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ORIGINAL RESEARCH

Evaluation of Hemocompatibility of Titanium after Various Surface Treatments: An *in vitro* Study

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ABSTRACT

Objective: To evaluate the hemocompatibility of titanium after various surface treatments.

Materials and methods: A total of 27 disk-shaped specimens (3 × 10.0 mm) were prepared from a cylindrical rod of medical grade titanium. The disks were divided into three groups, of which one was considered as the control (mechanical surface polished surface). The other groups being sandblasted disks and anodized disks. Surface evaluation was done for sandblasted and anodized disks with scanning electron microscope. The specimens were placed in polystyrene culture plates and agitated with phosphate buffered saline for 5 minutes before they were exposed to blood taken from human volunteer. The materials were under 30 minutes agitation at 75 ± 5 rpm using an Environ shaker thermostated at 35 ± 20°C. The total hemoglobin from the initial sample was measured using automatic hematology analyzer. Percentage hemolysis, thrombin time, platelet adhesion and activation were assessed. Significant differences between different treated titanium materials were determined using Minitab® Version 15.1.1.0. A two-sample t-test was performed to find the p-values for different groups of data.

Results: After 30 minutes of agitation, cells began to spread on the test surfaces. There was a clear reduction in the number of platelets before and after exposure to titanium samples. Reduction of leukocytes was seen to a least extent on the anodized surface. Rough surface induced higher hemolysis than other groups. Platelet reduction and leukocyte reduction in all the three surfaces was quite higher than that obtained for reference plate. Surface variation has no significance on the thrombogenic capabilities of medical grade titanium ($p < 0.05$).

Significance: The hemocompatibility of medical grade titanium did not vary with different surface modifications.

Keywords: Anodization, Blood, Hemolysis, Leukocytes, Platelets, Titanium.

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INTRODUCTION

The biocompatibility of implant material in the human body is related to the interaction between the living cells and implant material surface. Not all implant material surfaces have truly long-term biocompatibility, even though many biomaterials have been used for clinical implantation.

Biocompatibility must be divided into cytocompatibility (cell and tissue compatibility) and hemocompatibility (blood compatibility).

Importance of Hemocompatibility

The incidence of thromboembolic complications and bleeding complications has been 1.5 to 3% per year in the USA,[1] and 58% of implanted mechanical heart valves have failed within 12 years in China.[2]

Recent research is roaming around the interaction of blood components with titanium oxide films. It is believed that the biocompatibility of titanium derives from characteristics of the naturally formed titanium oxide film on its surface.[3] However, this naturally formed oxide layer is very thin (about 10 nm) and has a high density of defects. Better biocompatibility has been reported by improving the quality of titanium oxide films.[4] Sunny and Sharma[5] reported that the ratio of absorbed proteins albumin/fibrinogen increased 7 times as the thickness of titanium oxide film was increased from several nm to 200 nm by anodic oxidation processes. Huang et al,[4] reported that the clotting time increased 1.5 times when the thickness of the titanium oxide film was increased from 10 to 250 nm by a thermal oxidation process. Ebert and Schaldach[6]fabricated a titanium oxide ceramic doped with tantalum and found that the activated partial thromboplastin time of the material was even longer than that of low temperature isotropic pyrolytic carbon (LTIC). Huang et al,[7] and Zhang et al,[8] have reported that both amorphous and crystalline titanium oxide films prepared by ion beam enhanced deposition possess better hemocompatibility than LTIC.

For endosseous implant fixation in bones, two methods were employed. One is bone cement fixation and the other is cementless implantation. Consequently, prostheses can be classified into cemented and cementless ones in accordance with the fixation methods in bone tissues. For cemented prostheses, the components are fixed to the bony implant bed employing bone cement based on poly (methyl methacrylate). Penetrating into the cancellous bone structure, the cement hardens within a few minutes resulting from an exothermal reaction. This leads to a continuous cement mantle that is well-anchored in the bone and lies closely against the implant.[9] This technique had its own disadvantages and drawbacks. Cementless prostheses with the optimal surface structure and composition to enable osseointegration can produce lasting mechanical interlocking between the implant and bone.[10] Rough surfaces, porous coatings and surfaces with osteo-conductivity and osteoinductivity in body fluids have been shown to be good surfaces for osseointegration.

