

Osseointegration potential of mesenchymal stem cells on porous heat treated and untreated 3D-printed Ti6Al4V scaffolds

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ABSTRACT

Hypothesis: Aseptic loosening of artificial joints and long bones implants occurs due to the loss of implant fixation. By developing an implant with a 3D porous structure at the bone-implant interface, the ingrowth of bone will permit better and stronger interlocking of the implant to prevent loosening. This study hypothesizes that the seeding of this three-dimensional (3D) scaffold structure, with mesenchymal stem cells (MSCs), will further improve the potential for osseointegration of the implants, as the existing bone may be able to unite with the developing bone.

Aim: The aim of this study is thus to investigate the effect that the thermal oxidization, or heat treatment process on the laser sintered 3D Ti64 scaffolds will have on the potential for adhesion, proliferation and differentiation of seeded mesenchymal stem cells *in vitro*.

Method: Titanium-6Aluminum-4Vanadium, or Ti-6Al-4V (Ti64), is one of the most commonly used implant materials. Testing at the University of Cape Town has shown that the heat-treatment of Ti64, at 600 °C for 20 hours, vastly improves the material's mechanical properties and tribological results. Porous scaffolds were manufactured and seeded with MSCs. All cell tests were done with rat and human MSCs on both untreated and heat treated Ti64 scaffolds.

Results and Discussion: The results of MSCs adhesion, growth and differentiation tests on both untreated and heat-treated titanium porous scaffolds, are presented and compared and show marginal difference in cell counts.

Conclusions: It is possible to seed patient's MSCs into porous titanium implants that are in contact with the host bone, to improve osseointegration and secure interlocking of the implant.

KEY WORDS: Osseointegration, MSCs differentiation, Porous titanium implants

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Introduction

Arthroplasty revision surgeries are due to failed of artificial joints and implants. The most frequent reason for revision surgery is due to aseptic loosening of the joint, followed by cases of dislocation [1]. Aseptic loosening of the joint occurs due to the loss of implant fixation to the bone, resulting in joint movement and instability. It is expected that by 2025 the number of hip arthroplasty revisions will be double that of 2005, illustrating the severity of this problem [2].

Titanium alloy Ti64, has become a widely-used implant in orthopaedic surgery due to its high strength and excellent biocompatibility [3,4]. Although Titanium (Ti) is a reactive metal, reacting with oxygen molecules from its surrounding environment (water, air, extracellular matrix), it instantly creates an electrically and chemically inert film of titanium oxide (TiO₂), of approximately 4 nm thick on the surface of the metal [5]. This film formation is unique to Ti metal and its alloys, thus creating a barrier preventing contact with and loss of metal ions into surrounding tissues and consequently avoiding any adverse reactions [3].

Thermal oxidization of commercially pure or alloyed Ti, is a heat treatment process, which increases the depth of the oxide layer. This structural change provides a much greater surface hardness, elastic modulus and wear resistance to the material, thereby further reducing the risk of failure of the implant [6,7,8,9]. A unique process of thermal oxidation or heat treatment of Ti64 has been developed at the University of Cape Town (UCT). It shows that by extending the heat treatment time, the oxide layer is increased to a depth of approximately 20 µm [6]. Further work at UCT, tested the heat treatment capabilities via loaded sliding wear tests, simulating the sliding of a knee joint of a 90 kg person [10]. Results showed zero wear or debris production after approximately 53,000 m of sliding, or 2,000,000 cycles. A clearly defined "diffusion zone" was also seen, showing the extent of the thickened oxide layer.

To reduce aseptic loosening by improving the initial fixation and stability of implants, many surgeons are moving away from cemented fixation implants, and rather use cementless implants, which allow for direct bone-to-implant osseointegration

[11,12]. Cementless implants have been seen to have a longer-term, stronger fixation that improves with time, than that of cemented implants [13,14]. Studies have shown that the rate of osseointegration on cementless implants is associated with the properties of the implant material [15,16,17], which can be modified to improve the biological response to the implant and further encourage new bone growth [18,19,20].

Rough surfaces are a physical characteristic of an implant that encourages bone anchoring and biomechanical stability by promoting the entrapment of fibrin proteins, adhesion of cells and their subsequent proliferation and differentiation into osteoblastic cells [17]. Some of the methods used to produce rough surfaces include sintering, surface blasting and/or surface etching, plasma spraying, fine wire networks or beading [2,17,21,22,23]. Introducing porosity to the implant material prevents stress shielding and encourages bone ingrowth. Solid implants have a much higher stiffness than that of bone which can create stress shielding between the bone and the implant and result in aseptic loosening. This is the phenomenon whereby bone is resorbed during remodelling and not replenished due to its lack of loading, as the bone loadings are being transmitted through the stronger implant material [24]. The ingrowth of bone into a porous material will depend on pore sizes, shape, number, and interconnections; however, there is no consensus on what these should be.

Additive Manufacturing (AM), of which Selective Laser Sintering (SLS) is one of rapid manufacturing method, is fast becoming, a leader in product development for its ability to construct roughened and porous surface modifications with micrometric precision. It also offers the advantage of producing patient specific implants, by using MRI images or CT data as an input for the computer aided design (CAD) model, resulting in fewer problems of ill-fitting implants [25]. The technique of SLS allows the "growth" of any design by subjecting Ti64 powder to a high-power laser beam for bonding, layer by layer, guided by CAD. Using this technique, a detailed 3D scaffold design could easily be developed at the implant surface, as it would be continuous with a solid core of the implant. This continuity would result in a stronger unit [25]. Ti64, 3D scaffold samples in this study, were manu-

