization of the physiological behavior of MG -63 cells in the range of passages five to thirty. Significant cell physiology processes during the first 24 hours, including cell morphology, availability of adhesion receptors, cell cycle phases, as well as the expression of the signaling proteins Akt, GSK3a/b, IkB - α, ERK1/2, p38-MAPK, and intracellular calcium ion mobilization, remained

stable over the entire range of passages P5-P30. It was concluded that due to stable characteristics in a wide range of cell culture passages, MG-63 cells can be considered as a suitable in vitro-model to analyze the biocompatibility and bio functionality of implant materials.[35]

25. **Liu Y et al in the year 2019**, reviewed the effect of PRF as a bone graft material on osteogenesis based on laboratory investigations, animal tests, and clinical evaluations. Modified PRF graft materials are further reviewed, including PRF combined with other bone graft materials, PRF combined with drugs, and a new-type PRF. It was concluded that PRF is an adjunct therapy for bone regeneration.[39]

26. **Thanasrisuebwong P et al in the year 2020**, examined two fractionation protocols producing yellow i-PRF and red i-PRF on periodontal ligament stem cells (PDLSCs). Cell proliferation and cell migration were examined with an MTT and trans-well assay. Osteogenic differentiation was analyzed using alkaline phosphatase and Alizarin red staining. The red i-PRF mobilizes healing-associated stem cells and promotes better proliferation of the mobilized cells, which is beneficial for bone regeneration. It was concluded that red i-PRF could be suitable for using in bone regeneration because it induced growth of bone regenerative cells without inducing premature mineralization.[41]

27. **Franz-Josef Strauss et al in the year 2020**, systematically assessed the effects of platelet-rich fibrin (PRF) on in vitro cellular behavior. Included studies show that PRF enhances proliferation, migration, adhesion, and osteogenic differentiation on a variety of cell types along with cell signaling

activation. Also, PRF reduces inflammation, suppresses osteoclastogenesis, and increases the expression of various growth factors in mesenchymal cells. The overall findings suggest that PRF induces cell proliferation, migration, adhesion, and differentiation along with possessing anti-inflammatory properties further supporting its therapeutic potential in wound healing and bone regeneration.[31]

28. **Zahra Kargarpour et al in the year 2020**, investigated the effect of soluble extracts of PRF membranes on in vitro osteoclastogenesis in murine bone marrow cultures. Osteoclastogenesis was evaluated by histochemical, gene expression, and resorption analysis. It was concluded PRF membranes inhibits the formation of osteoclasts from hematopoietic progenitors in bone marrow cultures. It was also implied that the favorable effects of PRF membranes in alveolar ridge preservation may be attributed, by the inhibition of osteoclastogenesis.[34]

29. **Gassling V et al in the year 2020,** aimed to compare PRF with the commonly used collagen membrane, Bio-Gides as scaffolds for periosteal tissue engineering. Cell vitality was assessed by fluorescein diacetate (FDA) and propidium iodide (PI) staining, biocompatibility with the lactate dehydrogenase (LDH) test, proliferation level with the MTT tests and scanning electron microscopy (SEM). It was concluded that PRF appears to be superior to collagen (Bio-Gides) as a scaffold for human periosteal cell proliferation. Also, PRF membranes are suitable for in vitro cultivation of periosteal cells for bone tissue engineering.[38]

30. **Lorenzo Bevilacqua et al in the year 2021,** conducted an invitro study which assessed the blood wettability of titanium implant surfaces favors primary osseointegration and tissue healing in peri-implantitis. They concluded that average wettability decreased after instrumentation, and increased after the PRF application to a superior level both to the first and second steps. The highest wettability was obtained after etching. This study concluded that PRF and phosphoric acid used for conditioning exposed implant surfaces can be used for the healing of peri-implant tissues. [26]

31. **Fabrice Clipet et al in the year 2021**, aimed to determine the relevance of Choukroun's platelet-rich fibrin (PRF) in dental implantology by evaluating the in vitro effects of soluble factors released by PRF clot. Cellular viability, cell proliferation, and gene expression were analyzed using PRF conditioned medium with cells lines SaOS2 (osteoblast), MRC5 (fibroblast), and KB (epithelial cell). It was concluded that PRF was useful in stimulating tissue healing and bone regeneration.[32]

32. **Rucha shah et al in the year 2021**, conducted an invitro study to check the osteoblastic response of osteoblast-like cell line (MG-63) with coating of injectable platelet-rich fibrin on titanium discs. Injectable-PRF (i-PRF) was prepared by centrifugation of blood at 700 rpm for 3 minutes without any anti-coagulant. Cell proliferation, alkaline phosphatase production, and were assessed in both groups at day 1, 7, 14, and 21. Mineralization was assessed at the end of day 21. It was concluded that coating of titanium discs with i-PRF causes increased proliferation, alkaline phosphatase production, and increased mineralization at day 1, 7, 14, and 21 in MG-63 osteoblast-like cells.[14]

33. **Blatt S, Thiem DG et al in the year 2021**, performed a study where four BSMs (bone substitute materials) of different origins (allogeneic, alloplastic, and xenogeneic) were biofunctionalized with PRF and compared to PRF in terms of platelet interaction and growth factor release. In vivo, it was concluded that vessel formation was increased for BSMs and PRF in comparison to the native control (allogeneic: $p = 0.046$; alloplastic: $p = 0.046$; and xenogeneic: $p = 0.050$). It was also concluded that the use of BSMs in combination with PRF triggers bony regeneration in clinical approaches.[40]

34. **Pascoal MD in the year 2021**, compared the resistance to the traction of membranes produced with the original L-PRF and A-PRF protocols. The prepared membranes were submitted to a traction test assessing the maximal and the average traction achieved for each membrane. The results showed that A-PRF obtained a value of 0.0288 N mm^2 and L-PRF 0.0192 N mm^2 ($p < 0.05$ using unpaired t test). It was concluded that A-PRF had a significant higher maximal traction score and higher average traction compared to L-PRF, indicating that it had a higher resistance when two opposing forces are applied.[12]

35. **Francesca Accioni et al in the year 2022**, reviewed latest trends in surface modification for dental implantology. According to this review implementation of organic or inorganic based coatings not only promote osseointegration but also act as excellent therapeutic agents. Growth Factors-Based Coatings are a group of molecules involved in the cell division and tissue proliferation. It was further added that main growth factors used in

implant coatings are bone morphogenetic proteins (BMPs) and vascular endothelial growth factor (VEGF).[8]

36. **Pedro Maria et al in the year 2022,** compared the tensile strength and analyzed the structural organization among the membranes produced by L-PRF (leukocyte platelet-rich fibrin), A-PRF (advanced platelet-rich fibrin), and A-PRF+ (advanced platelet-rich fibrin plus) that varied in centrifugation speed and time. L-PRF (n = 12), A-PRF (n = 19), and A-PRF+ (n = 13) membranes were submitted to a traction test, evaluating the maximum and average traction. A-PRF+ was the most porous platelet concentrate with the greatest fiber abundance and cell preservation. Thus, this study concluded that A-PRF+ produced membranes with significant higher maximum traction results, indicating a better viscoelastic strength when stretched by two opposing forces.[13]

MATERIALS AND METHODOLOGY

SOURCE OF DATA:

This in vitro study was conducted in Department of Prosthodontics and Crown & Bridge, KAHER's KLE Vishwanath Katti Institute of Dental Sciences (Surface treatment of the test specimens), Department of Mechanical Engineering, Gogte Institute of Technology, Belagavi, (For quantitative evaluation of surface roughness of test specimens), Department of physics, Shivaji university Kolhapur, (For qualitative evaluation of surface roughness and to evaluate mineralization) and KAHER's Dr. Prabhakar Kore's Basic Science Research centre, Belagavi,(For preparation of PRF and osteogenic analysis).

This research was intended to evaluate and compare the osteoblastic response of Advanced platelet-rich fibrin plus (A-PRF+) and Leucocyte platelet rich fibrin (L-PRF) coated titanium discs.

METHOD OF COLLECTION OF DATA:

INCLUSION CRITERIA:

- Identical titanium (Grade 5) disc shaped specimens measuring 10 mm in diameter and 2 mm in thickness. (ASTM B348).
- Specimens with surface irregularities. ($R_a < 4\mu m$).
- Dense and thick formed fibrin clots of L-PRF and A-PRF+.
- Specimens without internal and external porosities.

EXCLUSION CRITERIA:

- Non identical titanium discs with irregular diameter and thickness.
- Specimens with surface irregularities. (R_a> 4µm).
- Incompletely formed fibrin clots of L-PRF and A-PRF+.
- Specimens with internal and external porosities.

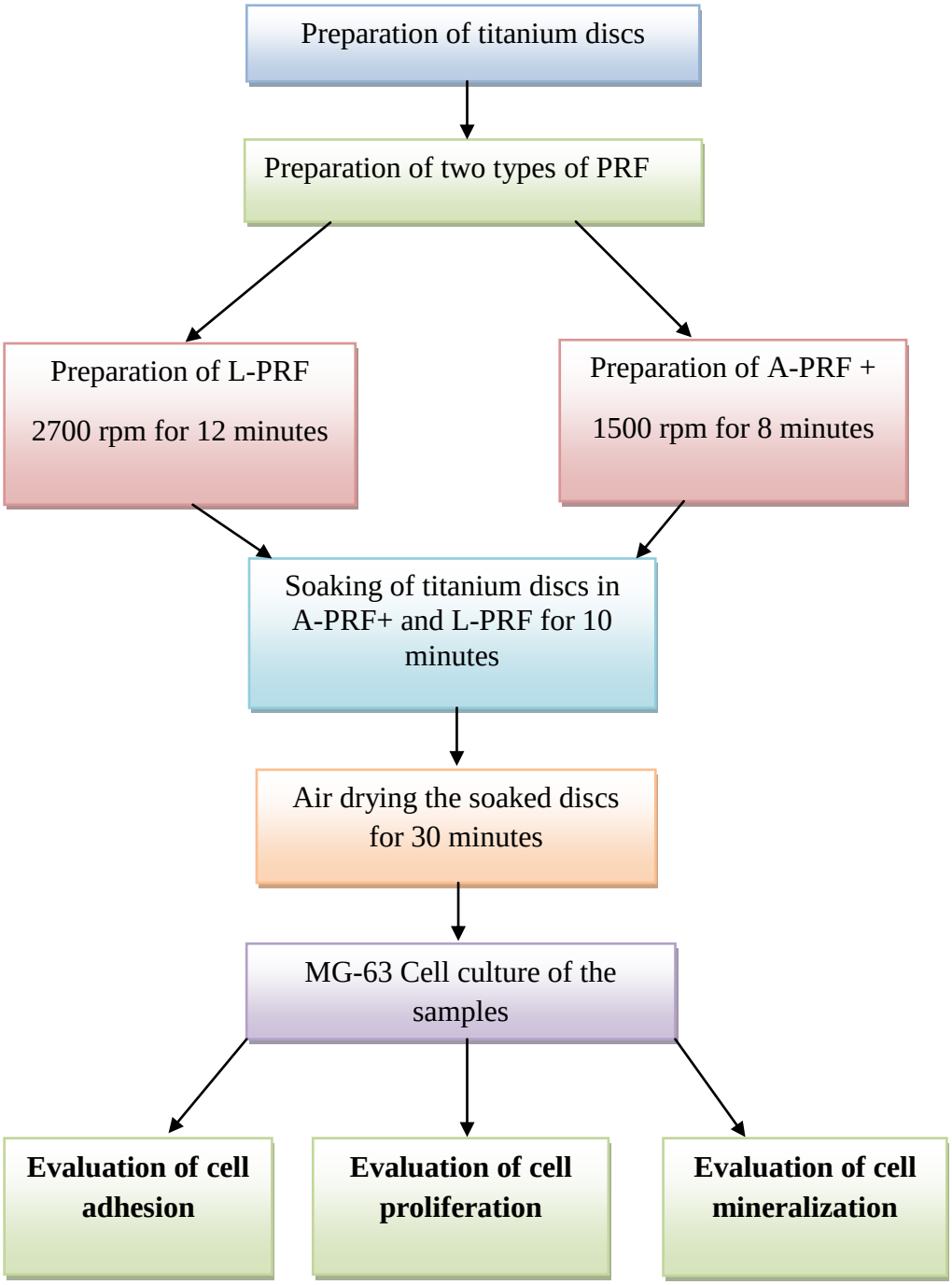
Table No. 1: - List of materials used in the study.

Material	Description	Manufacturer
Titanium Alloy	TYPE V (Ti-6Al-4V alloy)	Special Metals, Mumbai, India
Centrifuge tubes		Eppendorf, Mumbai, India
Phosphate buffer solvent (6.8 pH)	Sodium chloride, sodium dihydrogen orthophosphate dihydrate, potassium chloride, distilled water	Fisher Scientific, Pittsburgh, Pennsylvania.
Glacial Acetic acid	Product code: AS119	Hi Media, Mumbai, India
Distilled water	Batch No: -007M15	Rankem Chemicals, Avantor, India
Alizarin red S stain	Product code: CCK030	Hi Media, Mumbai, India
70% Ethanol	LOT No: -20151011	Changshu Hong sheng Fine Chemicals Co., Ltd.
Phosphate BufferSaline	LOT No.0000237353	Hi Media, Mumbai, India
Dulbecco's Eagle Medium	LOT No.0000284902	Hi media, Mumbai, India
Trypsin EDTA	LOT No.0000297540	Hi media, Mumbai, India
MTT Reagent	LOT No.0000173715	Hi Media, Mumbai, India
Tryphan Blue	LOT No: - 2024334	Hi Media, Mumbai, India
MG- 63 Cell Line		NCCS, Pune, India

Table No. 2: - Armamentarium used for evaluation of surface characteristics, & assessment of osteogenic potential of the test specimen.

Material	Description	Manufacturer
Centrifugation Machine	Model no :5418R	Eppendorf, Germany
Profilometer	Contact profilometer Model: - Surtronic S-128	Taylor Hobson,Brazil.
Scanning Electron Microscope	Model: JOEL JSM-6360	Japan
Laminar Air Flow	Model: - Vertical	Quesst International, Bangalore.
Micropipette	Model No: - 299932	Riviera Glass Pvt, Ltd., Mumbai, India
Tissue Culture Plate	24 well plate	Tarsons, Korea
Hemocytometer		KLEU/BSRC
Microscope	TCM400	LABOMED
CO2 Incubator	-	Eppendorf, Germany.
Micro Titre PlateReader	-	KLEU/BSRC/HF/2012-13

METHODOLOGY:



Preparation of specimen –

METHODOLOGY:

A total of 243 identical commercially available titanium Grade V, 10 mm x 2 mm (ASTM B348) disc shaped specimens were subdivided into three groups; one control and two study groups. (Figure 1).

1. Control group - Titanium Discs without PRF (platelet rich fibrin)
2. Study group 1 – Titanium discs treated with L-PRF (Leucocyte rich platelet rich fibrin)
3. Study group 2 -Titanium discs treated with A-PRF+ (Advanced platelet rich fibrin)

These identical titanium discs were sandblasted for 1 minute at a constant pressure of 4kg/cm^2 with 50 µm alumina. The discs were ultrasonically cleaned using acetone for 180 seconds to remove any residual contaminants.

1. Surface analysis –

The specimens in each group were numbered from 1 to 27. The specimens were subjected to surface roughness evaluation quantitatively and qualitatively. The quantitative analysis of surface roughness of the specimens was carried out using contact stylus profilometer (Surtronic S-128 – Taylor Hobson). An average roughness profile (R_a) was evaluated for each specimen to describe the overall roughness of the surface. Each disc was placed on a flat surface with surface to be tested facing upwards. The profilometer determined the surface profile along 3 lines on the surface by means of a tracking device. The disc specimens were mounted and diamond point-

stylus was made to run with a transverse length of 4 mm and cut off length of 0.8 mm and the arithmetical average value of all absolute distances of the roughness profile from the center line within the measuring length were calculated (Figure 2). Each group had 27 test specimens with their R_a measured by the profilometer. To evaluate the surface roughness qualitatively, random specimens from each sub group were examined under field emission Scanning Electron Microscope (SEM, Carl Zeiss). The SEM photographs were made at 100X, 500X and 1000X magnification for better visualization (Figure 3). The specimens were rinsed with distilled water, air dried, and fixed onto an aluminum cylinder (13 mm in diameter and 10 mm in height). The topographic observations of the polished surface were compared with each other as a complement to the quantitative results obtained by surface roughness assessment.

2. Preparation of L-PRF and A-PRF+ and coating of the titanium discs.

A single healthy volunteer (with no history of anticoagulant drug usage), between the age of 18 to 45 years participated in this study. Blood samples (9 cc) of peripheral blood was collected in sterile clear Eppendorf plastic tubes without any anticoagulant coating, under standard ambient conditions at 20 ± 2 °C and was placed immediately in a preprogrammed centrifuge machine (Eppendorf centrifuge 5418 R). L-PRF was prepared according to the original technique with centrifugation at 2700 revolutions per minute rpm (408 g), for 12 minutes (Figure 4a), and for the A-PRF+ preparation 1500 rpm (126 g) for 8 minutes (Figure 4b). The tubes were balanced by opposing tubes to equilibrate the centrifugation forces. After centrifugation, the tubes were placed in a sterile tube holder and the blood sample with clot was allowed to rest for approximately 4-8 minutes before extracting the clot from tube. The blood concentrate obtained after centrifugation had 3 distinct layers; red blood cell base at

the bottom, a PRF clot in the middle and an acellular plasma supernatant layer at the top. (Figure 5a and b). L- PRF and A-PRF+ clot was removed from the tube with a sterile tweezer. The fibrin clot was separated from the red blood cell fragment approximately 2mm below the dividing line using a scissor and was placed in two separate sterile petri dishes. Coating of titanium discs was done by immersing discs in respective PRF petri dishes for 10 minutes and then air dried for 30 minutes (Figure 6).

3. <u>Assessment of Osteogenic Potential</u>:

A total of the 243 titanium discs were used to assess the osteogenic potential. 81 titanium discs were coated with L-PRF which made up the study group 1, 81 titanium discs coated with A-PRF+ which made the study group 2, while the remaining 81 titanium discs without any PRF coating acted as a control. Out of 243, 81 titanium discs were used to assess the cell attachment (n_1=27, n_2=27, n_3=27) ,81 titanium discs were used to assess the cell proliferation (n_4=27, n_5=27, n_6=27) and the remaining 81 titanium discs were used to assess the cell mineralization (n_7=27, n_8=27, n_9=27) by Alizarin red S staining. Cell attachment was evaluated at 24 hours ($24n_1$=9 to $24n_3$=9), 48hours ($48n_1$=9 to $48n_3$=9) and 72 hours ($72n_1$=9 to $72n_3$=9). Similarly, cell proliferation was evaluated at 24 hours ($24n_4$=9 to $24n_6$=9) ,48hours ($48n_4$=9 to $48n_6$=9) and 72 hours ($72n_4$=9 to $72n_6$=9) and cell mineralization was evaluated using alizarin red S staining at day 7($7n_7$ =9 to$7n_9$ =9), day 14 ($14n_7$ =9 to$14n_9$ =9) and day 21($21n_7$ =9 to $21n_9$ =9) respectively. MG-63 cell lines (osteoblast like cells) were used to assess the osteogenic potential (Figure 7).

a) Cell attachment assessment -

The tryphan blue exclusion test was used to assess the number of viable cells. The viable cells with an intact cell membrane when mixed with dyes take up the dye and will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. The MG 63 cells were cultured onto specimens in the control and study group. (Figure 8a). 5 x 10^5 cells were seeded on each well on non-coated and coated titanium discs with LPRF and APRF+. Assays was performed in 12 well plates. The cell attachment assay was performed by considering control as non PRF coated discs (n_1=27) and test group as L-PRF coated discs (n_2=27) and APRF+ coated discs (n_3=27).

