EFFECT OF PHOTOFUNCTIONALIZATION ON THE BIO-ACTIVITY OF TITANIUM DISCS – AN IN VITRO STUDY

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Abstract

Surface treatment of titanium dental implants is generally done to enhance the bone formation over implants after surgical placement. Due to lengthy storage and shelf life, surface-treated dental implants eventually tend to lose their properties. The recently proposed idea of photo functionalization seeks to reverse these features. This process involves the surface treatment using UV radiation of different wavelengths. This current study was designed to assess and compare the photo-functionalization effects on laser-treated titanium discs utilising UV-A light at 400-320 nm and UV-C light at 250 nm in vacuum and non-vacuum. The titanium disks (diameter – 10mm and thickness – 2mm) were cut from commercial titanium rods. Laser treatment was performed by a Nd:yttriumaluminumgarnet(YAG) laser with the 1.06 µm wavelength, a 200 µs pulse duration and a pulse energy of 50J. Samples were numbered as1, 2,3….to 48 and random selection of 12 samples were assigned into four groups - only laser treated (control group),laser with UV-A, laser with UV-C under non-vacuum and laser with UV-C under vacuum condition. The two parameters protein adsorption assay and osteoblastic cell culture were studied on the titanium discs to check for surface property following photo-functionalization. The mean and standard deviation obtained for absorbance of MG-63 cells at 24 and 72 hours and protein adsorption at 2 and 24 hours shows that there is significant difference between the control, UVA, UVC non-vacuum and UVC vacuum groups. The results show a lot of diversity amongst the various groups. Tukey's HSD Post Hoc Tests were used to compare multiple groups within the groups. The titanium disc that had been UV-C treated under vacuum conditions outperformed the other groups in terms of surface characteristics and the difference was statistically significant.

Keywords: Implant, Photo-functionalization, Osseo-integration, UV radiation, Titanium, Laser.

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1. Introduction

Compared to conventional bridges and dentures, dental implants are currently one of the better treatment alternatives for individuals who have one or more missing teeth since the restorations are more comfortable and seem more natural.[1,2] Only successful osseo-integration can ensure the success of dental implants. The main requirements for the establishment of the future prosthetic phase are primary stability and adequate osseo-integration of the implant. Prior to secondary stability being attained, primary stability aids in the mechanical adaption of the implant to the host bone.[3] The size and quality of the bone, the implant's design, and the surgical method all play a role in how successfully implants are placed.[4]
It has been demonstrated that different implant surface treatments improve their performance, with laser-treated implants having superior bioactivity and a higher success rate.[5-8] various surface modification techniques including mechanical, chemical and physical ones can be used to improve the characteristics. This allows for the creation of certain surface topographies and roughness, the removal of surface contaminants and the improvement of adhesion properties.[9] However, titanium naturally loses some of its properties after being stored for a while. The notion of photo-functionalization has been applied to recover the lost properties.
"Photo-functionalization" is described as "the treatment of titanium with ultraviolet (UV) light having specific wave length and strength that induces the proven physicochemical and biological effects". After photo-functionalization, the osseo-integration around the implant surfaces is improved because the titanium surfaces' hydrophilicity, electrostatic optimisation and surface hydrocarbons are all improved.[10-17] Under vacuum and non-vacuum conditions changes are observed in the irradiating effect of UV radiation.[18]
There were few studies which proved the greater hydrophilicity and bio-activity of photo-functionalized implants using UV radiation of different wavelengths such as UV-A type which ranges from 400nm to 315nm, UV-B type which ranges from 315nm to 280nm and UV-C type which ranges from 280nm to 200nm.[13,17] No studies described the difference in the UV radiation effect under vacuum and non-vacuum conditions.[19]
The current study was designed to evaluate and compare the photo-functionalization effects on laser-treated titanium discs utilising UV-A light at 400-320 nm and UV-C light at 250 nm in vacuum and non-vacuum.

Experimental:

2. Materials And Methods

The use of titanium for various purposes in dentistry are of utmost important due to their biocompatibility and osteogenic potential. Surface treatment of pure titanium enhances implant bone integration.[6,9,11] In this study the effect of photofunctionalization over the laser treated titanium disks using two different UV radiation wavelengths under non-vacuum and vacuum conditions were examined. Protein adsorption assay and osteoblastic cell culture were evaluated to check their osteogenic potential.[13,17,19]

Sample preparation
From commercially available titanium (Ti6Al4V) rods 48 disc samples of 10mm diameter and 3mm height were milled manually(INDMAS Engineering Works). For cutting, semi-synthetic liquid coolant (KYOCERA) was used to avoid overheating which may alter the physico-chemical properties of the titanium metal rod. The 48 samples obtained were finished and treated with laser. Followed by the samples were introduced to photofunctionalization technique using UV-A type and UV-C type radiation under non-vacuum and vacuum conditions.