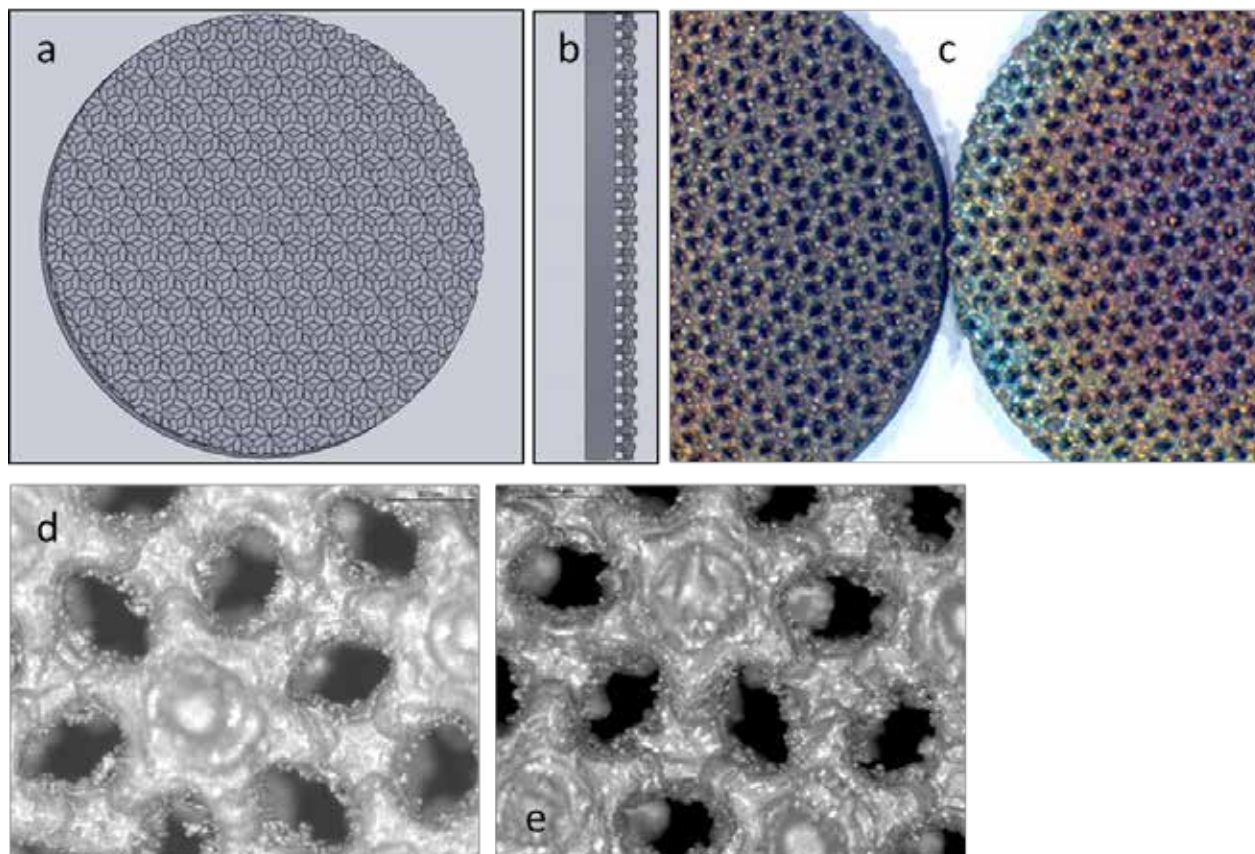


Figure 1 Structural design of Ti64 scaffolds: (a) top view; (b) side view; (c) naked-eye appearance of the untreated Ti64 substrate (left) and the heat treated Ti64 substrate (right); (d) surface topography of the untreated Ti64 scaffold structure; (e) surface topography of the heat treated Ti64 scaffold structure (Published with the permission of ATTRI Orthopaedics (PTY) Ltd)

factured by SLS process.

Early osseointegration of titanium alloy implants is crucial for their biological fixation. The adhesive and differentiation abilities of mesenchymal stem cells on Ti64 scaffolds have previously been established by various groups [21,22,26]. In this study, it is hypothesised that if a Ti64 scaffold is impregnated with the patient's own mesenchymal stem cells, this might improve the osseointegration of the implant, as mesenchyme cells have the capability to differentiate into osteoblastic cells and produce bone. The seeded cells adhering to the scaffold will potentially be able to undergo normal bone remodelling processes with the ingrowth of host bone allowing for a secure interlocking of the implant.

Methods, Materials and Preparation

Titanium scaffold disks

The scaffold surface, on one side of the Ti64 disks, was made up of 0.7 mm thickness struts forming inverted tetrahedral shapes, (**Fig. 1a**) and (**Fig. 1b**). These interconnected shapes formed pores of approximately 0.5 mm across corners, and have increased the surface area of the disks to be seeded, by 200%. This scaffold shape was chosen because it is used extensively on implants at the bone-implant interface to promote osseointegration. The disks/scaffolds were manufactured at the Centre for Rapid Prototyping and Manufacturing (CRPM) of the Central University of Technology (CUT), in Bloemfontein, South Africa, using an EOSINT M280 machine. They were manufactured by SLS from Ti64 powder using a 200 W sol-

