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Plasma of Argon affects the earliest biological response of different implant surfaces: an in vitro comparative study.

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Abstract

Aim: The aim of this in vitro study was to evaluate the early cell response and proteins adsorption elicited by the Argon plasma treatment of different commercially available titanium surfaces, using a chair-side device.

Materials and methods: Four hundred fifty sterile 4 mm diameter disks, made of grade 4 titanium, with three different surface topographies (machined, plasma sprayed, and zirconia-blasted and acid etched) were allocated to receive four different testing treatments (2% and 10% protein adsorption and cell adhesion with MC3T3-E1 and MG-63). Furthermore, the specimens were randomly divided to undergo Argon plasma treatment (10 W, 1 bar for 12 minutes) in a plasma reactor, UV light treatment (positive control group), or receive no treatment (control group). Pretreatment surface analyses using a scanning electron microscope and profilometer images were also performed.

Results: Profilometric analysis demonstrated that the evaluated specimens perfectly suit the standard parameters. The use of plasma of Argon was capable of affecting the quantity of proteins adsorbed on the different surfaces, notwithstanding their roughness or topographic features at a low Fetal Bovine Serum (FBS) concentration (2%). UV light treatment attained similar results. Moreover, both the plasma of Argon and the UV light demonstrated to significantly increase the number of osteoblasts adherent at 10 minutes in all tested surfaces.

Conclusions: This in vitro study highlights the potential biologic benefits of treating implant surfaces using plasma of Argon or UV, irrespective of the roughness of the titanium surface.

Key words: Plasma of Argon, UV light, surface energy, protein adsorption, bone cell adhesion.

Introduction

Osseointegration was originally defined as a direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant (Brånemark et al. 1985). It was histologically described as new bone in contact with the implant surface and without the formation of fibrous tissue around the implant (Albrektsson and Johansson 2001). Nowadays, osseointegrated implants are used widely in the oral and maxillofacial fields (Albrektsson et al. 2008). Clinically oriented healing processes of oral implants were first described by Schroeder (Schroeder et al. 1976) and Brånemark (Brånemark et al. 1977). The surgical trauma arising from implant placement induces the production of free radicals and oxygenated derivatives at the titanium surface, which lead to a thickening of the titanium dioxide (TiO_2) layer of the surface. Calcium and phosphorus ions from the bone matrix are then incorporated within the TiO_2 porous layer, making a highly dynamic amorphous layer from 20–40 to 500 nm thick at the bone–titanium interface (Albrektsson et al. 1983; Dohan Ehrenfest et al. 2010). This inorganic chemical modification might stimulate bone regeneration and increase the biochemical interlocking between bone matrix proteins and surface materials (Coelho et al. 2009). Osseointegration is not only dependent on conditions for bone repair, but also on the biomaterial used and its reactions. The importance of surface roughness, topography and chemistry has already been universally accepted (Sul et al. 2002; Sul et al. 2009). Several cell functions such as attachment, spreading and migration are significantly modulated by the surface features and are currently being elucidated from a cell biology and molecular perspective (Lavenus et al. 2015; Vallee et al. 2014; Ogino et al. 2015).

Cell adhesion to an artificial material mainly depends on the physico-chemical properties of the material surface. Indeed, cell adhesion is mediated by molecules of extracellular or provisional matrix (fibronectin, vitronectin, collagen, laminin or fibrin), which are spontaneously adsorbed to the surfaces from blood, other body fluids and culture media. With too hydrophobic surfaces, these molecules are adsorbed in a denatured and rigid state. On the other hand, too hydrophilic materials prevent the adsorption of proteins. Moderately hydrophilic and positively charged surfaces may, in

contrast, promote good adhesion levels (Bacakova et al. 2011). The extension and strength of cell adhesion plays a role in regulating the activity of proliferation and differentiation of cells (Parizek et al. 2013), hence the relevance of proper in vitro experimental settings for predicting the actual in vivo performance of bone interface materials

Nowadays, clinical research is focusing on more rapid and less invasive procedures, as well as, on enhancing the activity of bone forming cells at the tissue implant interface. This desire for ‘‘bioactivity’’ has been addressed using a variety of different approaches. Acceleration of osseointegration may depend on the removal of negative tissue conditions or optimization of the biomaterial rather than on an actual increase in the rate of bone response. Potentially accelerated osseointegration has been indicated by results from experiments with hydroxyapatite coating, using moderately roughened implants, hyperbaric oxygen treatment or anodization of c.p. titanium with artificially enhanced oxide layers (Oh et al. 2005).

