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ASTM-F86 passivation increases trace element release from Ti6Al4V into culture medium

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There is growing concern regarding the possible effects of prolonged body exposure to trace amounts of metals. The high corrosion resistance of titanium and its alloys is resulting in their more widespread use. Passivation of these metals using HNO_3 according to ASTM F86 is based on the assumption that this will produce an increased resistance to corrosion, but its effects do not appear to have been examined at the trace element level. We have used sensitive graphite furnace atomic absorption spectrophotometry to examine *in vitro* the influence of such passivation on the release of Ti, Al and V from Ti6Al4V and solid cpTi into serum-containing culture medium during incubation for 9 days. We detected a highly significant increase in the trace levels of Ti, Al and V in medium samples from passivated Ti6Al4V compared to those obtained from non-passivated Ti6Al4V; in addition, there was a significant time-related decrease in these levels, as well as in the levels observed in medium samples from the non-passivated Ti6Al4V controls and from passivated and non-passivated cpTi.

1. Introduction

Titanium and its alloys are used for a wide spectrum of biomedical devices due to their relative resistance to corrosion, and thus greater degree of biocompatibility, compared to stainless steel and cobalt chromium alloys. In order to increase corrosion resistance, an ASTM surface passivation protocol was developed for stainless steel and cobalt chromium alloy surgical implants [1], which is one of several possible passivation treatments [2]. Such passivation procedures have also been widely adopted for, in particular, orthopaedic titanium alloy implants.

Due to the considerable clinical success of bone interfacing implants over the last three decades, such devices are implanted into increasingly young recipients thereby extending their potential exposure to the biological milieu. Topographical modifications designed to improve implant retention increase the area available for metal ion release from implant components. However, while it has been recognized for a number of years that the tissue fluids of the human body can be highly corrosive [3], the effects of prolonged human exposure to Ti, Al, and V are unknown.

It is now accepted that corrosion products of metal implants can be found at sites distant from that of the implant [4] and are thus capable of generating both local and systemic effects [5]. Particulate debris, which is frequently found in association with prosthetic joint replacements, may also be a source of metal ion release in addition to the pathologies associated with phagocytosis by macrophages. Thus metal ion release is associated with both corrosion of bulk implant surfaces and degradation of particulate debris. Recent clinical work [6] demonstrated increased serum Ti levels in patients with loose hip implants, while other studies have found histological evidence of Ti6Al4V particle phagocytosis by macrophages [7], and increases in serum Ti six months following surgery for total hip replacement [8].

Indeed, metal ion release from implant surfaces may be of even greater concern due to the growing awareness of the crucial role played in many biological systems by a variety of elements in trace amounts, and the impact which any disturbance in this delicate balance could produce. In the case of Ti and Al, there is mounting evidence that these metal ions can affect cell function *in vivo* [9] as well as cell proliferation and synthesis of extracellular matrix *in vitro* [10, 11]. Furthermore, it has been shown [12] that Ti and V ions can inhibit apatite formation *in vitro*, and this could have important implications for mineralization at bone–metal implant interfaces *in vivo*. Thus the need for further investigations of the corrosion behaviour of Ti and its alloys is clearly evident.

To the best of our knowledge no controlled study has been performed, to date, on the precise effect of HNO_3 passivation treatment on Ti, Al, and V metal ion release from the surfaces of solid cpTi and Ti6Al4V. The purpose of this investigation was therefore to measure the release of these ions, from custom made cpTi and Ti6Al4V containers, into culture

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medium which we employed for *in vitro* modelling of the establishment of bone/implant interfaces [13, 14]. We have used a sensitive graphite furnace atomic absorption spectrophotometry (GFAAS) analysis system, developed in our laboratory [15, 16], which incorporates rigorous techniques to minimize contamination and achieve data in the low parts per billion (ppb) range.

2. Materials and methods

2.1. Fabrication of metal wells

Custom metal wells were machined from 4 cm lengths of 0.5″ and 15 mm diameter cpTi and Ti6Al4V rod stock respectively using a 0.375″ ball end milling cutter in order to obtain a standardized well surface. The final drilled volume of each well unit was 2.5 ml. These metal wells were cleaned and passivated in a Class 100 laboratory facility as described below.

