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Paper

IN VITRO ACTIVITY OF FORCED PULSED WATERJET (FPWJ) MODIFIED TITANIUM

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ABSTRACT

The objectives of the project was to determine (1) whether the FPWJ technique to roughen the surface of industrially relevant metals is effective on biocompatible metals (for example, Titanium), and 2) could generate a progressive micro- and nano-topography with bioactive abilities for biomedical applications.

The preliminary results have clearly shown that the FPWJ can modify the surface of titanium and by varying the treatment parameters, various gradients of roughness can be achieved on the metal. These surfaces were successfully tested with MG63 osteoblastic cells, a cell model widely used to mimic the activity of human bone cells, and found out that on specific treated surfaces (those characterized by the minimum roughness), cell proliferation at 3 days was significantly enhanced (+20%) as compared to untreated cold rolled titanium.

1. INTRODUCTION

Each year, over 100,000 Canadians require knee or hip replacement. Current technology uses a titanium alloy to attach the implant to the bone, though problems arise at their interface due to improper surface preparation. To attach the device to the patient's bone, the surface of the alloy is typically roughened by chemical etching and grit blasting with aluminum oxide particles to promote cell growth on the device [Carreon, et al]. However, as shown in **Figure-1**, the particles could typically get imbedded into the substrate of the metal. Once inside the patient, the particles can become dislodged and find their way into the artificial joint and the surrounding tissue causing wear [Carreon, et al], cell death and the loosening of the implant [Lass, et al]. This has become the