At the same time, alteration of the physical surface characteristics (increasing surface energy and therefore hydrophilicity) was shown to positively affect early bone responses: exposure to ultraviolet light (Sawase et al. 2008), alkali treatment (Tugulu et al. 2010), and acid etching with subsequent storage in saline solution (Rupp et al. 2006), or using plasma cleaning (Coelho et al. 2012; Duske et al. 2012) can be rendered hydrophilic dental implants surfaces.

Plasma can be categorized as either thermal (hot) or cold. At the same time, according to the pressure, plasma can categorized as vacuum or atmospheric pressure (Fridman 2008). Plasma, when produced with vacuum, is an electrically neutral, ionized gas composed of ions, electrons, neutral particles, vacuum ultraviolet and ultraviolet irradiation, free radicals and chemically reactive neutral particles. If Plasma is produced under normal pressure conditions, no vacuum ultraviolet radiation is produced. When plasma is produced in a vacuum chamber, atmospheric gases have been evacuated below 0.1 torr. These low pressures allow a relatively long free path of accelerated electrons and ions, preserving the integrity of materials (Moisan et al. 2002), removing all chemical

traces left from former treatments, and effectively producing cleaner and better controlled surfaces than with other preparation methods (Aronsson et al. 1997).

From a physic-chemical point of view, plasma treatment increases the surface energy and, therefore, reduces the contact angle promoting cells spreading (Duske et al. 2012). Additionally, plasma treatment was demonstrated to activate the surfaces at the atomic and molecular level, producing hydrophilic surfaces and enhancing their wettability (Swart et al. 1992). Appropriate plasma processes render surfaces hydrophilic, and modify the oxide layer that interacts with proteins and cells of surrounding tissue. Thus, plasma application can lead to an improved adhesion of tissue (Zhao et al. 2005). Similar bioactive effects were demonstrated using UV light treatment (Aita et al. 2009).

The aim of this in vitro study was to evaluate the early cell response and proteins adsorption elicited by the Argon plasma treatment of different commercially available titanium surfaces, using a chair-side device. As a positive control UV light treatment was chosen. The null hypothesis was that there were no differences in quantity of proteins adsorbed on titanium surfaces, among the different experimental groups.

Material and Methods

The study was designed to evaluate the effect of Plasma of Argon treatment on the earliest biological response of different implant surface modifications. A priori power analysis was estimated by referring to a similar clinical study, which investigated the same topic (Canullo et al. 2013). Based on these data, mean cell adhesion values of 181±37 and 135±26 at 2 hours (P = .0039) was projected by setting effect size dz = 1.438, error probability a = 0.05, and power = 0.95 (1-b error probability), resulting in 12 sample from each sub-group (G* Power 3.1.7 for Mac OS X Yosemite, version 10.10.3).

Four hundred fifty serially numbered, sterile 4 mm diameter disks (Sweden & Martina, Padua, Italy), made of grade 4 titanium, with three different surface topographies, i.e, machined (MAC), plasma sprayed (TPS) and zirconia-blasted and acid etched (ZRT), were used in the present study. The titanium disks were divided into three sub-groups of 78 samples each according to the surface topography. ~~Three computer-generated randomization lists (Random Number Generator Pro 2.08 for Windows, Segobit Software, http://www.segobit.com/) were used to randomly allocate~~ The titanium disks were divided into three additional sub-groups, consisting in an equal number of 36 titanium disks each, to be used as culture substrates and receiving the four different testing treatments (2% and 10% protein adsorption and cell adhesion with MC3T3-E1 and MG-63). The remaining six titanium disks for each sub-group (total of 18 titanium disks) were used for the surface analysis (scanning electron microscope and profilometer images). Twelve titanium disks for each sub-group were ~~randomly~~ allocated as test group and underwent Argon plasma treatment (10 W, 1 bar for 12 minutes) in a plasma reactor (Plasma R, Sweden & Martina). Twelve titanium disks for each sub-group were treated using UV light treatment (Toshiba, Tokio, Japan) for 3h (15W) at ambient conditions [intensity: 0.1mW/cm^2 (λ=360±20nm) and 2mW/cm^2 (λ=250±20nm)], as described by Aita et al. 2009. The remaining 12 non-treated titanium disks of each sub-group were used as controls. ~~All the computer-generated randomization lists were prepared in advance by an external investigator, not involved in the study and an independent consultant prepared all of the~~

~~envelopes/containing numbers for randomization, which were opened immediately before the testing procedures.~~ A flow diagram of the ~~randomization~~ sequence is reported in figure 1.