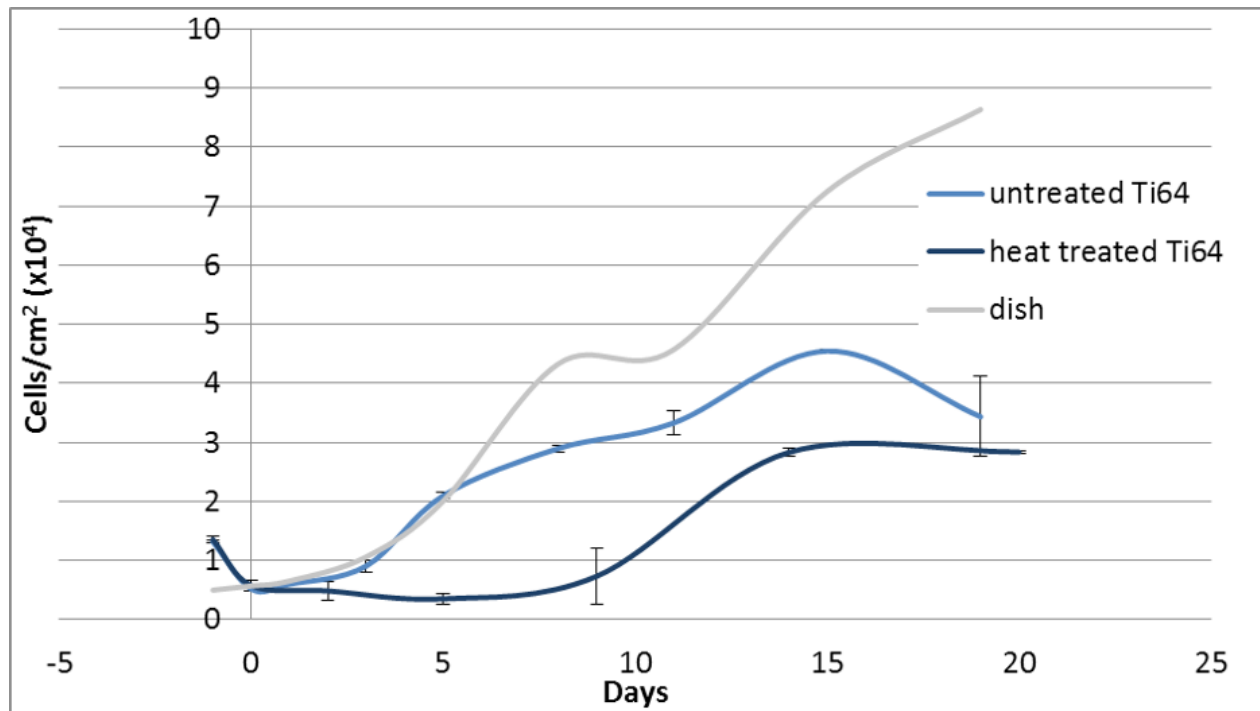


Figure 2. Proliferation of rMSCs on untreated Ti64 (n=4), heat treated Ti64 (n=3) and tissue culture dishes (n=1). No statistical analysis was done for growth on tissue culture dishes

id state fibre laser in a protective argon atmosphere supplied at 100 lt/min under 400 kPa pressure. After manufacturing, the scaffolds were etched to prepare the surface for cell culture work. The etching was done by submerging the scaffolds in Kroll's reagent (6% nitric acid, 2% hydrofluoric acid, 92% distilled water) for 30 seconds, followed by a thorough rinsing under tap water. The scaffolds were sonicated for five minutes in a water bath to loosen any trapped residue, and were finally rinsed with distilled water.

Thermal oxidation of scaffolds was done at the Department of Mechanical Engineering at UCT. The heat treatment was done in air at 600 °C for 20 hours in a Naber D2804 furnace on a clean ceramic brick. After completion, the scaffolds were cooled to room temperature before removing them from the furnace. After the heat treatment process, a pleochroic rutile surface was seen on the scaffold disks, whereby the substrate appears to have had a colourimetric change, (Fig. 1c). This is a form of naturally occurring TiO₂, and due to its presence one can confirm the success of thermal oxidation of the substrate. The roughened

topography obtained through the sintering development process in the untreated image (Fig. 1d) was unchanged in the heat treatment image (Fig. 1e). This suggests that the heat treatment process only changes the chemical microstructure due to the formation of the oxide.

Tissue culture

Rat mesenchymal stem cells (rMSCs) were obtained from the Cardiovascular Research Unit at UCT at a passage number 2. The cells were isolated from bone marrow, and MSC lineage was confirmed by testing for osteogenic differentiation using Alizarin red stain, and adipogenic differentiation using Nile red stain. Culture media included Dulbecco's Modified Eagle Medium (DMEM) (Gibco), supplemented with 10% Foetal Bovine Serum (FBS) (Hyclone), 2% antibiotics (concentration of 100 U/ml for both Penicillin and Streptomycin) and 0.01% Fungizone.

Human mesenchymal stem cells (hMSCs) were obtained from the Institute of Cellular and Molecular Medicine at the University of Pretoria at passage