The culture medium was removed after 24 hours, 48hours and 72 hours' time interval and the wells were washed thrice using Phosphate-buffered solution (PBS) at 37ºC to eliminate unattached cells. Tryphan blue was added to the cell suspension. All the cells along with the discs were transferred into a facon tube and centrifugation was done twice with the discs and later without the discs. One milliliter of media was added to the obtained cell pellet and the adherent cells were enzymatically counted using a hemacytometer (Figure 8b). The unstained cells in the first set of 16 squares were counted followed by moving onto the next set. By trypan blue assay cell obtained were counted and entered in an excel spread sheet for data analysis. Cell attachment was expressed as the number of cells.

b) Cell Proliferation assessment –

The MTT solution was prepared using 5 mg MTT reagent in 1 ml of Phosphate Buffer Saline (PBS – pH 7.4). During the log phase of cell growth, MTT assay was performed. MTT assay was performed for 24 hours, 48 hours and 72 hours initially in 24 well plates and after MTT dye, it was transferred to 96 well plates. Initially on 24 well plates, markings were done by considering control, LPRF and APRF+. A 50µl of 1×10^5 cells/ml cell suspension was seeded into each well microtiter plate and final volume was made up to 1500 µl by adding DMEM media.100µl of the above dilutions was added to the wells and incubated for 72 hours, in presence of 5 % CO_2, at 37^0C into CO_2 incubator. After 72 hours, 200µl of 5 mg/ ml MTT reagent was added to the wells. The plate was wrapped in silver foil as MTT is photosensitive and incubated for four hours in a dark place at room temperature (Figure 9). The supernatant was removed without disturbing the precipitated Formazan crystals and 1000 µl of DMSO was added to dissolve the crystals formed. The optical density (OD) was measured using microtiter plate reader at a wavelength of 570 nm at 24,48 and 72 hours (Figure 10).

$$\text{Formula: Surviving cells (\%)} = \frac{\text{Mean OD of test compound}}{\text{Mean OD at control (untreated cells)}} \times 100$$

The cell attachment and cell proliferation in study and control group was evaluated as percentage (%), tabulated and subjected to statistical analysis to draw the conclusion from resultant data.

c) Cell mineralization

i. Alizarin Red S staining:

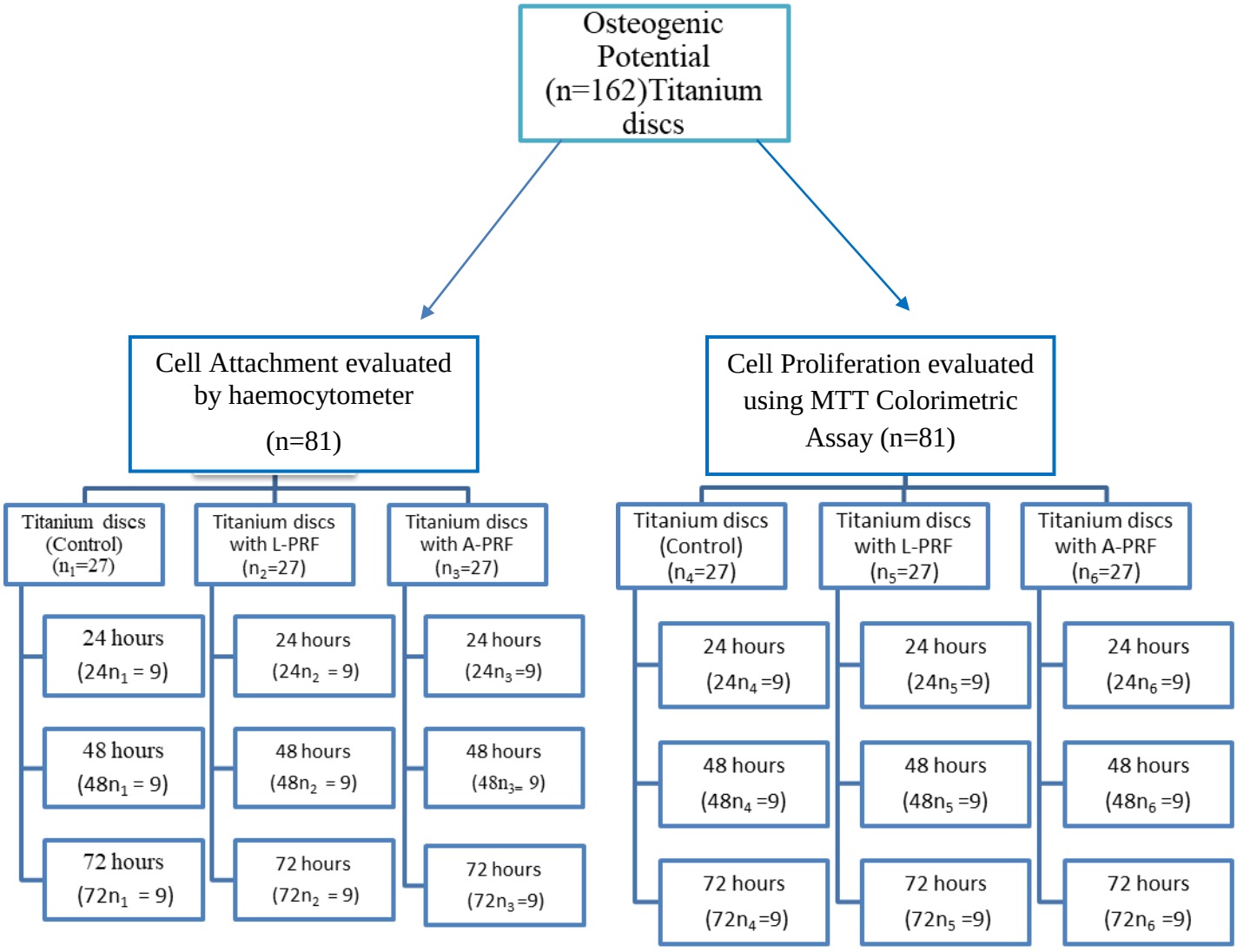
The osteogenic potential of LPRF and APRF+ on titanium discs was evaluated by Alizarin red assay on 7^{th}, 14^{th} and 21^{st} day. The assay was set up in 24 well plates. MG63 cells were seeded with density of $1x10^5$ on sterilized LPRF and APRF+ coated titanium discs. On each of the particular day, the supernatant media was removed and the discs along with cells were washed with PBS and fixed with 95% ethanol (200 µl) for 15 minutes at 4^0C. The cells were stained with 2% Alizarin red S (100µl, Ph 4.1-4.3) for 15 minutes (Figure 11). The formation of red staining was observed and was solubilized with 33% of glacial acetic acid (300 µl). The absorbance was measured by spectrophotometer at 415nm. The OD reading was calculated as OD of test with OD of control multiplied by 100.

$$\text{Absorbance} = \frac{\text{Mean OD of test compound}}{\text{Mean OD at control}} \text{ X } 100$$

The qualitative assessment was done by examining the staining under inverted light microscopy to observe calcified deposits (Figure 12) along with scanning electron microscopy to evaluate mineralized crystals at day 14 and 21 (Figure 13, 14) and the quantitative analysis was done by colorimetric assay.

ii. Scanning electron microscopy

Scanning electron microscopy of study group1 (L-PRF) and study group 2 (APRF+) with MG63 cell cultures undergoing differentiation was performed at day 14 and day 21 of culture since mineralization starts after a week of culture. The PRF coated titanium discs were first fixed with 100 µl 4% paraformaldehyde for 1 hour, then dehydrated in ethanol and gold sputter-coated with a 20 nm gold layer, SEM images were captured at accelerating voltages between 15 and 25 kV. Fixing was done with 100 µl 4% paraformaldehyde followed by scanning electron microscopy at day 14 and day 21 to evaluate mineralization .


Osteogenic Potential (n=162)Titanium discs
Cell Attachment evaluated by haemocytometer (n=81)
Cell Proliferation evaluated using MTT Colorimetric Assay (n=81)
Titanium discs (Control) (n_1=27)
Titanium discs with L-PRF (n_2=27)
Titanium discs with A-PRF (n_3=27)
Titanium discs (Control) (n_4=27)
Titanium discs with L-PRF (n_5=27)
Titanium discs with A-PRF (n_6=27)
24 hours ($24n_1$ = 9)
48 hours ($48n_1$ = 9)
72 hours ($72n_1$ = 9)
24 hours ($24n_2$ = 9)
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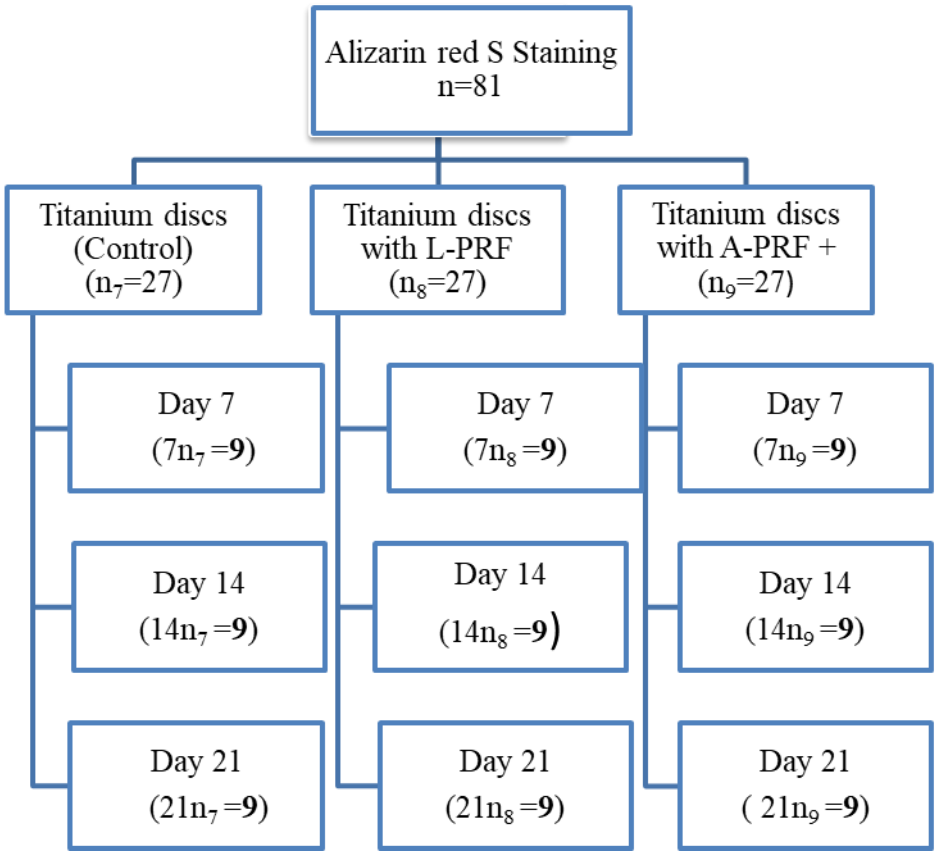
Alizarin red S Staining
n=81
Titanium discs
(Control)
(n_7=27)
Titanium discs
with L-PRF
(n_8=27)
Titanium discs
with A-PRF +
(n_9=27)
Day 7
($7n_7$ =**9**)
Day 7
($7n_8$ =**9**)
Day 7
($7n_9$ =**9**)
Day 14
($14n_7$ =**9**)
Day 14
($14n_8$ =**9**)
Day 14
($14n_9$ =**9**)
Day 21
($21n_7$ =**9**)
Day 21
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Day 21
($21n_9$ =**9**)

FIGURE NO 1: DISC SHAPED TITANIUM SPECIMENS

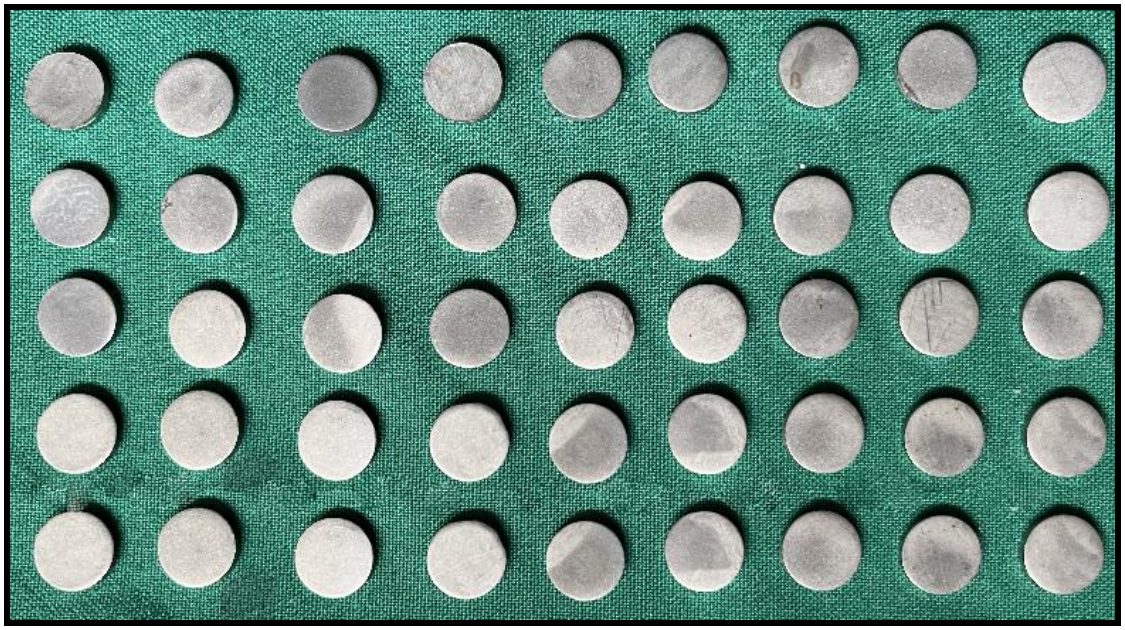


FIGURE NO 2: QUANTITATIVE EVALUATION OF SURFACE ROUGHNESS OF SPECIMEN USING PROFILOMETER

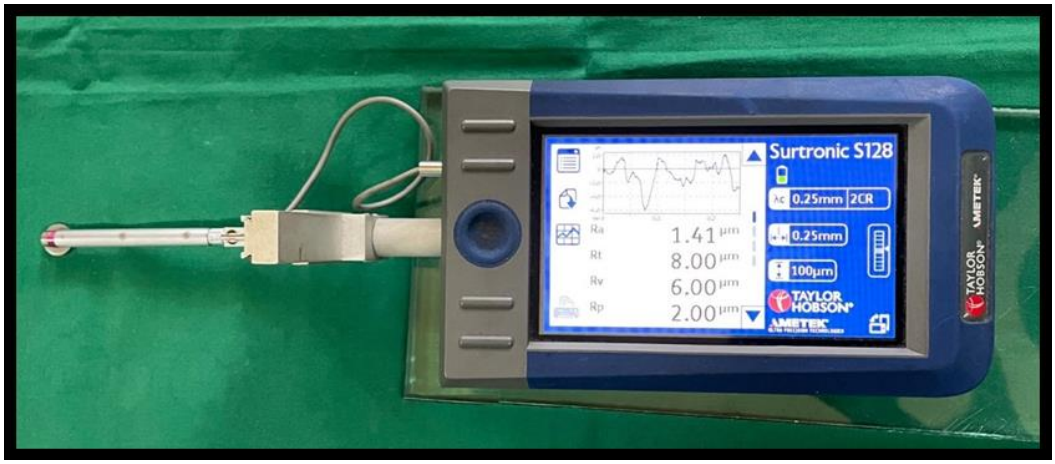
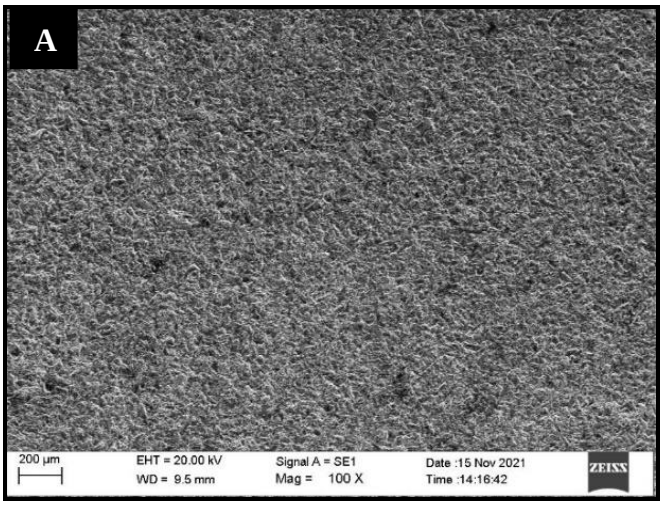


FIGURE NO 3: QUALITATIVE EVALUATION OF SURFACE ROUGHNESS OF SPECIMEN USING SCANNING ELECTRON MICROSCOPY.