These samples were numbered as1, 2,3….to 48 and random selection of 12 samples were assigned into four groups laser treated (control group), UV-A,UV-C under non-vacuum and UV-C under vacuum condition.

Laser modifications

Using Neodymium; Yttrium Aluminum Garnet (Nd: YAG) laser type, power - 4.0 kW; frequency - 3 kHz all the samples were subjected to radiation. All the samples were cleaned ultrasonically in organic solvent acetone (CH3COCH3) and 70% ethanol and followed by rinsing in distilled water before laser treatment.10 All disks were rinsed ultrasonically in distilled water, air-dried at room temperature and then stored in a sealed autoclavable pouch and autoclaved before use.[5,6,8]

Photofunctionalization

Photofunctionalization was performed for 12 minutes using a UV light A (400-315nm) for group 2 and UV light C (280-100nm) in the device under non-vacuum condition for group 3 and vacuum (-1 in Hg) condition for group 4.
UV light specifications – Philips 8W TL and TUV BULB and OREVA 1 feet holder.
UV chamber specifications: Dimensions - 500mm (W) 1150mm (D) ×1300mm (H) (approx.)

Grouping:

The distribution of samples, based on the Golden rule.
Group 1 - 12 samples (laser treated titanium disks)
Group 2 - 12 samples (laser treated titanium disks treated with UV-A light)
Group 3 - 12 samples (laser treated titanium disks treated with UV-C light under non vacuum)
Group 4 - 12 samples (laser treated titanium disks treated with UV-C light under vacuum)
48 no of samples is listed in table 1.

Evaluation of osteogenic potential

Protein Adsorption Assay

In a 24-well plate bovine serum albumin (BSA) solution was introduced over each sample. After 2 and 6h of incubation at 37 $^{\circ}$C, the free protein was removed and bicinchoninic acid added and left at 37 $^{\circ}$C for 30 min. Quantification of protein was analysed in microplate reader (SpectraMax M5, USA) at the range of 562 nm.[13]

Osteoblastic Cell Culture

MG-63 cells were cultured in a protein medium with foetal bovine serum in addition with penicillin and streptomycin at 37°C and the atmosphere is maintained in a humid condition with 95% of air and 5% of CO2. At 70-80% confluence, cell density in a culture medium were initially trypsinized and then counted. The samples were sterilized in 70% ethanol and washed using 50 ml sterile phosphate buffered saline (PBS). After that the PBS was completely removed from discs and air dried for 10 min and placed in 24-well plate. 50µl of cells were introduced over the disc and incubated for 8-hrs at 37°C under a humidified atmosphere of 95% air and 5% CO2. After 8-hrs of incubation, 500µl of complete medium was slowly added to all the wells to cover the surface of the discs and the plate was further incubated for 48-hrs.After 48-hrs incubation, 50µl of resazurin dye (prepared in PBS) was added to all the wells. Live cells through its metabolic activity, breaks resazurin into the resorufin products which is used as an oxidation-reduction indicator. On addition of the dye to the wells were further incubated for 4-hrs at 37°C under a humidified atmosphere of 95% air and 5% CO2. The fluorescence reading was taken at 590 nm using biotek multi-mode plate reader.[13] From reading, the percentage of cell viability were calculated using the following formula:
Percentage growth= 100 × [(T-B)/(C-B)]
Where
T is test,
C is untreated control,
B is Media Blank (Without cells)

3. Results

The mean, standard deviation and the combined mean and standard deviation obtained for absorbance of MG-63 cells at 24 hours between and within groups is tabulated in Table 2. It shows the one- way ANOVA analysis of the mean values and the significant difference between and within the groups. The F value was calculated from the mean value, from which the P value was calculated. The P <0.05, was value considered to be significant statistically. Here set A, B, C and D were statistically significant.

Multiple group comparison within the groups was done using Tukey's HSD post Hoc test. The significance value P <0.05, was regarded as significant statistically. From the values obtained, it was appreciated that there was significant variation detected between set D and A and set D and B and set D and C sets in MG-63 cells absorbance rate at interval of 24 hours.

The mean, standard deviation and the combined mean and standard deviation obtained for absorbance of MG-63 cells at 72 hours between and within groups is tabulated in Table 3. It shows the one-way ANOVA analysis of the mean values and the significant difference between and within the groups. The F value was calculated from the mean value, from which the P value was calculated. The P <0.05, was value considered to be significant statistically. Here set A, B, C and D were statistically significant.