2.2. Cleaning and passivation procedure

All cleaning and passivation was performed using an ultrasonic cleaner (Cavitator, Mettler Electronics Corp., USA). The metal wells, in groups of four, were inverted over individual polypropylene pegs within a 1 l polypropylene container. Particular care was taken to ensure that the entire cavity of each well was filled with solution during cleaning, rinsing, and passivation and that no air bubbles became trapped during inversion. Double distilled high purity (18.0 MΩ cm) water (DDH_2O), produced using a Corning Mega Pure System DF, was employed for cleaning and passivation solutions as well as for all rinsing; Decon was obtained from BDH. The protocol was as follows:

1. Cleaning: sonication in 2% Decon solution for 1 h;
2. Rinsing: sonication in three rinses of DDH_2O (each 5 min);
3. Passivation: sonication in 34% HNO_3 (1 h);
4. Final rinsing: sonication in five rinses of DDH_2O (each 5 min).

Steps 3 and 4 were used only for passivated samples; the 34% HNO_3 was prepared by mixing 40 ml of 70% HNO_3 with 60 ml of DDH_2O. Following the final rinse of step 4, each well was individually double packed in sealed autoclaving bags; all units were then subjected to steam sterilization at 121 °C for 30 min.

2.3. Culture medium stock solution and storage

Sufficient incubation medium (800 ml) was prepared for the entire experiment, and consisted of α-Minimal Essential Medium (α-MEM, Gibco) with 15% foetal bovine serum (Gibco); 10% antibiotics, consisting of Gentamicin (Gibco), Penicillin G and Amphotericin B (both from Sigma); 10^{-8} M dexamethasone, 10 m M β-glycerophosphate and 50 μg ml^{-1} ascorbic acid (all from Sigma); this was stored in a cleaned 1 l Teflon bottle at 4 °C. This medium was identical to that customarily used for our cell cultures; control and calibration medium samples were obtained from this stock and analysed in the same manner as the experimental samples.

Every possible precaution was taken to avoid contamination by extraneous sources of metal. Basic culture medium (α-MEM) was ordered in plastic, instead of glass, containers to reduce the danger of contamination introduced by glass. However, in some other instances no suitable plastic alternatives exist; our stock serum is supplied in glass bottles, as is the Penicillin G, Gentamicin, Amphotericin B, ascorbic acid, β-glycerophosphate, and dexamethasone used to supplement our medium. In addition, glass instead of plastic graduated cylinders had to be used, since the latter cannot be sterilized by autoclaving. All plastic and glass utensils used in these experiments were meticulously washed using our customary cleaning procedure previously described [15].

2.4. Analysis of trace concentration of Al, Ti, and V

The general analytical procedures used for the determination of trace levels of these elements has been described previously [15, 16]. For the purpose of these experiments some modifications were introduced, as detailed below. Wherever possible, "Ultrex" grade reagents such as hydrochloric, nitric, and perchloric acids as well as methyl iso butyl ketone (MIBK, used for iron extraction) from J. T. Baker Chemical Co. were used in these experiments. Additional high purity chemicals obtained from this supplier were: ammonium hydroxide, alkylaryl polyether alcohol (Triton TX-100), bromocresol green (a pH indicator); and standard atomic absorption solutions (1 mg ml^{-1}) for Al, Ti, and V. A Varian atomic absorption spectrophotometer (model 875) with graphite furnace GTA95 was employed for the atomic absorption measurements. The detection limits were as follows: Ti, 1.0 ng ml^{-1}; Al, 0.5 ng ml^{-1}; V, 0.1 ng ml^{-1}. All plastic and glass ware was cleaned according to the procedure previously described [15, 16]. All sample manipulation was performed in a Class 100 laboratory.

2.5. Determination of Al

Using 1.5 ml Eppendorf centrifuge tubes, 250 μl of the test sample was mixed with 500 μl of 1% Triton TX-100 solution; this mixture was agitated for 5 min in a shaker (Eppendorf, model 5432) and then centrifuged (Eppendorf, model 5414) for 2 min. For calibration 0, 20, 50, 100, 250, and 500 μl of a 50 ng ml^{-1} Al solution was added to a series of samples prepared by mixing 500 μl of 1% Triton TX-100 solution with 250 μl of low Al (stock medium) samples; these calibration samples were subjected to the same vibration and centrifugation procedures as used for the experimental specimens.

Trace concentration of Al was determined using GFAAS.

2.6. Determination of Ti and V

Samples were supplemented with iron, used as a coprecipitant for preconcentration of Ti and V, and digested as described in greater detail previously [16]. In brief, using graphite heating blocks, decomposition

was achieved in Pyrex test tubes as follows: 1.7 ml of a mixture consisting of concentrated nitric and concentrated perchloric acids (19 + 1) and 1.0 ml of sample was placed in this test tube, mixed carefully, and the tube then put in a graphite heating block. These samples were heated gradually, during a period of three days, up to a temperature of 230 °C; the heating was continued until the specimen had completely dried. Concentrated HCl (50 µl) was added to the dried samples, which were then covered and left overnight. On the following day, this residue was dissolved in 1000 µl of DDH_2O. Ti and V were co-precipitated on the iron hydroxide deposit, and subsequently extracted using methyl iso-butyl ketone (MIBK). Trace determination was then performed using GFAAS.