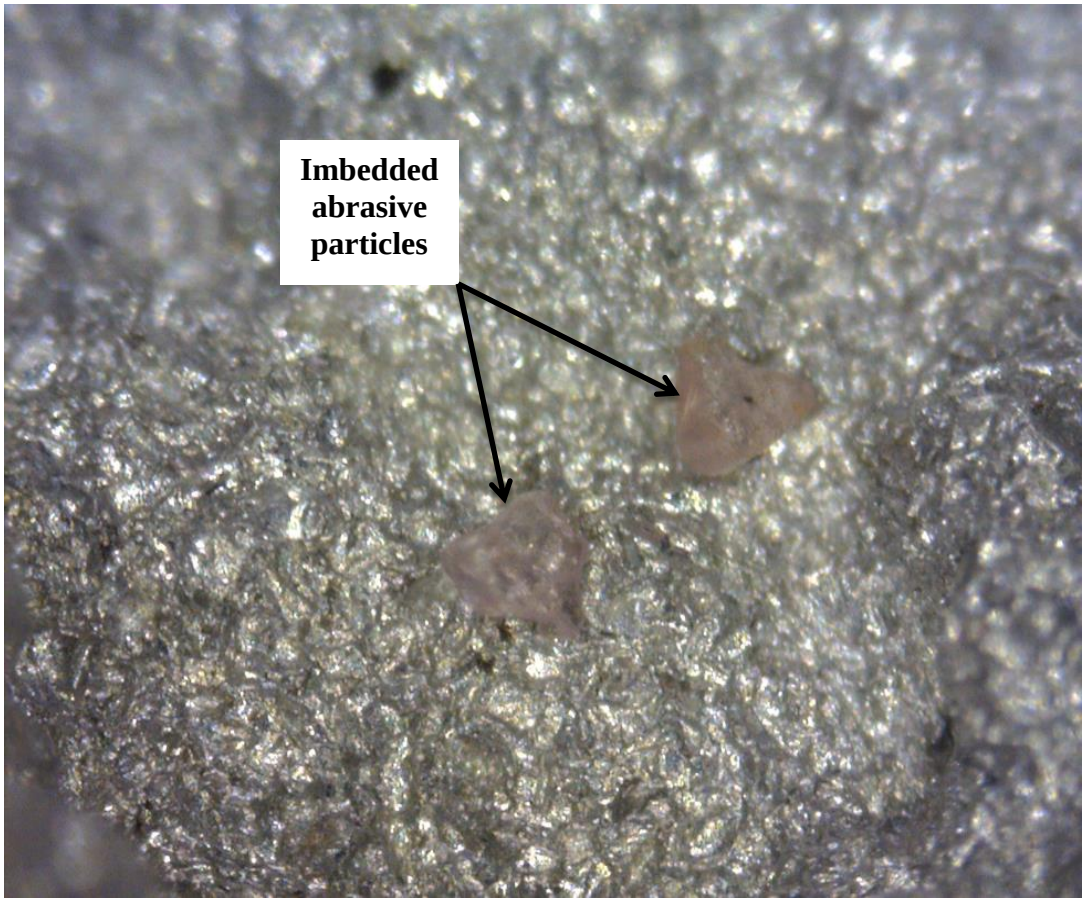

Figure 1. Photomicrograph showing the imbedded abrasive particles.

leading cause for revision surgery [Anon, Canadian Institute for Health]; in addition to the fact that Al exposure is associated with long-term health problems [Geetha, et al].

Metals remain the most important class of materials for bioimplants, with Ti-alloys, 316 stainless steel and CoCr-alloys representing the most commonly used in the industry. While these materials are by no means new, the fact that material or component related issues account for 28% of all recalled medical devices provides strong motivation for further materials development [Anon, Medical Device Recall Report]. The challenges are complex and multifold. The integration of

recent biocompatible metallic implants into living organisms must take into consideration the surface roughness and hydrophobicity to either promote or hinder cell adhesion, in addition to a predictable chemistry to ensure the chemical stability of the alloy and to prevent toxic elements from diffusing into the body. In the case of bone implants, osseointegration requires the foreign material to have roughness on multiple length scales. Micron and submicron roughness is necessary to match the cell dimensions and features of the inorganic bone matrix [Curtis and Wilkinson], while recent discoveries demonstrate the importance of nanoscale roughness [Webster and Ejiofor] that influences proteins and cell membrane receptors [Gittens, et al]. Therefore, to promote osseointegration of titanium implants to bone, the medical implant needs to have controllable feature sizes that span the micron scale down to the nano-scale in order to mimic the surrounding biological textures.

The present pilot investigation was undertaken based on the promising results obtained on the surface preparation of metals with the FPWJ [Vijay, et al and MacDonald, et al]. The FPWJ uses ultrasonic waves to modulate a continuous water stream into a series of pulses that create mechanical erosion by cyclic loading of the target [Vijay et al] without embedding any foreign material.

2. EXPERIMENTAL SETUP AND PROCEDURE

2.1 Sample Preparation and Visualization

Pure titanium samples provided by the University of Ottawa were surf prepped in the PurePulse booth, designed and manufactured by Pratt, USA. It is a totally automated turnkey system consisting of a booth (cell), a heavy duty turntable, NLB high pressure pump, an HMI integrated with the Fanutec Robotic system, a water collection and filtration system. A general view of the PurePulse booth with the turntable and the robot is depicted in **Figure-2**. The operating parameters were: Orifice diameter = 1.016mm, Pump pressure = 69MPa, Frequency of the FPWJ = 40kHz and standoff distance = 12.7mm. Initially nine samples were prepped at nozzle traverse speeds as listed in table 1at an index of 0.1mm (adjacent swaths) and are illustrated in **Figure-3**.

