Enhanced Osteoblast Proliferation and Corrosion Resistance of Commercially Pure Titanium Through Surface Nanostructuring by Ultrasonic Shot Peening and Stress Relieving

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This investigation was carried out to study the effect of a novel process of surface modification, surface nanostructuring by ultrasonic shot peening, on osteoblast proliferation and corrosion behavior of commercially pure titanium (c p-Ti) in simulated body fluid. A mechanically polished disc of c p-Ti was subjected to ultrasonic shot peening with stainless steel balls to create nanostructure at the surface. A nanostructure (<20 nm) with inhomogeneous distribution was revealed by atomic force and scanning electron microscopy. There was an increase of approximately 10% in cell proliferation, but there was drastic fall in corrosion resistance. Corrosion rate was increased by 327% in the shot peened condition. In order to examine the role of residual stresses associated with the shot peened surface on these aspects, a part of the shot peened specimen was annealed at 400°C for 1 hour. A marked influence of annealing treatment was observed on surface structure, cell proliferation, and corrosion resistance. Surface nanostructure was much more prominent, with increased number density and sharper grain boundaries; cell proliferation was enhanced to approximately 50% and corrosion rate was reduced by 86.2% and 41% as compared with that of the shot peened and the as received conditions, respectively. The highly significant improvement in cell proliferation, resulting from annealing of the shot peened specimen, was attributed to increased volume fraction of stabilized nanostructure, stress recovery, and crystallization of the oxide film. Increase in corrosion resistance from annealing of shot peened material was related to more effective passivation. Thus, the surface of c p-Ti, modified by this novel process, possessed a unique quality of enhancing cell proliferation as well as the corrosion resistance and could be highly effective in reducing treatment time of patients adopting dental and orthopedic implants of titanium and its alloys.

Key Words: osteoblast proliferation, surface nanostructure, c p-Ti, ultrasonic shot peening, corrosion

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Introduction

Titanium and its alloys are frequently used for dental implants because of their excellent combination of mechanical properties, chemical stability, and biocompatibility.[1,2] The predictability and success of dental implants is well established; however, the success rate has been reported to vary with sites and patients.[3] Therefore, modification in design as well as the surface of implants is essential for improving the process of osseointegration and reducing healing time, stress shielding, and force concentration, especially at bone sites that are less dense.[3–7] The major concern in the near future is to improve bioactivity of titanium implants, especially with respect to bone cells, to enhance the process of osseointegration and its effectiveness.

Several factors contribute to the process of osseointegration of dental implants and consequently the duration of healing. Surface characteristics of implants, such as topography, chemistry, charge, and wettability play a major role in biologic success of implants. Therefore, various surface modification processes have been developed and applied to titanium implants to achieve more biocompatible surface.[7,8]

Nanosize roughness on titanium implant surface through nanostructured coatings or compacting nanosize constituent particles and fibers has been observed to increase osteoblast functions, especially the adhesion, proliferation, and deposition of calcium-containing minerals on nanosize roughness, with respect to the conventional surfaces with micron size grains.[9–12] However, nanosize metal particles are known to be expensive and technique sensitive; therefore, alternative methods of creating nanosize surface roughness on titanium implants are highly warranted.

Fatigue life of commercially pure titanium (c p-Ti) has been reported to be improved by surface nanostructuring[13,14]; therefore, surface nanostructure would be an additional attribute to implants experiencing fatigue damage. Furthermore, in view of human tissue being nanostructured, a practical approach for the design of the next generation of implants, with nanostructured surface, is considered to be highly promising.[10] This study was undertaken to characterize surface microstructures of c p-Ti resulting from ultrasonic shot peening by steel balls as well as on subsequent annealing and to examine their effects on osteoblast proliferation and corrosion resistance in simulated body fluid (SBF).