Multiple group comparison within the groups was done using Tukey's HSD post Hoc test. The significance value P <0.05, was regarded as significant statistically. From the values obtained, it was appreciated that there was significant variation detected between set D and A and set D and B and set D and C sets in MG-63 cells absorbance rate at interval of 72 hours.

The mean, standard deviation and the combined mean and standard deviation obtained for protein adsorption at 2 hours between and within groups is tabulated in Table 4. It shows the one- way ANOVA analysis of the mean values and the significant difference between and within the groups. The F value was calculated from the mean value, from which the P value was calculated. The P <0.05, was value considered to be significant statistically. Here set A, B, C and D were statistically significant.

Multiple group comparison within the groups was done using Tukey's HSD post Hoc test. The significance value P <0.05, was regarded as significant statistically. From the values obtained, it was appreciated that there was significant variation detected between set D and A and set D and B and set D and C sets in protein adsorption at interval of 2 hours.

The mean, standard deviation and the combined mean and standard deviation obtained for protein adsorption at 24 hours between and within groups is tabulated in Table 5. It shows the one- way ANOVA analysis of the mean values and the significant difference between and within the groups. The F value was calculated from the mean value, from which the P value was calculated. The P <0.05, was value considered to be significant statistically. Here set A, B, C and D were statistically significant.

Multiple group comparison within the groups was done using Tukey's HSD post Hoc test. The significance value P <0.05, was regarded as significant statistically. From the values obtained, it was appreciated that there was significant variation detected between set D and A and set D and B and set D and C sets in protein adsorption at interval of 24 hours.

Graphical representation of comparison of MG63 cells absorbance at 24 and 72 hours among four sets along with control model in Graph 1.

Graphical representation of comparison of protein adsorption at 2 and 24 hours among four sets along with control model in Graph 2.

Graphical representation of comparison of MG63 cells viability in percentage at 24

and 72 hours among four sets along with control model in Graph 3.

Graphical representation of comparison of protein adsorption at 2 and 24 hours in percentage among four sets along with control model in Graph 4.

4. Discussion

The metal titanium and its alloys are best at integrating with host tissues and fostering osseointegration. Osseointegration was first defined by Branemark as the direct interaction between living bone tissue and the implant surface.[1,2] Due to its greater mechanical qualities and higher level of tissue compatibility, it is the most often utilised implant material..

Titanium as an implant material 1) Shows corrosion resistance property; 2) no intolerable reaction in the host and 3) has the necessary physical property.[2]

Though new alloys comprising niobium, iron, molybdenum, manganese, and zirconium are being researched, the most popular dental implant materials are commercially available pure titanium (cpTi) and Ti-6Al-4V alloy due to their better biocompatibility and mechanical qualities. When titanium is exposed to oxygen found in ambient air, a stable oxide layer forms, which is primarily what causes titanium to be hydrophobic.[5]

As rough surfaces need less time for integration and can greatly reduce treatment time, surface roughness is one of the most crucial factors for implant life. Surface roughness can be produced using either subtractive or additive processes. Physical, mechanical, and chemical procedures can be used to treat surfaces. In dental implants, it is utilised to alter the surface's topography and energy which improves wettability, cell proliferation, and the osseointegration process. The implant's biocompatibility and surface imperfections are crucial for better tissue acceptance and osseointegration.[7,8,9]

The light microscopic representation of bone produced at implant sites without an intervening fibrous connective tissue interface is one of the radiographic and clinical indicators of implant success.

Numerous cell lines have been utilised to explore interactions with implant biomaterials, including transformed Saos-2 and MG-63 cells generated from human osteosarcomas and non-transformed MC3T3-E1 cells produced from mouse calvaria. To study the impact of biomaterials and the accompanying cell response, MG 63 human osteoblast-like cells are used. Growth of osteoblastic cells is mostly influenced by the surface microarchitecture. Numerous osteoblastic genes, which are in charge of producing bone-forming cells, are found in MG63 cells. Following the addition of 1, 25-dihydroxyvitamin D3 (1, 25 (OH) 2D3), MG63 cells displayed an elevated alkaline phosphatase activity. The quantity of cells represents cell adhesion, spreading, and proliferation.[13]