The development of materials with improved hemocompatibility therefore is an ongoing task. The blood reactions are initiated by physical and chemical characteristics of the foreign surface. Definition and control of these parameters are prerequisite for any blood compatible surface. To an increasing extent, coating technologies combine the technological advantages of the bulk material with surface properties for appropriate performance in the biological ambiance.

The material of interest in the present topic is the medical grade titanium, which is mostly being the material of choice for dental implants. Not all implant material surfaces have truly long-term biocompatibility, even though many biomaterials have been used for clinical implantation. Therefore, various modification techniques have been considered to increase the surface biocompatibility of titanium (Ti)[10,11] which is one of the most popular implant materials for clinical applications. This present study is based on the evaluation of thrombogenic activities of various surface modified medical grade titanium.

MATERIALS AND METHODS

A cylindrical rod of medical grade titanium (grade 5) of diameter 10 mm was taken and was cut into slices of 3 mm height each. Three groups considered were smooth polished titanium pellets, roughened pellets and anodized pellets. Nine specimens from each group were considered. These pellets were exposed to blood from a human volunteer. Platelet poor and rich plasma was prepared by centrifugation. Blood parameters were analyzed using hematology analyzer (Sysmex-K 4500). The parameters taken into count in the present study were percentage hemolysis, thrombin time (TT) and prothrombin time measurement, platelet adhesion and activation. The specimens were sent for scanning electron microscope (SEM) analysis. Data analysis was done.

Preparation of Specimens

Specimens were cleaned by ultrasonic technique in ultrapure water.[21]

Mechanical Surface Polishing

The surface of the pellets in three groups were polished with silicon carbide grit papers starting from 240 and increasing to 400, 600, 800 and 1200. Intermittently after polishing with different sandpapers, the surface was washed with water to rinse off any particles generated while polishing. Ultrasonic cleaning in distilled and ultrapure water for about 5 minutes was done after polishing to clean the surface more effectively (Fig. 1).

Sandblasting

Some of the polished specimens were blasted with sand particles at a pressure of 90 psi. Ultrasonic cleaning in ultrapure water for about 5 minutes was done to ensure no sand particles were left on the surface. These were considered as Group II (Fig. 2).

Anodization

For anodization, titanium samples were connected to a direct current powered electrochemical cell which had a two electrode configuration.[12] A platinum mesh was used as the cathode and a titanium specimen was used as the anode. These platinum and titanium samples were connected to a direct current power supply (3645 Amperes) through copper wires. A constant voltage of 20 V was applied for 10 minutes according to previous studies.[13,14] The distance between the titanium anode and platinum cathode was kept constant at 1 cm. The electrolyte solution used in this study was 1.5%

Fig. 1: Polished samples

Fig. 2: Sandblasted samples

hydrofluoric acid and the anodization was conducted inside a Teflon beaker. During anodization, the electrolyte solution was constantly stirred with magnetic agitation to reduce the thickness of the double layer at the metal-electrode interface to obtain uniform local current densities on the titanium electrode.[15] After anodization, the specimens were again sonicated with acetone, 70% ethanol and distilled water, for 30 minutes each (Fig. 3).

Exposure of Materials with Blood

Blood from human volunteer was collected into the anticoagulant. The pellets from three groups were placed in polystyrene culture plates and agitated with phosphate buffered saline (PBS) for 5 minutes before they were exposed to blood (Fig. 4). To each plate, 5.0 ml blood was added and 1.0 ml was taken immediately for analysis and remaining 4.0 ml blood was exposed to the materials for 30 minutes under agitation at 75 ± 5 rpm using an Environ shaker thermostated at 35 ± 20°C (Fig. 5). Three empty polystyrene culture dishes were exposed with blood as reference.

Analysis of Blood Parameters

The blood count was analyzed in initial and 30 minutes samples using Hematology Analyzer (Sysmex-K 4500). The equipment calibration was verified using traceable standard reference control. Counts detected initially and on termination of exposure are noted. Percentage change in blood parameters is calculated for each sample.