Topography and surface analysis

Eighteen samples were located in a Scanning Electron Microscope (Zeiss EVO 50, Carl Zeiss AG, Oberkochen, Germany) to study the surface topography. Sterile forceps were used to avoid contamination. Forty field emission scanning electron microscope (FESEM) images were acquired on each sample, at low and high magnifications. The samples were further washed in distilled water and rinsed thoroughly in 70% and absolute ethanol. Then, the titanium disks were cleaned ultrasonically for 20 minutes in absolute ethanol and air dried under a laminar flow hood (Köunönen et al. 1992).

A non-contact 3D surface profiler (White light interferometer Talysurf CCI 3000, Taylor Hobson Limited, Leicester, England) was used to measure the surface roughness of MAC, TPS and ZRT samples. Ten measurements were conducted for each of the 18 titanium disks according to four amplitude parameters (Sa, Ssk, Sku and Sz). Sa is the arithmetic mean of the absolute values of the surface point departures from the mean plane within the sampling area. Ssk represents the deviation from the average baseline, where positive Ssk indicates a majority of peaks on the surface and negative Ssk indicates a majority of valleys. Sku describes the probability density sharpness of the profile. For surfaces endowed with low peaks and low valleys, Sku is less than 3, instead it becomes greater than 3 for surfaces with high peaks and low valleys. Sz is the maximum peak to valley height. Moreover a spatial and a hybrid parameter were chosen: Sds and Sdr%. The former represents the density of summits of the surface (pks/mm^2), while the latter corresponds to the ratio of the increment of the interfacial area of a surface over the sampling area (%).

The roughness values were calculated by filtering the surface profiles with a Gaussian filter. A 0.8-mm “cut-off” value was applied for filtering (Gadelmawlaa et al. 2002).

Protein adsorption

To quantify the amount of protein adsorbed onto the titanium disks, high and low protein concentration solutions (10% and 2% of Fetal Bovine Serum (FBS) in Phosphate Buffered Saline (PBS)) were used to incubate the titanium disks at 37°C for 30 minutes. Subsequently, the samples were washed twice with PBS and the adsorbed protein was eluted from the disks using Tris Triton buffer (10mM Tris (pH 7.4), 100mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 10% Glycerol and 0.1% SDS) for 10 minutes. Total protein amount was quantified using Pierce™ BCA Protein Assay Kit (Life Technologies, Carlsbad, California, USA) following the manufacturer's instructions.

Cell culture

To characterize in vitro the biological response, a pre-osteoblastic murine cell line MC3T3-E1 (ECACC, code 99072810) and a human osteoblastic cell line MG-63 (ATCC reference number: CRL-1427) were used. MC3T3-E1 and MG-63 were respectively maintained in Alpha MEM (Gibco Life Technologies, Milan, Italy) and Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Life Technologies, Milan, Italy) supplemented with 10% fetal bovine serum (Gibco Life Technologies, Milan, Italy), 100 U/ml penicillin, 100 µg/ml streptomycin. Cells were passaged at sub-confluence to prevent contact inhibition and were kept under a humidified atmosphere of 5% CO_2 in air, at 37°C.

Cell adhesion assay

Cell adhesion was evaluated on titanium disks using a 96 well plate as support. Cells were detached using trypsin for 3 minutes, carefully counted and seeded at 3 x 10^3 cells/disk in 100µl of growth medium on the disks with different roughness. The 96-well plates were kept at 37°C, 0.5% CO_2 for 10 min. Before and after fixation in 4% Paraformaldehyde in PBS for 15 min at room temperature, cells were washed two times with PBS and then stained with 1µM DAPI (Molecular Probes,

Eugene, California, USA) for 15 min at 37°C to visualize cells' nucleus. Images were acquired using a Nikon Eclipse T-E microscope with a 40× objective. The cells' nuclei were counted using the tool Analyze particles of ImageJ software (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/).