2.7. Control values

2.7.1. Stock Solutions

Concentrations of Al, Ti, and V in the stock culture medium were measured using modifications of our technique described previously [15, 16].

2.7.2. Laboratory environment and sample handling

In order to ascertain whether any potential sources of Al, Ti, or V contamination existed within the laboratory environment or as a result of sample handling, a set of wells was constructed from Teflon. These were washed to a trace element-free level and incubated with culture medium in the same manner as employed for the metal wells. The medium samples were harvested after incubation for 3 days and their Al, Ti, and V concentrations determined, with stock culture medium (see above) being used as a control.

2.8. Pilot study

A pilot study using three cpTi and three Ti6Al4V wells was carried out in order to establish an information base to assist in planning the final experimental protocol. From the results of this pilot study it was clear that there were differences in the variability as well as possible differences in the means among the groups. To achieve a more stable variance, the logarithmic transformation of the data was used in statistical analyses. The required sample size was estimated using the Student's "*t*"-test for the comparison of means obtained from those results (not shown) and an experimental protocol involving eight samples per factor was chosen. The factors were: two metals (cpTi and Ti6Al4V) and two surface conditions (passivated and non-passivated). Baseline trace levels of Al, Ti, and V were determined using stock, fully supplemented, culture medium.

2.9. Experimental protocol

The eight metal wells in each of the four groups (either passivated or non-passivated; consisting of cpTi or Ti6Al4V) were gripped externally and placed individually in the wells of a sterile, lidded, 24-well culture tray (one tray for each group) with cleaned sterile stainless steel forceps. Using a sterile plastic 25 ml pipette which had been rinsed twice by aspirating and then discarding two 25 ml aliquots of the same medium, 2 ml of this culture medium was inoculated into each cpTi or Ti6Al4V well.

Following incubation for 3 days under standard culture conditions (5% CO_2 in air; 37 °C; 100% humidity) the incubated medium was poured from each well into a cleaned and labelled polypropylene tube, which was then closed with a screw cap and additionally protected with Parafilm. All wells received further 2 ml inoculations, as before, and were incubated for another period of 3 days. This procedure was repeated once more, for a total of three time-points (each of 3 days). The collected culture medium samples were stored at − 70 °C prior to analysis.

2.10. Statistical analysis

Employing log transformations of the raw data for each element, Fisher analysis of variance (*F* test) was used to compare means by type of metal, whether passivated or not, and at three times after the experiment began. After log transformation, the observations for each metal satisfied the assumptions for the normal distribution. The means for each treatment metal combination ($N = 8$) have been expressed in the original units of measurement for publication.

3. Results

3.1. Control values

3.1.1. Stock solutions

Table I shows the concentrations of Al, Ti, and V in the basic medium (α-MEM), which are comparable to those considered to be within the "normal" levels of these elements in human blood [15, 16].

3.1.2. Laboratory environment and sample handling

Table II shows background levels of Al, Ti, and V in the laboratory environment or as a result of sample handling, measured using the Teflon wells. These results indicate that potential contamination from these sources was low enough to avoid influencing interpretation of the experimental values.

3.2. Experimental values

In this series of experiments the effect of two factors on Ti, Al, and V trace element release from solid cpTi and Ti6Al4V well units was tested: namely, the influence of passivation (using HNO_3 as prescribed by ASTM-F86), and that of time (up to 9 days). The influence of passivation, metal type, and time were tested for statistical significance using the *F*-test.

The effect of passivation on Ti corrosion was significantly different between Ti6Al4V and cpTi wells ($p = 0.0005$). In the Ti6Al4V wells passivation increased the corrosion dramatically ($p = 0.000\ 0396$,

TABLE I Concentration of Al, Ti and V in culture media

Data	Concentration ($ng\,ml^{-1}$)		
	Aluminium	Titanium	Vanadium
Mean	2.61	3.21	0.20
Stand. Dev.	0.318	0.497	0.067
Coeff of var.	0.122	0.155	0.337
Number	6	5	6

TABLE II Comparison of Al, Ti and V concentrations in control medium and medium from Teflon wells

Data	Concentration ($ng\,ml^{-1}$)					
	Al Control	Al Exp.	Ti Control	Ti Exp.	V Control	V Exp.
Mean	5.28	5.05	5.10	4.57	0.14	0.12
S.D.	0.472	0.336	0.093	0.895	0.037	0.016
C.V.	0.089	0.067	0.018	0.196	0.268	0.138
Number	4	11	4	11	5	14

Table IIIA) whereas in cpTi wells the amount of corrosion was not significantly different ($p = 0.459$, Table IIIB). There was a significant reduction in trace levels of Ti throughout the 9-day experimental period in all four categories ($p = 0.0001$). Control medium samples showed trace Ti levels which were, in general, considerably lower than those seen in these experimental samples, the exceptions being the non-passivated alloy and cpTi wells at 9 days.