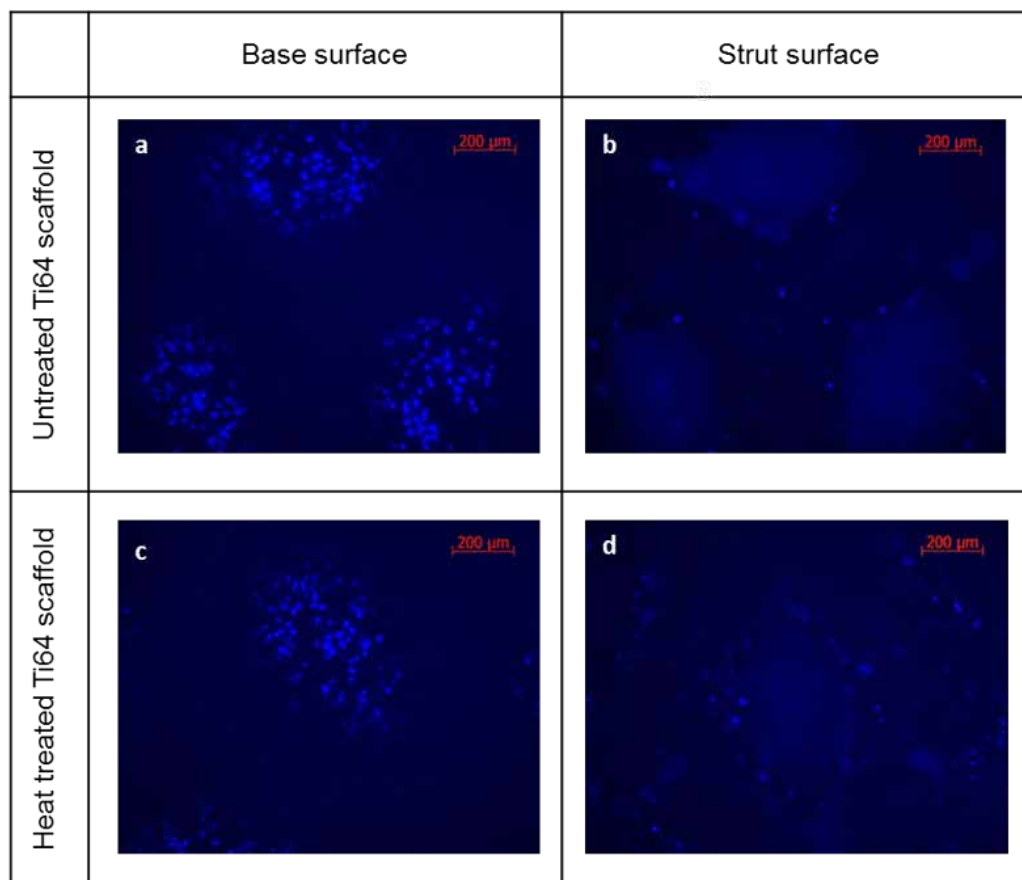


Figure 3 Fluorescence microscopy images of rMSCs locations on the Ti64 scaffolds. Cell nuclei have been stained blue with Hoechst LIVE stain. Images (3a) and (3b) represent the rMSCs on the untreated T64 scaffolds. Images (3c) and (3d) represent the rMSCs on the heat treated Ti64 scaffolds. (3a) and (3c) show the cells located at the base of the scaffolds, where the dark areas of the image are out of focus struts. (3b) and (3d) show the cells located on the struts of the scaffolds, where the unfocus area is the scaffold base.

number 3. The cells were isolated from adipose tissue, and MSC lineage was characterised by (i) their ability to adhere to plastic, (ii) their expression of the following markers: CD73, CD90, and CD105 (these markers were >95% positive); while lacking expression of CD34 and CD45 (<5%), as measured by flow cytometry, and (iii) their ability to differentiate into adipose, bone and cartilage lineages. Culture media included alpha Minimum Essentials Medium (αMEM) (Gibco), supplemented with 10% Foetal Bovine Serum (FBS) (Hyclone), 2% antibiotics (concentration of 100 U/ml for both Penicillin and Streptomycin) and 0.01% Fungizone.

Cells were thawed from liquid nitrogen storage

into standard culture medium in 75 cm³ tissue culture flasks. Confluent cultures were passaged and seeded to obtain a density of 5000 cell/cm² onto prepared Ti64 disks/scaffolds. The seeded cell-suspension formed a meniscus on top of the scaffolds which was incubated and left undisturbed for one hour before the scaffolds were flooded and submerged in standard culture medium. Cells were maintained in standard culture medium to observe cell growth on scaffolds, with the standard culture medium being changed every 2-3 days.

For differentiation experiments, the standard culture medium was changed to osteogenic medium two days after the seeding of cells onto the scaffolds

and replenished twice a week. Osteogenic media for the rat and the human cells included DMEM Gibco, supplemented with 10% FBS, 1% antibiotics, 0.01% Fungizone, as well as osteogenic supplements 0.01% dexamethasone (Sigma D4902), β -glycerophosphate (Sigma 50020) and ascorbic-2-phosphate (Sigma 40752).

Cell counting

The standard culture medium was removed and the scaffolds were rinsed twice with Phosphate Buffer Solution (PBS). The Scaffolds were transferred to a new culture dish and submerged in Trypsin/Edetic Acid (EDTA), having concentrations of 0.05%/0.02% respectively. The trypsin reaction was neutralized with standard culture medium. Using forceps, the scaffold disks were held vertically and rinsed with neutralized medium by pipetting through the scaffold to release adherent cells. The scaffold disks were then turned on their flat side (scaffolds up) and the rinsing was repeated using fresh standard culture medium. Cell suspension was centrifuged to form a pellet of cells which was re-suspended for counting using a haemocytometer.

Cell staining

The standard culture medium was suctioned off and scaffolds were washed twice with PBS before being submerged and incubated in 0.5 μ g/ml of Alizarin Red S stain in standard culture medium for 1 hour at 37 °C. The standard culture medium was then again suctioned off, washed twice with PBS and then incubated in 4 μ g/ml Hoechst Live stain for 15 minutes at 37 °C. Stained scaffolds were observed under a Zeiss Axiovert 200M Fluorescence microscope.

Results

Culture and differentiation of rat MSCs on Ti64 scaffolds

To determine the seeding efficiency, cells were harvested 24 hours after seeding and counted. Routinely a 40% seeding efficiency was obtained, (which was considered when seeding), to reach the recommended cell density of 5000 cells/cm² [27,28,29,30]. In osteogenic differentiation experiments, scaffolds were seeded to a recommended cell density of 10000 cells/cm².

To determine the proliferation of cells on the scaffolds, untreated and heat-treated scaffolds were seeded and harvested at various time intervals for up to 3 weeks along with a control of cells on a tissue culture dish, (**Fig. 2**). The growth on the Ti64 scaffolds was slightly slower, showing a three-day doubling time on both the untreated and heat treated Ti64, compared with the two-day doubling time of the cells on the dishes.

To identify the location of cells within the scaffolds, the cells nuclei were stained with Hoechst LIVE stain and visualised under fluorescence microscopy one day after seeding. The results show that cells could adhere to both the base and the struts of both untreated and heat-treated scaffolds, (**Fig. 3**). However, more of the cells settled on the base of the scaffold, leading to earlier the early-plateau in cell growth compared with the dishes. The density of cells that settled on the struts of the scaffolds was inconsistent between experiments and between individual scaffolds.

To establish the extent of differentiation of the rMSCs, 48 hours after seeding untreated and heat treated scaffolds, the growth medium was changed to osteogenic medium to induce differentiation. Cells were stained with Alizarin red for imaging at various time points of differentiation of cells on the untreated Ti64, heat treated Ti64 and tissue culture plates as a control for up to 4 weeks, (**Fig. 4**). The rMSCs showed differentiation on both the untreated and heat treated Ti64 scaffolds, but minimal differentiation on the struts of either of the scaffolds.