Percentage Hemolysis

The total hemoglobin from the initial sample was measured using automatic hematology analyzer (Sysmex-K 4500). The free hemoglobin liberated in to the plasma after exposure was measured in each sample using Shimadzu spectrophotometer and the percentage hemolysis was calculated using the formula [free hemoglobin (Hb)/total Hb)] × 100.

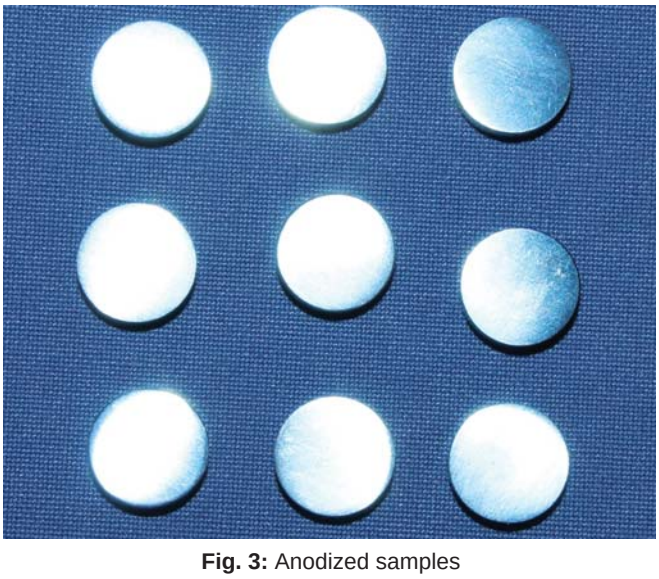

Fig. 3: Anodized samples

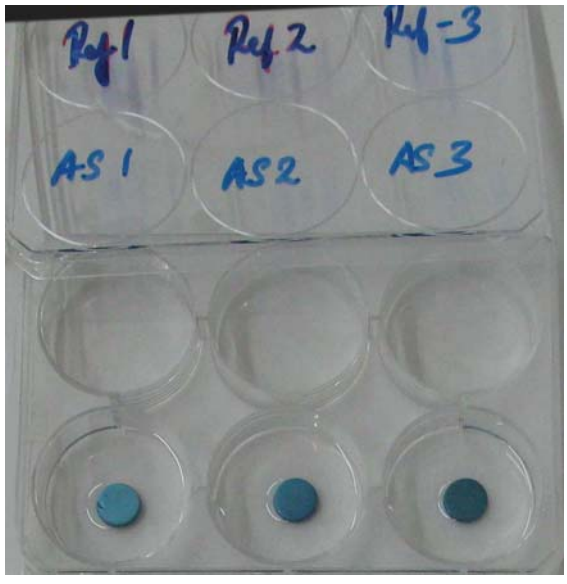

Fig. 4: Samples placed in polystyrene culture plates and agitated with phosphate buffered saline for 5 minutes

Fig. 5: Addition of blood to specimens

TT and Prothrombin Time Measurement

Platelet poor plasma was prepared by centrifuging blood from a healthy adult volunteer for 15 minutes. A total of 2 ml platelet poor plasma were put into contact with the test samples at a temperature of 37°C for 10 minutes in a silanized beaker. The TT measurements were performed by adding 200 ml TT reagent to 200 ml platelet poor plasma in a test tube and then evaluated with a clotting tester. The prothrombin time measurements were carried out by adding 100 ml platelet poor plasma to 200 ml prothrombin time reagent in a test tube and then evaluated with the same clotting

tester. Three tubes for each kind of sample, for both the TT and prothrombin time tests, were measured to obtain mean values. Through this, the leukocyte change was observed.