The effect of passivation on Al trace release from Ti6Al4V wells was also highly significant ($p = 0.000\,001$, Table IVA), while this procedure had no significant effect on Al levels in medium samples obtained from cpTi wells ($p = 0.3248$, Table IVB). Since Al is not a component of cpTi, this latter result is to be expected; the Al levels in these specimens were similar to those of the unincubated control medium samples. There was a time-dependent significant decrease in Al release from the Ti6Al4V wells ($p = 0.0001$).

Vanadium trace levels in medium obtained from

TABLE IIIA Concentration of Ti in fully supplemented culture media from Ti6Al4V wells

Ti6Al4V Wells	Titanium concentration ($ng\,ml^{-1}$)						
	Controls	Not passivated			Passivated		
		1	2	3	1	2	3
Mean (N = 8)	4.98	12.60	11.05	5.51	23.34	24.65	10.49
± SD	0.977	3.850	1.601	1.943	8.497	8.419	3.674
± SEM	0.345	1.361	0.566	0.687	3.004	2.977	1.299

TABLE IIIB Concentration of Ti in fully supplemented culture media from cpTi wells

Ti Wells	Titanium concentration ($ng\,ml^{-1}$)						
	Controls	Not passivated			Passivated		
		1	2	3	1	2	3
Mean (N = 8)	4.98	23.70	12.65	6.44	15.74	16.64	8.74
± SD	0.977	12.892	5.275	2.495	3.354	4.940	2.983
± SEM	0.345	4.873	1.865	0.882	1.186	1.747	1.055

TABLE IVA Concentration of Al in fully supplemented culture media from Ti6A14V wells

Ti6Al4V Wells	Aluminum concentration ($ng\,ml^{-1}$)						
	Controls	Not passivated			Passivated		
		1	2	3	1	2	3
Mean (N = 8)	3.48	8.93	5.70	4.52	16.88	9.66	6.61
± SD	0.392	1.187	0.707	0.384	4.574	2.750	1.407
± SEM	0.139	0.420	0.250	0.136	1.617	0.972	0.497

TABLE IVB Concentration of Al in fully supplemented culture media from cpTi wells

Ti Wells	Aluminum concentration ($ng\,ml^{-1}$)						
	Controls	Not passivated			Passivated		
		1	2	3	1	2	3
Mean (N = 8)	3.48	4.10	4.70	5.22	4.13	5.52	4.15
± SD	0.392	0.677	1.039	1.096	0.523	2.784	0.397
± SEM	0.139	0.239	0.367	0.387	0.185	0.984	0.140

TABLE VA Concentration of V in fully supplemented culture media from Ti6A14V wells

Ti6Al4V Wells	Vanadium concentration ($ng\,ml^{-1}$)						
	Controls	Not passivated			Passivated		
		1	2	3	1	2	3
Mean (N = 8)	0.25	6.20	2.79	0.59	21.10	10.10	4.22
± SD	0.082	2.191	1.129	0.334	8.828	5.697	2.003
± SEM	0.029	0.775	0.399	0.118	3.121	2.014	0.708

TABLE VB Concentration of V in fully supplemented culture media from cpTi wells

Ti Wells	Vanadium concentration ($ng\,ml^{-1}$)						
	Controls	Not passivated			Passivated		
		1	2	3	1	2	3
Mean (N = 8)	0.25	0.51	0.26	0	0.37	0.17	0.33
± SD	0.082	0.199	0.018	0	0.167	0.051	0.213
± SEM	0.029	0.070	0.006	0.	0.059	0.018	0.075

Levels of V at the 3rd time-point for the "Not passivated" group did not reach the detection limit, and are therefore recorded as zero.

passivated Ti6Al4V wells were also significantly higher ($p = 0.000\,001$, Table VA) compared to levels in medium samples obtained from the non-passivated alloy wells. The decrease in V release with time was observed in both passivated and non-passivated alloy wells, and was significant at $p = 0.0001$. As was the case with Al, passivation did not affect V levels in media obtained from cpTi wells ($p = 0.3931$, Table VB) since V is not a component of these; V trace levels here were similar to those of the unincubated control samples, except for the drop below the detection limit (0.1 ppb) at the last time-point in the non-passivated group.