This study was done on titanium discs. The surface modification included the process of photofunctionalization using UV light with two different wavelength UV-A and UV-C under vacuum and non-vacuum condition.[13,17] Under a scanning electron microscope, the impact of these surface changes was qualitatively investigated, and their osteogenic potential was assessed at the bimolecular level. These surface alterations are contrasted with titanium discs that underwent laser treatment rather than UV treatment. The outcomes demonstrated a considerable impact of UV light treatment on cell metabolism on the titanium surface treated with a laser. Uneven 3D surfaces can be produced by the laser source. When compared to other laser sources, Nd: YAG can only modify the implant surfaces 3-D structurally at the micro and nano meter scale.[6] This method is preferred for complicated surface geometry. Accurate control over the width and depth of the therapy are another benefit of laser technology. Due to the fact that this method allows for implant surface treatment without direct contact, there will be no contamination.[7] These techniques

make them interesting for geometrically complex biomedical implants. The UV treated and laser treated samples were subjected to check their osteogenic potential from cell viability at 24 and 72 hours and protein adsorption at 2 and 24 hours period. Samples in group B were irradiated under 15 W UVA at 360 nm. Samples in group C and D were irradiated under 15 W UVC lamp at 250 nm under non-vacuum and vacuum condition. The vacuum pressure is created using electrical vacuum pump and the negative pressure is created up to -1 in Hg. These samples were treated with UV light under ambient conditions for 12 minutes for each groups.

In this laboratory study, comparison of three different groups were done. The results showed that though the cells are binding on the surface of the disc, it does not show any toxicity on the tested cell line, MG-63. Finally, the reports of the present study revealed that the significance level of the four groups by multiple comparisons between the groups for MG-63 cells absorbance at 24 and 72 hours and protein adsorption assay at 2 and 24 hours. The results obtained indicates significant variation among the various groups. Multiple group comparisons within the groups were done by using Tukey's HSD Post Hoc Tests. UVC treated titanium disc under vacuum condition shows better results than the other groups and it is statistically significant when compared to other groups.

Other markers which signifies the bone formation were not evaluated with enough power in this study. Further studies are needed for clear demonstration of osteogenic gene expression as well as for cell metabolism.

Various surface modifications were described in the literature. The clinical effect of photofunctionalization on MBL remains unclear due to the shortage of available studies.[20] Some surface modifications claims to be better in comparison to other modification, which suggests the need for standardization. Recent advancements in the materials for dental implant such as the combination of zirconium and titanium, zirconium and PEEK are seen. Future studies are required in the surface treatment of these materials to obtain better osseointegration and reduce the healing time especially in diabetes patients whose risk of implant failures are higher.[21]

5. Conclusion

This in-vitro study was conducted to evaluate and compare the effect of surface modification of commercially pure titanium using ND-YAG laser treatment with photofunctionalization using UV radiation on their osteogenic potential and effect of the cell toxicity to the surface topography. With certain limitations of the study the following conclusion were made:

1. ND: YAG laser modified surface showed increased confluence of osteogenic MG-63 cells and increased protein adsorption.
2. All the four groups – laser without UV treatment and laser with UV-A, UV-C non vacuum and UV-C vacuum treatment showed adherence of human osteosarcoma cells MG-63.
3. ND-YAG laser with UV treated samples showed better adherence of MG-63 cells and adsorption of proteins in comparison with UV untreated group of titanium discs.
4. Among all the groups, ND -YAG laser treated samples with UV-C vacuum group showed increased adherence of MG-63 cells and adsorption of proteins.
5. Laser treatment of commercially pure titanium with photofunctionalization using UV-C radiation under vacuum condition can increase the bone implant interface by providing a clean and contamination free surface for osseointegration.

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up for UV treatment and titanium sample preparation.

