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ORIGINAL RESEARCH

Cytocompatibility by MTT Assay and Platelet Adhesion of Ti and Ti-6Al-4V coated with Hydroxyapatite in different Plasma Gas Atmospheres

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ABSTRACT

Aim: This study was performed to evaluate the biocompatibility of pure titanium and Ti-6Al-4V metals coated with hydroxyapatite (HA) by plasma spray using different plasma gas atmospheres.

Materials and methods: The cell viabilities for each HA-coated sample in an atmosphere of argon, argon–hydrogen, nitrogen, and nitrogen–hydrogen were studied using MTT assay and platelet adhesion test.

Results: The mean cell viabilities by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay of samples coated with HA in argon–hydrogen plasma atmosphere showed maximum cell viability at different time intervals compared with other coating atmospheres of argon–hydrogen, nitrogen, and nitrogen–hydrogen. A statistically significant value of cell viability ($p < 0.001$) was observed between and within the groups of argon, argon–hydrogen, nitrogen, and nitrogen–hydrogen plasma gas atmosphere. The platelet adhesion study showed agglomerates of platelet cells in some isolated regions of HA for all atmospheres.

Significance: The results obtained in this study can serve as a guide for the development of new Ti-based HA-coated implants in different plasma gas atmospheres.

Keywords: Cytocompatibility, Hydroxyapatite, MTT assay, Plasma coating, Platelet adhesion, Titanium, Ti-6Al-4V.

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INTRODUCTION

Commercially, pure titanium (CP-Ti) and Ti-6Al-4V are the most suitable metallic materials as dental and medical implants because these materials exhibit good mechanical properties and have corrosion resistance.[1] However, vanadium in Ti alloys may dissolve and form harmful metal ions, which have been detected in tissue close to the titanium implant.[2,3] Materials of good biological properties show a high rate of bone growth and high corrosion resistance in high-chloride-containing body fluid environment. Corrosion of implant material causes minute corrosion products to accumulate in adjacent tissues and stimulate allergy in patients.

The efficiency of metallic implants for biomedical applications can be improved by coating with biocompatible materials. Bioconductive material hydroxyapatite (HA) [$Ca_{10}(PO_4)_6(OH)_2$, (HA)] is widely used as a coating on the surface of the metals or alloys because they chemically connect the metal/alloy implant and bone. Hydroxyapatite promotes chemical osteointegration through the formation of a light bond with bone due to its similarity to CaP minerals found in bone tissues. Specifically, the biocompatibility of the HA and the osteoconductivity behavior of this ceramic material were confirmed and employed in medicine for more than 25 years.[4]

Hydroxyapatite coatings have been applied by a variety of methods: Dip coating,[5,6] electrophoretic deposition,[7,8] hot isostatic pressing,[5] ion beam sputtering,[9] ion beam dynamic mixing,[10] plasma spraying,[11] conventional flame spraying,[12,13] and high-velocity oxy-fuel combustion spraying.[14] Among these, plasma spray appears to be most favorable in terms of chemical control, biocorrosion resistance,[15,16] process efficiency,[2] and the degree to which the surface fatigue resistance is reduced.[17]

In plasma-sprayed coatings, the hot gas jet created by plasma arc expands, entrains powder particles, heats the particles, and accelerates them toward the substrate, where they impact, deform, and solidify to form a coating. The high degree of particle melting and relatively high particle velocity of plasma lead to higher deposit densities and bond strength compared with most flame and electric arc spray coatings. The high droplet/substrate adhesion is achieved due to the high particle velocity and deformation that can occur on impact. The inert gas plasma

jet, ignoring ambient air mixing effects, contributes to lower oxide content than other thermal spray processes.

The ideal HA coating for orthopedic and dental implants would be one with low porosity, strong cohesive strength, good adhesion to the substrate, a high degree of crystallinity, and high chemical purity and phase stability. Depending on the specific application, different materials are used. In some applications, osseointegration behavior is the deciding factor, whereas for some materials in contact with blood, the prevention of platelet adhesion and subsequent clotting is the main focus. For example, endosseous implants initially come into contact with blood. Thus, the nature of the interaction between the blood and implanted endosseous implants may influence bone healing events in the peri-implant healing compartment. Furthermore, mechanical properties like wear, hardness, and elastic modulus can have an important influence on biocompatibility. In the present investigation, studies on the following were conducted:

- Does the coated surface have an influence on the cell viability?
- Do the HA-coated metal substrates modulate platelet activity?