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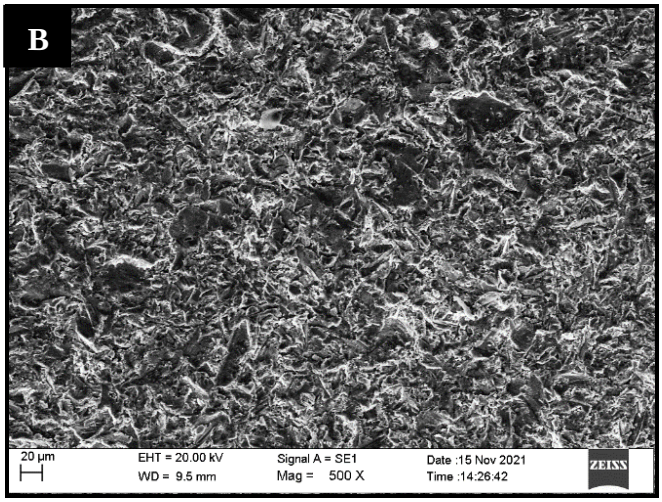

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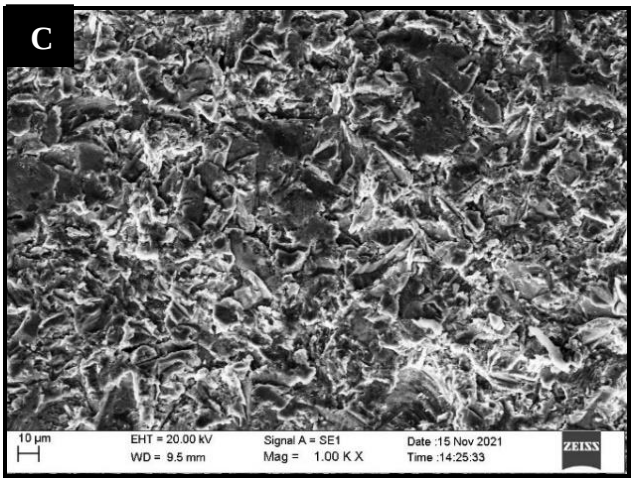


C:1000X

FIGURE NO 4: PROTOCOL FOR PREPARATION OF PLATELET RICH FIBRIN

A) LPRF **B) APRF +**

FIGURE NO 5: PLATELET RICH FIBRIN

A) LAYERS OF PRF **B) TYPES OF PRF**

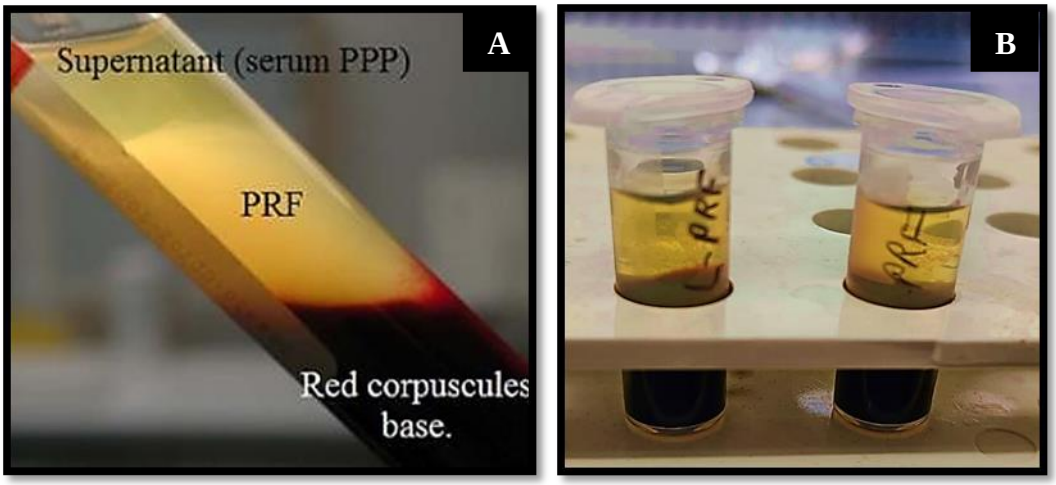


FIGURE NO 6: COATING OF TITANIUM DISCS WITH PLATELET RICH FIBRIN BY DIPPING METHOD

FIGURE NO 7: MG-63 CELL LINES USED TO ASSESS OSTEOGENIC POTENTIAL

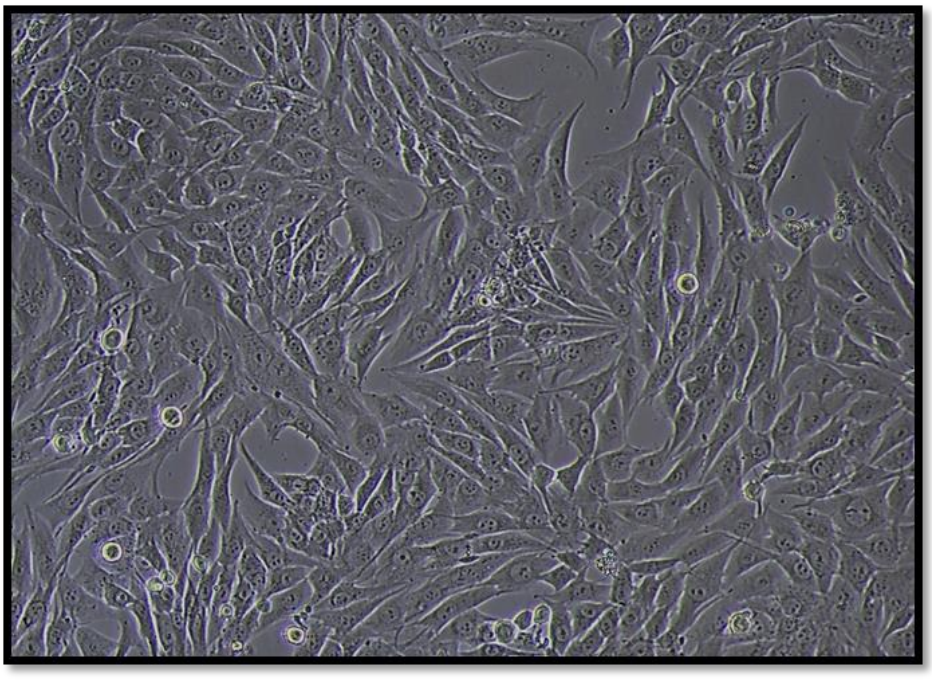
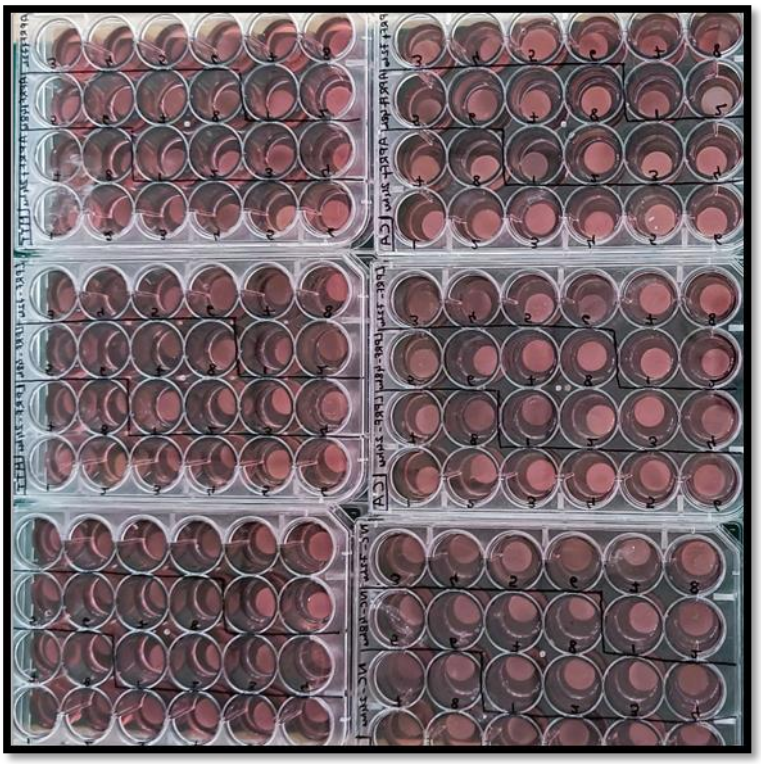


FIGURE NO 8:

A) SPECIMENS SEEDED WITH MG-63 CELLS FOR ASSESSING CELL ATTACHMENT



B) EVALUATION OF ATTACHED CELLS ON HEMOCYTOMETER

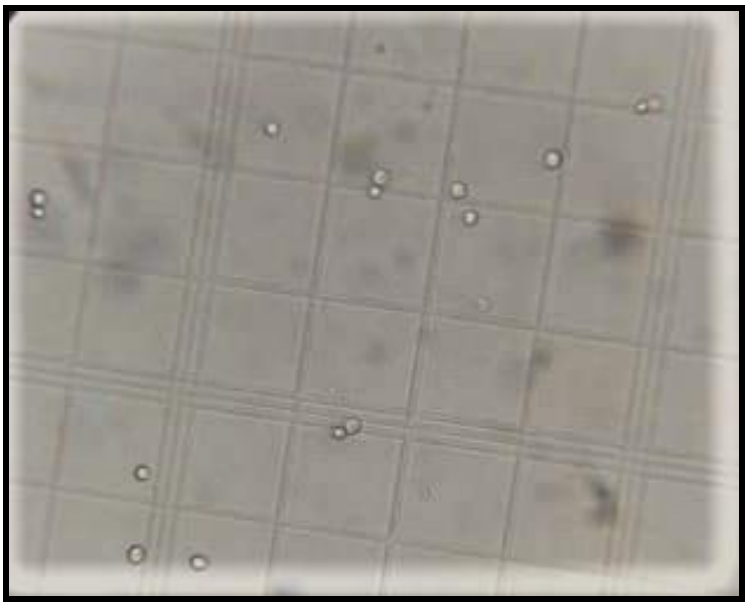


FIGURE NO 9: WELL PLATE WRAPPED IN ALUMINIUM FOIL FOR MTT ASSAY TO ASSESS CELL PROLIFERATION

FIGURE NO 10: MTT ASSAY TO ASSESS CELL PROLIFERATION OF THE STUDY AND CONTROL GROUPS AT 24,48 ,72 HOURS

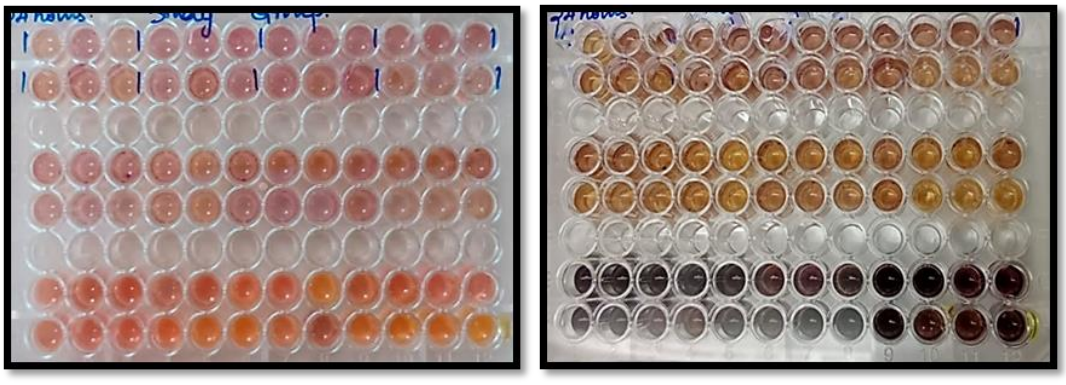
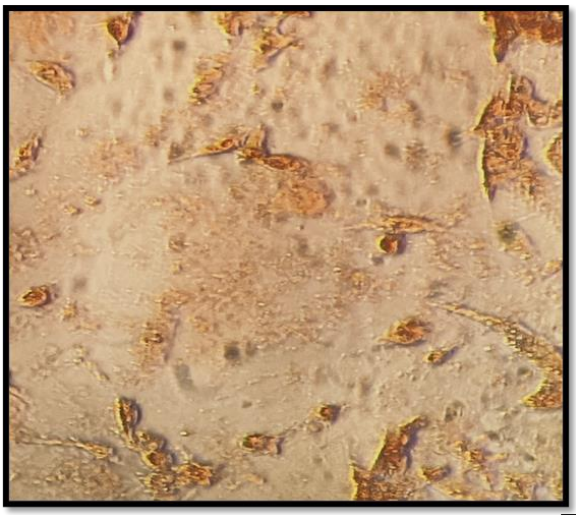


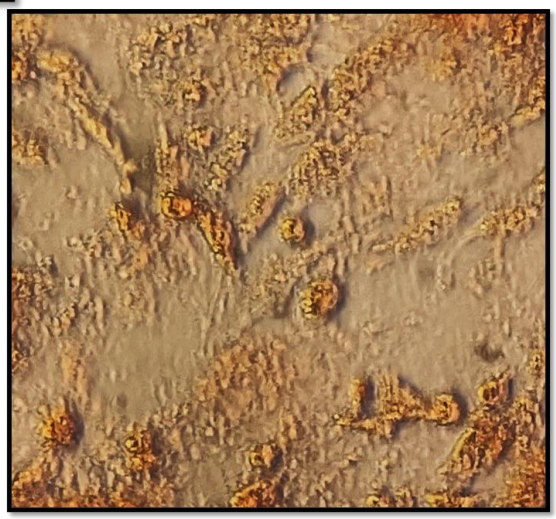
FIGURE NO 11: STAINING OF MG-63 CELLS BY ALIZARIN RED -S TO ASSESS CELL MINERALIZATION OF THE STUDY AND CONTROL GROUPS AT DAY 7, DAY 14 AND DAY 21.

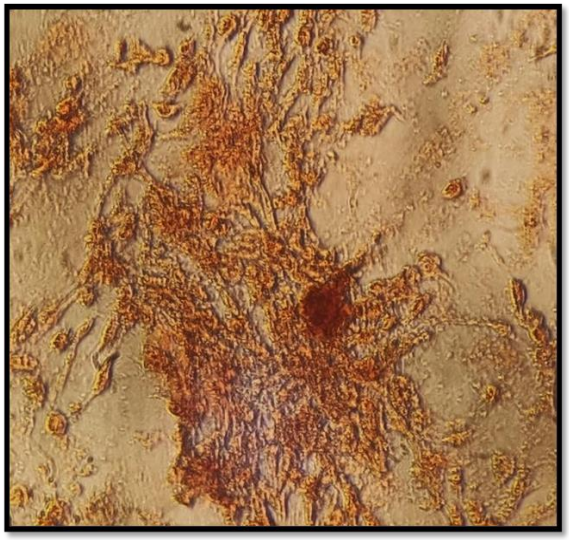
FIGURE NO 12: QUALITATIVE EVALUATION BY ALIZARIN RED – S STAINING TO ASSESS CELL MINERALIZATION OF THE STUDY AND CONTROL GROUPS USING INVERTED LIGHT MICROSCOPE (4X)



CONTROL

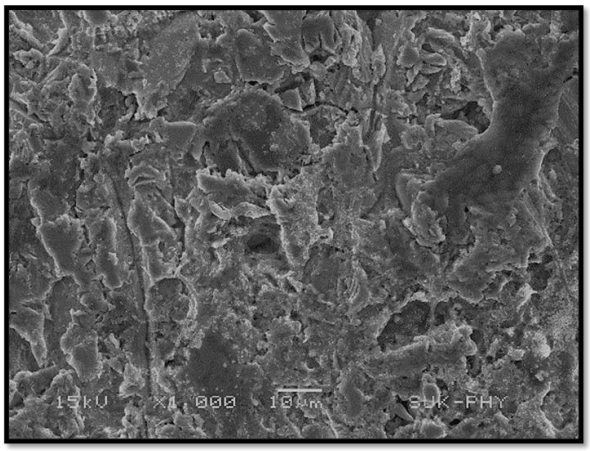
STUDY GROUP 1





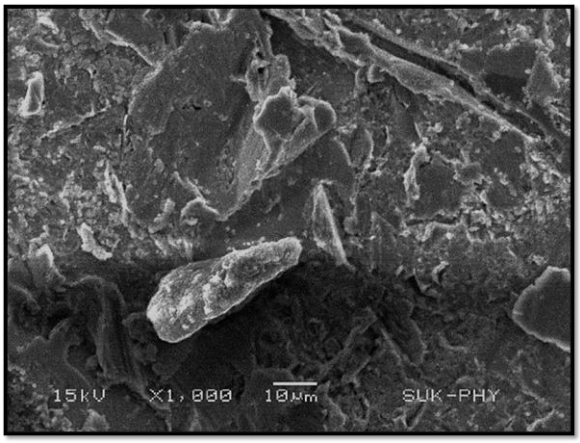
STUDY GROUP 2

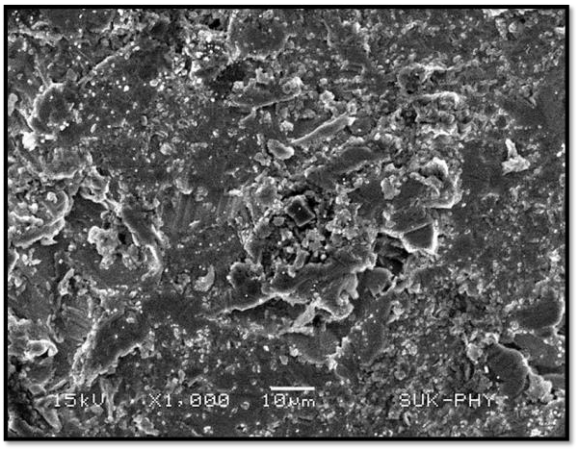
FIGURE NO 13: QUALITATIVE EVALUATION OF CELL MINERALIZATION OF THE STUDY AND CONTROL GROUPS USING SCANNING ELECTRON MICROSCOPY AT DAY 14 (1000X).



CONTROL

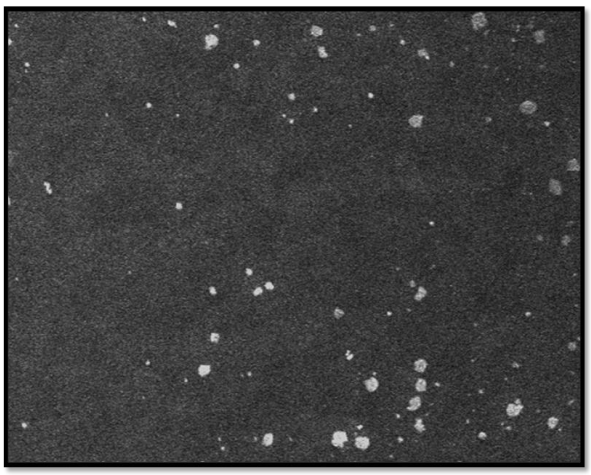
STUDY GROUP 1





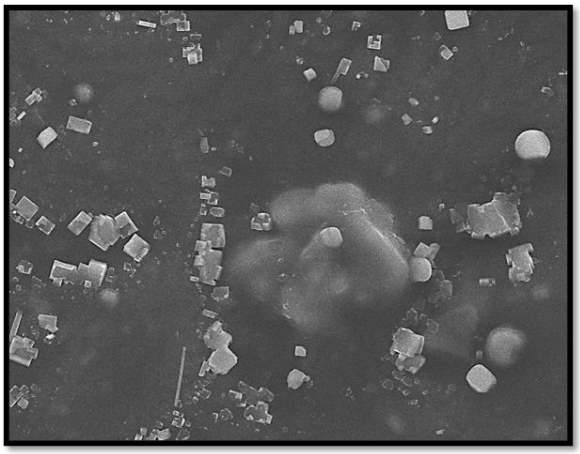
STUDY GROUP 2

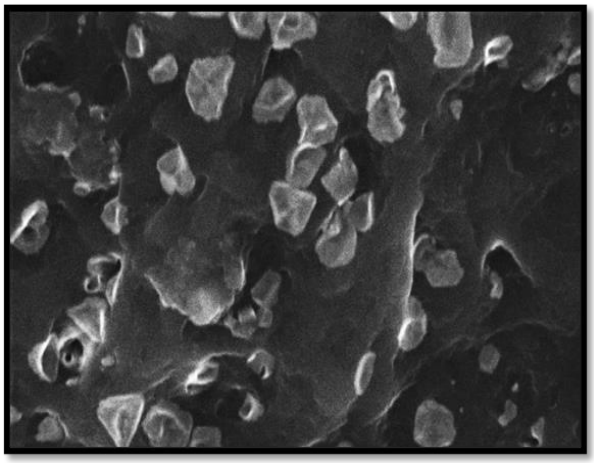
FIGURE NO 14: QUALITATIVE EVALUATION OF CELL MINERALIZATION OF THE STUDY AND CONTROL GROUPS USING SCANNING ELECTRON MICROSCOPY AT DAY 21 (1000X).