At the end of the culture period, at day 26, a semi-quantitative analysis of osteoblast activity was performed on 6 different microscopy images of the base of the untreated and heat treated scaffolds. The comparison of the semi-quantitative results, (data not shown), for the untreated and heat-treated scaffolds showed an increase in the number of cells on the heat-treated Ti64, yet a slightly greater number of calcium nodules on the untreated Ti64.

Culture and differentiation of human MSCs on Ti64 scaffolds

The seeding efficiency of the human MSCs were tested in a similar manner as for the rat MSCs. That is, a recommended cell density of 5000 cells/cm² was obtained [27,28,29,30].

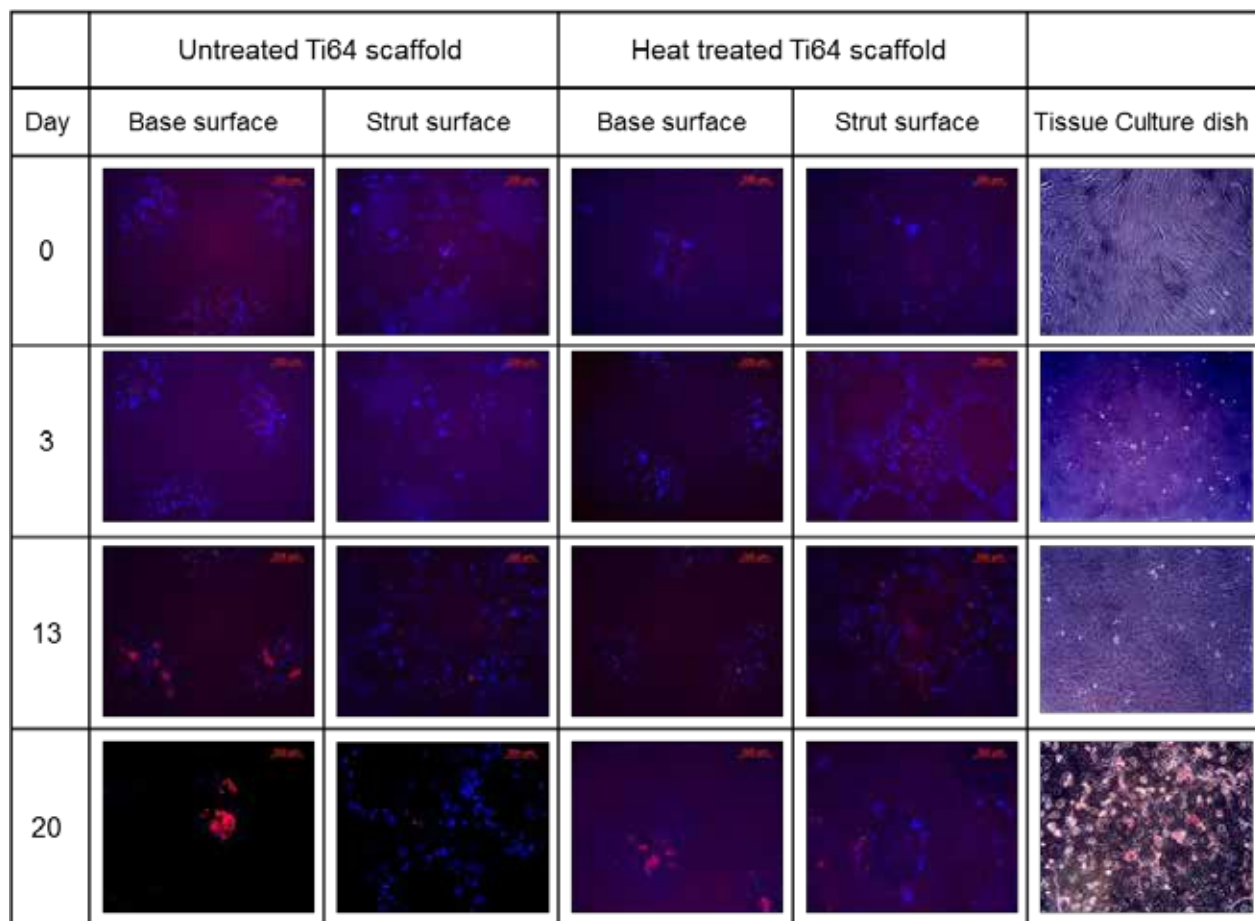


Figure 4 Differentiation timeline of rMSCs on untreated Ti64 scaffolds, heat treated Ti74 scaffolds and tissue culture dishes. Cells on scaffolds were visualised through fluorescence microscopy, and cells on tissue culture dishes were visualised through phase contrast microscopy. Alizarin red stained calcium deposits red. Cell nuclei were counter-stained blue with Hoechst

Tests were carried out in a similar manner to that of the rMSCs, to determine the growth and proliferative potential of the hMSCs on the untreated and the heat treated Ti64 scaffolds, (**Fig. 5**). The growth on the heat treated Ti64 scaffolds was slightly slower, showing a six-day doubling time compared with a four-day doubling time on the untreated Ti64. Cell proliferation was slightly slower on Ti64 scaffolds in general, when compared with the doubling time of three days of the cells on the dishes.

To understand the location of cells within the scaffolds, the cells nuclei were stained with Hoechst LIVE stain and visualised under fluorescence microscopy one day after seeding. The results show that cells could adhere to both the base and the struts of both

scaffolds, (**Fig. 6**). However, as with the rat MSCs, more of the cells settled on the base of the scaffold and the density of cells that settled on the struts of the scaffolds was inconsistent.

To establish the extent of differentiation of the hMSCs, 48 hours after seeding untreated and heat treated scaffolds, the growth medium was changed to osteogenic medium to induce differentiation. Cells were stained with Alizarin red for imaging at various intervals of differentiation of cells on the untreated Ti64, heat treated Ti64 and tissue culture plates as a control, up to 4 weeks, (**Fig. 7**). The hMSCs showed differentiation on both the untreated and heat treated Ti64 scaffolds, but minimal differentiation on the struts of either of the scaffolds.