Platelet Adhesion and Activation

Platelet-rich plasma was prepared from citrate anticoagulated blood by centrifugation at 124 gm for 30 minutes. A total of 100 µl/cm^2 of the platelet-rich plasma were distributed on each sample and incubated for 45 minutes at 37°C in humidified air. Then the supernatant plasma was aspirated, and the samples were washed in phosphate buffered solution (PBS). The adherent thrombocytes were lysed in 50 µl 1% Triton-X 100. The lactate dehydrogenase activity in this lysate is proportional to the number of lysed cells. The activity was determined photometrically. The values are normalized to the number of platelets in the whole 100 ml aliquot platelet-rich plasma.

Sample Preparation Protocol for SEM

The treated specimens were fixed in 2.5% glutaraldehyde, 4% paraformaldehyde solution for 30 minutes and rinsed with PBS for 30 minutes. They are washed with ultrapure distilled water for 5 minutes. The process is repeated twice. Then, the cleaned samples are dehydrated with 50% ethanol, 70% ethanol, 80% ethanol, 90% ethanol, 95% ethanol, 100% ethanol, for 15 minutes in each of the solutions. They are rinsed with ethanol and hexamethyl disilazane reagent in 1:1 ratio for 15 minutes. Critical point drying with hexamethyl disilazane reagent is done for 15 minutes. The specimens were allowed to dry and gold sputtering of the surface was done for SEM imaging.

Data Analysis

Significant differences between different treated titanium materials were determined using Minitab® Version 15.1.1.0. A two-sample t-test was performed to find the p-values for different groups of data. Results obtained were validated with student's t-test. Mean and standard deviation for the data were also calculated using Minitab®. Analysis of variance test is administered to check whether the average significant difference among rough smooth and anodized types of platelet count in blood samples.

RESULTS

The highest average platelet reduction was seen on smooth surface medical grade titanium (20.9%), followed by anodized surface (18.83%), and the least was in rough surface (16.1%) (Table 1). The average percentage reduction in reference was 7.8 ± 0.79 (Table 2). The p-value for the platelet reduction comparing the three groups of surface modifications was 0.125 (Table 3), which meant that the results were not significant (Fig. 6).

The average percentage reduction of leukocytes was observed to be least in anodized surface (12.433%), followed by smooth and rough surface samples (13.507%, 13.467% respectively) (Table 4). Average percentage change in reference was 1.60 ± 1.39. The least p-value was shown by smooth surface material (0.003), which shows that this type of surface has more significant results in relation to the leukocyte count (Table 5). The p-value for the leukocyte change comparing the three groups of surface modifications was 0.952 (Table 6), which meant that the results were not significant (Fig. 7).

There were very minor differences with respect to the hemolytic effect of the three groups. Highest is being shown by rough surface followed by anodized and smooth surface groups (0.03, 0.027, 0.023 respectively). Average percentage hemolysis in reference after 30 minutes exposure is 0.03 ± 0.00 (Table 7). The p-value showed to be 0.296 (Table 8), which meant that surface modification has no significance on the hemolytic effect of medical grade titanium (Fig. 8).

Table 1: Platelet count in blood samples before and after exposure to materials

Sample type	*Initial count (×10^8)*	*Final count (×10^8)*	*Percentage reduction*
Smooth surface a	9.23	7.5	18.74
Smooth surface b	9.25	7.2	22.16
Smooth surface c	9.21	7.2	21.80
Rough surface a	8.88	7.44	16.2
Rough surface b	9.48	8.12	14.4
Rough surface c	9.28	7.64	17.7
Anodized surface a	9.24	7.16	22.5
Anodized surface b	9.28	7.80	16
Anodized surface c	9.16	7.48	18

Table 2: Paired t-value and p-value for platelet count before and after exposure of materials

Sample type	*Difference mean*	*Difference SD*	*Paired t-value*	*p-value*
Smooth surface a Smooth surface b Smooth surface c	1.9	0.174	19.17	0.003
Rough surface a Rough surface b Rough surface c	1.48	0.144	17.77	0.003
Anodized surface a Anodized surface b Anodized surface c	1.74	0.3055	9.90	0.01

SD: Standard deviation

Table 3: Analysis of variance test and significance values for platelet count before and after exposure of materials

Type	*Mean*	*Standard deviation*	*F-value*	*p-value*
Smooth	20.9	1.879	3.01	0.125
Rough	16.1	1.652		
Anodized	18.83	3.329		