Materials and Methods

Ultrasonic shot peening

Ultrasonic shot peening is a process of cold working, in which small spherical peening media with sufficient hardness are stroked onto the substrate surface. Each shot striking the metal surface acts as a tiny peening hammer, causing severe plastic deformation and formation of nanostructure on the metal surface. The impact directions of the balls onto the sample surface are random. Indigenous c p-Ti (grade 2) was procured from M/S Mishra Dhatu Nigam (MIDHANI, Hyderabad, India) in the form of a plate of 10-mm thickness. A disc of 65-mm diameter and 4-mm thickness was machined from the plate. One side of the disc was mechanically polished on emery papers from 1/0 to 4/0 grades and finally on Selvyt cloth with a slurry of alumina powder in water as abrasive media. The polished surface of the disc was subjected to ultrasonic shot peening, with stainless steel balls of 8 mm diameter, to achieve optimum combination of nanoscale surface roughness, surface area, and associated energy. Shot peening was carried out in a vibrator generator (Model SNC 1 SNC, Advanced Technology Co Ltd, Chengdu, China) at a frequency of 50 Hz, for 45 minutes, to create nanostructure at the surface. Using this technique, a very thin region of nanostructure was created at the surface of the c p-Ti disc. The thickness of the shot peened disc was reduced from 4 mm to 2 mm by machining from the other side, opposite to the shot peened one, and square samples of $10 \times 10 \times 2$ mm size were prepared. Fifty percent of these samples, prepared from the shot peened disc, were annealed at 400°C for 1 hour, in a vacuum-sealed (approximately 10^{-4} Torr) silica tube. Identical square-shaped samples were prepared also from the as received (unshot peened) material to compare their osteoblast culture and corrosion resistance in SBF with those of the shot peened as well as with the subsequently annealed ones.

Microstructural characterization

The microstructures of c p-Ti in the as received, shot peened, and shot peen-annealed conditions, were

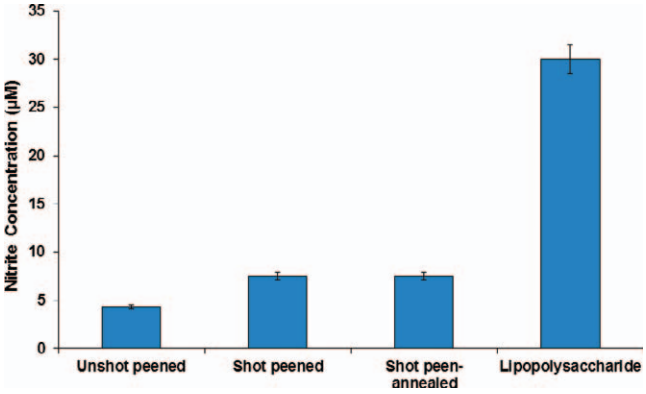


Figure 1. Nitrite concentration production at different surfaces as a marker of inflammation.

examined using an optical microscope (Leitz Metallux-3, Wetzlar, Germany), an atomic force microscope (Nanoscope IV Controller, Veeco, The Helios, Singapore), and a scanning electron microscope (Quanta 200F, Eindhoven, Netherland).

Cell adhesion

A human osteoblast cell line, MG 63, procured from the National Centre for Cell Sciences(Pune, India), was cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with heat-inactivated fetal calf serum (FCS) (10%), penicillin (100 U/mL), streptomycin (100 U/mL), and gentamycin (20 μg/mL). FCS was from Biological Industries (Haemek, Israel); all other chemicals, unless mentioned otherwise, were procured from Sigma- Aldrich Chemicals (St Louis, Mo). The cells were seeded into 25 cm^2 culture flasks (Nunc, Roskide, Denmark) and were allowed to grow in a controlled humidified incubator (Model 3121, Thermo Forma, Marrietta, Ohio) with 5% CO_2 and 98% humidity at 37°C. Before seeding of the cells, all of the samples of c p-Ti, in different conditions, were soaked in Extran MAO_3 phosphate-free detergent solution (Merck GmbH, Darmstadt, Germany) and subsequently autoclaved at a pressure of 15 $lb/inch^2$ for 30 minutes. Osteoblasts were seeded at a density of 0.5 $\times$ 10^6 osteoblasts/well onto the surface of each test sample, which was kept in a 12-well plate (Nunc, Roskide, Denmark).

Viability/proliferation assay

Cytoproliferative activity of osteoblasts was measured by 4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) assay. Osteoblasts (0.5 $\times$ 10^6 cells/well) were seeded onto 10 $\times$ 10 $\times$ 2 mm surfaces in 12-well culture plates in 1 mL complete DMEM and incubated for different durations of 24, 48, and 72 hours. After completion of the incubation period, 50 μL of 5 mg/mL MTT solution was added to the monolayers and incubated for 4 hours at 37°C. The MTT reaction was terminated by addition of 0.04 N HCl in isopropanol. The MTT formazan formed was measured spectrophotometrically (540 nm) using an EMax spectrophotometer (Molecular Devices, Sunnyvale, Calif), and the relative intensities were plotted. Each experiment was performed 4 times in triplicate, and standard deviation and variance were calculated. Proliferation of adherent cells was distinguished among the different groups by using parametric 2-way analysis of variance (Bonferroni test) as a function of time.