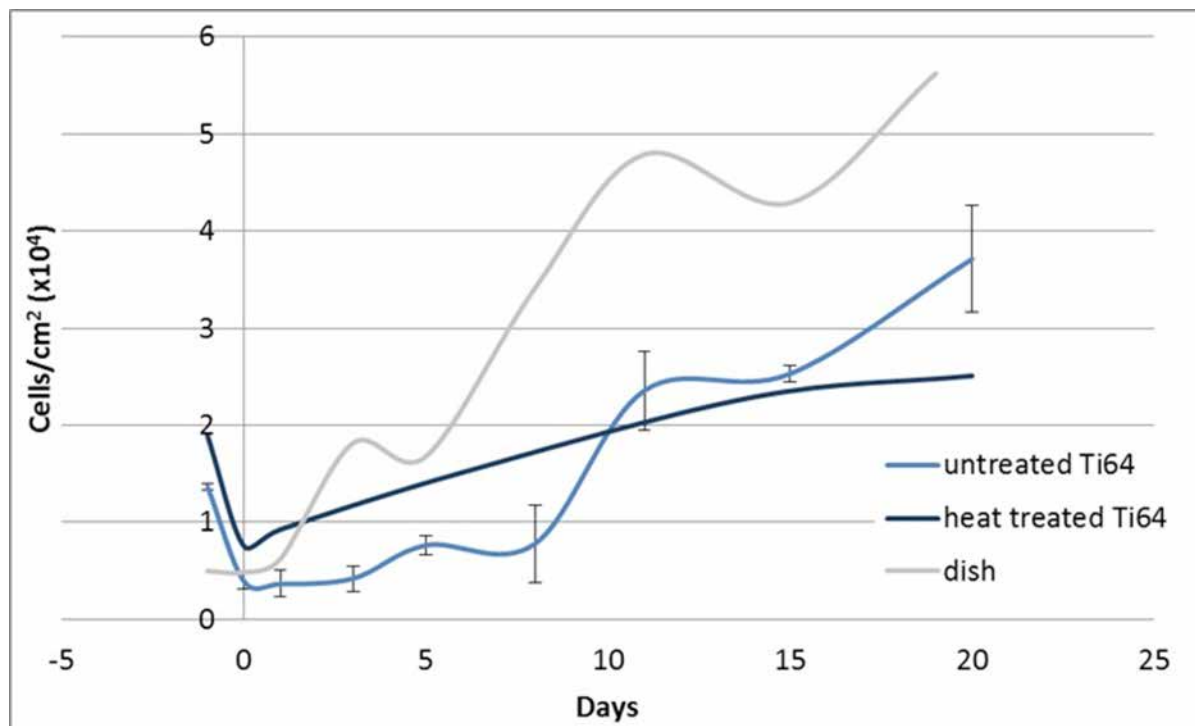


Figure 5 Proliferation of hMSCs on untreated Ti64 (n=2), heat treated Ti64 (n=1) and tissue culture dishes (n=1). No statistical analysis was done for growth on heat treated Ti64 scaffolds or on tissue culture dishes

At the end of the culture period, at day 26, a semi-quantitative analysis of osteoblast activity was performed on 6 different microscopy images of the base of the untreated and heated scaffolds. The comparison of the semi-quantitative results, (data not shown), for the untreated and heat-treated scaffolds showed an increase in the number of cells on the untreated Ti64, yet a greater number of calcium nodules on the heat treated Ti64, opposite of what was seen for the rat MSCs.

Discussion

The results in this study show that the heat treatment of the Ti64 does not have a considerable effect on the cells ability to proliferate and differentiate into osteoblasts. This was shown through the assessment of cell adhesion, growth and differentiation on untreated Ti64, compared with that on the heat treated Ti64.

The heat treatment was seen to make minimal difference to the number of adhering cells in this study. For both the rat and the human MSCs test results, the adherence of cells was within 10% of one another for

the untreated and heat treated Ti64. This agreed with the results from previous studies [31], which found no significant difference in the number of attached cells on the untreated and the heat treated Ti64 surface at all inspected time points over a 24-hour period.

The growth rate of the rat and the human MSCs appeared to be slightly longer on the heat treated Ti64, suggesting that the heat treatment producing the titanium oxide (TiO₂) layer on the substrate, possibly marginally slows the rate of cell growth. Saldaña et al. [32] cultured human primary bone cells on untreated and heat treated (at 500 °C and 700 °C) Ti64 disks in standard culture medium for up to seven days. Their results showed a much faster overall growth of cells on both substrates, with no noteworthy change in cellular growth on either heat treated Ti64 substrate. They concluded that heat treatment of Ti64 substrate does not affect the cell growth [32]. Differences between this study and that of Saldaña et al.'s, may explain the differing growth results:

- The cells used by Saldaña et al, were primary bone-cells harvested from bone, which may possess