CONTROL

STUDY GROUP 1

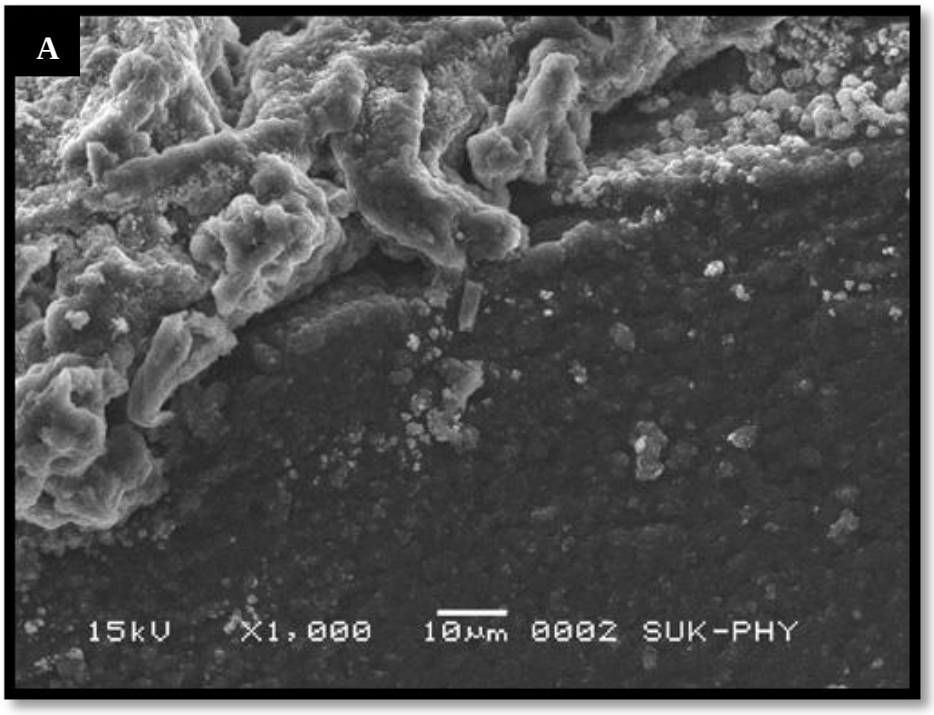


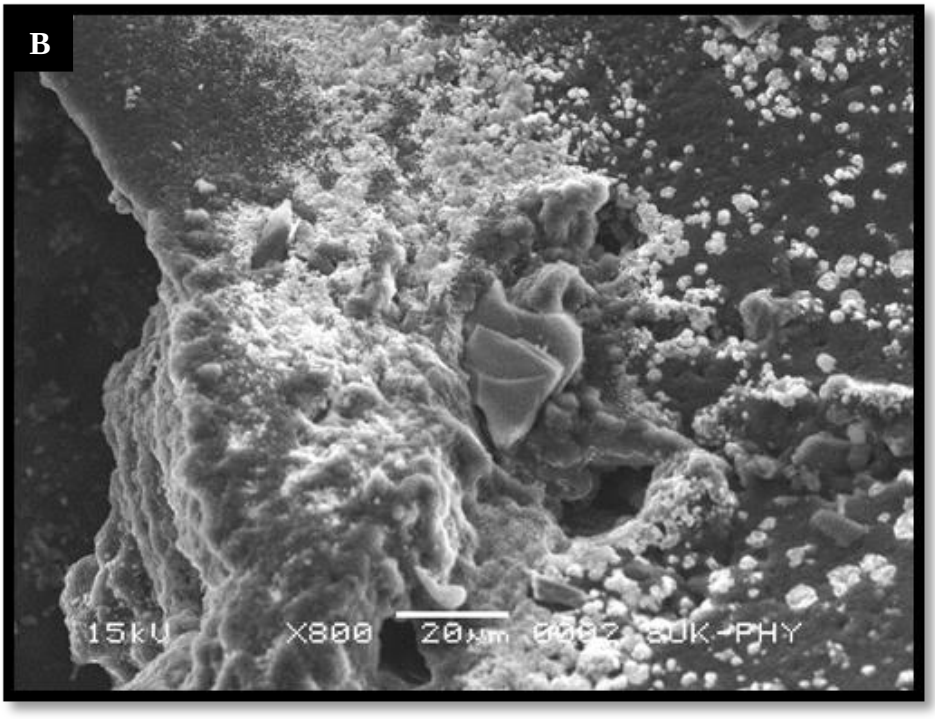


STUDY GROUP 2

FIGURE NO 15: PLATELET RICH FIBRIN LAYER WITH NETWORK OF FIBRIN OBSERVED UNDER SCANNING ELECTRON MICROSCOPE.

A)1000X and B) 800X





RESULTS

This present study evaluated and compared the osteoblastic efficacy of Advanced platelet-rich fibrin plus (A-PRF+) and Leucocyte platelet rich fibrin (L-PRF) coated titanium discs. Osteogenic potential was evaluated by assessing the cell attachment, cell proliferation of MG-63 cells on PRF coated titanium discs at 24hours, 48hours, 72-hour time intervals and mineralization was assessed at day 7, day 14 and day 21. For its osteogenic potential, the cell attachment was assessed using hemacytometer while cell proliferation was assessed by MTT Assay at 24hours, 48hours, 72 hours and cell mineralization was assessed by Alizarin red-S staining at day 7, day 14 and day 21.

CELL ATTACHMENT

Table 3: Shows the summary of cell attachment scores in three groups i.e., noncoated titanium disc, LPRF and APRF+ at three different time intervals (24hours, 48hours and 72hours).

Table 4: Shows the comparison of the noncoated titanium discs, LPRF and APRF + discs at three-time intervals (24hours, 48hours and 72hours) with cell attachment scores by Two-way ANOVA.

Table 5 and Graph 1: Shows the pair wise comparison of three groups with cell attachment scores by Tukeys multiple posthoc procedures.

Table 6 and graph 2: Shows the pair wise comparison of three-time intervals (24hours, 48hours and 72hours) with cell attachment scores by Tukeys multiple posthoc procedures.

Table 7 and graph 3: Shows summary of the pair wise comparison of interactions between three groups, and three-time intervals (24hours, 48hours and 72hours) with cell attachment scores by Tukeys multiple posthoc procedures.

Graph 4 and graph 5: Depicts time trend graphs showing relationship between three groups and three-time intervals with respect to cell attachment.

CELL PROLIFERATION

Table 8 and graph 6: Shows the comparison of study group and control group with percentage of cell proliferation at different treatment time intervals of 24, 48, 72 hours.

Table 9 and graph 7: Shows the comparison of three groups at three-time intervals (24hours, 48hours and 72hours) with cell proliferation scores by Two-way ANOVA.

Table 10 and graph 8: Shows the pair wise comparison of interactions between three groups at three-time intervals (24hours, 48hours and 72hours) with cell proliferation scores by Tukeys multiple posthoc procedures.

Graph 9 and graph 10: Depicts time trend graphs showing relationship between three groups at three-time intervals with respect to cell proliferation.

CELL MINERALIZATION

Table 11: Shows summary of Alizarin red assay scores of three groups (noncoated titanium disc, LPRF and APRF +) at three-time intervals (Day 7, Day 14, Day 21).

Table 12: Shows the Comparison of three groups and three-time intervals (Day 7, Day 14, Day 21) with Alizarin red assay scores by Two-way ANOVA.

Table 13 and graph 11: Shows pair wise comparison of three groups with Alizarin red assay scores by Tukeys multiple posthoc procedures.

Table 14 and graph 12: Shows pair wise comparison of three-time intervals (Day 7, Day 14, Day 21) with Alizarin red assay scores by Tukeys multiple posthoc procedures.

Table 15 and graph 13: Shows pair wise comparison of interactions between three groups and three-time intervals (Day 7, Day 14 Day 21) with Alizarin red assay scores by Tukeys multiple posthoc procedures.

Graph 14 depicts a time trend graph showing effects of three groups on different time intervals with respect to alizarin red assay.

The resultant values of osteogenic potential by means of cell attachment (number of cells), cell proliferation (%) of specimens and alizarin red assay (%) in Study Groups and Control Group were subjected to statistical analysis to draw conclusion from the experimental data. Descriptive statistical measures such as mean, standard deviation was computed for all the groups. Comparison of three groups that is noncoated titanium disc, LPRF and APRF + at three-time intervals (24hours, 48

hours and 72hours) with cell attachment scores, cell proliferation and alizarin red assay (day 7, day 14 and day 21) was done by Two-way ANOVA. Pair wise comparison of three groups with cell attachment scores, cell proliferation and alizarin red assay was done by Tukeys multiple posthoc procedures. To study the effect of three groups i.e., noncoated titanium disc, LPRF and APRF + on different time intervals (24hours, 48hours and 72hours) with cell attachment scores, cell proliferation and alizarin red assay (day 7, day 14 and day 21), a time trend graph was plotted. The p value of <0.05 was considered statistically significant.

The mean and standard deviation was calculated for the study groups (titanium discs coated with LPRF and APRF +) and control group (noncoated titanium disc).

CELL ATTACHMENT

Pair wise comparison of three groups with cell attachment scores were carried out by Tukeys multiple posthoc procedures. The mean values of the cell attachment of the number of cells in the study group 1 LPRF coated titanium discs was (870000 ± 365447), study group 2 APRF+ (1351481±298805) compared to control group (1453388±72693), thus showing statistically significant difference between them (Table 5 and graph 1).

The mean and standard deviation for the cell attachment on (titanium discs coated with , LPRF and APRF +) and control group (noncoated titanium disc) was calculated by Tukeys multiple posthoc test .The mean values of the cell attachment of the number of cells in the study group 1 LPRF coated titanium discs was (1360000 ± 2783.88) at 24 hours, 48 hours (1704444 ±54108.9) and 72 hours (990000 ± 1.22)

and study group 2 APRF + coated titanium discs was (1380000 ± 2783.88) at 24 hours, 48 hours (1430166.7±316.23) and 72 hours (1550000 ± 1.22) were statistically significant (p = 0.0001*) as compared to the control group showing an increased cell attachment in the study group at all three-time intervals. (Table 7 & Graph 3).

CELL PROLIFERATION

The mean and standard deviation of cell proliferation for the study groups (titanium discs coated with, LPRF and APRF +) and control group (noncoated titanium disc) was calculated by Two-way ANOVA and pairwise comparison between them was done by tukeys multiple posthoc test.

The mean values of the cell proliferation in the study group 1 (LPRF) was (97 ± 0.11) at 24 hours, 48 hours (100 ±1.22) and 72 hours (116 ± 1.41) , study group 2 (APRF +) was (307 ± 1.87) at 24 hours, 48 hours (250 ±1.47) and 72 hours (307± 1.87) were statistically significant (p = 0.0001*) as compared to the control group showing an increased cell proliferation in the study groups .The results concluded that APRF+ coated titanium discs showed increase in proliferation of MG63 cell lines from 24 hours to 72 hours than compared to LPRF, thus showing that APRF+ helps in accelerated proliferation of MG63 cell lines when compared to LPRF (Table 10 & Graph 8).

CELL MINERALIZATION

The mean and standard deviation of alizarin red assay for mineralization for the study groups (titanium discs coated with, LPRF and APRF +) and control group (noncoated titanium disc) was calculated by Two-way ANOVA and pairwise comparison between them was done by tukeys multiple posthoc test.

The mean values of the alizarin red staining for the study group 1 (LPRF) was (48 ± 2.83) at day 7 , day 14 (86±1.22) and day 21 (124 ± 1.22) , study group 2 (APRF +) was (65 ± 2.24) at day 7, day 14 (132 ±1.22) and day 21 (145± 1.87) were statistically significant (p = 0.0001*) as compared to the control group showing an increased calcium deposits in the study groups .The results concluded that the APRF+ coated titanium discs showed highest calcium rich deposits than compared to LPRF coated titanium discs, thus APRF+ can help in enhanced mineralization (table 15 and graph 13).

CELL ATTACHMENT

Table 3: Summary of cell attachment scores in three groups that is noncoated titanium disc, LPRF and APRF + at three-time intervals (24hours, 48hours and 72hours)

Main	Levels	Mean	SD	SE	95% CI for mean	
					Lower	Upper
Groups	Titanium disc	870000.00	365447.04	70330.32	725433.97	1014566.03
	L PRF	1351481.48	298805.49	57505.14	1233277.97	1469685.00
	APRF+	1453388.89	72693.50	13989.87	1424632.30	1482145.48
Times	24hours	1070000.00	432434.34	83222.03	898934.67	1241065.33
	48hours	1491537.04	160769.70	30940.14	1427938.66	1555135.41
	72hours	1113333.33	324429.44	62436.47	984993.32	1241673.35
Interactions	Titanium disc with 24hours	470000.00	2783.88	927.96	467860.12	472139.88
	Titanium disc with 48hours	1340000.00	2783.88	927.96	1337860.12	1342139.88
	Titanium disc with 72hours	800000.00	1414.21	471.40	798912.94	801087.06
	L PRF with 24hours	1360000.00	2783.88	927.96	1357860.12	1362139.88
	L PRF with 48hours	1704444.44	54108.94	18036.31	1662852.63	1746036.26
	L PRF with 72hours	990000.00	1.22	0.41	989999.06	990000.94
	APRF+ with 24hours	1380000.00	2783.88	927.96	1377860.12	1382139.88
	APRF+ with 48hours	1430166.67	316.23	105.41	1429923.59	1430409.74
	APRF+ with 72hours	1550000.00	1.22	0.41	1549999.06	1550000.94

Table 4: Comparison of three groups that is noncoated titanium disc, LPRF and APRF + at three-time intervals (24hours, 48hours and 72hours) with cell attachment scores by Two-way ANOVA

Sources of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value	p-value
Main effects					
Groups	5242969191358.00	2	2621484595679.0	7968.3672	0.0001*
Times	2903483635802.00	2	1451741817901.0	4412.7713	0.0001*
2-way interaction effects					
Groups*Times	3003965049383.00	4	750991262346.00	2282.7424	0.0001*
Error	23687022246.00	72	328986420.00		
Total	11174104898789.0	80			

*$p<0.05$

This table concluded that there was a significant difference in the cell attachment scores between the Study and Control Groups.

Table 5: Pair wise comparison of three groups with cell attachment scores by Tukeys multiple posthoc procedures

Groups	Titanium disc	L PRF	APRF+
Mean	870000.00	1351481.48	1453388.89
SD	365447.04	298805.49	72693.50
Titanium disc	-		
L PRF	P=0.0001*	-	
APRF+	P=0.0001*	P=0.0001*	-

*p<0.05

Graph 1: Pair wise comparison of three groups with cell attachment scores

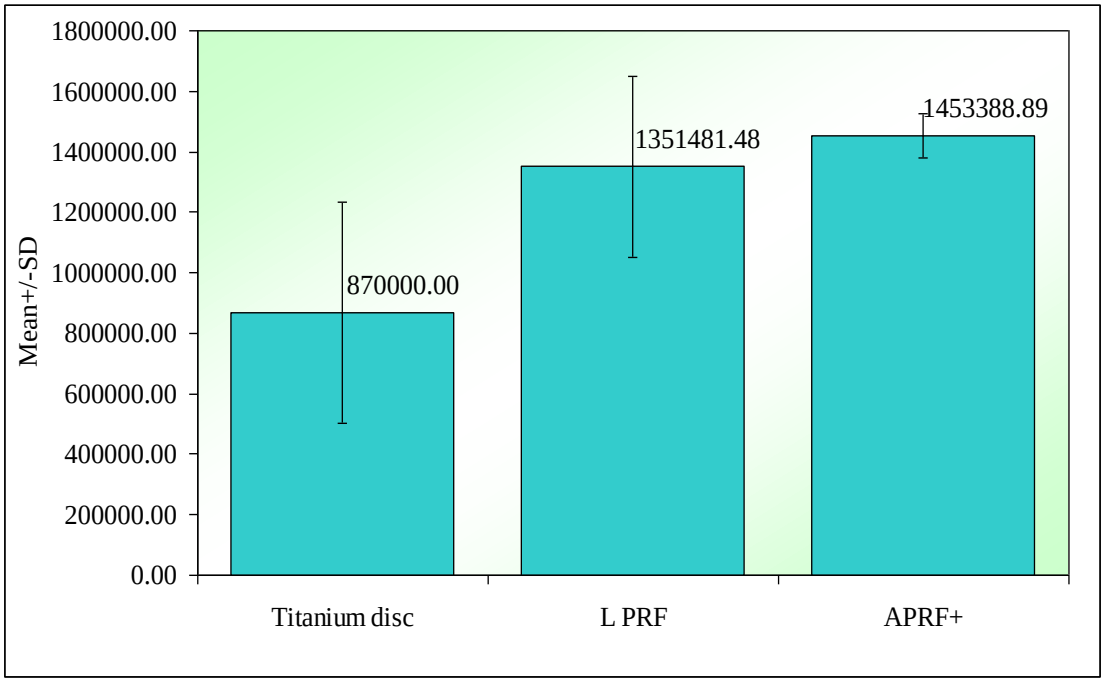


Table 6: Pair wise comparison of three-time intervals (24hours, 48hours and 72hours) with cell attachment scores by Tukeys multiple posthoc procedures

Times	24hours	48hours	72hours
Mean	1070000.00	1491537.04	1113333.33
SD	432434.34	160769.70	324429.44
24hours	-		
48hours	P=0.0001*	-	
72hours	P=0.0001*	P=0.0001*	-

*p<0.05

This table demonstrates statistically significant difference in the cell attachment between the three groups that is noncoated titanium, LPRF and APRF + at 24,48,72 hours. Also, there was a statistically significant difference in the cell attachment between 24-48- and 24-72-hour time intervals between the groups.