The properties of the coatings are controlled by regulating various plasma spray parameters. Some basic parameters include power, current, distance between nozzle and substrates, plasma work gas rate, and feed rate. The plasma working gas composition remains the same during the plasma spray process and is not generally a basic parameter that changes during the coating process. However, it is considered to be an important parameter that has an influence on coating properties.[18] For example, argon mixed with hydrogen gives a good coating. But without hydrogen, the powder particles bounce back from the flame instead of entering it. This is because of the high velocity and viscosity of the argon gas. Plasma work gas composition also influences the thickness of the coating. For example, nitrogen as carrier gas results in the thicker coating as compared with the use of argon gas. Therefore, one can expect the plasma gas composition to also play a major role in the properties of HA coating.

A strict plasma control is required to optimize the property of the coating. Many investigators[2,16,19-26] have studied various processing parameters to understand the phase constitution, porosity, degree of crystallinity, OH^- content, microstructure, and surface roughness of the HA coating by regulating the basic spray parameters.

It was therefore planned to study the effect of plasma working gas composition on HA coating keeping in mind the basic parameters of plasma spray technique. The coatings were made on Ti and Ti-6Al-4V metal substrates. The plasma gas compositions selected in the present study were argon, nitrogen, argon–hydrogen, and nitrogen–hydrogen. The aim of this study was to evaluate the biocompatibility of pure titanium and Ti-6Al-4V metals coated with HA by plasma spray using different plasma gas atmospheres.

MATERIALS AND METHODS

Plasma Coating

Plasma spray processing is the process of utilizing plasma flame in order to spay various coating materials onto metal substrates. In this plasma is a stream of gas, i.e., made luminous as a result of heat. This luminous plasma flame is used to coat the powder onto the substrate metal by fusion. In the present study, plasma coating of HA procured from Plasma Biotal Limited, UK (Capital 30 Batch P253/0116) was given on CP Ti (ASTM B 348 Gr. 1), hereafter called Ti 12, and Ti-6Al-4V (ASTM B 348 Gr. 5), hereafter called Ti 31 metal substrates. The metallic samples of dimension 100 × 6 × 4 mm were prepared from the rods of Ti 12 and Ti 31 received from Mishra Dhatu Nigam Ltd., Hyderabad, India. The coating was done at Spraymet Surface Technologies Pvt. Ltd., Bengaluru, India. The surface of the substrate metal was roughened before coating for better bonding between the metal substrate and HA coating. Surface roughening was done with grit blasting with Al_2O_3 of grit size 24. The roughened Ti 12 and Ti 31 samples were then washed with water, followed by alcohol before plasma coating.

A plasma spray process used a direct current (DC) electric arc to generate a stream of high-temperature ionized plasma gas. The arc was struck between a tungsten cathode and a copper anode within the torch. The torch was fed with a continuous flow of inert gas, which was ionized by the DC arc, and was compressed and accelerated by the torch nozzle so that it issues from the torch as a high-velocity (in excess of 800 m/sec), high-temperature (12,000–16,000 K) plasma jet. The coating material in the powder form was carried in an inert gas stream into the plasma jet where it was heated and propelled toward the substrate. The coating was carried out separately using four gas atmospheres: Argon, argon–hydrogen, nitrogen, and nitrogen–hydrogen. Here argon and nitrogen served as primary gas and hydrogen acted as a secondary gas. A pressure of 0.76 MPa for the primary gas (argon and nitrogen) and 0.069 MPa for the secondary gas of 10% volume (hydrogen) was applied to get the desired plasma jet. The stand-off distance between the substrate and the jet was maintained at 8 cm till achievement of the desired coating thickness of 100 µm.

Toxicity Evaluation

In vitro evaluation of any biomaterial is very important in order to understand the cell viability of the material

before actual usage. In the present study of toxicity evaluation, specimens of dimension 6 × 4 × 4 mm (n = 6) were cut using low-speed Isomet Buehler precision cutting instrument from all HA-coated and -uncoated samples for cell viability by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma Chemical Co.).

MTT Assay

The test was carried out according to the protocol described by Mosmann.[27] It is a simple colorimetric test for cell proliferation and survival, which is used for the measurement of cytotoxicity. The assay involves the ability of viable cells to convert a soluble tetrazolium salt MTT into the purple formazan end product by mitochondrial dehydrogenase enzymes. The purple color reaction is used as a measure of cell viability.