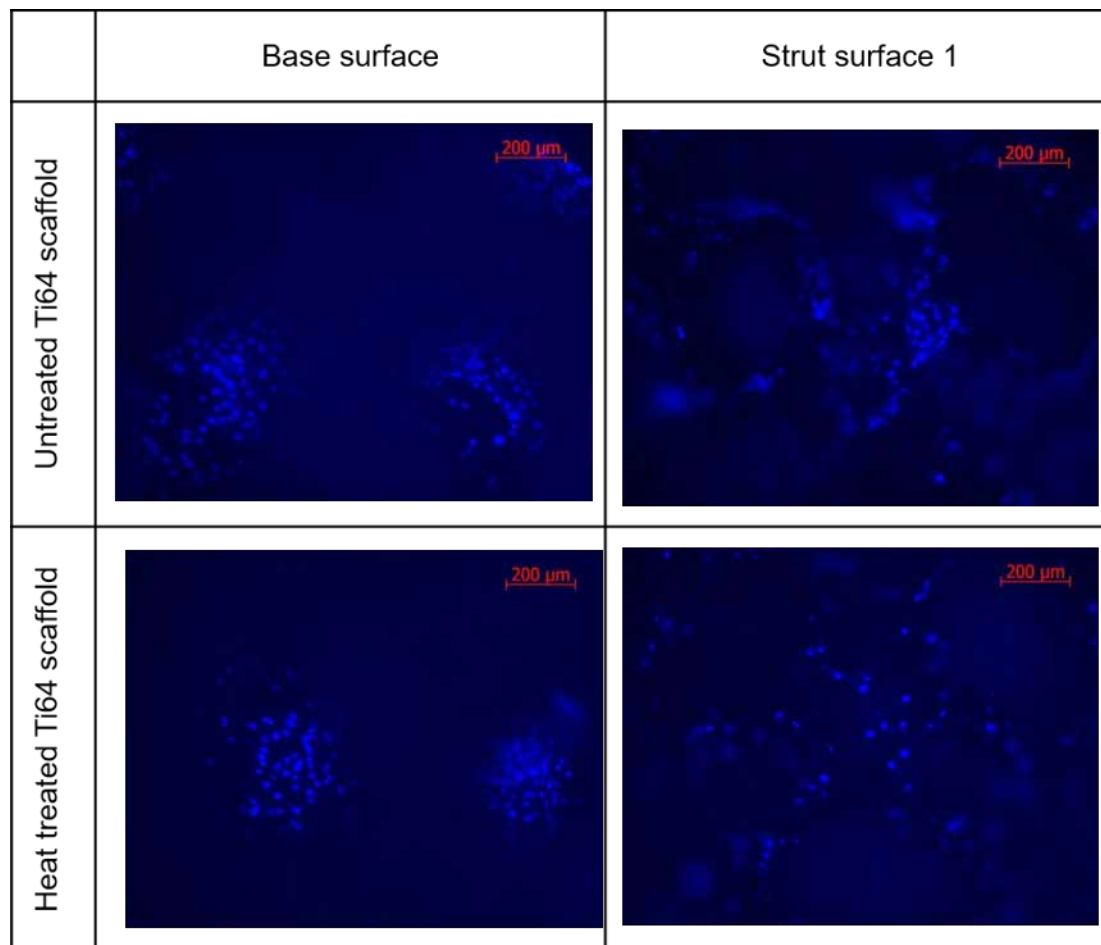


Figure 6 Fluorescence microscopy images of hMSCs locations in the Ti64 scaffolds. Cell nuclei have been stained with Hoechst LIVE stain. Images (6a) and (6b) show the hMSCs on the untreated scaffolds. Images (6c) and (6d) show the hMSCs on the heat treated Ti64 scaffolds. (6a) and (6c) show cells adhered to the base of the scaffolds, where the dark areas of the image are out of focus struts. (6b) and (6d) show cells adhered to the struts, with the out of focus area being the base of the scaffold.

different growth properties to that of mesenchymal stem cells harvested from adipose tissue.

- This study cultured cells on 3D Ti64 scaffolds, whereas Saldaña et al.'s study was carried out on flat Ti64 disks. This would suggest that the location of cells may affect the cells' growth which will be discussed.

The imaging results of differentiation show similar timelines of differentiation occurring on both the untreated and heat treated Ti64 for both the rat and the human cells, (Fig. 4) and (Fig. 7). The semi-quantitative analysis of differentiation images, showed the extent of differentiation to be marginally higher

for the rMSCs on the untreated Ti64, and marginally higher on the heat-treated scaffolds for the hMSCs. The semi-quantitative results obtained for the human cells showing slightly more differentiation occurring on the heat treated Ti64 over 26 days, aligns with the previous work done [32,33,34]. Saldaña et al. [32] measured the levels of differentiation markers such as alkaline phosphatase activity, osteocalcin, and osteoprotegerin and found higher expressions and visualisation of mineralized nodules on the heat treated Ti64 after only two days in differentiation medium. Their work concluded that the heat treatment would improve the biocompatibility of the Ti64. Nishiguchi