Statistical Analysis

Data were recorded on Microsoft "Excel 2007" (Microsoft Inc., Redmond, WA. USA). A descriptive analysis was performed with presentation of data using means ± standard deviations (SD). Due to the nonparametric nature of the data collected, differences between groups were analyzed using the Mann–Whitney–Wilcoxon test, by means of GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). All of the statistical comparisons were conducted with a 0.05 level of significance.

Results

Topography and surface analyses

The three surfaces tested are depicted in figure 2. Machined samples show the typical marks left by the milling process (Fig. 2A,B), while both TPS (Fig. 2D, E) and ZRT (Fig. 2G,H) surfaces display the proper features of roughened implant surfaces, as it is expected for plasma sprayed and zirconia-blasted and acid etched (ZB/AE) titanium samples.

The tridimensional analysis of the titanium disks is represented in figure 2 (C,F,I), while Sa, Ssk Sku and SZ values are reported in Table 1. Comparing Sa and Sz values, TPS samples resulted rougher than MAC and ZRT, in a statistically significant way. Also, from Ssk and Sku parameters, it can be noticed that TPS surface is the most irregular. Indeed, the Ssk parameter assumed a positive value only for the TPS surface, meaning that the profiles have high valleys. This is consistent with the Sku value of TPS (4.14 with a remarkably high standard deviation), which is representative of a surface with high peaks and low valleys. It is noteworthy that both MAC and ZRT samples showed Sku close to 3, as it is expected for perfectly random surface patterns.
The Sdr value of the MAC sample was significantly lower respect to the ZRT. As for the Sds parameter, TPS surface had significantly higher value than those of MAC and ZRT surfaces.

Protein adsorption

Outcomes at 2% and 10% concentrations are summarized in Table 2. Plasma of Argon for 20 min and UV light for 3h were capable of affecting the quantity of proteins adsorbed on the different surfaces, notwithstanding their roughness or topographic features at a low FBS concentration (2%), the differences between test and control disks being statistically significant. However, not statistically significant differences could be detected at a higher FBC concentration (10%).

Cell adhesion

The treatment with plasma of Argon for 20 min and UV light for 3h increased in a statistically significant way the number of osteoblasts adherent at 10 minutes in all surfaces (Table 3).

Discussion

Traditionally, implant surfaces have been both cleaned and sterilized by radiofrequency plasma devices with high temperature plasma sources (Coelho et al. 2009). Non Thermal Plasmas (NTPs) can achieve similar effects at ambient temperatures. Although the effects of NT Plasma of Argon have been reported previously, both in vitro and in vivo, as for cell spreading, wettability (Duske et al. 2012) and early osseointegration in animal models (Giro et al. 2013), this paper focused on different surfaces at the early stage of biological interaction, to the authors' knowledge, for the first time. In the present study, the positive effect of NT Plasma of Argon in terms of osteoblast adhesion and protein adsorption was demonstrated. As substrates, machined, plasma sprayed and blasted and acid-etched surfaces were chosen to represent some of the most clinically used dental implant surfaces.

The relevance of surface roughness in enhancing bone interlock is well known (Le Guéhennec et al., 2007). Thus, in order to increase the surface roughness and, consequently, the osseointegration, various surface modification techniques were proposed, such as: plasma-spraying, blasting with ceramic particles, acid-etching and anodization (Aparicio et al., 2011). The different topographies of the analyzed samples are consistent with literature, notably the roughness values measured on the TPS samples (Kubies et al., 2011).

Upon implantation, the first interaction between an intra-bony biomaterial and its recipient is conceivable as the contact of water molecules and salt ions, followed by blood proteins (MacDonald et al. 2002). These proteins may affect the eventual behavior of the cells recruited within the healing site. Thus, to simulate the protein adsorption occurring in vivo, a protein adsorption assay was implemented, following and adapting previous publications (Majumdar 2015; Nishimoto 2008; Yang 2003). Two different concentrations of FBS were chosen as the lowest limit

allowing cell survival (2%) and the standard concentration usually adopted in cell cultures. At 2% FBS, Argon based TPS and UV light treatment were able to increase, in a statistically significant way, the amount of adsorbed protein, while the same effect could not be detectable when a supra physiological concentration (10% FBS) was assayed.