Exponentially growing V79 cells (10^4) were seeded into 96-well plates and incubated for 24 hours at 37°C in CO_2. Eight wells each served as a control, blank, and rest of the wells were used for different test samples. The culture medium was removed and substituted with fresh medium with different test samples labeled as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 for Ti 12 argon, Ti 31 argon, Ti 12 argon–hydrogen, Ti 31 argon–hydrogen, Ti 12 nitrogen, Ti 31 nitrogen, Ti 12 nitrogen–hydrogen, Ti 31 nitrogen–hydrogen, Ti 12, and Ti 31 respectively, for 24, 48, and 72 hours. After that, 100 µL of the MTT stock (1 mg/mL) was added to each of the 96 wells followed by 4-hour incubation at 37°C in a 5% CO_2 atmosphere. The test samples were removed from the medium and purple-colored precipitate of formazan was solubilized by dissolving in a 100 µL buffer consisting of 23% sodium dodecyl sulfate in 50% N,N-dimethyl formamide (pH 4.7). Greater magnitude of optical density (OD) due to intense purple coloring is regarded as showing higher cell viability. After 5 to 10 minutes of incubation at 37°C, the ODs were read on a multiwell spectrophotometer (Tecan, Austria) at 540 nm wavelength. Percent viability was calculated as follows:

$$\text{Percent viability} = \frac{\text{Average of test (OD)} - \text{Average of blank (OD)}}{\text{Average of control (OD)} - \text{Average of blank (OD)}} \times 100$$

Platelet Adhesion Study

The activation of platelet in contact with any biomaterial affects the healing process, and it is a function of microtexture, composition, and other parameters. Platelet adhesion is an initial, crucial, and complex matter. Activation and adhesion of platelet play a fundamental role in the development of thrombosis. For the platelet adhesion study, specimens of dimension 6 × 4 × 4 mm were cut from uncoated Ti 12 and Ti 31 and also from HA plasma-coated samples of argon, argon–hydrogen, nitrogen, and nitrogen–hydrogen (n = 6). The uncoated Ti 12 and Ti 31 samples' surface was polished by initially grinding with successively finer Emery papers and finally polished on a disk using lavigated alumina. All the test samples were cleaned in acetone and ethyl alcohol solution for 5 minutes, and finally rinsed with distilled water and dried.

Methodology of Platelet Adhesion Blood adhesion tests were conducted in a class 10,000 clean room.[28] The samples were evenly distributed in two culture dishes; 200 mL blood (containing 3.8 mL wt% citrate solution, blood/citrate acid = 9:1) extracted from a healthy adult was centrifuged to form a platelet-rich plasma (PRP) and erythrocyte. The PRP contained about 30–35 × 10^7 platelets/mL. Then the blood was poured into culture dishes. After culturing at 37°C for 30 minutes and 3 hours respectively, the PRP was taken out of the wells. A phosphate-buffered solution was added to the wells and gently rinsed 2 to 3 times to get rid of platelets adsorbed nonspecifically on the surface. Then the samples were soaked in 2% glutaraldehyde for 1 hour and 5% glutaraldehyde for 12 hours to fix the platelets, which adhered specifically on the surface. After rinsing with distilled water, the samples were subsequently dehydrated through 50, 75, 90, and 100% ethanol–water solutions twice for 10 minutes each. After dehydrating, the samples were subsequently dealcoholized through 50, 75, 90, and 100% isoamyl acetate water solution twice for 10 minutes each. The samples were dried at a critical point overnight. After sputter coating with platinum, the surfaces of the samples were observed under scanning electron microscopy, and the photographs of the platelets were randomly taken from the scanned field.