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Figures And Tables

Figure 1. Dimension of the titanium sample

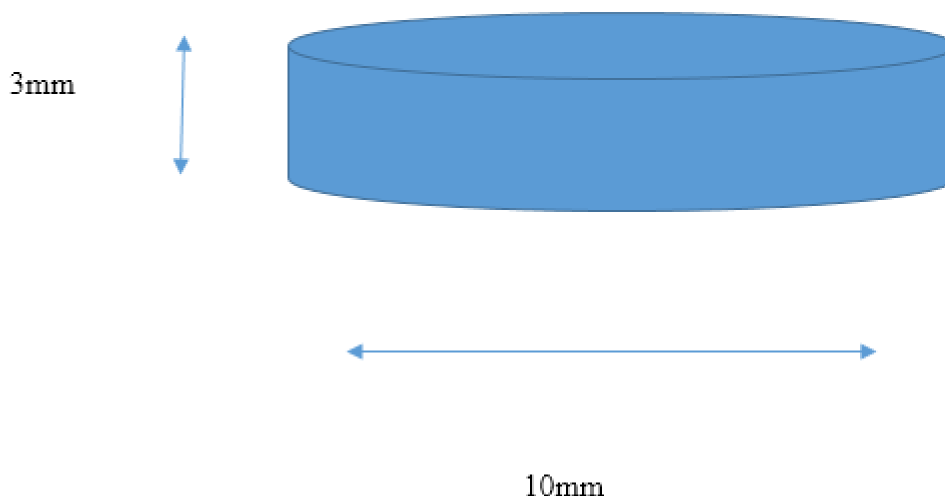


Figure 2. Machined titanium discs

Figure 3. Laser treated titanium discs

Figure 4. Photofunctionalization chamber