To summarize, HNO_3 passivation performed as recommended by ASTM F86 guidelines significantly increased Ti, Al, and V trace element levels in serum-containing medium incubated in Ti6Al4V wells, compared to levels detected in such samples obtained from non-passivated Ti6Al4V wells. There was a significant time-related reduction, during the 9-day incubation period, in release of Ti, Al, and V from Ti6Al4V wells (both passivated and non-passivated); also of Ti from both groups of cpTi wells.

4. Discussion

The pilot study showed that the levels of Ti, Al, and V were considerably higher from Ti6Al4V, incubated for 3 days with serum-containing culture medium, compared to the trace levels of these elements found to be present in control medium samples. A parallel trend was observed with the release of Ti from cpTi wells. A more detailed study was then undertaken, with appropriate statistical analysis, of the influence of two factors on these phenomena: first, the effect of the ASTM F86 prescribed surface treatment of metallic surgical implants (using HNO_3); and second, the effect of time (9 days) on this process.

Passivation treatment applicable to certain stainless steel and cobalt alloys (identified in ASTM F86-91, Section 7.2) with HNO_3 (Section 7.2.1) may also be used (Section 7.3) for other implant metal surfaces such as those of titanium and titanium alloys (referred to in Section 2.1). The concentration of nitric acid recommended for this procedure is described as follows: "20 to 40 volume % nitric acid" (Section 8.2.1).

Our results clearly showed (Tables IIIA, IVA and VA) that trace element release of the constituent elements of Ti6Al4V was significantly increased by HNO_3 passivation, compared to the non-passivated control wells. The level of significance in these experiments was high: $p = 0.000\,0396$ for Ti; $p = 0.000\,001$ for both Al and V levels. The corrosion of the cpTi well units was not affected by this passivation process, as shown by the fact that there was no significant difference in trace Ti levels found in medium samples obtained from passivated and non-passivated cpTi wells (Tables IIIB, IVB, and VB).

There was a significant decrease, during the 9-day experimental period, in trace element levels found in medium samples obtained from all the experimental groups. A possible explanation for this, is that such reduction is due to a thickening of the protective oxide layer on the wells. It has long been recognized that, owing to their strong affinity for oxygen, titanium [3] and its alloys [17] readily repair damage to the passive surface film. A further effect which may influence this phenomenon is that initial ion release may be dominated by ion exchange events at the solid–liquid interface which occur before thermodynamic equilibrium of the surface protein adsorption and desorption events has been reached. Once the latter is achieved, further ion exchange events may be inhibited. Clearly, more refined experimental protocols are needed to identify these events.

These findings were completely contrary to our expectations, since passivation is generally assumed to reduce corrosion by producing a more stable protective surface oxide layer. Corrosion is a surface phenomenon; thus the interpretation of these unexpected

results requires careful analysis of the effect of the ASTM-F86 passivation protocol on the surfaces of solid cpTi and Ti6Al4V. It is possible that the significant increase in Ti, Al and V trace levels which we have detected here in association with Ti6Al4V passivation, is caused by a thinning of its protective oxide layer due to the acid passivation treatment. This would correlate with our previous findings on the comparative thicknesses of oxides on cpTi and Ti6Al4V [18].

Replacement of defective skeletal and dental components by prosthetic devices manufactured from titanium and its alloys is widespread owing to their relative resistance to corrosion, and thus greater degree of biocompatibility, compared to stainless steel and cobalt chromium alloy (for which ASTMF-86 was originally recommended). For these and other reasons, such implant corrosion requires urgent consideration. Detailed surface analysis is required to elucidate possible explanations for these observations; we are now in the process of performing these investigations. Armed with such information, there is an increased possibility of developing rational, precisely defined, methodologies to address the issue of cpTi and Ti6Al4V implant corrosion *in vivo*.

5. Conclusions

Passivation of Ti6Al4V using the ASTM-F86 protocol increases Ti, Al and V release into serum-containing culture medium, compared to unpassivated control wells, during a 9-day incubation period at 37 °C in an atmosphere of 5% CO_2 in air and 100% humidity. The levels of Al and V in medium obtained from both passivated and non-passivated Ti6Al4V wells decreased throughout the re-feeding sequence, but generally failed to reach the levels found in non-incubated control samples which approached those obtained from passivated and non-passivated Ti wells.

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