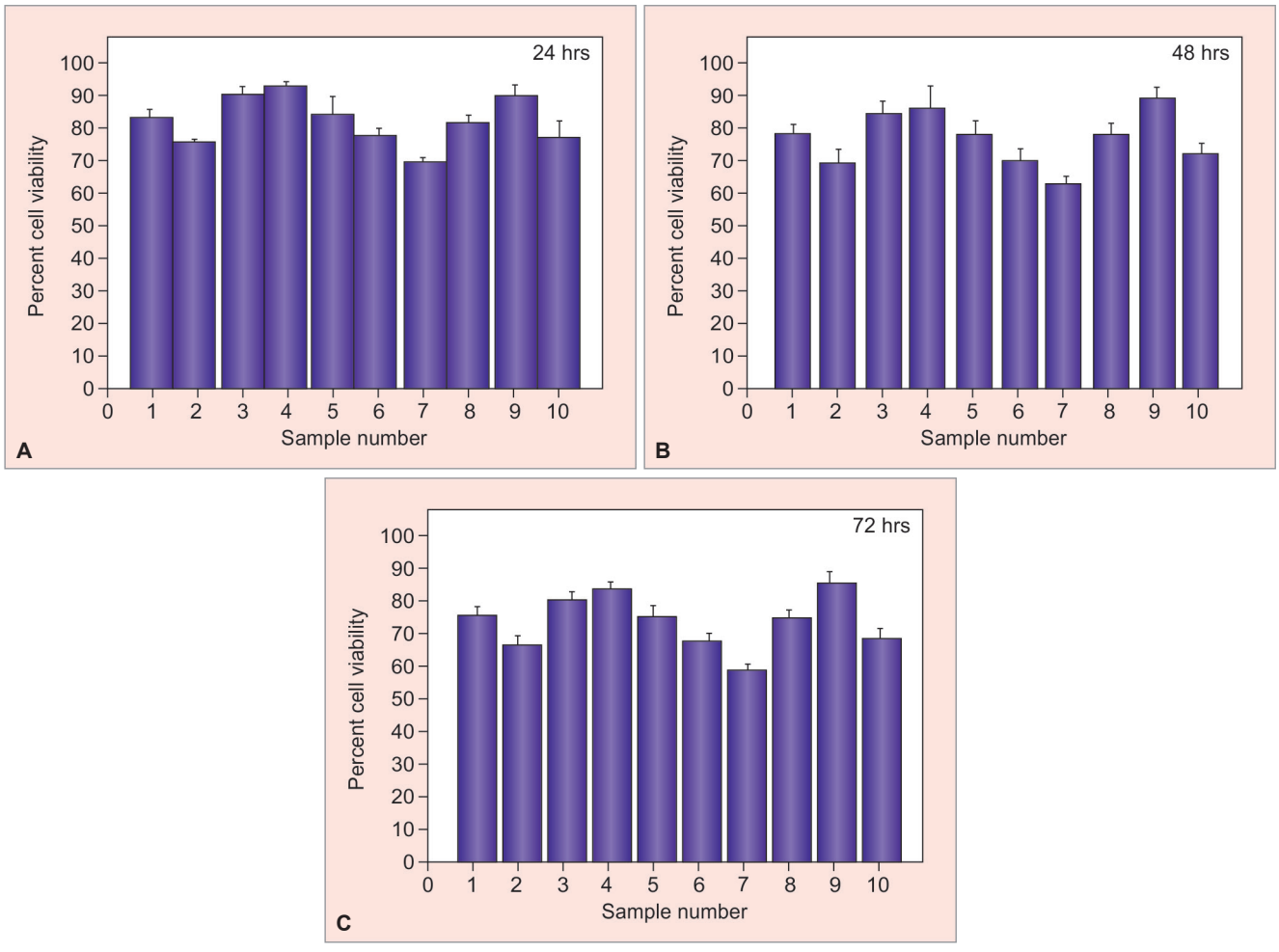
RESULTS

Toxicity Evaluation by MTT Assay

The aim of this study was to evaluate the cytotoxicity of uncoated and coated Ti 12 and Ti 31 in different gas atmospheres. The results of cell viability at 24, 48, and 72 hours are presented in Graphs 1A to C. The statistical analysis was done by students unpaired t-test using Statistical Package for the Social Sciences (SPSS) vession 17.0 software, which is presented in Table 1. Maximum cell viability was observed for the argon–hydrogen atmosphere at different time intervals.

Platelet Adhesion Study

The platelet adhesion on coated and uncoated samples exposed to 30 minutes and 3 hours is shown in sets of Graphs 1A to C, Figs 1A to H, and 2A to H. Platelet



Graph 1A to C: Toxicity evaluation of HA-coated and -uncoated specimen at 24, 48, and 72 hours

adhesion was found in some areas of the coating that were soaked in PRP for 30 minutes and 3 hours. Uncoated samples showed more platelet activation as compared with coated samples, with an increase in the soaking period.

DISCUSSION

Toxicity Evaluation by MTT Assay

The decomposition of HA owing to extremely high-temperature subjection during plasma coating causes complicated phase compositions in the coatings including crystalline and amorphous HA, calcium phosphates, such as tricalcium phosphate and tetracalcium phosphate, and calcium oxide. These impurities can lower the bioactivity of HA coating. The MTT assay is one such test through which the toxicity evaluation of the material can be tested by cell viability measurement.

Graphs 1A to C shows the comparative cytotoxic effect of various test samples' treatment on V79 cells *in vitro* at 24, 48, and 72 hours. There was time-dependent decrease in the cell death as indicated by a decrease in OD in those wells treated with different test samples. The percent cell viability was found to be more in the samples coated with HA in the argon–hydrogen atmosphere on Ti 12 (sample code 3) and Ti 31 (sample code 4) compared with other test samples. The uncoated Ti 12 (sample code 9) also showed higher cell viability compared with other groups (argon, nitrogen, and nitrogen–hydrogen) at the tested time periods. Lower values of cell viability of samples coated in the atmospheres of argon, nitrogen–hydrogen, and nitrogen were observed.