Measurement of nitrite production

The concentration of nitrite, the stable end product of nitric oxide, in culture supernatants was determined by Griess' reaction. Nitrite content was quantified by extrapolation from sodium nitrite standard curve in each experiment (Figure 1). Osteoblasts were plated onto different surfaces and incubated under standard culture conditions for 12 hours; the supernatants were collected and nitric oxide was estimated by titrating for nitrite using Griess' reagent. Bacterial lipopolysaccharide (100 ng/mL/10^6 cells) was taken as the positive control.

Corrosion resistance

Electrochemical potentiodynamic polarization study was carried out in Ringer solution (NaCl 9 g/L, KCl 0.42 g/L, $CaCl_2$ 0.48 g/L, $NaHCO_3$ 0.2 g/L) at a pH of 7.2, using a Gamry Potentiostat, (Gamry PC4, Warminster, Penn). Salt concentration in the Ringer solution corresponded to that in the body fluid. A conventional 3-electrode system with high-density graphite as counter electrode, saturated calomel electrode as reference electrode, and the test specimen as working electrode, was used. The anodic polarization curves were recorded, at a scan rate of 1 mV/s. The polarization scan was conducted in the voltage range from −500 to +1500 mV with reference to the open circuit potential. Tafel extrapolation was used to determine corrosion parameters.