Figure 4. Photofunctionalization chamber

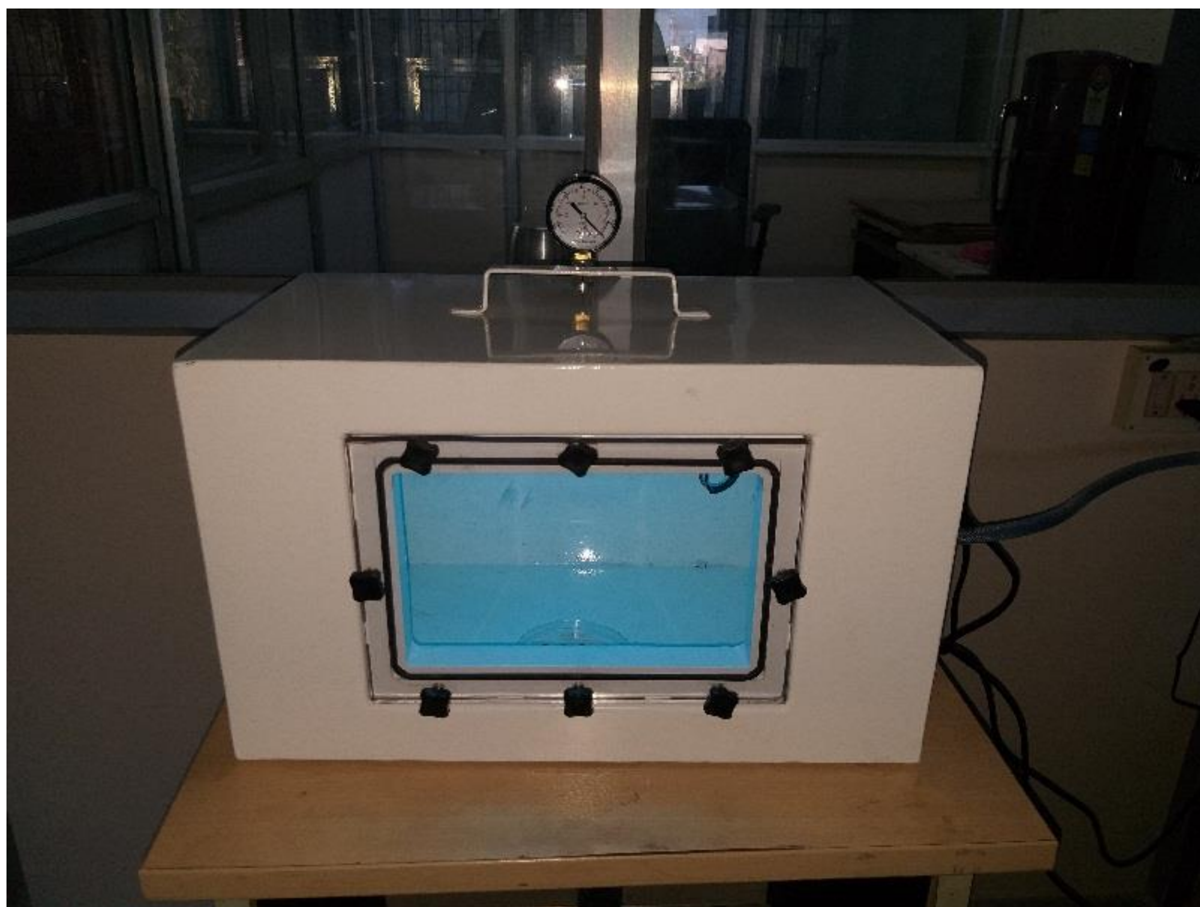


Figure 5. Electronic vacuum pump

Figure 6. UV treated titanium discs

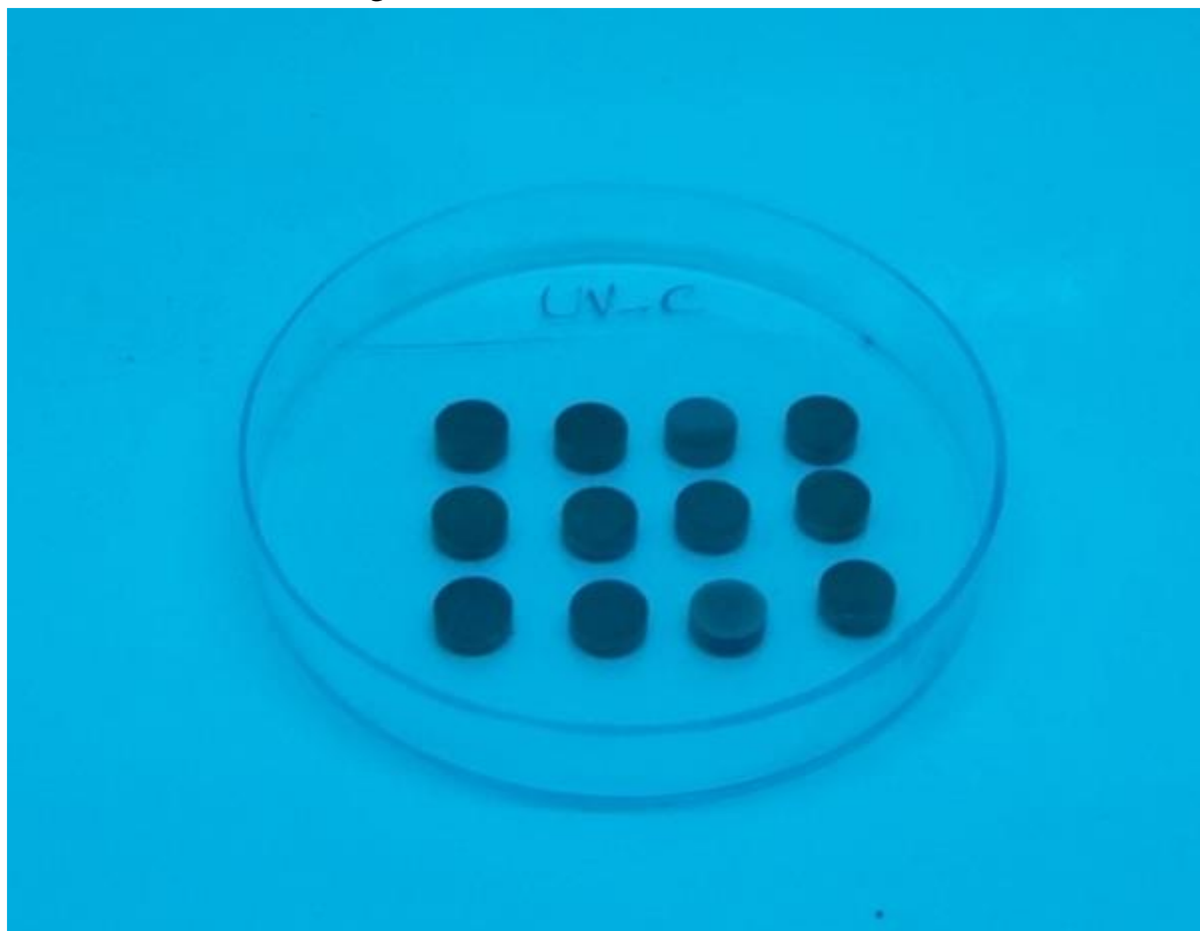


Figure 7. Plate-1 MG 63 cells seeded on test discs after incubation with Resazurin