The statistical analysis of the results as shown in Table 1 showed statistically high significant value ($p < 0.001$) for all coating atmospheres at different time intervals within and between the groups. The mean cell viability was found to be highest in the case of HA coated in the argon–hydrogen atmosphere, which is above 90% at 24 hours and above 80% at 48 and 72 hours. However, there was time-dependent decrease in cell death in all the samples coated with HA in different atmospheres as well as uncoated Ti 12 and Ti 31.

The reason for showing high cell viability in the case of argon–hydrogen plasma coating atmosphere may be attributed to the inert nature of primary plasma gas

Table 1: Statistical analysis descriptive

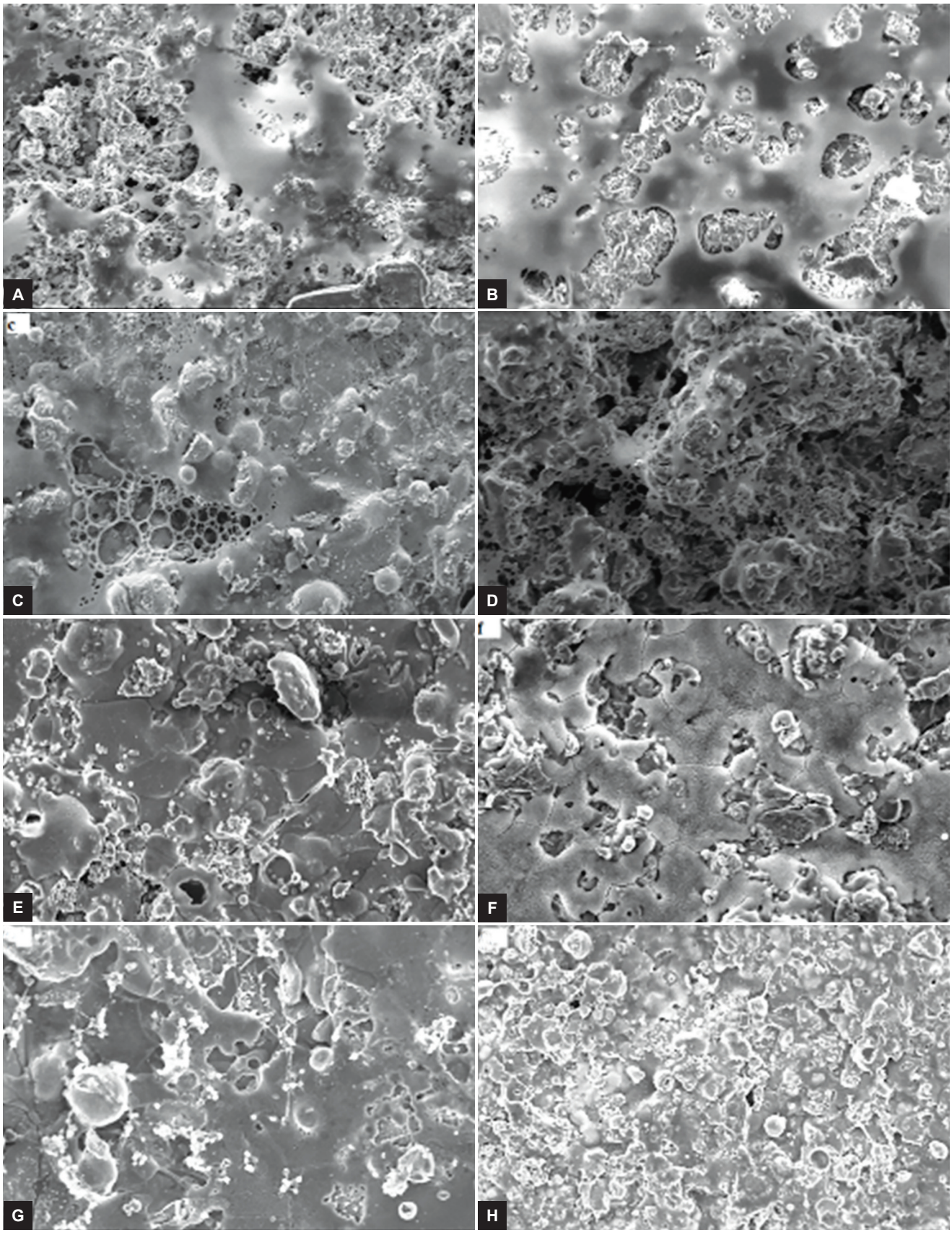
Groups	Class		n	Mean	Std. Deviation	F-value	p-value
I	Ar-Ti 12	24 hours	6	83.500	1.049	128.478	<0.001
		48 hours	6	78.167	0.753		
		72 hours	6	75.833	0.753		
	Ar-Ti 31	24 hours	6	76.500	1.049	166.689	<0.001
		48 hours	6	70.833	0.753		
		72 hours	6	67.000	0.894		
II	Ar/H-Ti 12	24 hours	6	90.500	1.049	203.790	<0.001
		48 hours	6	84.167	0.753		
		72 hours	6	81.000	0.632		
	Ar/H-Ti 31	24 hours	6	93.000	0.632	180.326	<0.001
		48 hours	6	88.833	0.753		
		72 hours	6	85.167	0.753		
III	NTi 12	24 hours	6	84.333	1.033	103.941	<0.001
		48 hours	6	78.667	0.816		
		72 hours	6	76.500	1.049		
	N-Ti 31	24 hours	6	77.833	0.753	263.922	<0.001
		48 hours	6	70.833	0.753		
		72 hours	6	68.167	0.753		
IV	N/H-Ti 12	24 hours	6	69.500	1.049	280.574	<0.001
		48 hours	6	62.667	0.816		
		72 hours	6	58.333	0.516		
	Ni/H-Ti 31	24 hours	6	83.000	0.894	136.311	<0.001
		48 hours	6	78.667	0.816		
		72 hours	6	75.167	0.753		
V	Ti 12	24 hours	6	90.833	0.753	30.064	<0.001
		48 hours	6	88.833	0.753		
		72 hours	6	86.667	1.211		
	Ti 31	24 hours	6	78.667	0.816	121.250	<0.001
		48 hours	6	73.000	1.549		
		72 hours	6	68.667	0.816		

