



Polarization of hydroxyapatite: Influence on osteoblast cell proliferation

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ABSTRACT

Hydroxyapatite (HA) has been used clinically to treat bone defects. However, modifications of the surface properties of HA could improve and control bone matrix deposition and localized host tissue integration. The aim of this study was to investigate the effect of developing a surface charge on HA discs with respect to osteoblast activity *in vitro*. HA discs (12 mm × 2 mm) were sintered in either air or water vapour. The HA discs were then electrically polarized (positive and negative surfaces) or non-polarized (controls) and seeded with MC3T3-E1 cells. Polarized HA sintered in water vapour was shown to retain six times more charge than polarized HA sintered in air. Picogreen analysis demonstrated that at 4 h cell number was significantly higher on the negatively and positively charged HA surface (water sintered) in comparison to the non-charged water and air-sintered HA controls. At 7 days there was a significant increase in cell number on the negatively charged HA (air sintered) sample in comparison to the negatively charged water vapour sintered HA sample and the non-charged water vapour sintered control sample. Also at 7 days, the picogreen data showed a significant increase in cell number on the positively charged water-treated HA sample in comparison to both the air- and water-treated HA non-charged control HA samples. An alamarBlue assay at 7 days demonstrated significant cell metabolic activity on the charged surfaces (both positive and negative) in comparison to the non-charged HA and the tissue culture plastic controls. This study demonstrated that all of the HA discs tested supported cell viability/attachment. However, cell attachment/proliferation/metabolic activity was significantly increased as a result of developing a charge on the HA surface.

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1. Introduction

Drawbacks to current repair strategies for patients suffering from bone disease/trauma include tissue availability and donor site morbidity. Bone tissue engineering is an emerging technique that offers potential solutions to these problems. Scaffolds may be used to support and encourage cellular activity and promote faster healing. Hydroxyapatite (HA) is a calcium phosphate ceramic that has been used clinically and has been shown to have bioactive, osteoconductive and biocompatible properties [1–3]. It may be possible, however, to further enhance HA with respect to bone integration using treatments that have the potential to improve cell proliferation and thus improve implant integration and wound healing.

Recently, studies have demonstrated that polarizing sintered bioceramics, and thus creating a residual permanent charge on opposite sides of the material, can influence the response and activity of the cells it supports [4–6]. It has been shown that sintering HA samples under water vapour improves the conductivity of the samples when compared to those sintered in air [7,8]. The presence of water vapour in the atmosphere during sintering restricts

the dehydration of OH⁻ ions and, as a result, due to the rotation and movement of OH⁻ ions within the sub-lattice channels, protonic conductivity is increased [9]. In air, partial dehydration of the OH⁻ ions leaves vacancies within the structure, and this predominantly determines the conductivity. HA has an ionic crystal structure with an OH⁻ ion sub-lattice that is essentially one-dimensional [10]. The chemistry of calcium phosphates is extremely complex, with a series of possible related inter-substitutions giving rise to various “impure” and/or calcium deficient apatites. The process that dominates the polarization effect in HA has been termed “nomadic polarization”, and is brought on by protonic mobile charges due to partial dehydration in the OH⁻ sub-lattice. This applies not only to protons but also to large negative ions, such as OH⁻ ions [10–13]. The monoclinic to hexagonal phase transition between 200 and 250 °C also allows the protons to rotate and orientate themselves to become aligned with the OH⁻ channels [14]. At a still higher temperature, e.g. 350–450 °C, the OH⁻ ions acquire enough thermal energy to reorientate themselves independently of their OH⁻ ion neighbours in the chain. This can also be aided by an applied electric DC field.

A number of studies have investigated the effect of electrical charges and voltages on the biological response to polarized HA. However, there is no consensus in the literature as to whether po-

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sitive or negative surface charges are more beneficial to this response. A number of authors report an increase in cell numbers recorded on negatively charged HA surfaces [15,16] with up to 10 times more cells found attached to the charged surface when compared to the electrically neutral surfaces [17]. In contrast to this, increased cell numbers have been found on positively charged HA surfaces compared to negatively charged HA surfaces 2 days after seeding [18], with fewer cells counted on the negative surfaces compared to the neutral surfaces. After 4 days no difference was observed between the positively and negatively charged surfaces [18].