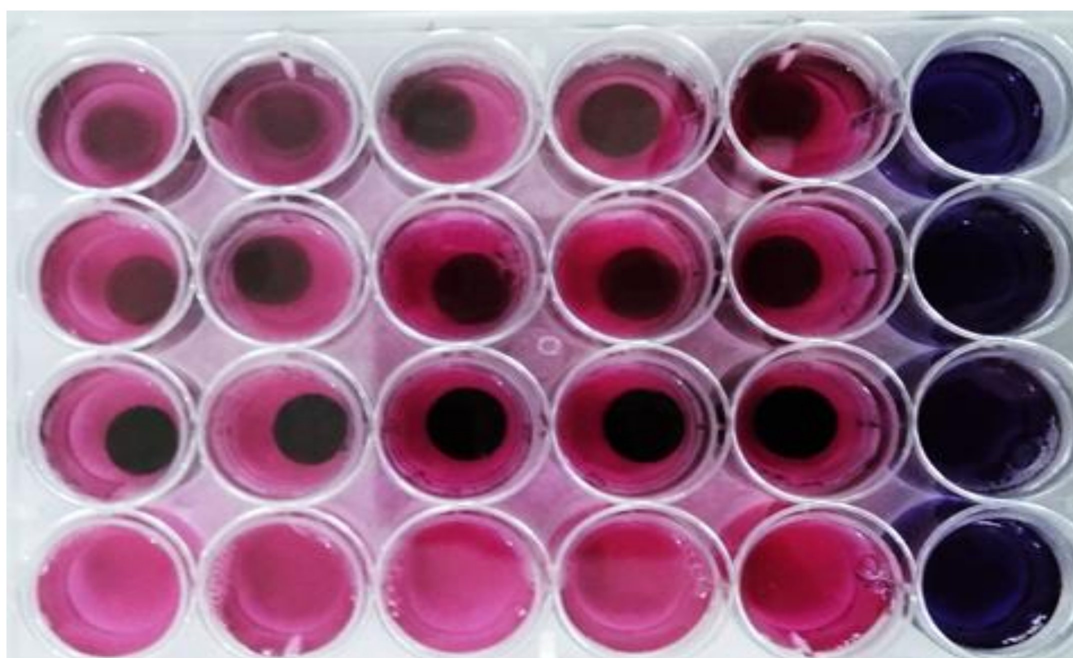


Figure 8. Plate 2 MG-63 cells seeded on test discs

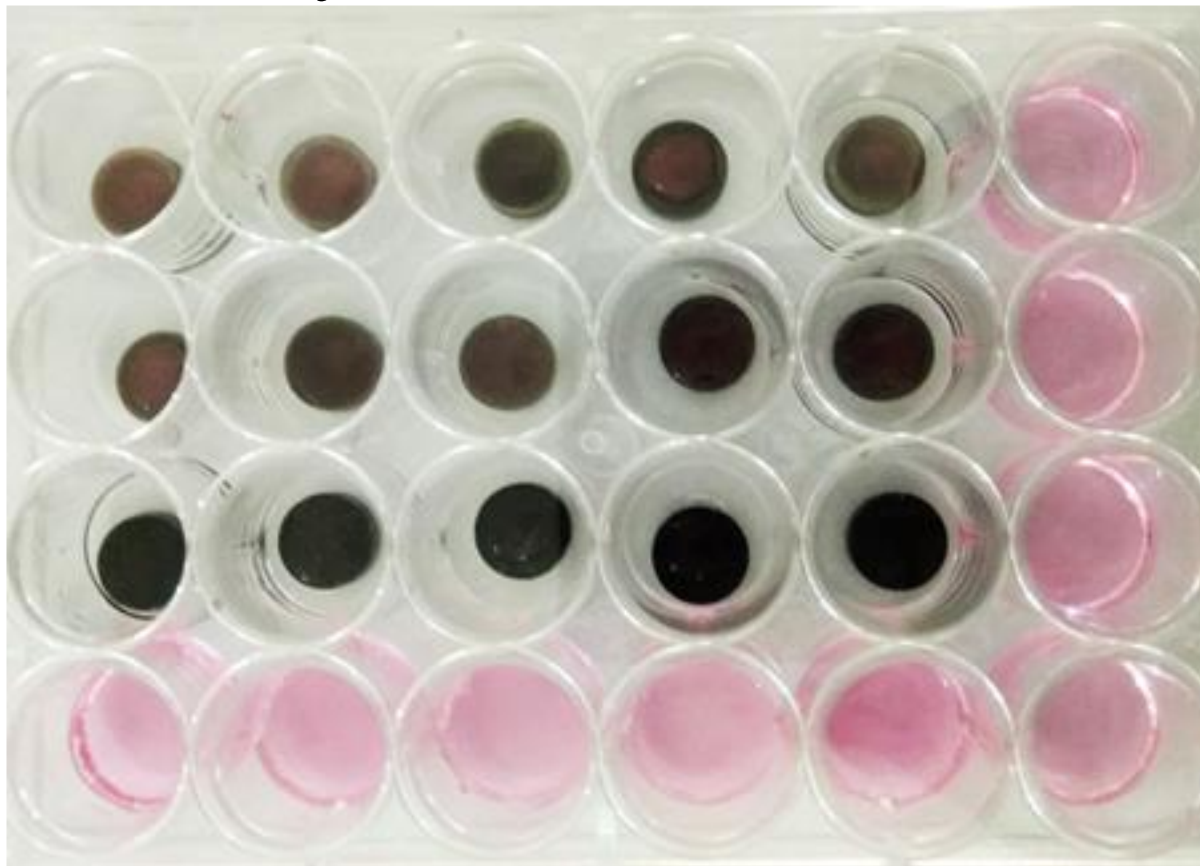
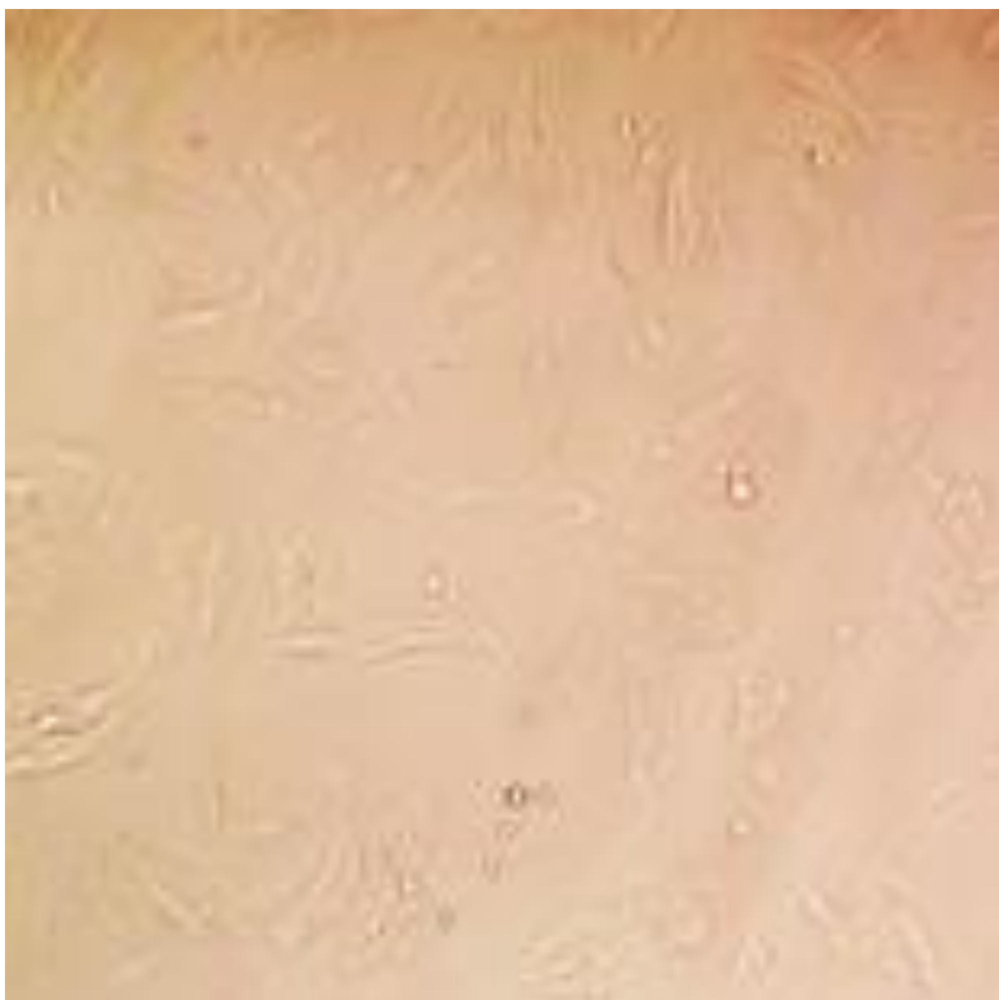


