Cathodic Voltage Preconditioning of Ti-6Al-4V in Media Affects MC3T3 Pre-Osteoblast Cell Viability

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Statement of Purpose: It has recently been reported that reduction electrochemistry at metallic biomaterial surfaces can adversely affect cell viability[1,2]. This observation raises significant questions about the standard paradigm of metallic biocompatibility where primary factors relate to the toxicological consequences of ion and particle release. It is known that cathodic voltages can occur in vivo that may generate reduction currents and adverse surface reactions that have not been previously understood. This includes the possibility of reduction of proteins, amino acids, and other species in the extracellular environment. In an attempt to understand the underlying mechanisms involved, this study tests the hypothesis that cathodic voltage preconditioning of Ti surfaces, in the presence of cell culture media constituents, will adversely affect subsequent cell viability when cultured either on the conditioned surface or on a fresh surface with the conditioned media.

Methods: Ti6Al4V discs were wet polished to 600 grit, sonicated, washed with 70% ethyl alcohol and UV sterilized for 30 minutes. The discs were placed into a custom made electrochemical cell culture chamber with electrical contacts to the Ti6Al4V disc to be as a working electrode, and graphite counter and chlorided silver wire reference electrodes.

The intent of these experiments was to culture cells on conditioned Ti surfaces in fresh media, or on fresh Ti surfaces in conditioned media to determine how sensitive and responsive the cells were to either the surface or the media to changes induced by reduction electrochemical reactions. Preconditioning was performed by applying -1000 mV for 24 hr to the Ti surface in the presence of different solutions (described below). After 24 hr, the conditioned samples and conditioned media were kept sterile and separated. The conditioned Ti surfaces were placed in fresh media and cells were cultured with no voltage for 24 hr. The conditioned solutions were used to culture cells on fresh Ti surfaces with no voltage.

The conditioned solutions explored were: 1) Alpha Minimal Essential Media (AMEM, Cellgro, VA) at -1000 mV for 24 hr, 2) AMEM with 10% fetal bovine serum (FBS) (Gibco, NY), 1% Penicillin-streptomycin Glutamine (PSG) (Gibco, NY) at -1000 mV for 24 hr and 3) Phosphate Buffered Saline (PBS) at -1000 mV for 24 hr. Note, for PBS only the Ti surfaces were assessed after conditioning, not the solution. Thus, six separate test conditions (including control) were explored: 1) control culture on Ti with fresh growth media at open circuit potential (OCP).) 2) Fresh Ti in conditioned AMEM/ 10%FBS/ 1%PSG, 3) fresh Ti in conditioned AMEM (into which fresh FBS/PSG were added prior to cell culture), 4) Conditioned Ti in PBS, 5)Conditioned Ti surface in AMEM/10% FBS/1% PSG with fresh media plus 6) Conditioned Ti surface in AMEM alone with fresh media.(n=3, n=2 for group 2). MC3T3-E1 subclone 4 (ATCC, VA) pre-osteoblasts were used in all experiments. Approximately 10,000 cells were placed onto the surfaces and allowed to settle and attach over 20 minutes after which media was added into the 6 well plates to bring the total volume of media to 5 ml and the tests were run without voltage for 24 hr. After 24 hr, the viability of cells were evaluated using Live/Dead cytotoxicity assay for mammalian cells (Invitrogen, CA). Fluorescent images were acquired with fluorescent microscope (Axiovert CFL 40, Zeiss) using Image-Pro Plus. ImageJ (NIH) was used to count cells. Images of conditioned surfaces were acquired using a scanning electron microscope (SEM) (Jeol JSM-5600, Japan). Analysis of Variance (ANOVA) and Tukey-Kramer post –hoc tests of fraction viable was performed with $p<0.05$ being statistically significant.

Results: The results are summarized in Fig. 1. Fresh surfaces with conditioned media (AMEM with or without 10%FBS/1%PSG) showed small effects on cell viability with percent viable in the 93 to 95% range. Conditioned Ti surfaces in fresh media showed dramatic decrease in cell viability with between 95% (for AMEM/FBS/PSG) and 99% (AMEM alone) of cells dead at 24 hr.) Preconditioning Ti in PBS had little to no effect (98% viable) and controls were approximately 99% viable.

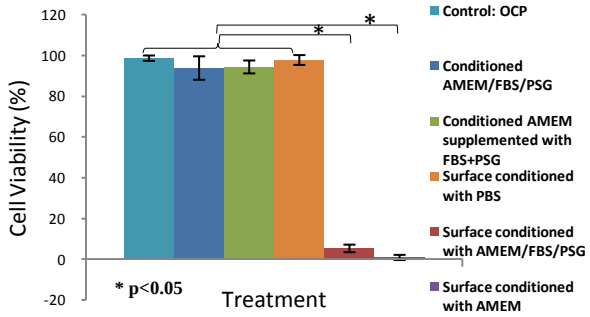

Figure 1. Percent cell viability versus conditioning treatments. Note the large loss of viability on conditioned surfaces and little loss on fresh surfaces in conditioned media.

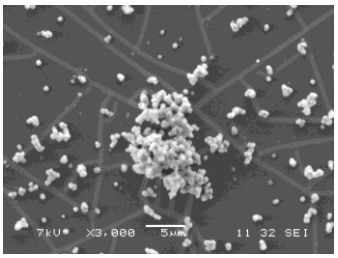

Figure 2: SEM micrograph of Ti surface after conditioning. Note the precipitates and film covering the surface.

SEM analysis showed precipitated species from the AMEM alone, whose composition is, as yet, undetermined.

Discussions and Conclusions: This study demonstrates that preconditioning reduction reactions in the presence of amino acids, sugars and inorganic salts at Ti surfaces can alter the surface and induce cell death even in the absence of voltage. Clearly reduction reactions at implant surfaces are a significant factor in affecting metallic biocompatibility. **References:** (1) Kalbacova et al, Biomaterials 28 (2007) 3263–72; (2) Ehrensberger et al., 2009 JBMR-A (accepted)