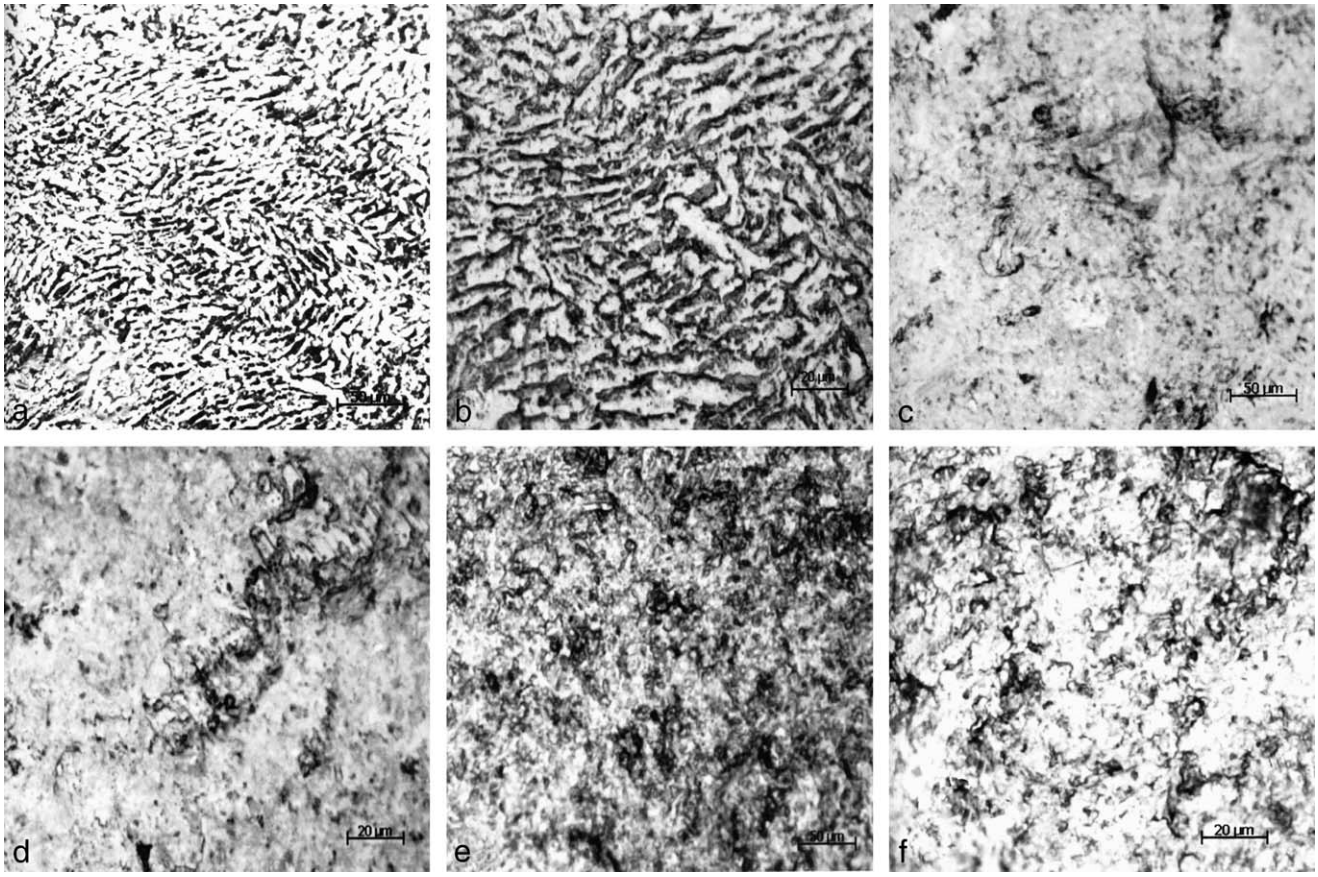


Figure 2. Optical micrographs showing microstructure of commercially pure titanium in different conditions: (a, b) unshot peened, (c, d) shot peened, and (e, f) shot peen-annealed.