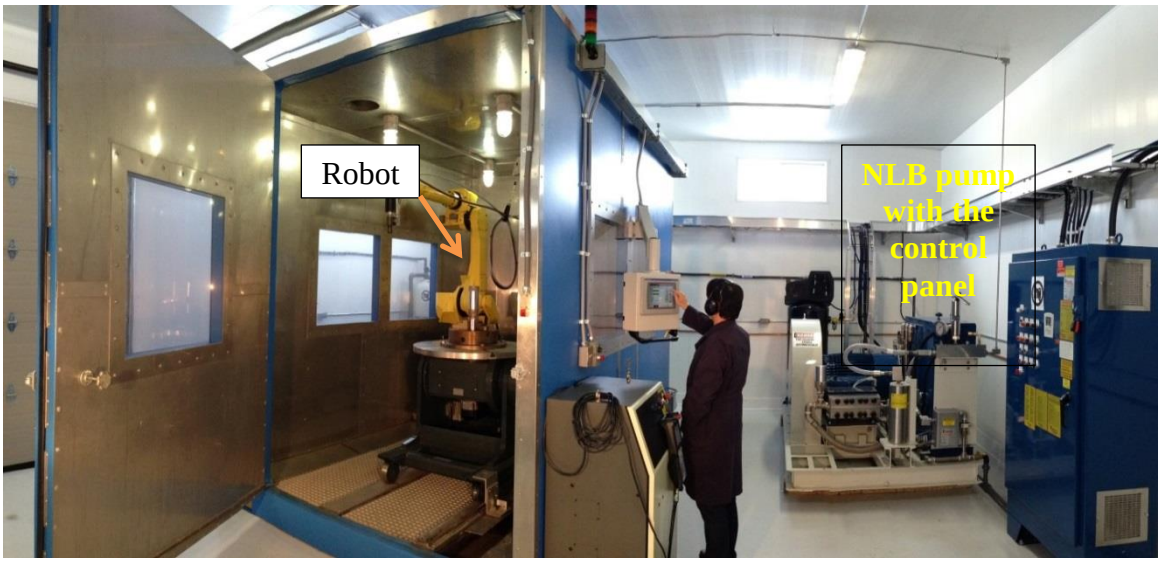

Figure 2. A general view of the PurePulse booth showing the Fanutec robotic system and the NLB pump.

2.2 Surface Visualization and Analysis

Figure 3. Overview of the Ti samples after surface preparation

A JSM-7500F Field Emission Scanning Electron Microscope (FESEM, JEOL, Japan) was used to image treated samples in order to confirm progressive roughening through varied transverse speeds.

3D topographical reconstruction was achieved with the VHX-2000 digital optical microscope (Keyence Corp., Osaka, Japan) which records multiple images at various focus depths prior to generation of a 3D model. Surface roughness was recorded using the SRG-4000 portable profilometer (PHASE II, NJ, USA) equipped with a diamond stylus.

For the reasons explained below (Section 4), the next set of four samples was prepped respectively at the traverse speeds of: 0 (control sample, that is, untreated original sample), 400, 350, 300 and 250mm/s (**Figure- 4**).

Table.1. Traverspeeds of treated samples.

Sample #	1	2	3	4	5	6	7	8	9
Traverse speed (mm/s)	400	350	300	250	225	200	175	150	120

Figure 4. Second set of four samples.

2.3 Cell Culture and Analysis

MG-63 human osteosarcoma (ATCC, USA) cells were grown in Dulbecco's Modified Eagle Medium (DMEM, GIBCO) containing 10% fetal bovine serum (FBS), 20 U mL^{-1} penicillin and 20 U mL−1 streptomycin (ThermoFisher Scientific, USA) in a 5% CO2 incubator at 37°C. Both sets of samples were treated with MG63 Cells, an human bone-derived osteosarcoma cell line that presents with a fibroblast morphology (biological terms are explained in the Glossary). Cells were seeded onto treated samples that were previously sterilized in 70% EtOH. After a 3-day incubation, cells were fixed with 4%-PFA. Nuclei visualization was achieved with NucBlue® Fixed Cell ReadyProbes® Reagent (ThermoFisher Scientific, USA) and actin was visualized with Alexa Fluor® 555 conjugated to Phalloidin (ThermoFisher Scientific, USA).

Cells were imaged using an AxioObserver.D1 inverted microscope (Carl Zeiss, Germany) and driven by AxioVision 4.8 (Carl Zeiss, Germany). For viability assessment, a total of 16 images per disc were captured in a 4 × 4 grid pattern across the disc using a 10× A-Plan 0.24 Ph1 objective (Carl Zeiss, Germany). Images were processed using a custom pipelines in the open-source software package, CellProfiler (Broad Institute).

3. EXPERIMENTAL RESULTS

Preliminary analysis of cell (MG63) proliferation across all treatments is depicted in **Figure-5** and representative fluorescent images of extremes shown in **Figure-6**. Surface visualization is shown in **Figure-7** with analysis via 3D reconstruction including roughness shown in **Figure-8**. Cell

morphology from further experiments, specific to Con1-4, can be seen in **Figure-9** with proliferation analysis displayed in **Figure-10**.