Graph 2: Comparison of three time intervals (24hours, 48hours and 72hours) with cell attachment scores

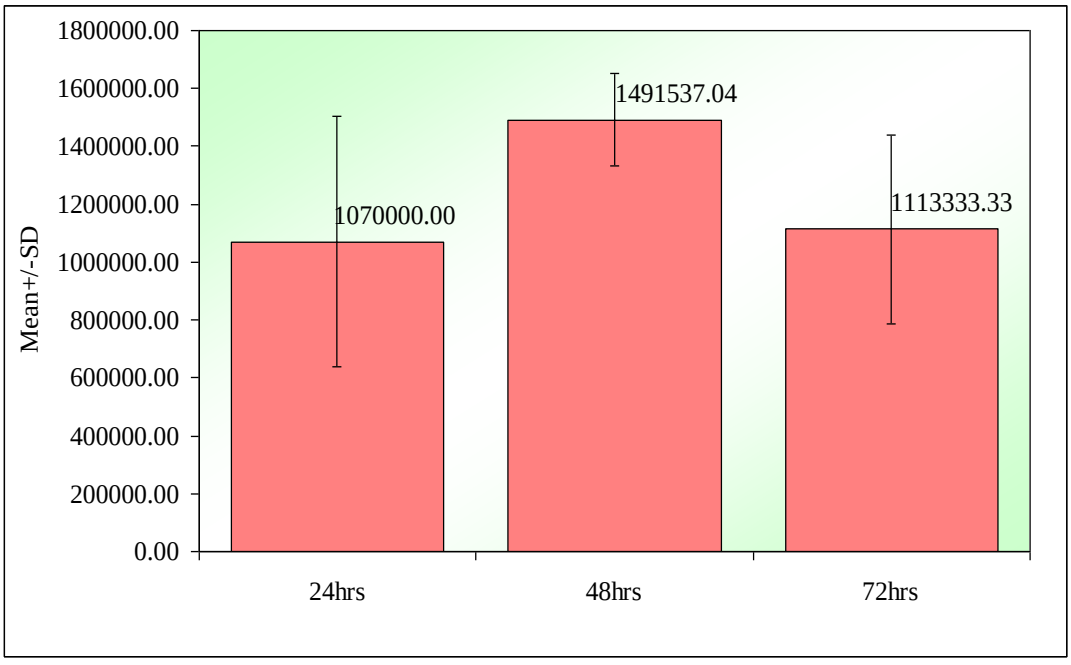


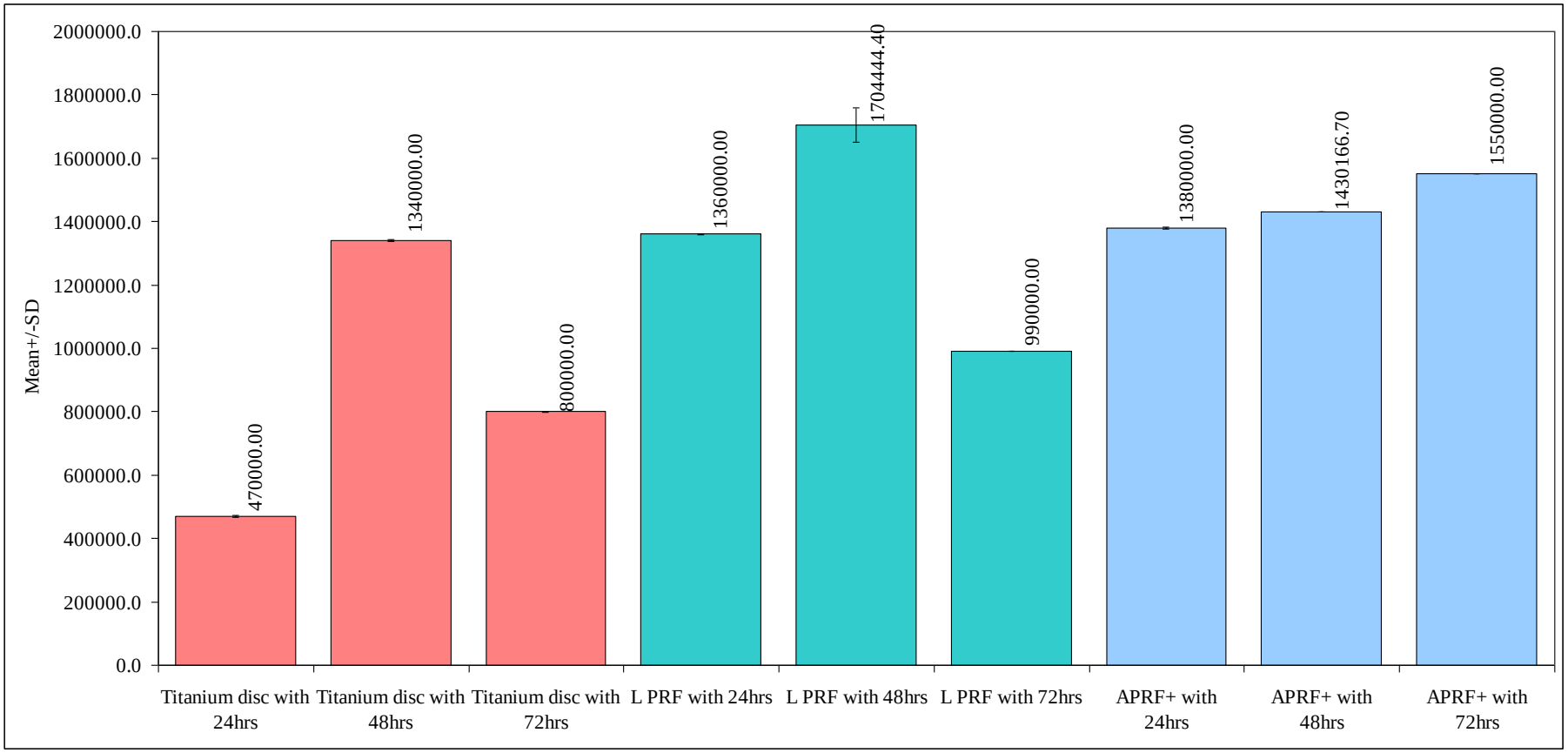
Table 7: Pair wise comparison of interactions between three groups and three time intervals (24hours, 48 hours and 72hours) with cell attachment scores by Tukeys multiple posthoc procedures

Interactions	Titanium disc with 24hours	Titanium disc with 48hours	Titanium disc with 72hours	L PRF with 24hours	L PRF with 48hours	L PRF with 72hours	APRF+ with 24hours	APRF+ with 48hours	APRF+ with 72hours
Mean	470000.0	1340000.0	800000.0	1360000.0	1704444.4	990000.0	1380000.0	1430166.7	1550000.0
SD	2783.88	2783.88	1414.21	2783.88	54108.94	1.22	2783.88	316.23	1.22
Titanium disc with 24hours	-								
Titanium disc with 48hours	p=0.0001*	-							
Titanium disc with 72hours	p=0.0001*	p=0.0001*	-						
L PRF with 24hours	p=0.0001*	p=0.3335	p=0.0001*	-					
L PRF with 48hours	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	-				
L PRF with 72hours	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	-			
APRF+ with 24hours	p=0.0001*	p=0.0005*	p=0.0001*	p=0.3335	p=0.0001*	p=0.0001*	-		
APRF+ with 48hours	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	-	
APRF+ with 72hours	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	-

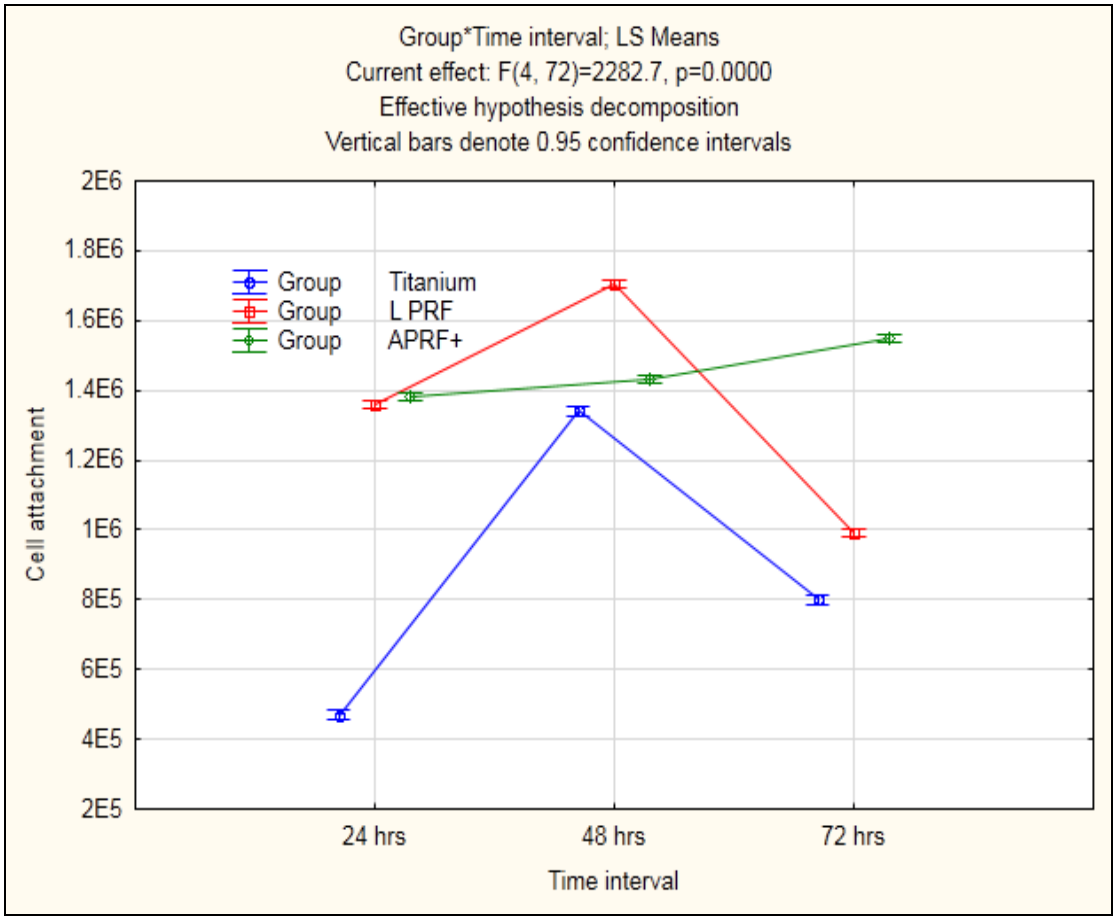
*p<0.05

This table shows Pair wise comparison of interactions between three groups (non-coated titanium discs, LPRF and APRF +) at three-time intervals (24hours, 48hours and 72hours) with cell attachment scores by Tukeys multiple posthoc procedures. This table also concludes that after seeding $5x10^5$ cells per well, it was found that at 24 hours a greater number of attached cells in APRF+ group (13,80,000 cells) were present whereas at 48 hours maximum number of cells were seen in LPRF group (17,00,000 cells) and at 72 hours maximum number of cells were attached to APRF+ coated discs (15,50,000 cells). Thus APRF+ coated discs have more propensity of attachment of MG63 cells.

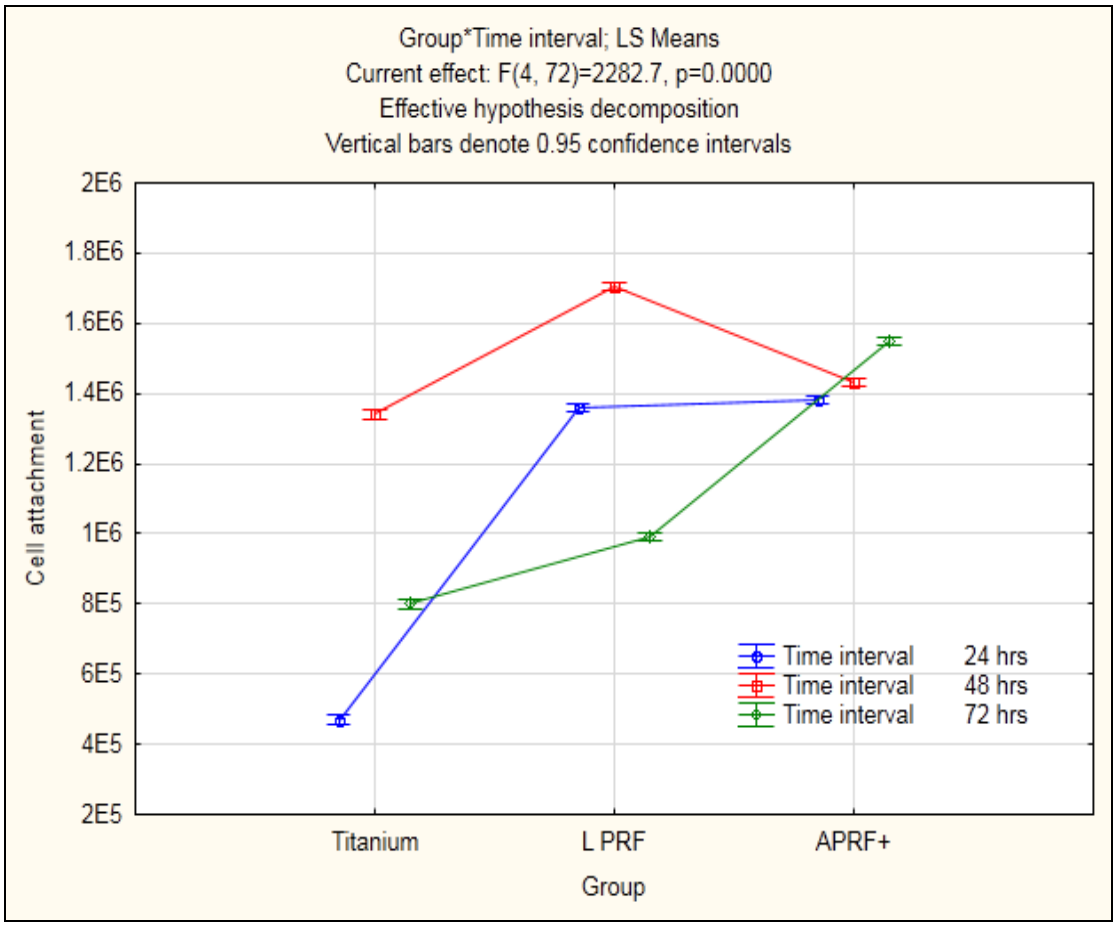
Graph 3: Comparison of interactions between three groups and three-time intervals (24hours, 48hours and 72hours) with cell attachment scores



Graph 4: This is a time trend graph showing effects of different time intervals on three groups with cell attachment scores



Graph 5: This is a time trend graph showing effects of three groups on different time intervals with cell attachment scores



CELL PROLIFERATION

Table 8: Comparison of study groups and control group with percentage of cell proliferation at different treatment time intervals of 24, 48, 72 hours

Main	Levels	Mean	SD	SE	95% CI for mean	
					Lower	Upper
Groups	Titanium disc	100.00	0.00	0.00	100.00	100.00
	L PRF	104.50	8.42	1.62	101.17	107.83
	APRF+	288.00	27.43	5.28	277.15	298.85
Times	24hours	168.17	100.05	19.25	128.59	207.75
	48hours	150.00	72.07	13.87	121.49	178.51
	72hours	174.33	95.84	18.44	136.42	212.25
Interactions	Titanium disc with 24hours	100.00	0.00	0.00	100.00	100.00
	Titanium disc with 48hours	100.00	0.00	0.00	100.00	100.00
	Titanium disc with 72hours	100.00	0.00	0.00	100.00	100.00
	L PRF with 24hours	97.50	0.11	0.04	97.41	97.59
	L PRF with 48hours	100.00	1.22	0.41	99.06	100.94
	L PRF with 72hours	116.00	1.41	0.47	114.91	117.09
	APRF+ with 24hours	307.00	1.87	0.62	305.56	308.44
	APRF+ with 48hours	250.00	1.41	0.47	248.91	251.09
	APRF+ with 72hours	307.00	1.87	0.62	305.56	308.44

This table demonstrated that there was no statistical significant difference seen in the cell proliferation for the study group at 24-48 and 48-72 hours however statistically significant difference was seen at 48-72 hour time interval.

Graph 6: Comparison of three groups with cell proliferation scores

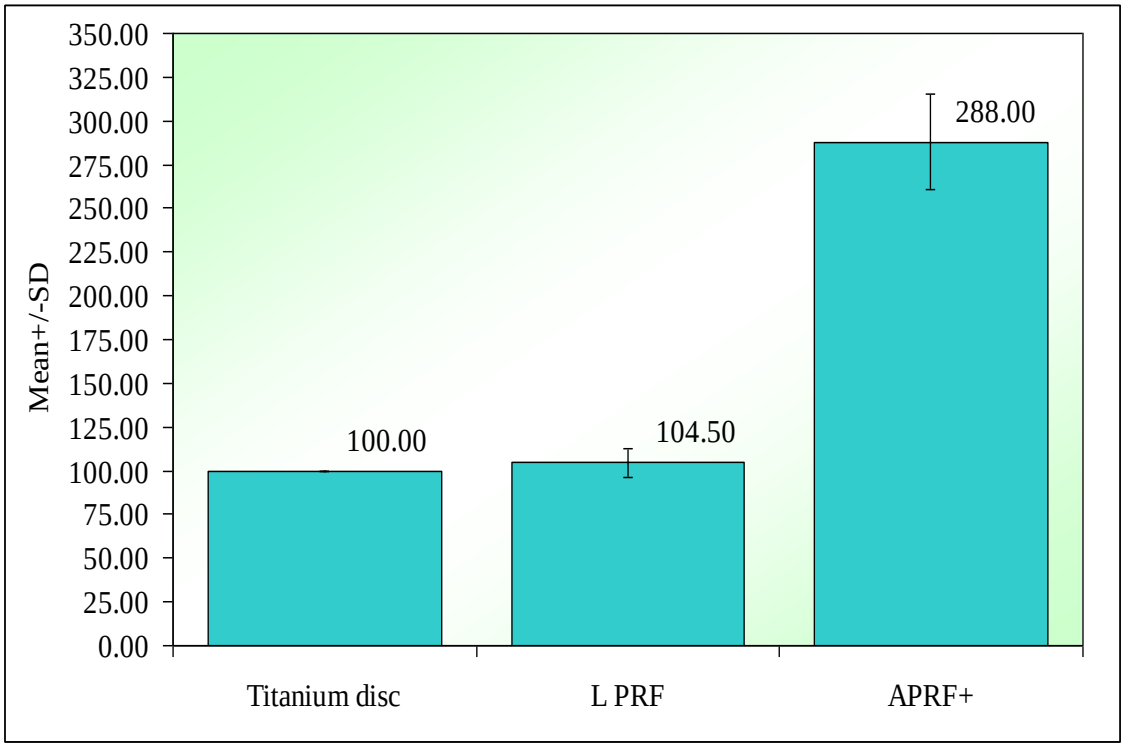


Table 9: Comparison of three groups and three-time intervals (24hours, 48hours and 72hours) with cell proliferation scores by Two-way ANOVA

Sources of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value	p-value
Main effects					
Groups	621328.5000	2	310664.2500	223454.8050	0.0001*
Times	8641.5000	2	4320.7500	3107.8322	0.0001*
2-way interaction effects					
Groups*Times	12666.0000	4	3166.5000	2277.6024	0.0001*
Error	100.1000	72	1.39		
Total	642736.1000	80			

*p<0.05

Graph 7: Comparison of three-time intervals (24hours, 48hours and 72hours) with cell proliferation scores