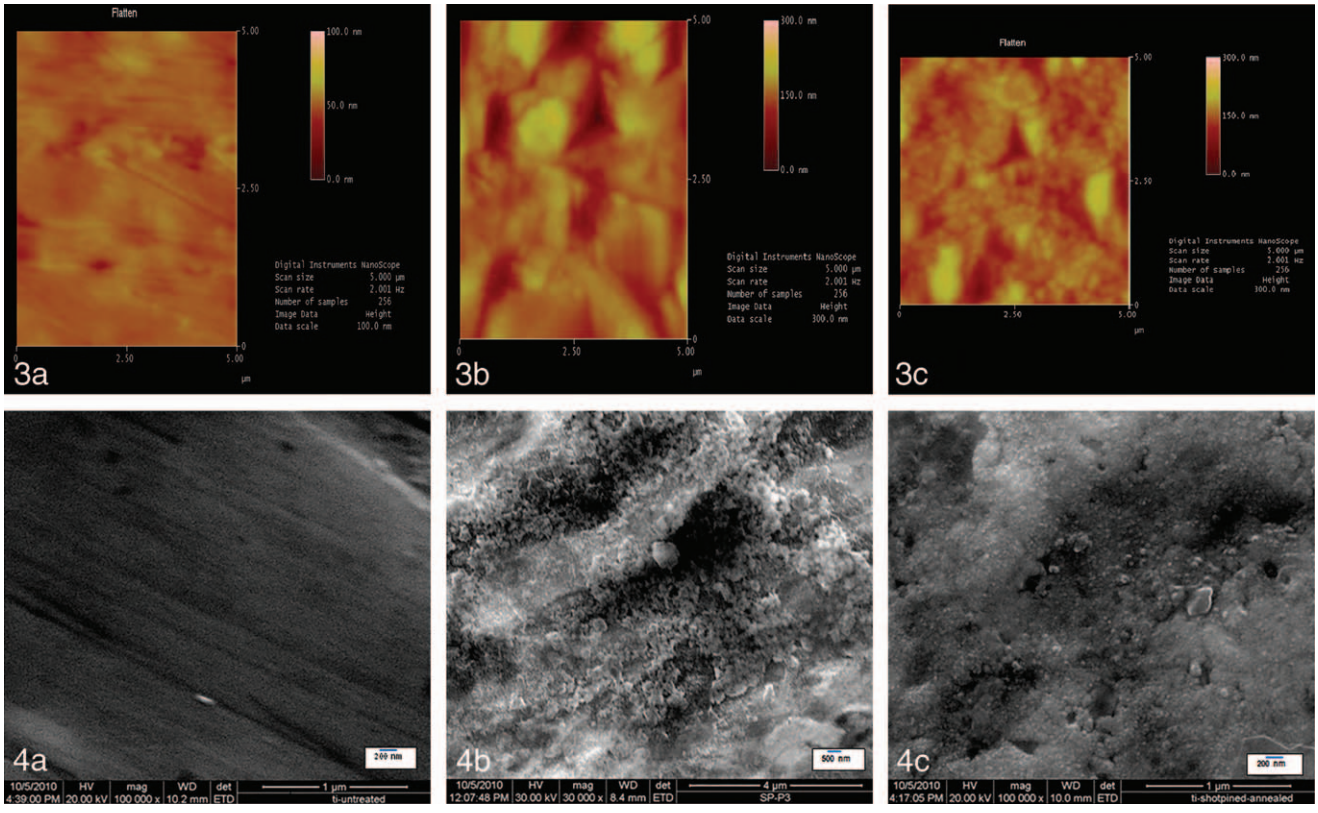
Results

Microstructure

The microstructures of c p-Ti in the 3 conditions are shown by the optical micrographs in Figure 2. Grains of approximately 20 μm mean intercept length may be seen in the as received condition (Figure 2a and b). Refinement of grains resulting from shot peening is quite obvious in the shot peened condition (Figure 2c and d). Similar refined microstructure can also be seen in the shot peen-annealed condition (Figure 2e and f). Microstructures in the above 3 conditions, recorded by atomic force microscope and scanning electron microscope, are displayed in Figures 3 and 4, respectively. As expected, the microstructure in the as received condition was featureless, whereas nanosize grains were seen with inhomogeneous distribution in the shot peened condition. Relatively more distinct nanosize grains, with increased number density and more uniform distribution were seen in the shot peen-annealed condition. The average size of the nanosize grains was in the range of 14–20 nm. The roughness of the surface was highest in the shot peened condition and was followed in decreasing order by those of shot peen-annealed and the as received ones.

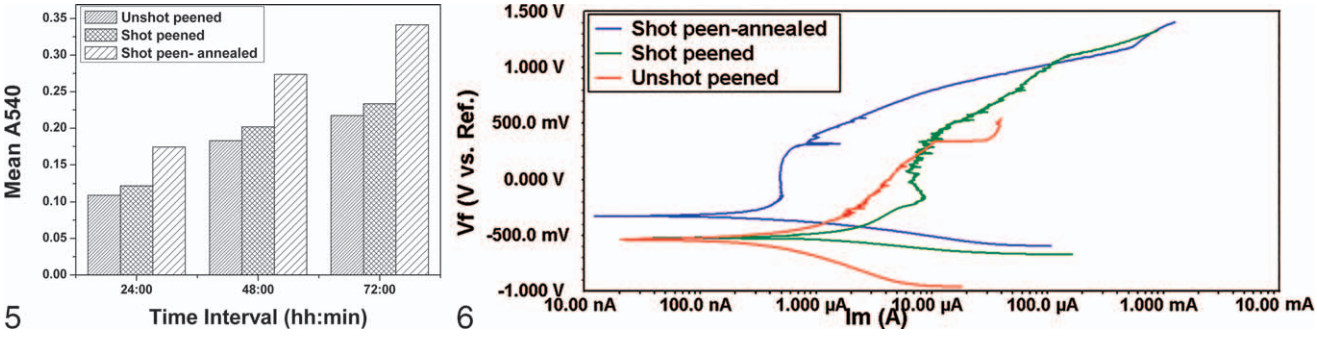
Cell growth behavior

The growth behavior of human osteoblast cell line MG 63 on the as received samples was analyzed using MTT assay at 24, 48, and 72 hours of incubation. It may be seen from the bar diagrams in Figure 5 that, in general, there was increase in cell proliferation in all 3 cases, with increase in duration of proliferation. Further, there was only marginal increase in cell proliferation from shot peening; however, proliferation was enhanced significantly by the subsequent annealing treatment. This relative trend of cell proliferation, on the surface of the 3 different categories of samples, was maintained for all 3 durations of proliferation (24,



Figures 3 and 4. Figure 3. Atomic force micrographs showing surface features of commercially pure titanium in different conditions. (a) unshot peened: smooth surface without nanocrystals; (b) shot peened: relatively rough surface and nanocrystals in some regions; and (c) shot peen-annealed: large number of distinct nanocrystals with sharp grain boundaries. **Figure 4.** Scanning electron micrographs showing surface features of commercially pure titanium in different conditions. (a) unshot peened: smooth surface without nanocrystals; (b) shot peened: relatively rough surface and nanocrystals in some regions; and (c) shot peen-annealed: large number of distinct nanocrystals.