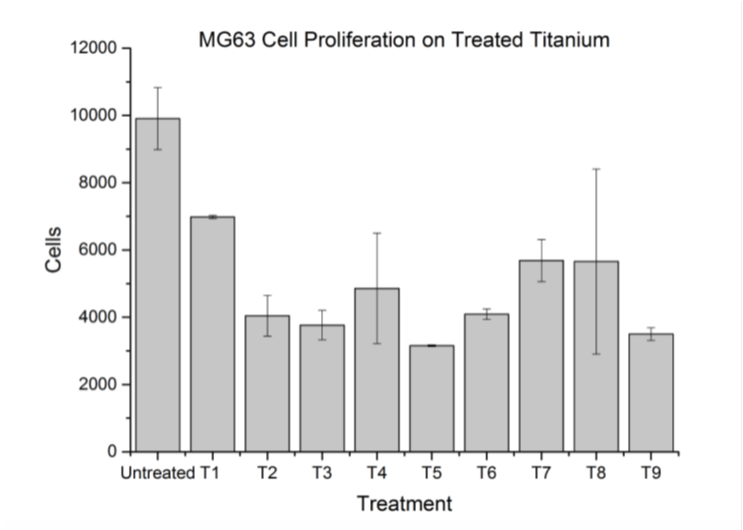

Figure 5. Initial broad cell proliferation study for samples in Table 1.

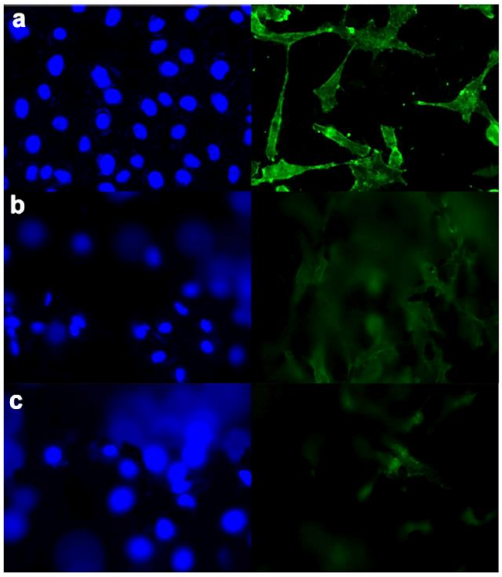

Figure 6. Fluorescence staining of MG63 cells from initial broad assessment showing (a) untreated Titanium, (b) Condition #5 and (c) Condition #9.
Blue: Hoescht stain for nuclei; Green: Phalloidin stain for F-actin