argon and the reducing nature of the secondary gas hydrogen. In such inert and reducing atmosphere, there is less chance of material undergoing chemical changes due to oxidation.

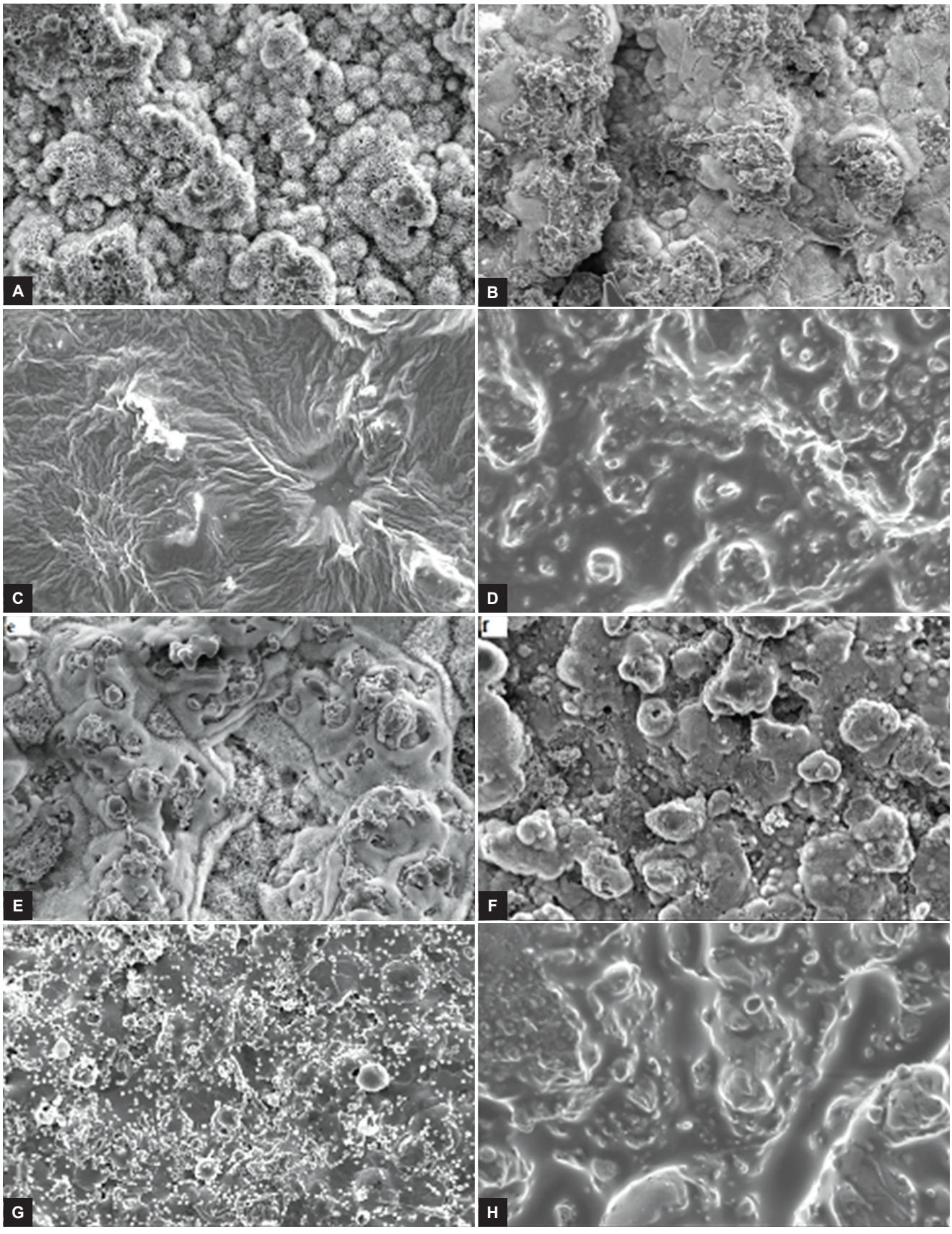
Platelet Adhesion Study

Many studies[29-31] have shown that HA-coated endosseous implants exhibit more implant bone formation and more bone contact at early healing times than uncoated ones. In order to determine the change in potential blood compatibility because of structural changes that have taken place during the coating at different atmospheres, platelet adhesion studies were selected. Platelet adhesion is one of the most important steps during blood coagulation on the artificial surface. This test is used to evaluate the antithrombus of biomaterials through observing the state of platelets on the surface of biomaterials. If the substance used is biocompatible, then the platelet adhering to it should be minimal or negligible. This is because the platelets always adhere to substances which are foreign in nature. This is a protective phenomenon to remove any foreign body. Platelets activate the coagulation system in which the fibrinogen is covered with fibrin, which is seen as fibrin thread. It has also been reported[32] that