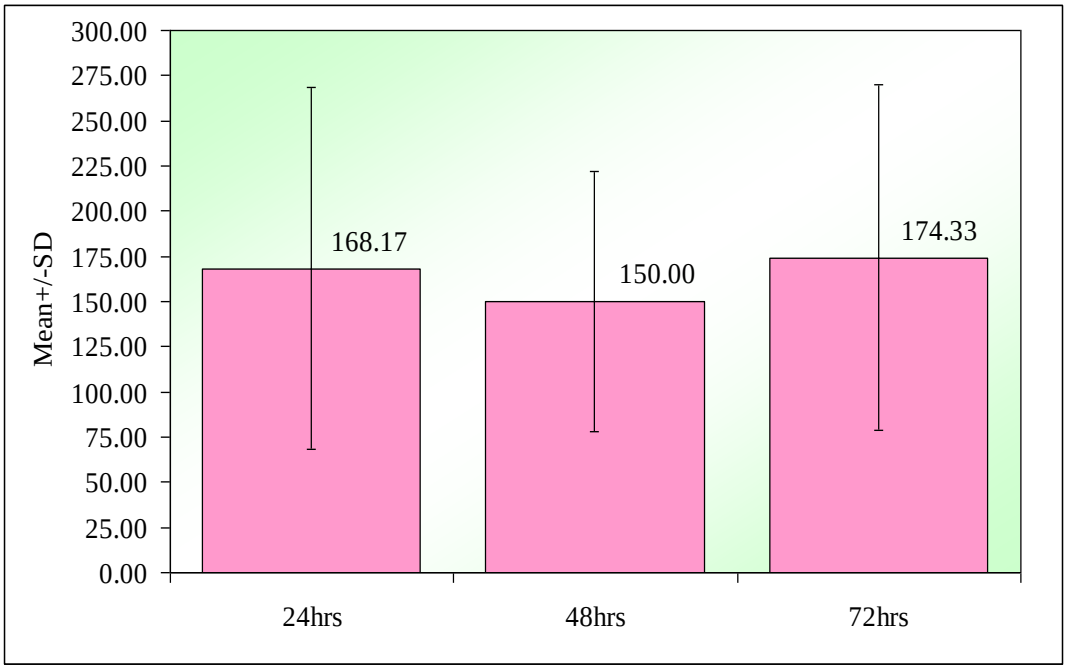
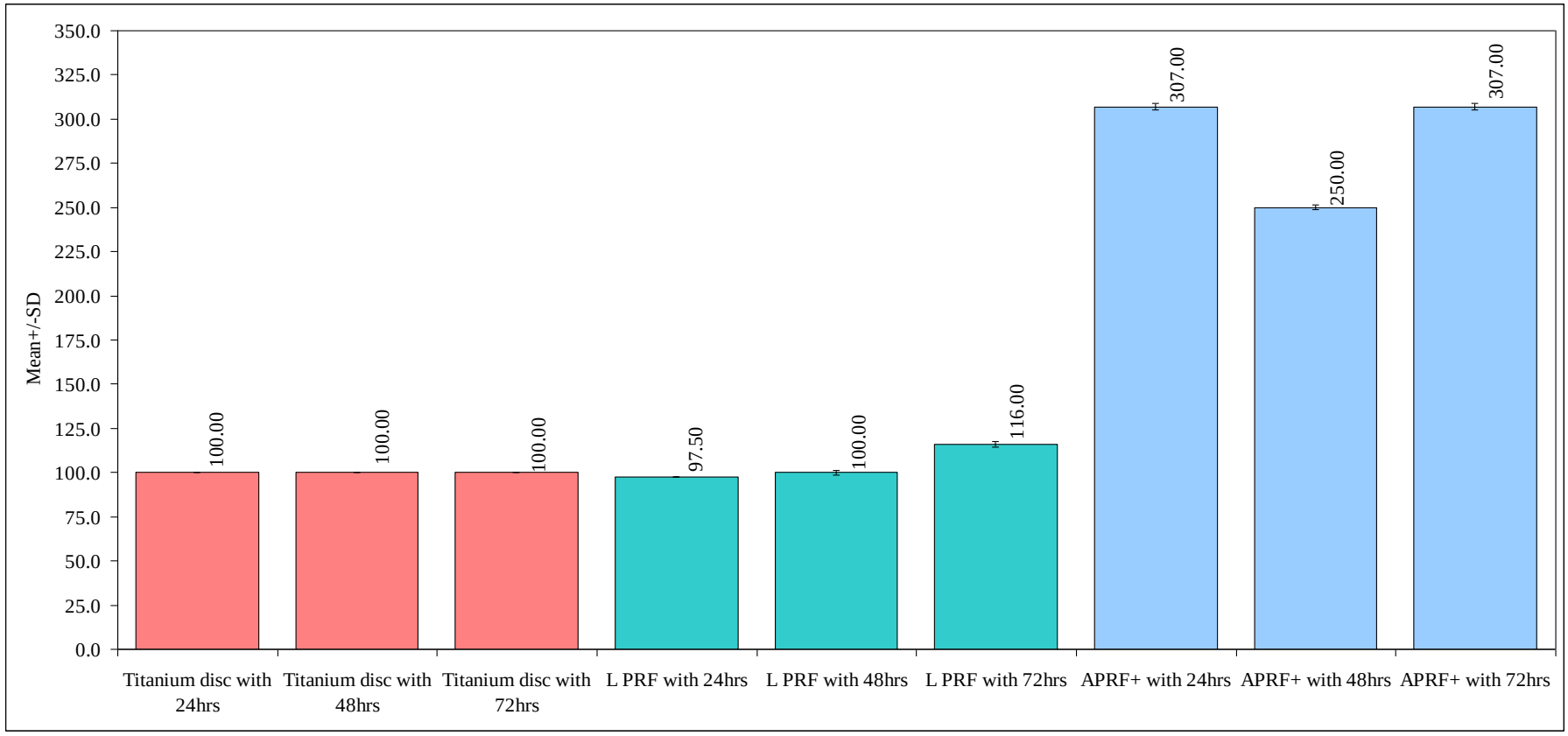


Table 10: Pair wise comparison of interactions between three groups and three-time intervals (24hours, 48hours and 72hours) with cell proliferation scores by Tukeys multiple posthoc procedures

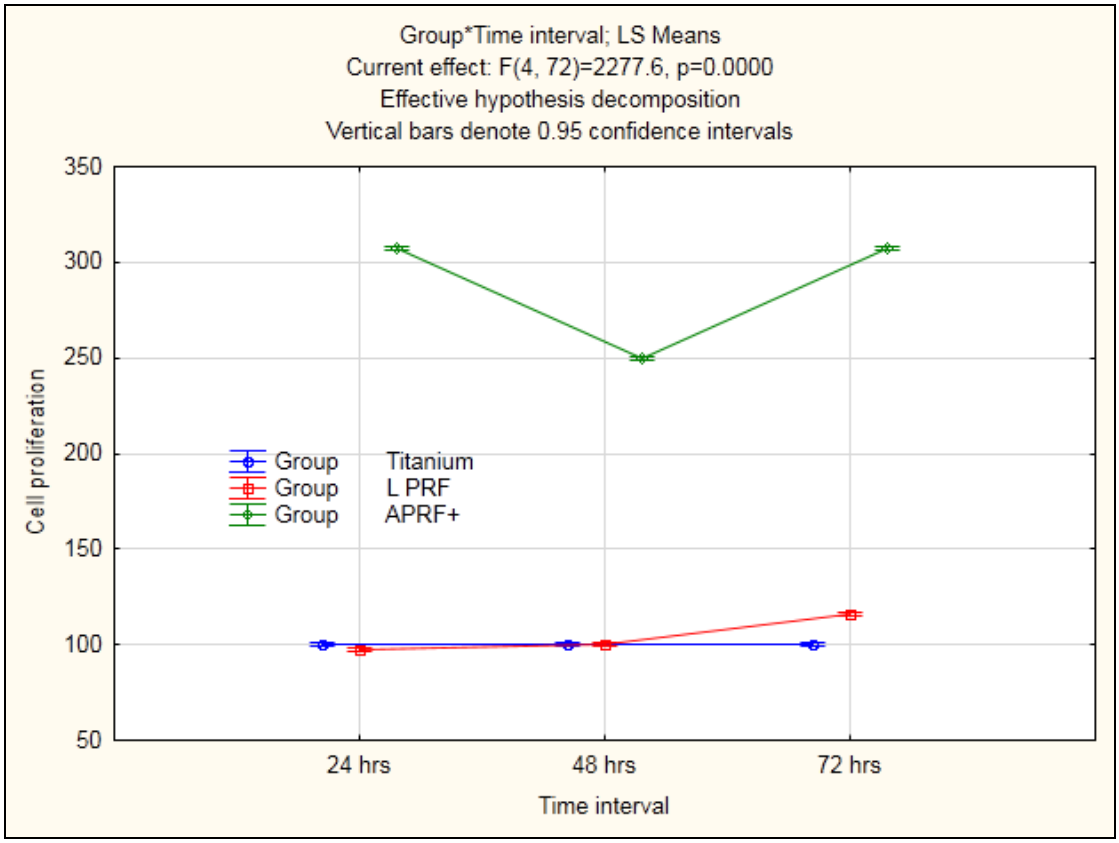
Interactions	Titanium disc with 24hours	Titanium disc with 48hours	Titanium disc with 72hours	L PRF with 24hours	L PRF with 48hours	L PRF with 72hours	APRF+ with 24hours	APRF+ with 48hours	APRF+ with 72hours
Mean	100.0	100.0	100.0	97.5	100.0	116.0	307.0	250.0	307.0
SD	0.00	0.00	0.00	0.11	1.22	1.41	1.87	1.41	1.87
Titanium disc with 24hours	-								
Titanium disc with 48hours	p=1.0000	-							
Titanium disc with 72hours	p=1.0000	p=1.0000	-						
L PRF with 24hours	p=0.0009*	p=0.0009*	p=0.0009	-					
L PRF with 48hours	p=1.0000	p=1.0000	p=1.0000	p=0.0009*	-				
L PRF with 72hours	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	-			
APRF+ with 24hours	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	-		
APRF+ with 48hours	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	-	
APRF+ with 72hours	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=1.0000	p=0.0001*	-

*p<0.05

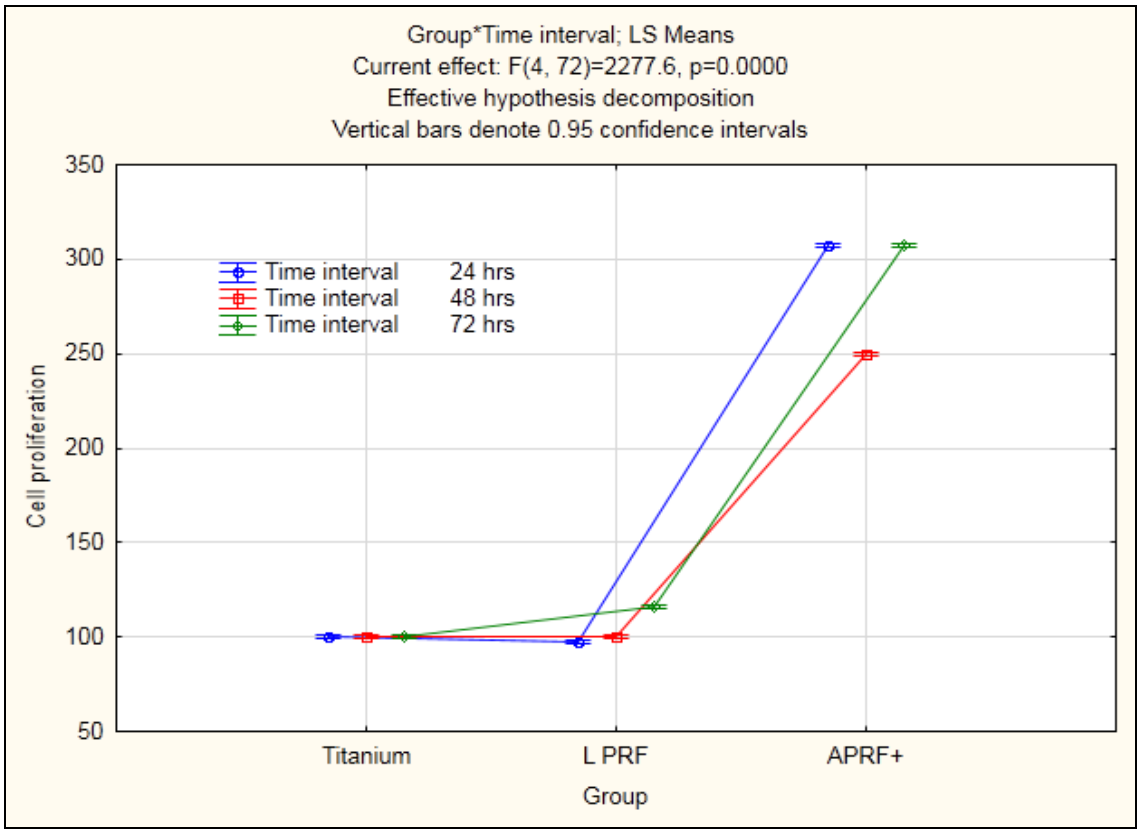
Graph 8: Comparison of interactions between three groups and three-time intervals (24hours, 48hours and 72hours) with cell proliferation scores



Graph 9: This is a time trend graph showing effects of different time intervals on three groups with cell proliferation scores



Graph 10: This is a time trend graph showing effects of three groups on different time intervals with cell proliferation scores



CELL MINERALIZATION

Table 11: Summary of Alizarin red assay scores of three groups (noncoated titanium disc, LPRF and APRF +) at three-time intervals (Day 7, Day 14, Day 21)

Main	Levels	Mean	SD	SE	95% CI for mean	
					Lower	Upper
Groups	Titanium disc	100.00	0.00	0.00	100.00	100.00
	L PRF	86.00	31.67	6.10	73.47	98.53
	APRF+	114.00	35.76	6.88	99.85	128.15
Times	Day 7	71.00	22.15	4.26	62.24	79.76
	Day 14	106.00	19.64	3.78	98.23	113.77
	Day 21	123.00	18.78	3.61	115.57	130.43
Interactions	Titanium disc with Day 7	100.00	0.00	0.00	100.00	100.00
	Titanium disc with Day 14	100.00	0.00	0.00	100.00	100.00
	Titanium disc with Day 21	100.00	0.00	0.00	100.00	100.00
	L PRF with Day 7	48.00	2.83	0.94	45.83	50.17
	L PRF with Day 14	86.00	1.22	0.41	85.06	86.94
	L PRF with Day 21	124.00	1.22	0.41	123.06	124.94
	APRF+ with Day 7	65.00	2.24	0.75	63.28	66.72
	APRF+ with Day 14	132.00	1.22	0.41	131.06	132.94
	APRF+ with Day 21	145.00	1.87	0.62	143.56	146.44

Table 12: Comparison of three groups and three-time intervals (Day 7, Day 14, Day 21) with Alizarin red assay scores by Two-way ANOVA

Sources of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value	p-value
Main effects					
Groups	10584.0000	2	5292.0000	2268.0000	0.0001*
Times	37962.0000	2	18981.0000	8134.7143	0.0001*
2-way interaction effects					
Groups*Times	21204.0000	4	5301.0000	2271.8571	0.0001*
Error	168.0000	72	2.33		
Total	69918.0000	80			

*$p<0.05$

Table 13: Pair wise comparison of three groups with Alizarin red assay scores by Tukeys multiple posthoc procedures

Groups	Titanium disc	L PRF	APRF+
Mean	100.00	86.00	114.00
SD	0.00	31.67	35.76
Titanium disc	-		
L PRF	P=0.0001*	-	
APRF+	P=0.0001*	P=0.0001*	-

*p<0.05

Graph 11: Comparison of three groups with Alizarin red assay scores

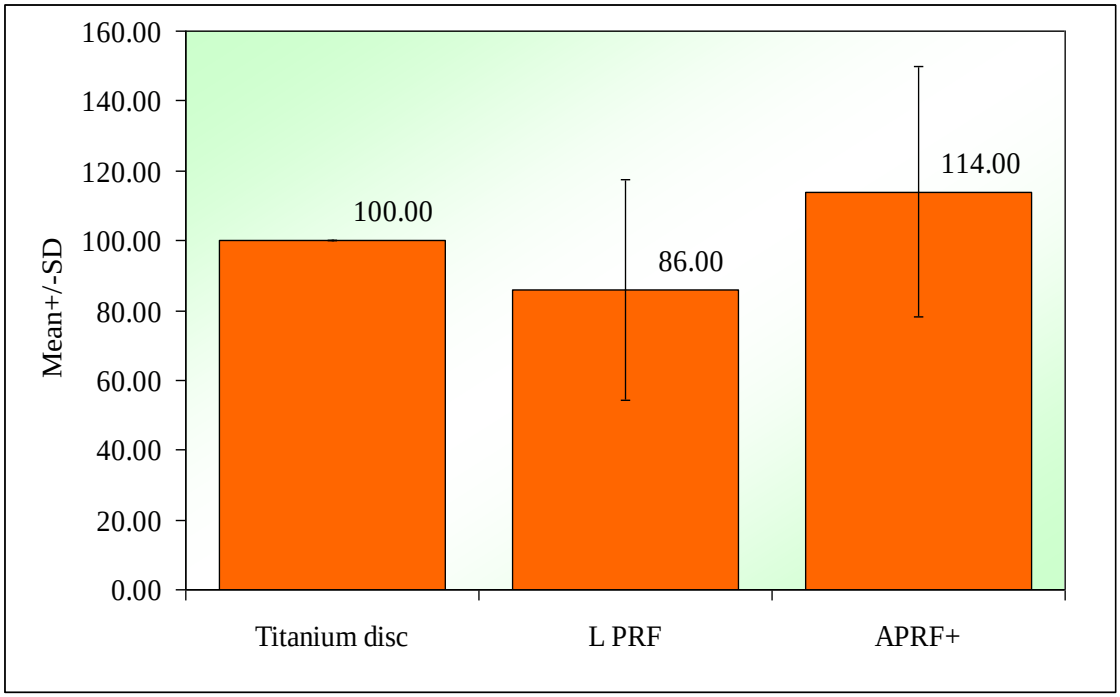


Table 14: Pair wise comparison of three-time intervals (Day 7, Day 14, Day 21) with Alizarin red assay scores by Tukeys multiple posthoc procedures

Times	Day 7	Day 14	Day 21
Mean	71.00	106.00	123.00
SD	22.15	19.64	18.78
Day 7	-		
Day 14	P=0.0001*	-	
Day 21	P=0.0001*	P=0.0001*	-

*p<0.05

Graph 12: Comparison of three-time intervals (Day 7, Day 14, Day 21) with Alizarin red assay scores