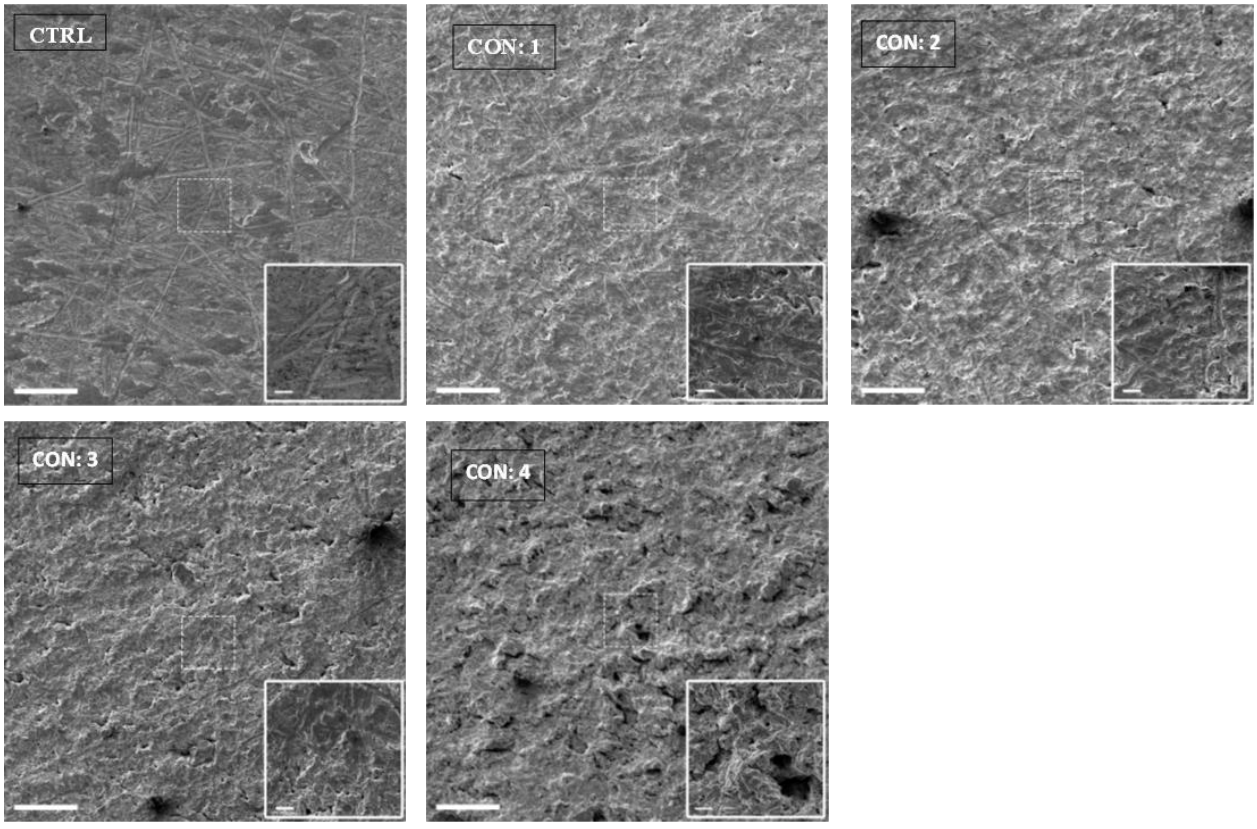

Figure 7. Images of the substrates taken with a scanning electron microscope (SEM). Primary Magnification: 1020X, scale bar = 100:m. Inset magnification: 1000X, scale bar = 10:m.