an increase in the complexity of surface microstructure enhances platelet adhesion activation on titanium. For this reason, polishing the surface of a titanium metal to a mirror surface has been expected to be the reasonable method to improve thromboresistance.[33]

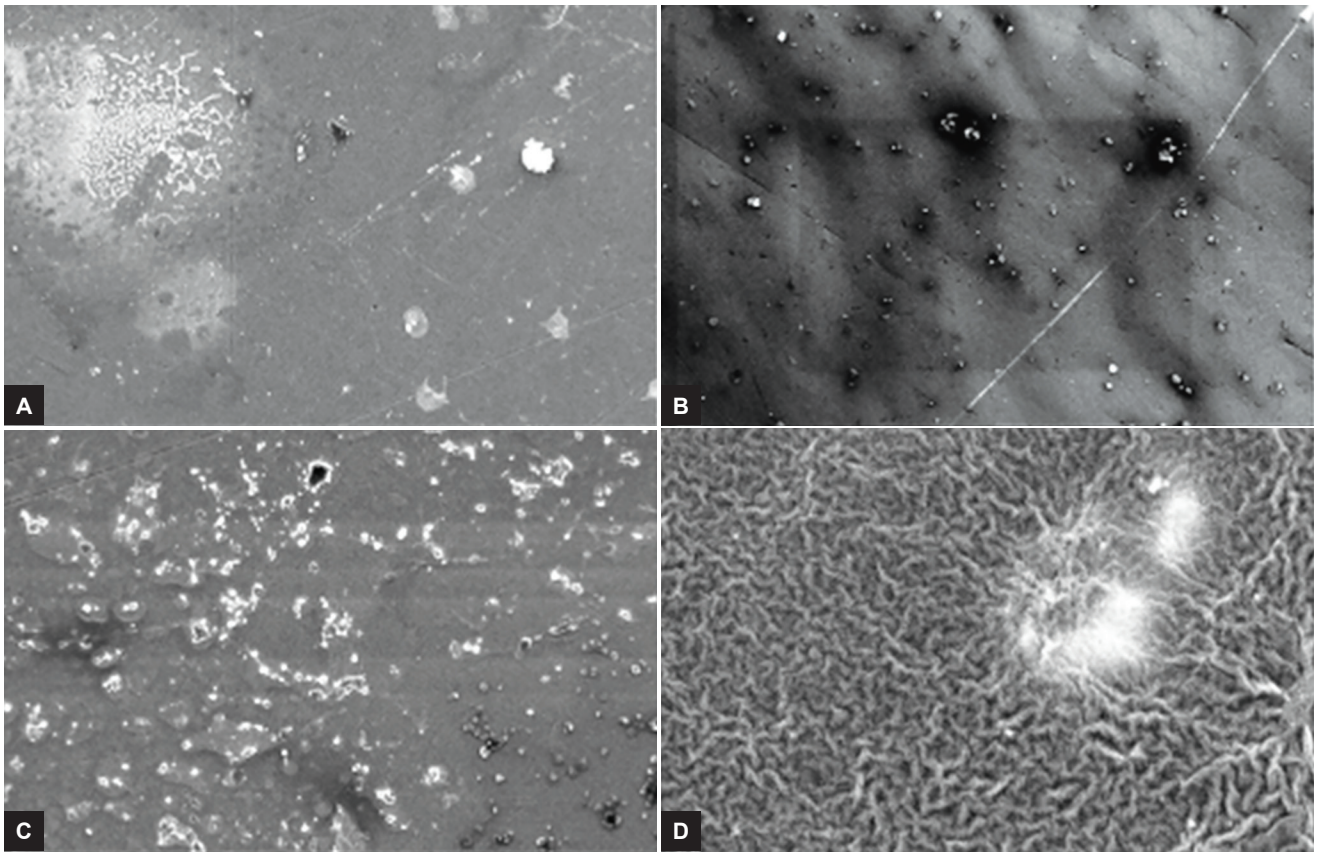
The present study involves the influence of plasma gas composition on the blood compatibility of HA coating. Upon scanning, the entire surface of all the coated samples soaked in PRP for 30 minutes as well as 3 hours showed only some isolated regions of platelet adhesion. Some of the isolated regions where platelets were found in coated samples that were dipped for a period of 30 minutes in PRP are presented in Graphs 1A to C. Figures 1A to H show the platelet adhesion after 3 hours of dipping. From these figures, it can be seen that there was no significant difference in the platelet activation at 30 minutes and 3 hours of soaking period. The platelets were observed in the form of agglomerates with distorted structure. This can be attributed to the rough and complicated structure of HA. Platelet adhesion was also found in the case of uncoated metal samples as shown in Figures 2A to H. Since these samples were highly polished, the platelets