Data reported confirmed increased protein adsorption reported in literature after treatment of titanium disks by means of both Plasma of Argon (Shibata et al. 2002) and UV light (Aita et al. 2009).

These data are in accordance with the mechanism of action envisaged for plasma of Argon. Indeed, the NTP application has been reported to cause a substantial increase in surface energy (in both polar and disperse components), which is likely due to the removal of the adsorbed Carbon species from the surface (Baier 1986; Baier 1987; Baier and Meyer 1988).

Analogous mechanisms were proposed for the UV light treatment in a series of papers by Ogawa and coworkers (Aita et al., 2009; Att et al., 2012; Hori et al., 2010; Ishijima et al., 2015; Miyauchi et al., 2010; Suzuki et al., 2009; Yamada et al., 2010).

It must be highlighted that a saturation effect could be evoked to explain the similarity of response between test and control groups at high protein concentration (Majumdar et al., 2015; Nishimoto et al., 2008; Yang et al., 2003).

The observation mentioned above concerning the protein adsorption is also supported by the cell adhesion assay performed, where the cell number resulted significantly higher on the NTP and UV treated surfaces compared o the non-treated ones. In fact, in vitro cell adhesion is mediated by molecules, mainly proteins that are spontaneously adsorbed to the surfaces from culture media (Bacakova et al. 2011). Hydrophilic and positively charged surfaces are known to promote good adhesion levels, whilst highly hydrophobic surfaces hinder cell adhesion by the adsorption of proteins in a denatured and rigid state (Bacakova et al. 2011).

Two osteoblastic cell models were adopted in the present study. Being the most used osteoblast cell line endowed with a pre-osteoblastic phenotype (Quarles et al. 1992; Wang et al.

1999), MC3T3-E1 cells represent, despite their murine origin, a reliable in vitro model and a viable alternative to primary human osteoblasts for biomaterial interface research (Czekanska et al. 2012). MG-63 cells display an immature osteoblast phenotype and are preferably used for short term studies as inconsistent data were reported in literature on their mineralization capabilities (Czekanska et al. 2012).

The present study presented similar results in terms of cell adhesion compared to previously reported outcomes showing that Plasma treatment of the titanium implant surface can positively affect osteoblast adhesion, thus enhancing its adsorption on titanium (Swart et al. 2002; Junker et al. 2009; Tavares et al. 2009; Huang et al. 2011).

Similarly, it was observed that UV treatment increases osteoblast adhesion (Aita et al. 2009). However, it must be highlighted that similar results in protein adsorption and cell adhesion were obtained through different time exposure to the different bio-activating processes.

Taken together, the results presented might suggest the activation of the implant surface that may speed up and qualitatively enhance the osseointegration process. However, in vivo experiments are needed so as to confirm this preliminary data and settle the rationale of a treatment that might be clinically relevant in case of bone reparative-deficiencies.

Conclusions

Within its limitations, the present study highlights potential benefits of treating implant surfaces using plasma of Argon or UV. This positive effect is displayed irrespectively of the roughness of the titanium surface.

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Tables

Table 1. Mean roughness values ± SD

	Sa (um)		Ssk		Sku	
MAC	0.44±0.01	vs. TPS p<0.0001 vs. ZRT p<0.0001	-0.22±0.07	vs. TPS p<0.0001 vs. ZRT p=0.0070	2.9±0.09	vs. TPS p<0.0001 vs. ZRT p=0.6069
TPS	4.44±0.26	vs. MAC p<0.0001 vs. ZRT p<0.0001	0.29±0.19	vs. MAC p<0.0001 vs. ZRT p=0.0001	4.14±0.33	vs. MAC p<0.0001 vs. ZRT p<0.0001
ZRT	0.95±0.03	vs. MAC p<0.0001 vs. TPS p<0.0001	-0.08±0.12	vs. MAC p=0.0070 vs. TPS p=0.0001	3.01±0.19	vs. MAC p=0.6069 vs. TPS p<0.0001
	SZ (um)		**Sdr (%)**		**Sds (pks/mm2)**	
MAC	3.72±0.17	vs. TPS p<0.0001 vs. ZRT p<0.0001	2.85±0.18	vs. TPS p=0.0078 vs. ZRT p<0.0001	55275±970	vs. TPS p<0.0001 vs. ZRT p=0.0043
TPS	53.63±4.22	vs. MAC p<0.0001 vs. ZRT p<0.0001	1072±134	vs. MAC p=0.0078 vs. ZRT p=0.0081	88973.5±234	vs. MAC p<0.0001 vs. ZRT p<0.0001
ZRT	10.38±0.31	vs. MAC p<0.0001 vs. TPS p<0.0001	24.31±1.8	vs. MAC p<0.0001 vs. TPS p=0.0081	53021.2 ±197	vs. MAC p=0.0043 vs. TPS p<0.0001