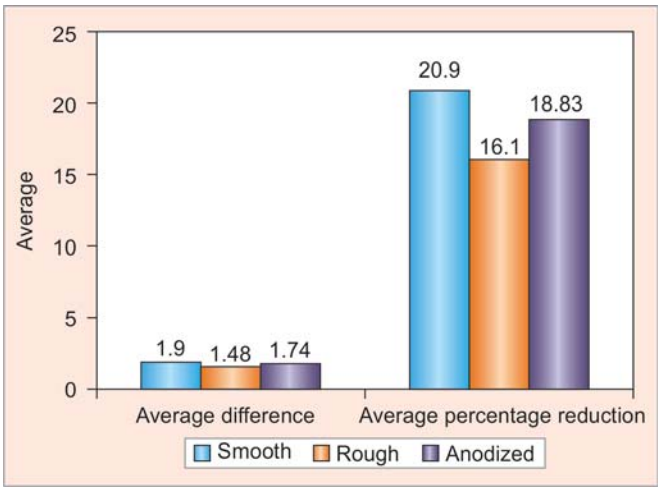

Fig. 6: Average difference and average percentage reduction of platelet counts

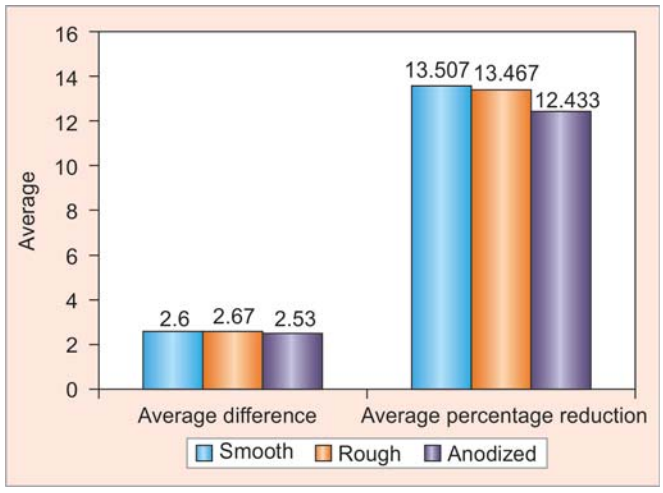

Fig. 7: Average difference and average percentage reduction of leukocyte counts

Table 4: Leukocyte count in blood before and after exposure to materials

Sample type	*Initial count ($\times 10^6$)*	*Final count ($\times 10^6$)*	*Percentage reduction*
Smooth surface a	19.6	17.1	12.75
Smooth surface b	19.5	16.6	14.87
Smooth surface c	18.6	16.2	12.9
Rough surface a	19.6	17.2	12.2
Rough surface b	20.0	16.8	16.0
Rough surface c	19.6	17.2	12.2
Anodized surface a	19.6	18.4	6.1
Anodized surface b	20.8	16.4	21.2
Anodized surface c	20.0	18.0	10.0

Table 5: Paired t-value and p-value for leukocyte count before and after exposure of materials

Sample type	*Difference Mean*	*Difference SD*	*Paired t-value*	*p-value*
Smooth surface a Smooth surface b Smooth surface c	2.6	0.265	17.02	0.003
Rough surface a Rough surface b Rough surface c	2.67	0.462	10	0.01
Anodized surface a Anodized surface b Anodized surface c	2.53	1.66	2.63	0.119

SD: Standard deviation

Table 6: Analysis of variance test and significance values for leukocyte count before and after exposure of materials

Type	*Mean*	*Standard deviation*	*F-value*	*p-value*
Smooth	13.507	1.183	0.05	0.952
Rough	13.467	2.194		
Anodized	12.433	7.839		

A SEM image of polished and anodized titanium is shown in Figures 9 and 10. The image clearly depicts the development of nanostructured pores. A higher magnification at 2000× SEM image of polished and anodized titanium taken at 100KX is shown (see Figs 9 and 10). Diameter of the pores were measured and found to be between 50 and 60 nm.