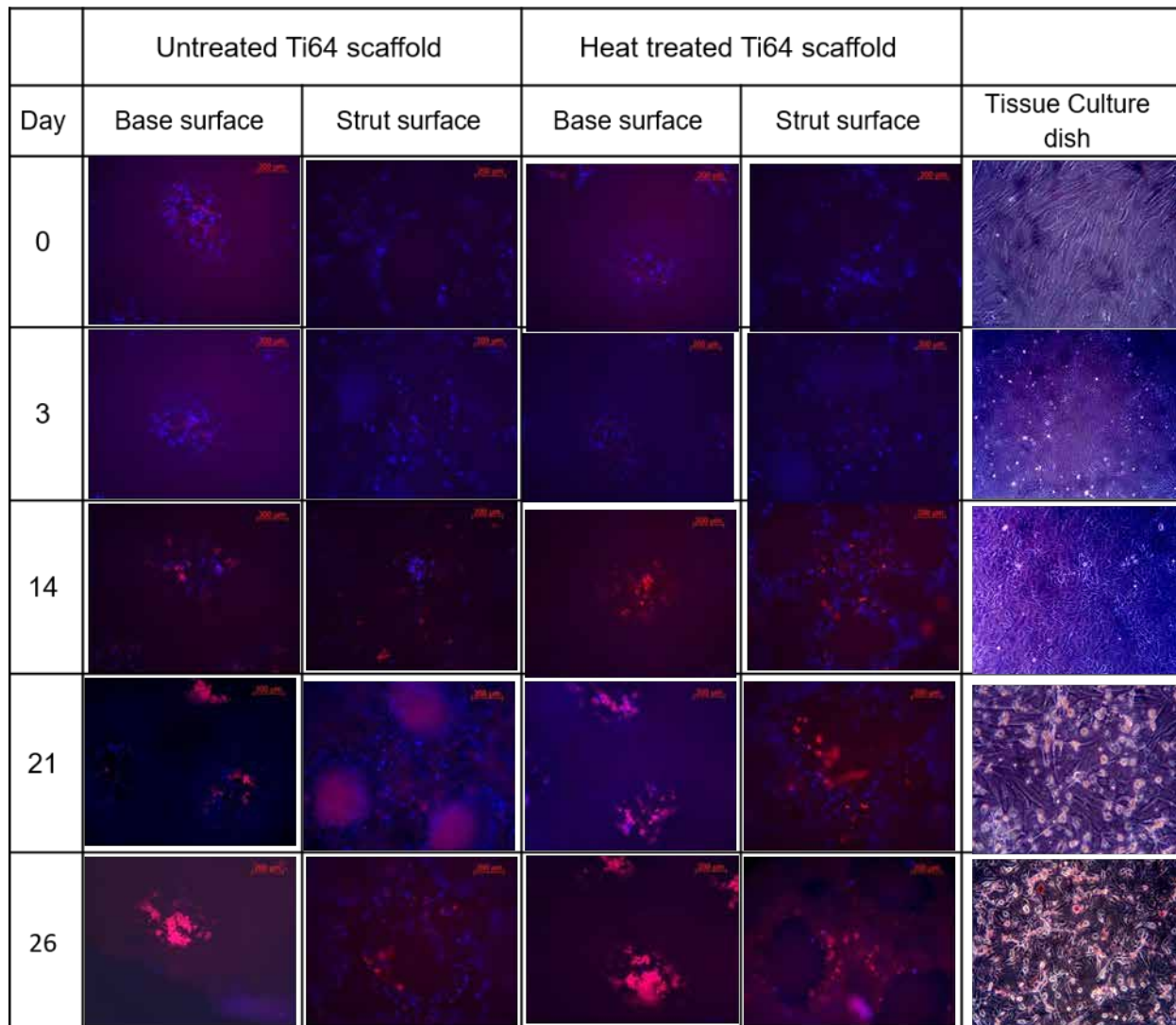


Figure 7. Differentiation timeline of hMSCs on untreated Ti64 scaffolds, heat treated Ti64 scaffolds and tissue culture dishes. Cells on untreated and heat treated Ti64 scaffolds were visualised through fluorescence microscopy, and cells on tissue culture dishes were visualised through phase contrast microscopy. Alizarin red stained calcium deposits red. Cell nuclei were counterstained blue with Hoechst.

et al. [33,34] repeatedly showed the improved rate and extent of bone formation on alkali and heat treated Ti64 in vivo, 12 weeks after implantation in the femur bone of beagle dogs. Their work proposed that the alkali and heat treatments of Ti64 made the substrate osteoconductive.

It was noted that the location of the cells within the three-dimensional scaffolds, as well as the cells' source and age, had an important contributing factor on the cells' performance.

For both the rat and the human MSCs tests, and on both the untreated and heat treated Ti64 scaffolds, a greater number of cells were seen to have adhered to the base of the scaffolds as opposed to the struts. The scaffolds in this study were designed to have a pore size of 500 μm , thus it may be suggested that it is highly possible that the cells would pass through the pores and accumulate at on the base of the scaffolds, (Fig. 3) and (Fig. 6). The location of cells has already been shown to possibly affect the growth of

the cells, where cells reached confluency and plateau earlier than if they were more sparsely seeded (**Fig. 2**) and (**Fig. 5**). Correspondingly, it was interesting to note that the sparsely seeded cells on the struts of the scaffolds, were reluctant to differentiate and rather remained as mesenchymal stem cells. This was possibly related to the cell density on the struts or the role that initial cell attachment has on potential proliferation and differentiation. The reorganization of the cytoskeleton at contact interfaces during attachment, result in morphological and behavioural changes in the cell that have lasting effects on the cells growth and differentiation capabilities [35].

The second contributing factor of the cells' performance was the source and age of the cells [36,37]. In vitro tests [36] showed that hMSCs were found in highest concentration in Adipose Tissue (AT), and that AT derived cells showed no significant difference in their osteogenic differentiation potential compared with Bone Marrow (BM) derived MSCs, making them an attractive source for MSCs. However, in vitro tests [37] also showed that although MSCs could differentiate into various lineages, BM derived MSCs still showed the highest levels of osteogenesis, and AT derived MSCs showed the highest levels of adipogenesis.

The work from Kern et al. [36], showed that the MSCs have a limited proliferation capacity in that as the cells age, the in vitro doubling time of cells is hindered. For AT derived MSCs, they reached proliferative senescence after passage seven. Sakaguchi et al. [37] also reported the slower doubling time of MSCs the longer they remained in culture; however, they noted that although the doubling time was slower, the BM derived MSCs retained proliferative ability up to the tenth passage, whereas AT derived MSCs lost theirs at the eighth passage. Considering these previous results, the passage 6 human cells used in this study may also show adhesion and proliferation that is lessened than the potential of younger cells to


be used in future applications.

It was pointed out that not only the in vitro aging of the cells is important, when considering the effects on cell proliferation, but also the age of the patient [38]. MSCs obtained from patients 66 years and older, the cell maximal life span and the rate of proliferation were greatly reduced compared with MSCs obtained from patients between the age of 18 and 29 years [38]. In this study, although the age of the donor, combined with the length of time in culture had vast effects on the proliferation capabilities of the cells, it did not appear to affect the differentiation capabilities. The reduced presence of osteogenesis is thus due to the reduced presence of viable cells, rather than the lack of differentiation of cells.

Conclusions

Based on the discussions on previous and current researches presented in this study, it is an important factor to consider the age of the patients intended for joint or bone replacements. Older patients may require higher volumes of MSCs and shorter term in vitro culturing, for therapies to be effective.

Further, in summary, the following conclusions can be drawn:

- Heat treatment of Ti64 producing an oxide layer (TiO₂), does not adversely affect the cells' capabilities.
- Ti64 can be considered as an osteoconductive material.
- Cell differentiation is also dependent on the cell's location on the scaffolds or porous structures. 

Conflict of interest disclosure

The authors declared no conflicts of interest.

Financial support

The project was supported financially by the Postgraduate fund of UCT (University of Cape Town) and by ATTRI Orthopaedics PTY Ltd

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