48, and 72 hours). Statistical analysis of the data of osteoblast proliferation for the 3 conditions is presented in Tables 1a through c. It may be seen that there was statistically significant difference ($P < .001$), in particular, in the shot peen-annealed condition. The increase in osteoblast proliferation in the shot peened and shot peen-annealed condition, with respect to that of the untreated condition, is presented in Table 2. It may be seen that while the increase in cell proliferation was almost 10% for the shot peened, it was enhanced to almost 50% for the shot peen-annealed condition. The improvement in



Figures 5 and 6. Figure 5. Histograms showing increasing osteoblast growth on surface of commercially pure titanium from unshot peened to shot peened, and shot peen-annealed at different time intervals of 24, 48, and 72 hours. Growth is significantly higher in the shot peen-annealed condition than in the shot peened and as received conditions, at all the time intervals. **Figure 6.** Tafel plots for commercially pure titanium in different conditions.

TABLE 1(A)

Statistical analysis of cell proliferation data: mean values at different time intervals in the 3 conditions

Group	At 24 Hours	At 48 Hours	At 72 Hours
Unshot peened (n = 12)	1.02 ± 0.01	1.25 ± 0.01	1.28 ± 0.29
Shot peened (n = 12)	1.75 ± 0.09	2.20 ± 0.12	2.36 ± 0.05
Shot peen-annealed (n = 12)	2.22 ± 0.05	3.03 ± 0.03	3.36 ± 0.06

TABLE 1(B)

Statistical analysis of cell proliferation data: intercondition *P* values at different time intervals

Intercondition Comparison	At 24 Hours	At 48 Hours	At 72 Hours
Unshot peened vs shot peened	.007	<.001	<.001
Unshot peened vs shot peen-annealed	.002	<.001	<.001
Shot peened vs shot peen-annealed	.001	.001	<.001

TABLE 1(C)

Statistical analysis of cell proliferation data: *P* values for each condition at different time intervals

Individual Condition Comparison	Unshot Peened	Shot Peened	Shot Peen-Annealed
24 vs 48 h	<.001	<.001	<.001
24 vs 72 h	<.001	<.001	<.001
48 vs 72 h	<.001	<.001	<.001

cell proliferation on these surfaces was almost comparable for the 3 time intervals of cell proliferation.

Corrosion behavior

The important parameters characterizing corrosion behavior of c p-Ti in the 3 states in SBF, at 37°C, are recorded in Table 3 with chi-square values for the respective conditions. It may be seen that corrosion rate was increased by almost 327% in the shot peened condition with respect to the as received material. On the other hand, the corrosion rate was reduced by 41% following annealing of the peened specimen as compared with that of the as received material. Thus, it is obvious that highest corrosion resistance was exhibited in the shot peen-annealed condition. Tafel plots for the 3 conditions of c p-Ti are shown in Figure 6. It may be seen that corrosion potential had become nobler, and corrosion current was reduced for the shot peen-annealed condition. These features are indicative of the presence of a strong oxide layer with effective barrier action on the surface of the shot peen-annealed specimen, which was formed from annealing at 400°C. It is obvious from the shape of the plot, that the high rate of dissolution in the shot peened condition was due to cathodic depolarization, which is indicative of weak passive film, with less effective barrier action on the shot peened surface. It may be inferred from the Tafel plot for the as received condition that passive film on the as received specimen was relatively stronger than that on the shot peened surface.

DISCUSSION

It is seen that a nanostructure (14–20 nm) was created at the surface of c p-Ti by ultrasonic shot peening and it was not only stable but became more prominent following subsequent annealing at 400°C. The observed effect of shot peening as well as that of subsequent annealing on osteoblast proliferation may be understood in terms of the surface topography, form, and stability of oxide film, and the residual stresses associated with the surface, in respective conditions. In the shot peened condition, the surface was nanostructured; oxide film could be presumed to be amorphous like that of the untreated surface, as shot peening was carried out at an ambient temperature. The film would have been relatively weaker and less uniform in thickness as compared with that on the untreated surface because of adverse effect of

Table 2

Percent increase in total cell number on modified surfaces with respect to the unshot peened surface

Condition	24 Hours	48 Hours	72 Hours
Shot peened	11.6%	9.9%	7.4%
Shot peen-annealed	59.7%	49.0%	57.0%

dislocations with high density and residual stresses in the substrate on bonding between the passive oxide film and the substrate.