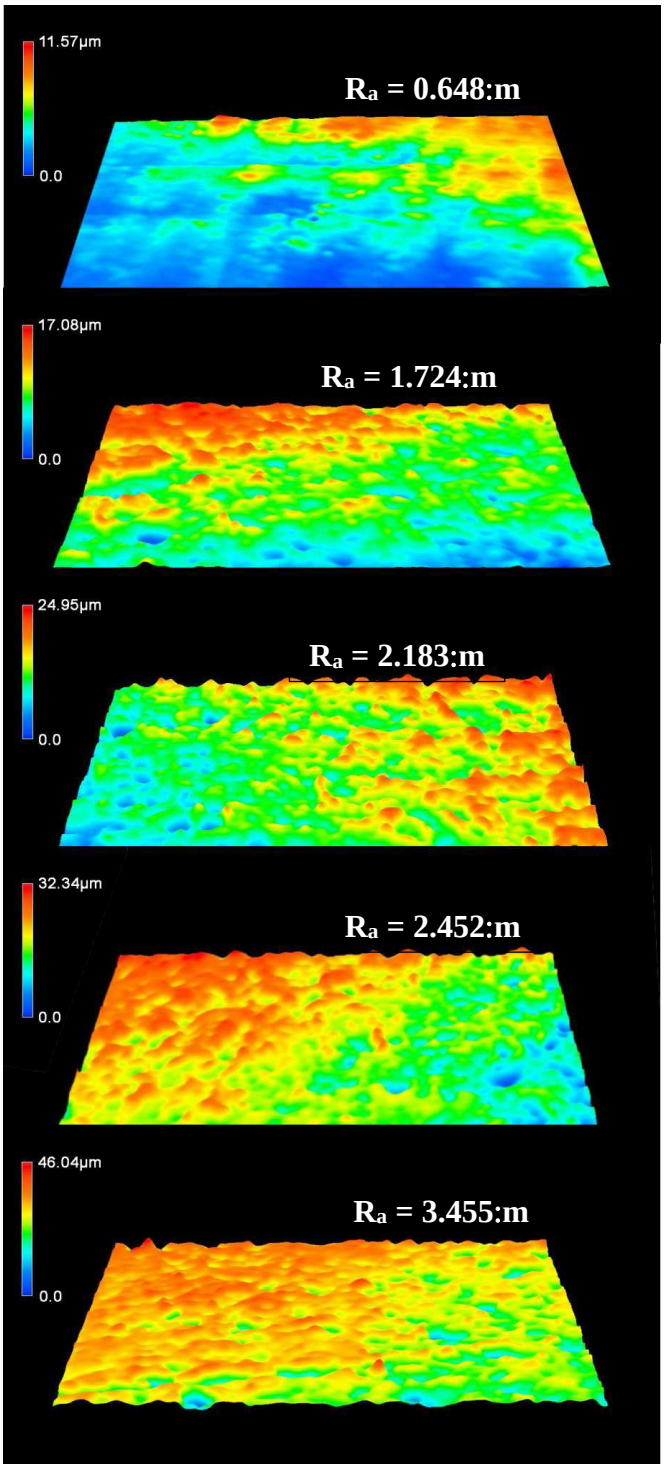

Figure 8. Three dimensional reconstruction of topography of samples including mean roughness values.

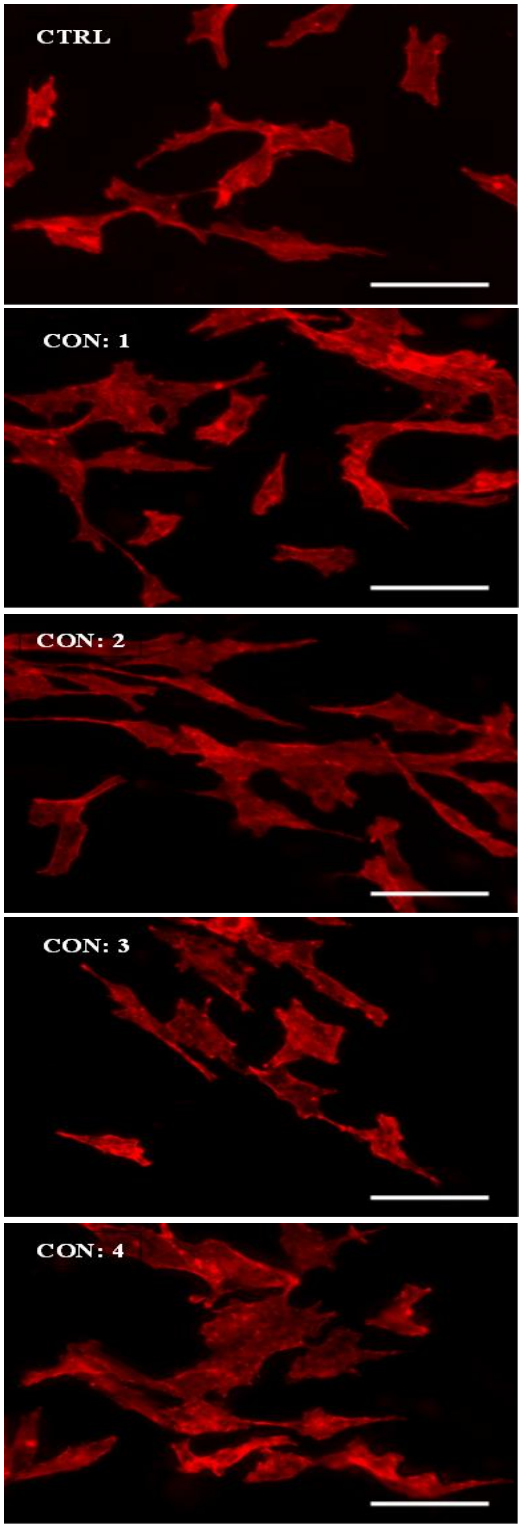

Figure 9. Typical fluorescence images of MG63 cells culture on the samples. Scale bar = 200:m.