Table 7: Percentage hemolysis in plasma samples after exposure

Sample type	*Percentage hemolysis*
Smooth surface a	0.02
Smooth surface b	0.02
Smooth surface c	0.03
Rough surface a	0.03
Rough surface b	0.03
Rough surface c	0.03
Anodized surface a	0.03
Anodized surface b	0.02
Anodized surface c	0.03

Table 8: Analysis of variance test and significance values for hemolysis counts after exposure of materials

Type	*Mean*	*SD*	*F-value*	*p-value*
Smooth	0.0233	0.00577	1.5	0.296
Rough	0.03	0.000		
Anodized	0.02667	0.00577		

SD: Standard deviation

DISCUSSION

In the provocative article, 'The Catastrophe Revisited: Blood Compatibility in the 21st Century', Buddy Rutner[16] aptly describes the inability of the biomaterial community, despite 50 years of intense research, to agree on an exact definition of the term 'blood compatibility'.[17]

He proposes five hypotheses, why progress toward a clear understanding of blood compatibility is so slow, as are follows:

- *Hypothesis 1:* It is impossible to manufacture material that is completely blood compatible.
- *Hypothesis 2:* We do not understand the biological processes of blood compatibility.

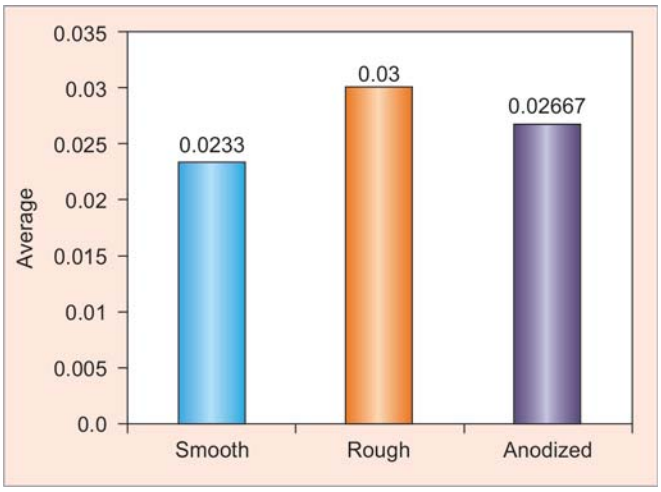

Fig. 8: Average percentage hemolysis in plasma samples after exposure

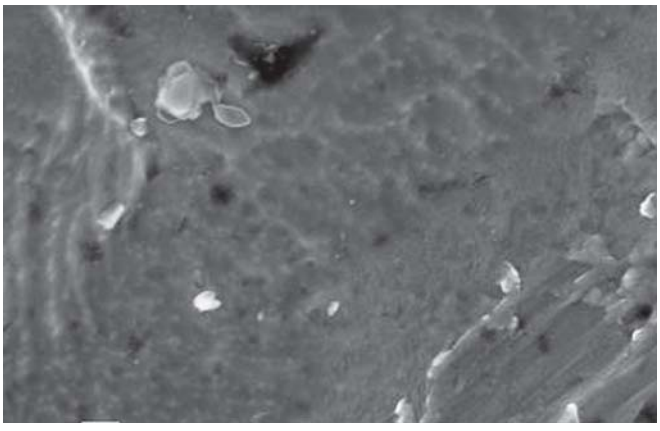

Fig. 9: Scanning electron microscope image of sandblasted sample at 2000×

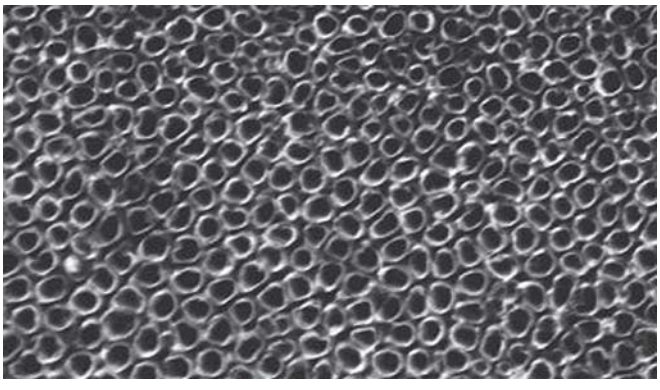

Fig. 10: Scanning electron microscope image of anodized samples at 2000×

- *Hypothesis 3:* We do not know how to test blood compatibility.
- *Hypothesis 4:* Certain materials of natural origin appear to have a better blood compatibility, but we do not know why?
- *Hypothesis 5:* In the meantime, better blood compatible materials are available but regulatory requirements and economic conditions prevent implementation in clinical practice.

From International Standard ISO 10993

Unfortunately, the issue of hemocompatibility of medical products is often neglected. Neither the American Food and Drug Administration (FDA) nor the European Directives or the German Central Authority for Health Protection with Regard to Medicinal Products and Medical Devices (ZLG) has defined any clear qualitative and quantitative guidelines for the blood compatibility of plastic materials. Even the implementing standard ISO 10993-4 (testing for interactions with blood) is relatively unclear and ambiguous. There is a need to give a detailed and a clear idea about hemocompatibility which plays a very important role in titanium and dental implantology.

Most commonly used methods for testing are as follows:

1. Blood, plasma, serum extraction and heparinization.
2. Static models with serum.[18]
3. Chandler loop model.[19]
4. Heart-lung machine model.[20]
5. Flow cytometric examinations.

It is important to remember, there is no guidance currently endorsed by the FDA. But, there are tests the FDA has been requiring. Nelson Labs recommends manufacturers contact the FDA directly to determine which of the test trends will be most suitable for their device.