The passive titanium oxide film on c p-Ti, formed at room temperature, is known to be amorphous[15,16] and to undergo transformation to crystalline form (anatase) at 276.10°C.[16] In light of these facts, little role of the passive oxide film could be visualized in enhanced cell proliferation of approximately 10% in the shot peened condition. A high density of dislocations and considerably high magnitude of residual stresses would have been there in the shot peened region resulting from severe plastic deformation caused by ultrasonic shot peening.[17] A peak subsurface compressive residual stress of 480 MPa was observed by Jiang et al[17] in a 0.8-mm thick sheet of c p-Ti blasted by sand particles of 200–300 μm diameter. Furthermore, the sheet was bent into a curved shape on only one side due to compressive stresses associated with the surface when the sheet was sand blasted. . In the present investigation, the thickness of the shot peened disc was kept at 4 mm to avoid bending following ultrasonic shot peening with stainless steel balls of 8-mm diameter. However, the magnitude of the residual stresses associated with the shot peened surface may be visualized easily. Residual stress at the surface has been found to be detrimental for cell proliferation.[18] In sharp contrast, as mentioned above, surface morphology of nanoscale has been found to be highly effective in promoting cell proliferation. Materials with nanoscale particles/grains (<100 nm) are considered quite unique in the sense that they simulate dimensions of bone constituents.[10]

It is now well established that surfaces with micro and nano topography are more conducive to proliferation of osteoblast and formation of good quality bone.[19–21] Surface roughness has been found to affect contact angle/wettability.[22] Alloy Ti6Al4V, coated with hydroxyapatite, had the lowest contact angle and best wettability. Overall bone-to-implant contact is the basis for biomechanical anchorage of dental implant. A statistically significant difference ($P < .001$) was found in the implant-to-bone contact percentage in machined (conventional) and resorbable blast material implants.[23] Resorbable blast material implant is characterized by ultraclean surface with macro roughness, created by blasting the implant surface with coarsely ground (180–425 μm) particles of calcium phosphate, a resorbable material, followed by specific passivation to remove the embedded particles.[24] In view of the above facts it is obvious that the observed increase of approximately 10% in cell proliferation on the shot peened surface was essentially due to the surface nanostructure countering the deleterious effect of surface residual stresses on cell proliferation. The marked enhancement of almost 50% in cell proliferation in the shot peen-annealed condition was essentially from the stress recovery and partly from the crystallized form of the oxide layer. The crystallized form of surface oxide on c p-Ti has been shown to be highly effective in the spreading and proliferation of cells.[25,26] While there was drastic decrease in corrosion resistance due to shot peening, there was marked increase in corrosion resistance following subsequent annealing treatment. The drastic decrease in corrosion resistance of c p-Ti in the shot peened condition, despite nanostructure at the surface, would have been from less protective passive film, as mentioned above. There would have been much variation in thickness of the passive oxide film due to inhomogeneity in

Table 3

Corrosion parameters for different conditions of commercially pure titanium in Ringer solution

Material Condition	Corrosion Potential (mV)	Corrosion Current (nA/cm^2)	Corrosion Rate (mpy) × E^{-3}	Chi-square
Unshot peened	−469.0	51.10	17.33	0.03623
Shot peened	−522.0	216.0	74.10	0.04458
Shot peen-annealed	−329.0	30.10	10.22	0.02430

dislocation density and residual stresses in the substrate. The marked increase in corrosion resistance in the shot peen-annealed condition was due to formation of a strong oxide layer on the surface, with effective barrier action, against the transfer of metal ions to electrolyte. Relatively higher corrosion resistance in the shot peen-annealed condition than in the as received state would have been from adherent and strong oxide film on the shot peen-annealed surface from the significantly large increase in grain boundary area.[27] In the present investigation, corrosion behavior of c p-Ti characterized by potentiodynamic polarization was similar to that observed in the earlier studies on c p-Ti[17] and 304 stainless steel[28] in 3.5% NaCl solution. In these studies, corrosion resistance was found to be low in the sand blasted condition and significantly improved in the subsequently annealed condition. The improved corrosion resistance of these materials in the sand blasted and annealed condition was attributed to surface nanostructure formed by subsequent annealing and consequent effective passivation.

Thus, it is obvious from this investigation that there is high potential of this novel process of surface modification, through nanostructuring, in enhancing osteoblast proliferation as well as corrosion resistance of c p-Ti without any modification in surface chemistry to avoid risk of deterioration in corrosion resistance. One of the possible limitations of this process with respect to other processes may be the relatively high initial cost of the processing unit.

Abbreviations

c p-Ti: commercially pure titanium
DMEM: Dulbecco's Modified Eagle's Medium
MTT: 4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide
SBF: simulated body fluid

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