Table 2. Protein Adsorption (Mean of absorbance at 562nm) At least 4 samples for each condition were used and at least 3 indipendent experiment were performed.

2% FBS		**MAC**	**MAC Plasma**	**MAC UV**
	Mean	**0.0481**	**0.0645**	**0.0724**
	Standard error	0.0059	0.0010	0.0051
	P value		**vs. MAC 0.0286**	**vs. MAC 0.0286**
		TPS	**TPS Plasma**	**TPS UV**
	Mean	**0.0790**	**0.1216**	**0.1280**
	Standard error	0.0053	0.0104	0.0139
	P value		**vs. TPS 0.0286**	**vs. TPS 0.0286**
		ZRT	**ZRT Plasma**	**ZRT UV**
	Mean	**0.0728**	**0.1013**	**0.0928**
	Standard error	0.0026	0.0078	0.0062
	P value		**vs. ZRT 0.0286**	**vs. ZRT 0.0286**
10%FBS		**MAC**	**MAC Plasma**	**MAC UV**
	Mean	**0.1035**	**0.1152**	**0.1180**
	Standard error	0.0076	0.0135	0.0149
	P value		**vs. MAC 0.4857**	**vs. MAC 0.6571**
		TPS	**TPS Plasma**	**TPS UV**
	Mean	**0.1725**	**0.1865**	**0.1930**
	Standard error	0.0146	0.0157	0.0144
	P value		**vs. TPS 0.3429**	**vs. TPS 0.3429**
		ZRT	**ZRT Plasma**	**ZRT UV**
	Mean	**0.1627**	**0.1810**	**0.1923**
	Standard error	0.0178	0.0200	0.0229
	P value		**vs. ZRT 0.6571**	**vs. ZRT 0.4857**

Table 3. Cell Adhesion (Mean of cell number/field). At least 4 samples for each condition were used and at least 3 indipendent experiment were performed.

MC3T3-E1			
	MAC	**MAC Plasma**	**MAC UV**
Mean	17.50	31.00	27.00
Standard error	1.26	2.80	3.11
P value		**vs. MAC 0.0286**	**vs. MAC 0,0286**
	TPS	**TPS Plasma**	**TPS UV**
Mean	30.50	52.50	59.75
Standard error	2.25	4.63	5.48
P value		**vs. TPS 0.0286**	**vs. TPS 0,0286**
	ZRT	**ZRT Plasma**	**ZRT UV**
Mean	31.50	54.25	49.00
Standard error	2.60	6.16	2.16
P value		**vs. ZRT 0.0286**	**vs. ZRT 0.0286**

MG-63			
	MAC	**MAC Plasma**	**MAC UV**
Mean	11.00	20.75	18.75
Standard error	1.08	1.93	1.31
P value		**vs. MAC 0.0286**	**vs. MAC 0.0286**
	TPS	**TPS Plasma**	**TPS UV**
Mean	20.00	33.50	35.00
Standard error	3.51	1.44	2.48
P value		**vs. TPS 0.0286**	**vs. TPS 0.0286**
	ZRT	**ZRT Plasma**	**ZRT UV**
Mean	23.75	36.00	32.75
Standard error	1.93	2.04	1.11
P value		**vs. ZRT 0.0286**	**vs. ZRT 0.0286**

Captions

Figure 1. A flow diagram of the ~~randomization~~ sequence.

Figure 2A-I. The three surfaces tested as visualized per Scanning Electron Microscope and tridimensional analysis: machined surface at low magnitude (A) high magnitude (B) and tridimensional analysis; TPS low (D), high (E) magnitude and tridimensional analysis (F); ZRT at low (E), high (F) magnitude and tridimensional analysis (I).