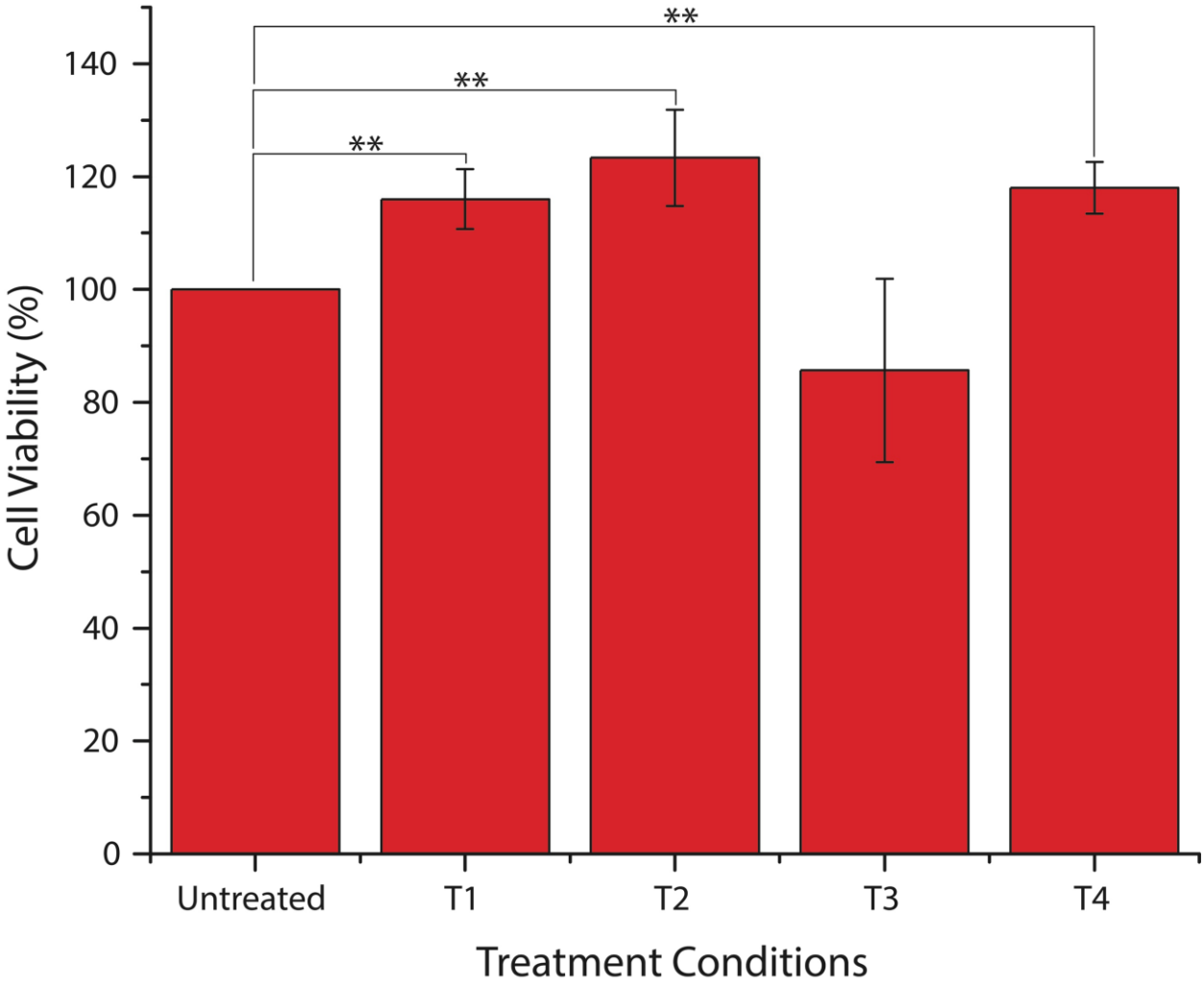

Figure 10. Cell viability of MG63 human osteoblast cells on untreated and treated samples from additional focused experiments. Results are expressed as a percentage change compared to untreated titanium.

4.0 DISCUSSION

Most of the commercial implants (e.g., Stryker) have proprietary treatment to make them characteristically rough at the implant-bone contact surface with each being unique from one company to another (and even from one model to another). As such, it would be impossible to replicate a "universal" implant surface to use as a platform for further enhancement of these surfaces. Also, since mechanically polished surfaces definitely do not reflect modern interfaces, we decided to use widely available commercially cold-rolled titanium, with no additional treatment, as our base to determine whether FPWJ treatment can be an alternative method to generate a texture with beneficial cellular effects.