One trend is Hemolysis testing—a test that must be performed on any device coming in contact with blood. The test looks for bursting red blood cells. The test is conducted by placing a device sample in blood to see if there are toxic reactions that impact the red blood cell. If there are none, then the sample is perceived to be safe. A second test—complement activation—is part of ISO 10993-4, but in the standard, the test only applies to devices with large surface areas that come in contact with blood. The FDA is now requiring this for all devices that contact the circulatory system. The most controversial test, and the only animal test of the three, is *in vivo* thrombosis. It is an expensive test and is not always reproducible. The test requires a sample medical device, along with another similar device known to be safe (predicate device), to be placed in animals to test its reaction. The *in vivo* thrombosis test is especially controversial since the results are not always accurate and there is a major push and trend in Europe, to discontinue all animal testing.

The hope is to make the standard more effective in result predictions instead of just for safety purposes. The goal is to get it to the point where tests are not used unnecessarily, but rather use acceptable protocols written directly into the standard. Not many studies were observed regarding the hemocompatibility of titanium, as were seen in case of the more frequent cytocompatibility. Both hold equal importance. More common and a well-versed term is biocompatibility of titanium which is defined as the

combination of cytocompatibility and hemocompatibility. This particular study was done to assess the hemocompatibility of medical grade titanium. Various surface modifications were considered, like roughened, smooth surface and anodized surface which were more nearer to the clinical implications.

Rough surface showed lesser average percentage reduction of platelets, followed by anodized and smooth (Table 2). The p-value showed that platelet reduction significant results on variation of surface (Table 3). Reduction of leukocyte count was least for anodized surface (Table 4). Rough surface induced higher hemolysis than other groups (Table 7). Platelet and leukocyte reduction in all the three surfaces was quite higher than that obtained for reference plate. Surface variation has no significance on the thrombogenic capabilities of medical grade titanium.

CONCLUSION

There were many adhered platelets on the anodized titanium and relatively reduced platelet aggregation on the rough surface and much lesser on the smooth surface. The deposited platelets exhibited an activated morphology, as demonstrated by extended pseudopodia and surface spreading. Despite its relatively large variation, no effect of the roughness on fibrinogen adsorption or blood platelet adherence was seen. The blood platelet adhesion on the surfaces in this study in general was very low, usually below 2.5% of the whole amount of blood platelets in the plasma sample. This renders this class of surfaces a promising candidate for good blood compatibility and low clotting activation.

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