Figure 9 . Control MG -63 cells after 24 hours and after 72 hours



TABLES:

Table 1. Distribution of samples for various surface treatments and for osteogenic potential

Parameters	Surface treatment			
Osteoblastic cell culture and Protein adsorption assay	Laser treated	Laser with UV-A	Laser with UV-C (non-vacuum)	Laser with UV-C (vacuum)
	Group 1 (n=12)	Group2 (n=12)	Group 3 (n=12)	Group 4 (n=12)

Table 2. Osteoblastic cell culture 24 hours

Absorbance - 24 Hours	**MEAN**	**SD**	**p- Value**
Set A	0.3617	0.00718	0.000***
Set B	0.3775	0.00754	
Set C	0.5483	0.00835	
Set D	0.5950	0.01087	
Cell control	0.6700	0.00000	

***$p < 0.001$

Table 3. Osteoblastic cell culture 72 hours

Absorbance - 72 Hours	**MEAN**	**SD**	**p- Value**
Set A	0.5250	0.00522	0.000***
Set B	0.6150	0.01000	
Set C	0.7950	0.01000	
Set D	0.8292	0.00793	
Cell control	0.9200	0.00000	

Table 4. Protein adsorption assay at 2 hours

Protein adsorption - 2 Hours	**MEAN**	**SD**	**p- Value**
Set A	0.21292	0.000996	0.000***
Set B	0.22517	0.001528	
Set C	0.26208	0.001084	
Set D	0.26850	0.001883	
Cell control	0.32000	0.000000	

Table 5. Protein adsorption assay at 24 hours

Protein adsorption - 24 Hours	**MEAN**	**SD**	**p- Value**
Set A	0.21275	0.000965	0.000***
Set B	0.22467	0.001614	
Set C	0.28183	0.000937	
Set D	0.29208	0.001084	
Cell control	0.32000	0.000000	