Throughout the initial visualization and analysis of the surface, we found that the FPWJ treatment did in fact create a progressive roughening in the cold-rolled titanium between all nine of the tested conditions. While untreated titanium gave an angle of 93º (with both distilled water and oil), all

treated samples showed high hydrophilicity, since droplets were not able to stand on such surfaces long enough for an angle to be measured. This is an encouraging data since surface energy is known to affect cells, and hydrophilic surfaces provide advantageous effect on cells. To determine these cellular effects, we utilized MG63 osteosarcoma-derived cells which present with a heterogeneous combination of mature and immature osteoblastic (bone like) features and well spread fibroblast morphology. Our initial broad study across these surfaces regarding cell proliferation determined a wide disparity and lack of trend (**Figure-5**). While our initial interest was in the micro- and nano-scale feature over an entire range from FPWJ treatment, we determined that conditions with a transverse speed < 250mm/s developed cavitations with such variable intra-cavitation features which subsequently left single-cell analysis impossible. As such, we decided to focus on those conditions with transverse speeds > 250mm/s in an effort to better determine a trend and thus understanding of the effects.

We again confirmed that there was progressive erosion in the topography of these conditions as the transverse speed was decreased confirmed with SEM (**Figure-7**) which was better visualized with 3D reconstruction (**Figure-8**) leading to measured surface roughness (Ra) ranging from 0.648µm in Control samples to 3.455µm by Condition 4. These samples were sterilized and MG-63 cells were deposited for an incubation of 3-days to determine cell proliferation and morphology. Initial observation saw no distinct changes in cell morphology (**Figure-9**) between control and treated samples. However, analysis of cell proliferation determined that there was indeed an effect. Treatments T1, T2 and T4 were found to present with enhanced proliferative capability showing an average 15-25% increase ($p < 0.01$) in measured cell proliferation (**Figure- 10**). Important to note is that these enhancements are compared to a control with absolutely no pre-treatment after its initial production during cold-rolling and as a result presented with an existing measured roughness (**Figure-7**, Panel A). Often, controls are pretreated to create a 'perfect' surface (e.g., mirror-polish finish) which would produce results with a greater difference between the initial condition and treatments, and can be less relatable to widely available materials. As such, this further strengthens the noteworthy change in our treated conditions after only a 3-day incubation.

5. CONCLUSIONS

We found that FPWJ treatments with transverse speeds <400mm/s are capable of producing contaminant-free topographies on cold-rolled titanium that enhanced the proliferative capabilities by 15-25% after three days after single-cell analysis. While this has been determined, additional benefits at the tissue level can be pursued with the macro-/nano-scale roughness potentially promoting osseointegration thus determining if enhancement is achieved at both the single-cell and tissue levels.

6. ACKNOWLEDGMENTS

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8. GLOSSARY

ANOVA: Analysis of Variance is a collection of statistical models used to analyze the differences among group means and their associated procedures (taken from Wikipedia).

F-actin: It is the skeleton of the cell showing the shape of the cell (**See Figure-6**)

Fibroblast: Fibroblasts and fibrocytes are two states of the same cells, the former being the activated state, the latter the less active state, concerned with maintenance and tissue metabolism (taken from Wikipedia).

Fluorescence Microscope: It is an optical microscope that uses fluorescence instead of reflection and absorption to study properties of inorganic or organic substances. In the life sciences, fluorescence microscopy is a powerful tool which allows the specific and sensitive

staining of a specimen in order to detect the distribution of molecules (In **Figure-6**, the nuclei were stained with the blue DNA dye (Hoescht) and the F-actin by the green dye (Phalloidin).

MG 63 Cells: These are a strain of osteoblasts propagated from one single osteoblast so every cell in the MG-63 strain should behave the same although this will change over multiple rounds of mitosis.

Morphology: It is a branch of biology dealing with the study of the form and structure of organisms and their specific features. For example, internal morphology includes aspects of the form and structure of the internal parts like bones.

Osteoblast Cells: These are the type of cells that have the potential to develop into bone.

Osteosarcoma Cell: It is a cancerous tumor in a bone. It is the most common form of primary bone cancer.