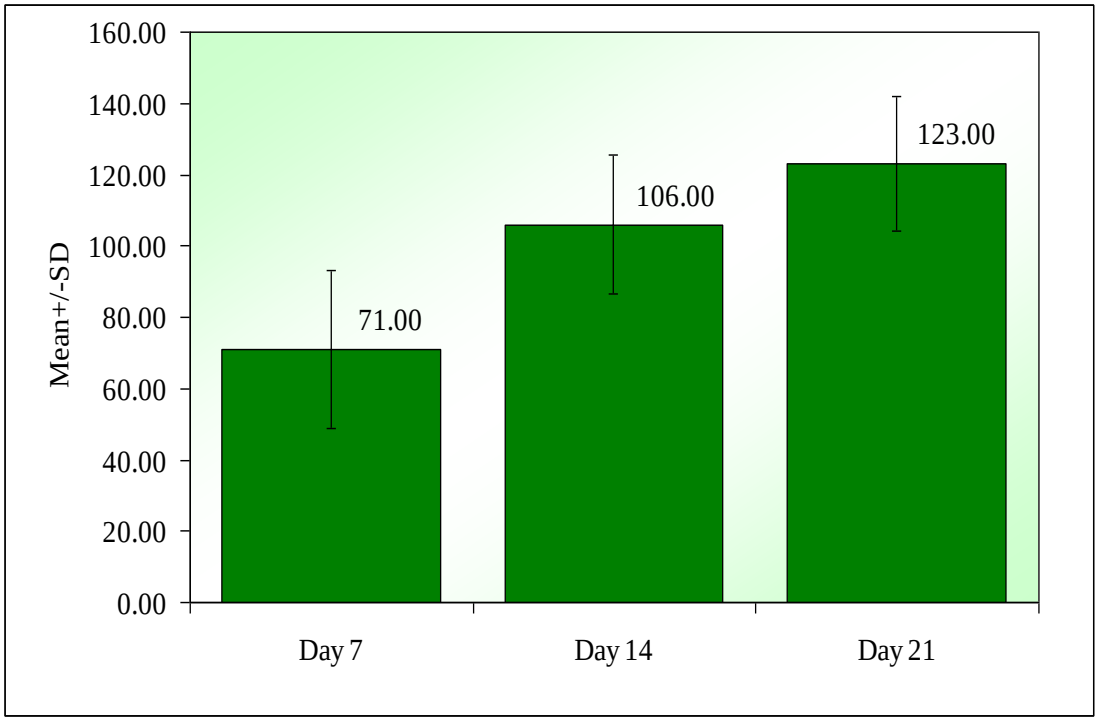
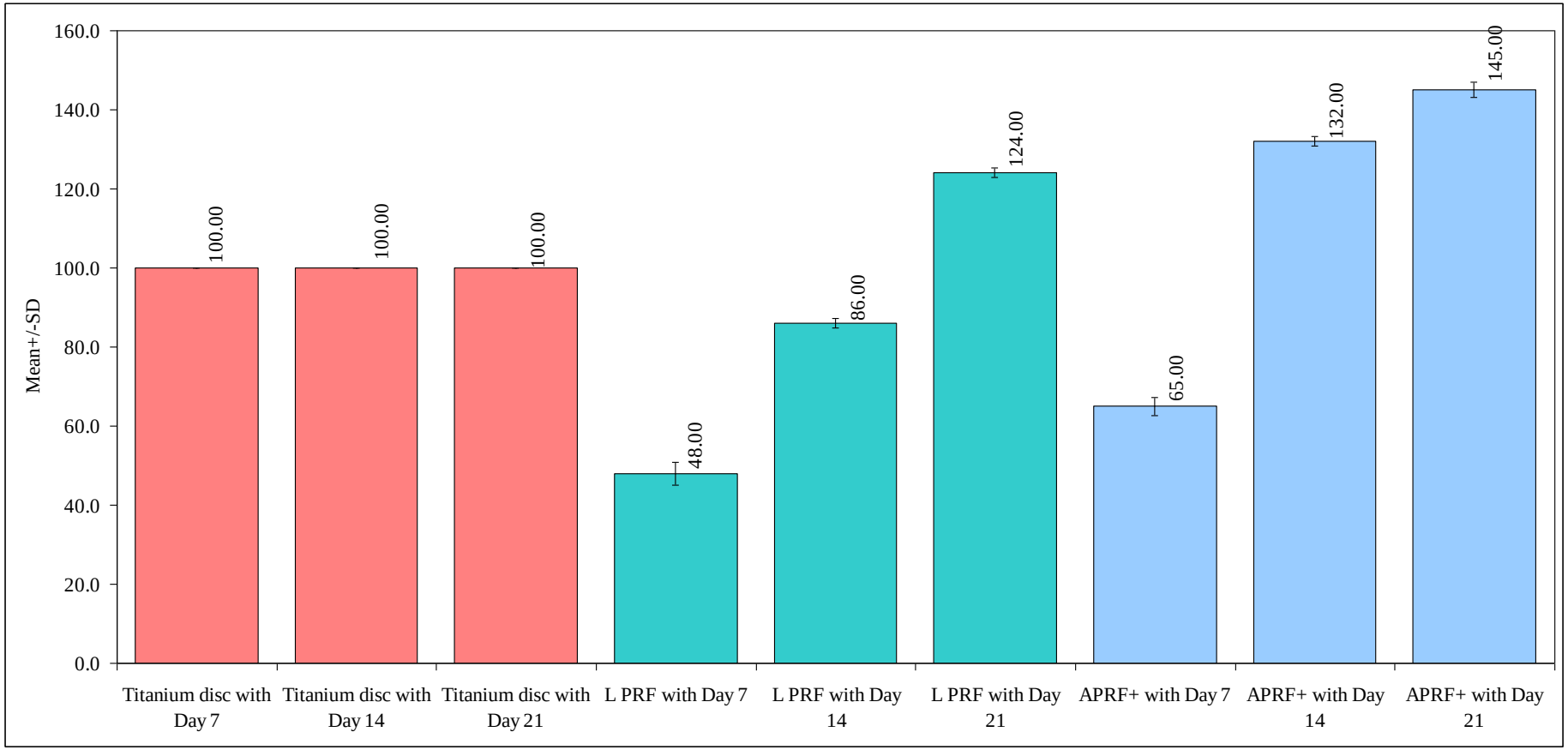


Table 15: Pair wise comparison of interactions between three groups and three-time intervals (Day 7, Day 14 Day 21) with Alizarin red assay scores by Tukeys multiple posthoc procedures

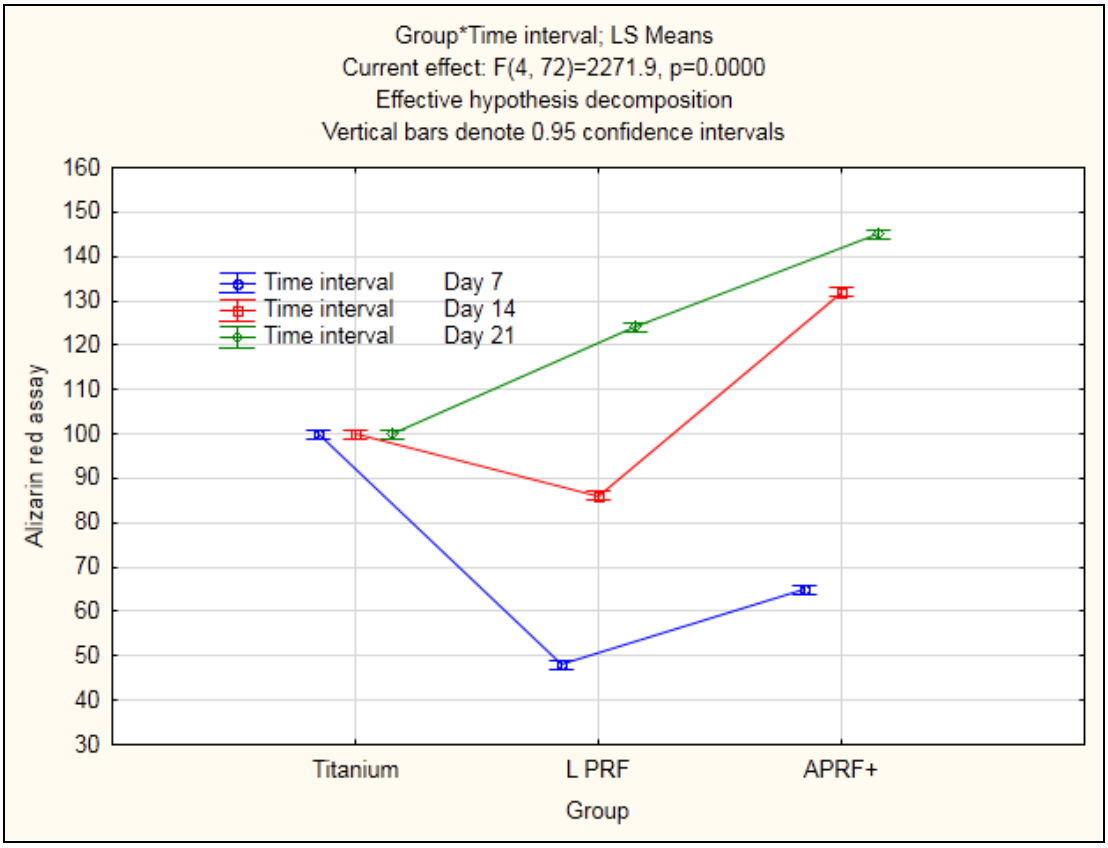
Interactions	Titanium disc with Day 7	Titanium disc with Day 14	Titanium disc with Day 21	L PRF with Day 7	L PRF with Day 14	L PRF with Day 21	APRF+ with Day 7	APRF+ with Day 14	APRF+ with Day 21
Mean	100.0	100.0	100.0	48.0	86.0	124.0	65.0	132.0	145.0
SD	0.00	0.00	0.00	2.83	1.22	1.22	2.24	1.22	1.87
Titanium disc with Day 7	-								
Titanium disc with Day 14	p=1.0000	-							
Titanium disc with Day 21	p=1.0000	p=1.0000	-						
L PRF with Day 7	p=0.0001*	p=0.0001*	p=0.0001*	-					
L PRF with Day 14	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	-				
L PRF with Day 21	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	-			
APRF+ with Day 7	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	-		
APRF+ with Day 14	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	-	
APRF+ with Day 21	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	p=0.0001*	-

*p<0.05

Graph 13: Comparison of interactions between three groups and three-time intervals (Day 7, Day 14 Day 21) with Alizarin red assay scores



Graph 14: This is a time trend graph showing effects of three groups on different time intervals with respect to alizarin red assay.



DISCUSSION

The primary objective of present-day dentistry is to reassure the patient, to achieve proper function, articulation, esthetics and overall well-being, irrespective of the breakdown, or trauma to the functional masticatory apparatus. In response to this primary objective, dental implants represent a great alternative for individuals with healthy oral hygiene, who have had tooth loss from periodontal disease, trauma, or any other cause.

Dental implants are surgical fixatures, biologically inert in nature, which are placed in the jaw bone beneath the gingiva as an alternative to infected or damaged tooth. The duration for bone healing and subsequent osseointegration of the dental implant differs from three months to six months or more.

Today, titanium materials differing from Grade 1 to 4 ASTM (American society for testing and materials) are commercially available as pure titanium or Ti-based alloys, known to be the utmost biocompatible substance to living tissue and for its minimal toxicity, resistance to corrosion and high mechanical resistance. [2,49]

Enhancement of dental implant osseointegration has been an area of research for more than four decades now. There have been number of experimental studies regarding type of materials, macrogeometry, microgeometry, the effect of drilling sequence and so on to intensify osseointegration. It is well acknowledged that the overall favorable outcome of implants depends on the quality and quantity of host bone, presence of sufficient primary stability at the time of implant placement, and formation of a direct bone to implant contact (BIC).[49]

Osseointegration consists of a sequence of bone modeling and remodeling processes and is defined as the actual anatomic and physiologic attachment of the surface of a load-bearing artificial implant with native bone.

The reliability of successful osseointegration is by the quality, distribution, and amount of bone present at the site of the dental implant.[49] However, osseointegration and BIC are mainly enhanced by implant surface characteristics, such as surface topography, chemistry, roughness and energy. Alberktsson and Wennerberg classified implant surfaces as smooth surfaces with a surface roughness (Sa less than 0.5 μm), slightly rough surface (Sa = 0.5–1 μm) , moderately rough surface (Sa = 1–2 μm), rough surface (Sa > 2 μm) and they concluded that the surfaces which are moderately rough might have applications clinically in contrast to rough and smooth surfaces. [21]

In the present study for the surface characterization, quantitative evaluation of all the disc-shaped specimens was done using Contact stylus Profilometer (Surtronic S-128 – Taylor Hobson) respectively, which is a contact stylus apparatus used to calculate surface profiles and roughness. This factor depicts the general surface roughness and is the average arithmetical numerical of complete profile roughness distances from the center line within the measuring length. Profilometer determined the profile along 3 lines on the surface by means of a tracking device. An average roughness profile (Ra) was evaluated for each specimen to describe the general roughness of the surface. The outcomes attained by surface profilometry showed that all tested specimens had a mean average surface roughness (Ra) within the range of 1.34 to 1.54 μm. To compliment the surface roughness, values resulted on quantitative

evaluation, the specimens were subjected to Scanning Electron Microscopy (100X, 500X, 1000X) for assessment of roughness value qualitatively.

The average estimate range of roughness found in the literature is 1–2 μm, [50] which coincided with average roughness values which was obtained from profilometric readings of the current study. According to Lukaszewska et al, surface texture affects bone forming cell morphology; on rough surfaces, cells have a smaller coverage area and are less dispersed than on smooth surfaces.

The rough surfaces display greater amount of filamentous lengthening and interdependence, which implies increased adhesive qualities in comparison to smooth surface. Higher cell-based vitality was seen with respect to roughened surfaces when compared to surfaces which are smooth. [51]

Lima et al suggested that although implants with a rough surface promote osseointegration more effectively than those with a machined surface, they still need to be handled cautiously since this surface treatment can have a negative influence or can modify the titanium oxide surface.[52] Hence standardizing the surface roughness of the titanium is crucial, so that a constant surface roughness could be utilized to assess the characteristics of the coating.

Although a high success rate for machined titanium implants with surface coating has been seen, various other surface treatments have been advocated and put forth till date, to improve cell attachment, cell differentiation, and bone apposition; allow bone fixation; limit the percentage of degradation in the body fluids; and function in a therapeutic way. [2,53]

Clinicians are increasingly looking into and using PRF, an autogenous biomaterial produced by centrifugation of the patient's blood, as an adjuvant autologous bioproduct to support bone and soft tissue regeneration. Neither bovine thrombin nor anticoagulant (or any other gel-forming substance) are needed for this procedure.

According to Kawase in the year 2015, he concluded that to achieve the best outcomes for tissue repair and regeneration, a framework (clot network), platelets, leukocytes, growth factors, and stem cells must interact. All of these necessary active elements of PRF, once combined and prepared appropriately, play a role in the synthesis of extracellular matrix, chemotaxis, angiogenesis (neo-vascularization), cell growth and differentiation, and other critical processes which are involved in repair and renewal.[11] So, due to the advantages of biological coatings, the application of PRF as a autologous coating is considered in the study.

Based on the type of substance used, biological coatings have been separated into two categories: inorganic as well as organic coatings. The potential of bioactive materials to mend bone is enhanced when they are coated onto core biometals. This results in the biomechanical benefits of a matrix like porous titanium. The porous structure encircles minor fissures and concentrates stress in a limited way. The element magnesium, growth factors, bone morphogenetic proteins (BMP) and HA (hydroxyapatite) are some of the substances utilized in therapeutic coatings. In this context, improving cell-protein adhesion, implant-host mineralization and antimicrobial behavior of the surface layer is the goal. [8]

Growth Factor based organic coatings consists of a substance that aids in tissue development and the division of cells. The combination of each growth factor's recognition of a particular membrane receptor either promotes or prevents cell division. The two primary growth factors used in implant coatings are vascular endothelial growth factor (VEGF) and bone morphogenetic proteins (BMPs). VEGF is a signal protein that has shown to activate gene and protein expression of vasculogenesis and has been found, during in vitro experiments, to enhance primary rat osteoblast proliferation and to increase alkaline phosphatase (ALP) activity. In addition, the in vivo experiment showed an important improvement in the activation of osteoblasts and endothelial cells. BMPs are proteins with a key role in inducing bone and cartilage formation by the regulation and promotion of osteogenic and bone mesenchymal stem cells. These features have led BMPs to be increasingly applied in dental coatings. [71]

The centrifuged blood sample is divided into three layers, which also initiates the coagulation process: an acellular plasma forms at the tube's top, fibrin clot that is substantially synthesized forms in the middle, and red blood cells are collected at the tube's bottom.[54]

Choukroun's PRF procedure protocol is 3,000 rpm/10 minutes and Dohan Ehrenfest’s group (leukocyte- and platelet-rich fibrin (L-PRF) standard protocol: 2,700 rpm/12 minutes .Choukroun’s advanced PRF (A-PRF),enriched with leukocytes: 1,300 rpm/ 8 minutes .Choukroun’s i-PRF (solution/gel): 700 rpm/3 minute and Advanced platelet rich fibrin plus (APRF+)1500 rpm / 8 minutes.[11]

According to Oncu et al, the growth factors in Choukroun's novel PRF formulation (A-PRF) released progressively over a maximum of 10 days. and stimulated significantly higher growth factor release over time when compared to Choukroun's standard PRF .[11]

Considerable concentrations of growth factors are made available within the first 20 minutes after preparation, so PRF membranes should therefore be put to immediate use following preparation.[56]

Fuijoka-Kobayashi et al. proposed A-PRF+, a new variation that shortens the rotation time while retaining the same force as the A-PRF procedure. So, the present study used A-PRF+, as very few studies were undertaken till date without any conclusive data and L-PRF was used because it is the gold standard in the field of autologous products. It is believed to have unique benefits even with the time reduction; it has a favorable effect on the preservation of cells within the clot that forms and, as a result, it further enhances the qualities of A-PRF+ in comparison to those previously discussed.[13] According to this protocol, 10 mL of the patient's blood is centrifuged at 1300 rpm for 8 min in glass tubes without any anticoagulants.

One of the main uses of PRF is to use it as a bioactive barrier, that facilitates natural tissue regeneration (NTR) and healing, by intimate contact with tissues both above and below it. PRF membranes are a collection of natural autologous membrane that can be utilized as standalone ,supplementary, or in conjunction therapies in comparison to heterologous membranes that are viewed as foreign objects by the tissues of the person being treated and obstruct the natural healing process of the tissue.[11,57]

According to Krasny et al suggested that PRF will remodel (biodegrade) more quickly in situ compared to a resorbable collagen membrane, but because growth factors and other matrix proteins release slowly from PRF, it will also strongly induce cells in the periosteum/gingival tissue.

Secondly membranes can be used as competitive barriers, which is the term used to describe the biological trait of PRF to prevent the migration of soft tissues further into the augmented or grafted region.[58,59]

Lastly, a PRF membrane is simpler to manipulate than a real blood clot, as it is made of a solid substance with a robust fibrin architecture. The triple-layered structure of fibrin gives the PRF membrane excellent volume, flexibility, and toughness, that is better suited for manipulation and suturing.[62,63]

The PRF membrane's elasticity enables the physician to punch it and allow for placing it around a healing or prosthetic abutment or even suture it.[63]

According to Sharma et al in the year 2011[64] , no in vitro cytotoxicity effects have been detected whatever the quantity of PRF used, thus increasing the predictability of wound healing and regeneration potential of tissues ,due to its extremely low risk of infection, compared to other commercially membranes .[48,65,66.]