Figs 1A to H: Platelet adhesion on HA coating after 30 minutes: (A) Ti 12 in argon atmosphere; (B) Ti 31 in argon atmosphere; (C) Ti 12 in argon–hydrogen atmosphere; (D) Ti 31 in argon–hydrogen atmosphere; (E) Ti 12 in nitrogen atmosphere; (F) Ti 31 in nitrogen atmosphere; (G) Ti 12 in nitrogen–hydrogen atmosphere; and (H) Ti 31 in nitrogen–hydrogen atmosphere



Figs 2A to H: Platelet adhesion on HA coating after 3 hours: (A) Ti 12 in argon atmosphere; (B) Ti 31 in argon atmosphere; (C) Ti 12 in argon–hydrogen atmosphere; (D) Ti 31 in argon-hydrogen atmosphere; (E) Ti 12 in nitrogen atmosphere; (F) Ti 31 in nitrogen atmosphere; (G) Ti 12 in nitrogen–hydrogen atmosphere; and (H) Ti 31 in nitrogen-hydrogen atmosphere



Figs 3A to D: Platelet adhesion on uncoated sample: (A) Ti 12 after 30 minutes; (B) Ti 31 after 30 minutes; (C) Ti 12 after 3 hours; and (D) Ti 31 after 3 hours

observed were found to be spherical compared with HA-coated samples. Uncoated samples showed increased platelet activation with time. More platelet activation at 3 hours of soaking in PRP was observed in the case of Ti 31 as compared with Ti 12, which can be seen from Figures 3A to D for Ti 12 and Ti 31 respectively.

Because of the complex nature and rough surface of HA, quantification with respect to cell count and size could not be carried out. In coated samples, the platelets were found to be distorted and in the form of agglomerations. Therefore, in the present study, only qualitative analysis was carried out. The results of the present study indicated that the samples coated under different atmospheres did not significantly affect the platelet adhesion and were effective in suppressing the adhesion to, and activation of platelets on, Ti 12 and Ti 31. No significant change in platelet activation was also observed in coated samples upon increasing the soaking period to 3 hours. The surface microtopography of the coating was responsible for platelet activation rather than the presence of HA or the atmospheres under which the HA coatings were carried out.

CONCLUSION

Taking into consideration the limitations related to the present study, the following can be concluded:

- Cell viability is better when HA is coated in the argon–hydrogen plasma atmosphere.
- The plasma coating atmosphere has a significant influence on cell viability.
- The plasma coating atmosphere has no influence on platelet activation.
- Platelet activation is dependent on the surface microtopography of the HA on the substrate metal.
- Increase in platelet activation with fibrin formation occurs in uncoated Ti 12 and Ti 31 with an increase in the soaking period with PRP.

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