PRF is currently the safest and most economical choice for patients and clinicians for improving healing and tissue (soft tissue and bone) regeneration outcome[67,] especially in patients with systemic conditions where healing is compromised (i.e., diabetics and smokers), or in surgically compromised situations (damaged flap). It promotes recovery of the soft tissue, stimulate the healing of a damaged flap and reduce the risks of flap necrosis after a surgery.

According to Oncu et al in the year 2015, assessed the impact of PRF prior to implant insertion and discovered that the test group's values for the implant primary stability using ISQ (implant stability quotient) were greater than those of the uncoated implants. This indicated that PRF may enhance stability of the implant during the early stages of osseointegration.[72]

So, in the present study, osteogenic potential for the specimens in the control and study group were evaluated by assessing their cellular adhesion, cell proliferation and cell minerilaization using MG-63 cells (osteoblast like cells) on the PRF (LPRF and APRF+)treated surfaces which acted as study groups and machined titanium discs which were control group.

MG-63 cell lines were used in the study as it grows more quickly than the subsequent subcultures and can maintain the distinct phenotypic morphology than the primary bone-forming lines making it a good in vitro model. These cell types are associated with initial cell material characterization that is known for its high proliferation potential due to its capacity to continuously divide and grow thereby finding its use in various invitro and biocompatibility studies [86,87]

In this study, cell adhesion was evaluated using the tryphan blue exclusion test, which is a simple and rapid technique measuring the cell viability and adhesion demarcating non-viable cells having blue cytoplasm and viable cells showing clear cytoplasm when mixed with dyes.

The results of the present study indicate that there was a higher cell adhesion in the PRF coated titanium surfaces at 24 hours, 48 hours and 72 hours. It was found out that, at 24 hours a greater number of attached cells in APRF+ group (13,80,000

cells), whereas at 48 hours maximum number of cells were seen in L-PRF group (17,00,000 cells) and at 72 hours, maximum number of cells were attached to APRF+ coated discs (15,50,000 cells). Thus APRF+ coated discs have more propensity of attachment of MG63 cells. In comparison to LPRF, APRF+ coated Titanium discs increased the MG63 cell lines proliferation from 24 hours to 72 hours, thus showing that APRF+ helps in accelerating growth of MG63 cell lines.

The essential finding of this investigation was that the PRF enhanced early adhesion of MG-63 to the titanium alloy surface, which is explained by the polyelectron complex, which hydrophilizes the surface, and by protein molecules reacting through functional groups, which is in line with research findings by Parfitt et al. [89], Zhao et al. [48], and Qu et al [36] showed that hydrophilic surfaces are more responsive to cell attachment using different kinds of models and materials.

The larger surface area and increased surface roughness could potentially be the cause of the increased cell attachment. According to research by Lukaszewska et al., surface architecture may also influence cell adhesion and proliferation on implants.[90] Another reason for higher cell attachment in PRF coated discs was the presence of growth factors in the PRF.

When compared between the time intervals within the groups there was a definite increase in the cell adhesion in the study group (A-PRF+ coated discs) between 24 hours – 48 hours, 48 hours -72 hours and 24 hours-72 hours showing a greater effect size as compared to the control group (non-coated discs).

Cell proliferation was assessed using MTT Assay which measures the cellular metabolic activity which specifies the differentiation, vitality of cells and cytotoxicity.

It is a colorimetric test that measures monolayer of cells plated on multiwell plates, dependent on mitochondrial respiration and indirectly serving to assess the cellular energy capacity of a cell.[88]

Titanium discs were sown with cells and evaluated at intervals of 24hours, 48hours and 72 hours. There was a higher cell proliferation on the specimens in the study group at all the time intervals tested, however these changes were not significant at 24hours-48hours and 24hours-72 hours between the study groups and control group. When compared between time intervals there were no statistically significant results at 24hours-48 hours, however, significant difference was seen at 24-72 hour time interval indicating that there was a functional maturation as well as proliferation at 48 hour time interval. No statistical change was seen between 48-72 hours which could be due to cell confluence in the study group.

Cell mineralization was evaluated both qualitatively and quantitatively using the Alizarin red S staining test, which is considered to be the benchmark for quantifying osteoblastic minerialization. The qualitative assessment was done by examining the staining under inverted light microscopy to observe calcified deposits (Figure 12) along with scanning electron microscopy to evaluate mineralized crystals at day 14 and 21 (Figure 13, 14) and the quantitative analysis was done by colorimetric assay.

By using Alizarin red S staining, the presence of extracellular matrix was evaluated in the control (non-coated discs, LPRF and APRF + coated discs at day 7, day 14 and day 21). The deposition and formation of red color extracellular matrix was depicted with red-colored matrix at the periphery of cells.[25]

The principle of this staining is that an alizarin red S-calcium complex is formed by calcium, which is a chelation process. The formation of an alizarin red S-calcium complex results in the production of a vivid red stain that is also visible to the naked eye.[92]

The results of this study with respect to mineralization showed that more extracellular matrix was seen in APRF+ when compared to non-coated and L-PRF coated disc both qualitatively and quantitatively. Platelet rich fibrin layer with network of fibrin was observed under scanning electron microscope. (Figure 15). The absorbance measured was 145 % of the APRF+ when compared to 124% of the LPRF group at day 21. Also the deposition and formation of red color extracellular matrix was seen in increased amounts qualitatively in APRF+ when compared to LPRF. The APRF+ coated titanium discs (Figure 13 ,14) showed highest calcium rich deposits than compared to L-PRF coated titanium discs, thus indicating that APRF+ can help in osseointegration of titanium implants, with a possible explanation for accelerated mineralization by the osteoblasts, due to presence of increase amount of growth factors in APRF+. [93]

In 2022, a study by Mara Simoes-Pedro et al., evaluated the mechanical and structural properties of three distinct platelet-rich fibrin membranes (L-PRF, A-PRF, and A-PRF+) and performed a tensile strength essay. They concluded that notably greater tensile strength was seen with the A-PRF+ protocol, making this type of membrane the most suitable for suturing and handling [13].

In a study conducted by Marco et al, they concluded that the organization of dense clot of L-PRF was found on implant surfaces which are micro roughened when assessed with surfaces which are not roughened in vitro .[9]

In 2021, Rucha Shah et al. conducted a study to assess the osteoblastic behavior of titanium discs coated with injectable PRF and came to the conclusion that covering titanium discs with i-PRF improves mineralization at days 1, 7, 14, and 21 in MG-63 osteoblast-like cells and promotes differentiation and alkaline phosphatase activity. [14] However, no studies were conducted to evaluate the osteoblastic efficacy comparing L-PRF and APRF+ and thus, this study was undertaken to evaluate the same.

Other PRF protocols were utilized in addition to the original L-PRF technique; nevertheless, the majority of research lacked sufficient details. These particulars are crucial since distinct methods, such as those pertaining to centrifugation time and g-force, can significantly alter properties like the production of growth factors or the concentration of live cells. For example, growth factor release and cell content are improved by lowering the g-force. This discovery is regarded as one of the key advancements in PRF that paved the way for the creation of novel procedures, such as liquid PRF (fluid-PRF), injectable PRF (i-PRF), and enhanced platelet-rich fibrin (A-PRF+).[31]

Kubesch et al. also showed that lower the speed of centrifugation, the faster the revascularization of PRF-based matrices, owing to the ability for cells to more readily penetrate through PRF, potentially owing to the lower fibrin density of the PRF clot. These lower centrifugation speeds have also been shown in a series of studies to release more growth factors over time when compared to the original high g-force protocols.[85]

To summarize this study, the APRF + group showed enhanced proliferation, cell attachment and mineralization compared to L-PRF. The potential cause could be that the synthesis stages and shorter centrifugation times used to prepare A-PRF+ led to an increase in PRF cell content, and also the release of different growth factors such as TGF-β1, PDGF, EGF and IGF greater in A-PRF+ compared to other types of platelets concentrates like Advanced PRF and L-PRF[29]

The investigative findings of this study, were in accordance with other in vitro studies, demonstrating that, in comparison to the shorter centrifugation treatment, the longer centrifugation protocol (2,700 rpm) results in (stronger) fibrin clot with less inter-fibrous gap and fewer cells. A-PRF+ (1500 rpm) generated a fibrin clot that was less dense and had a looser inter-fibrous structure with more cells in it. (Hauser, et al., 2013).[55]

Within the limitations of the present study, it is suggested that PRF showed osteoblastic activity, osteogenesis which is triggered by osteoblastic cells, is typified by a series of occurrences that include cell adhesion, cell differentiation and osteoblast phenotypic expression.

The study investigated the response of osteoblast like MG-63 cells which were cultured on to the characterized titanium disc surfaces with LPRF and APRF+.

The results showed that all discs, coated with PRF independent of the surface whether rough or not, increased osteoblastic activity with enhanced cell attachment, cell proliferation compared to the specimens in machined titanium surface.

Based on the above findings, this research revealed that the PRF increased osteoblastic activity when compared to non PRF coated titanium disc. Among study groups APRF + increased proliferation, attachment and mineralization. Coating titanium with PRF may represent an alternative method to increase osteoblastic activity and also promote early osseointegration.

SCOPE OF STUDY

- The present study evaluated the osteogenic response of titanium discs coated with LPRF and APRF + and the findings of the present study can be examined in vivo.
- Other implant materials such as zirconia and polyetheretherketone (PEEK), can be used to check the efficacy of PRF coating and its effect on osteoblastic activity.
- Since PRF is autologous and biocompatible, its combination with different bone graft materials can be assessed.
- Further investigations can be done on the different forms of PRF to determine their duration, growth factor release amount, dissolution rate, etc.
- Cell differentiation using other cell markers such as ALP, RANKL, VEGF can be done.
- Cell mineralization using Von Kossa staining as an alternative to Alizarin red S staining for the deposition of calcium deposits can be done.

LIMITATIONS OF STUDY

- This is an in vitro study; all the clinical conditions have not been studied.
- The study used MG-63 cells (osteoblasts like cells) which is an osteosarcoma cell line which lack coherence in the capacity of cell differentiation
- The PRF coating may not be uniform with the help of dipping method and an ideal coating method needs to be utilized to have strong adhesion to the substrate and conformation to a substrate with an arbitrary shape.

CLINICAL IMPLICATIONS

The results of the present study, showed that the use of PRF coating on titanium had significantly increased cell attachment, cell proliferation and mineralization and thus enhances osteoblastic activity.

The ease of preparation and cost-effectiveness of PRF membrane offers a huge advantage over other commercially available membranes. It is widely applicable in dentistry, while being financially realistic for the patient and the healthcare system.

PRF is currently the safest and most economical choice for patients and clinicians for improving healing and tissue (soft tissue and bone) regeneration outcome. PRF has no contraindication since it is from patients own blood it can be used in all kinds of patients, especially in patients with systemic conditions where healing is compromised (i.e., diabetics and smokers).

In the present study two types of PRF (LPRF and APRF+) were studied and compared, among which APRF+ showed increased osteoblastic activity. It was concluded that lower centrifugation speeds allowed for the faster revascularization of PRF-based matrices owing to the ability for cells to more readily penetrate through PRF, potentially owing to the lower fibrin density of the PRF clot.

With an aim of improving the success rate in cases of bone defects and in systemic conditions like osteoporosis, these PRF coatings on titanium would expedite the bone healing thereby enabling immediate or early loading protocols along with stimulating bone to permit implant placement in sites that lack sufficient residual alveolar ridge. With these advantages, PRF coating on titanium can be

considered as beneficial, promising in enhancing early osseointegration, just by reducing the centrifugation speed. However, further long-term in vivo investigations need to be carried out to understand the long-term benefits of APRF+ on bone healing, remodeling and osseointegration in clinical scenario's and to formulate better coating methods for uniform surface coating of PRF on titanium surfaces.

CONCLUSION

Within the limitations of this in vitro study the following conclusions were drawn.

On comparing the osteogenic potential, the cell attachment was higher on titanium surfaces which were treated with APRF + followed by LPRF and noncoated titanium discs at different time intervals. APRF+ coated titanium discs showed increase in proliferation of MG63 cell lines from 24 hours to 72 hours than compared to LPRF, thus showing that APRF+ helps in accelerating proliferation of MG63 cell lines. The APRF+ coated titanium discs showed highest calcium rich deposits than compared to LPRF coated titanium discs, at all the three-time intervals i.e. day 7, day 14 and day 21. Thus, indicating APRF + coated titanium discs showed increased cell attachment, cell proliferation and cell mineralization.

SUMMARY

The present in vitro study was carried out to evaluate the osteogenic potential of PRF coated (LPRF and APRF +) on titanium surfaces.

A total of the 243 identical commercially available identical Titanium Grade V ,10 mm x 2 mm (ASTM B348) disc shaped specimens were used. 81 titanium discs were coated with Leucocyte platelet rich fibrin (L-PRF) which made up the study group 1, 81 titanium discs coated with Advanced platelet rich fibrin plus (A-PRF+) which made the study group 2, while the remaining 81 titanium discs without any PRF coating acted as a control. Out of the total titanium discs which were 243, 81 titanium discs were used to assess the cell attachment ,81 titanium discs were used to assess the cell proliferation and the remaining 81 titanium discs were used to assess the cell mineralization by Alizarin red S staining.

All the specimens from each group were subjected to quantitative evaluation of surface roughness using Profilometer and one specimen from each group was subjected to Scanning Electron microscopy for qualitative assessment of surface roughness. A mean roughness profile was evaluated for each specimen to describe the overall roughness of the surface.

Both the types of PRF (LPRF and APRF +) were prepared and the discs in the study groups 1 and 2 were coated by dipping method simultaneously.

The osteogenic potential was evaluated by assessing the cell attachment, cell proliferation and cell mineralization on PRF coated and non-coated discs .Cell attachment and cell proliferation were evaluated at 24 hours, 48 hours and 72 hours

Cell mineralization was evaluated at day 7 ,day 14 and day 21 using alizarin red-S staining of MG-63 cells .The cell Attachment was evaluated using hemocytometer while Cell Proliferation was assessed using MTT Assay.

The resultant data were tabulated and then subjected to statistical analysis to draw conclusion from experimental data. ($p<0.05$)

The results showed that the cell attachment was higher for the PRF coated discs with the highest seen in the APRF + group at all three-time intervals as compared to noncoated titanium discs. There was a statistically significant difference in the cell attachment between the time intervals for the study groups (LPRF and APRF +). The cell proliferation was higher in the study groups (LPRF and APRF +) .and with statistically significant results at all three-time intervals. The APRF+ coated Titanium discs showed highest mineralization when compared to LPRF coated Titanium discs. Hence, within the limitations of the study we can conclude that PRF could be used as a surface coating rendering increased osteogenic properties and APRF + proved to have enhanced cell proliferation, cell attachment and cell mineralization, aiding in early osseointegration.

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ANNEXURE – I

ETHICAL CLEARANCE

KLE VK INSTITUTE OF DENTAL SCIENCES
A constituent Unit of KLE Academy of Higher Education and Research
Accredited 'A+' Grade by NAAC (3rd Cycle) & Placed in Category 'A' by MoE (GoI)
NEHRU NAGAR, BELAGAVI 10
Phone : 0831-2470362
FAX: 0831-2470640
Web: http://www.kledental-bgm.edu.in
E-mail: principal@kledental-bgm.edu.in

Approval Letter **Sl. No. :137**

Institutional Research and Ethics Committee KLE VKIDS

Registration No. EC/NEW/INST/2021/2435 - Research and Ethics Committee

To..... **REG. NO- IM0221004** **.....** **Date:20/04/2023**

Sub: IR&EC Approval of your application to conduct the study Protocol

The Institutional Research & Ethics Committee of KLE VKIDS, Belagavi has reviewed and discussed your application to conduct the research.

Protocol Title: Comparative Evaluation of Osteoblastic Response on Titanium Discs Coated with L-PRF (Leucocyte Platelet Rich Fibrin) and A-PRF+ (Advanced Platelet Rich Fibrin Plus) – An In Vitro study.....

The following documents were reviewed:

1. **Study Design and synopsis protocol** ✓
2. **Consent form** ✓

Discussion Points:

IR&EC of KLE VKIDS meeting was held on 19th/20th-2022.....in College Council Hall, KLE VKIDS, Belagavi. IR&EC considered your application letter dated: 11th/20th-2022..... IR&EC reviewed and discussed the above submitted study related documents. The decision on the protocol is as under:

1.	Approved - [We approve the trial to be conducted in its present form]	✓
2.	Approved with suggestions/conditions	
3.	Minor modification/ Amendments	
4.	Major modification for full board review	
5.	Disapproved	

The study is approved for the duration: 6 months/1 year/ more than 1 year

Conflict of Interest : Yes/ No ✓

Note: *It is to be noted that neither PI nor any of the proposed study team members were present during the decision-making procedures of the Institutional Research & Ethics Committee & members who are independent of the Investigator and the Sponsor of the trial have voted / provided opinion on the trial. The Institutional Research & Ethical Committee follows procedures that are in compliance with the requirements of ICMR (Indian Council of Medical Research) guidance related to GCP (Good Clinical Practice) and New Drugs and Clinical Trial Rules, 2019.*

Yours truly,

MEMBER SECRETARY
Research & Ethical Committee
KLE VK Institute of Dental Sciences
BELAGAVI.

Member Secretary, IEC KLE VKIDS

ANNEXURE- II

CERTIFICATION OF GRADE V TITANIUM DISCS

ISO 9001:2015 Certified
★ Optical Emission Spectrometry
★ PMI
★ Hardness Testing
★ Ultrasonic Flaw Detection
★ Ultrasonic Thickness Gauging
★ Dye Penetrant Testing

METAL TEST LAB
(Recognised By Government Deptts & Undertakings)
Office : Gr. Flr. Bhavnagari Bldg., 72, Nanubhai Desai Rd., Khetwadi Main Road, Mumbai - 400 004.
Phone : 6743 7546 • Mobile : 9224778882 / 9223371637 • E-mail : metaltestlab2016@gmail.com

TEST REPORT

T/C No : 1820 DATE 04/03/2022

PARTY NAME : SPECIAL METALS
125, C.P. TANK ROAD,
MUMBAI - 400 004.

REFERENCE : -
MATERIAL DESCRIPTION: TITANIUM DISC
GRADE : TI GR 5

%	C %	Si %	Mn %	P %	S %	Cr %	Mo %	Ni %	Al %
COMP	0.0600								6.10
REQD	– 0.0800	–	–	–	–				5.5000 6.7500

%	Co %	Cu %	Nb %	Ti %	V %	W %	Pb %	Fe %	N %
COMP				87.88	4.40		--	0.069	–
REQD	– –	– –	– –	– –	3.5000 4.5000	– –	– –	– 0.4000	– –

REMARK: THE ABOVE MATERIAL CONFIRMS TO TITANIUM GR. 5 W.R.T. ELEMENTS SPECIFIED.

For METAL TEST LAB

AUTHORISED SIGNATORY

1. The above Test Reports relate only to the sample submitted.
2. The above samples are not drawn by the laboratory.
3. The company or its partners shall in no way responsible for any financial liability due to any act of omission or error made.
4. No part of this Test Report shall be reproduced without the written permission of this laboratory.