The results of *in vivo* experiments are also contradictory. One study reports enhanced osteoblast activity resulting from both positive and negative polarization of HA plates implanted in calvarial bones of rats [6]. This was coupled with a decrease in osteoclast activity, which was attributed to the increase in bone formation on the negative surfaces due to the accumulation of Ca^{2+} ions on the surfaces. Two further studies implanted polarized HA in canine femora and rat tibiae, respectively [19,20]. In the first of these, the surface charge on polarized HA was shown to influence protein adsorption onto the HA surface *in vivo* layer which was thought to result in improved osteoconduction on both positively and negatively charged surfaces. The second study corroborated these results when an increase was found in the number of mature osteoblasts in bone formed on charged HA surfaces, irrespective of the polarity of the charge.

Some of the apparent contradictions between both *in vivo* and *in vitro* studies will arise from variations in the experimental methods used, cell types employed and analysis techniques utilized in addition to variations in animal models, implant sites and durations of implantation. Direct comparison of the results from these studies is therefore difficult. However, there is evidence that the surface charge, whether positive or negative, can affect the biological response.

This paper describes the *in vitro* response of MC3T3-E1 bone cells to polarized hydroxyapatite – in particular, analysis of short (4 h) and long-term (7 day) responses of cell attachment, proliferation and metabolic activity. A detailed electrical and compositional study of the materials used is reported elsewhere [21]. It was found that the polarization of the samples was enhanced by the use of water vapour during sintering; the level of charge measured in the HA discs sintered in water was found to be six times greater than that for samples sintered in air. The aim of this study was to investigate the effect of the charge retained in polarized HA discs, sintered in either air or water, on osteoblast local attachment and proliferative capability with respect to potentially improving clinical treatment strategies.

2. Materials and methods

2.1. HA sample manufacture

Dense hydroxyapatite discs (12 mm diameter \times 2 mm height) were fabricated using a commercially available precipitated calcium phosphate powder (TCP130, Thermphos UK Ltd.). After ball milling, the powder was sieved and compacts were cold pressed at 80 MPa for approximately 30 s, followed by sintering either in water vapour or air at 1300 °C for 4 h with a heating rate of 60 °C h⁻¹. Characterization of the materials has been reported elsewhere [21,22]. In summary, the phase composition indicates that the majority of the material (75%) remains as HA, with the remainder composed of TCP phases. For the air-sintered material an average grain size of 3.7 μm was measured, whereas the dense and porous materials sintered in water vapour had smaller average grain sizes of 2.3 and 2.4 μm , respectively. Typical density was

93% theoretical [22]. The microstructures appeared very similar in both cases, leading to the conclusion that it was reasonable to compare their electrical properties on an equal basis.

2.2. High-temperature polarization of HA ceramics

After sample preparation, specimens were placed between two platinum electrodes. This assembly was then placed in an electric furnace and heated to the desired polarization temperature of 400 °C. Once the polarization temperature had been reached a DC electric field of 3 kV cm⁻¹ was applied for 1 h. The electric furnace was programmed to cool at a rate of 2 °C min⁻¹ with the DC field still applied until room temperature was reached.

Once the samples had been polarization, the degree of polarization was determined from the thermally stimulated discharge current (TSDC). This involved reheating a polarized sample to depolarize the material and measuring the depolarization current density as a function of temperature using a Keithley 6514 electrometer. Table 1 defines the sample nomenclature used for the polarized and non-polarized control samples.

2.3. Cell culture

MC3T3-E1 osteoblast-like cells were cultured in culture medium consisting of alpha-minimal essential medium (Biosera L0476), 10% fetal calf serum (Biosera S1900), 1% L-glutamine (Biosera G7513 2 mM), 1% antibiotics and antimyotics (Biosera L0010, 100 U ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin), 50 μg ml⁻¹ ascorbic acid, 10 mM β -glycerophosphate and 10⁻⁸ M dexamethasone (Sigma–Aldrich).

HA discs were autoclaved at 120 °C for 25 min for sterilization prior to cell seeding, at a temperature where no thermally stimulated discharge current was observed and well below the polarization temperature (400 °C). They were then individually placed into the wells of a 24-well plate with either the negative or positive charged surface facing upwards. Cells were added to the top of each HA disc (to either a negatively charged surface, a positively charged surface or an uncharged control surface) at a density of 750,000 cells in a 150 μl culture medium suspension per HA disc. Samples were incubated for 1 h at 37 °C under 5% CO₂. One and a half millilitres of culture medium was added to each sample prior to further incubation. The culture medium was changed every 2–3 days.

2.4. Picogreen assay

At 4 h and 7 days after initial cell seeding, a picogreen assay was performed on the samples. The assay (P7589 Invitrogen) was performed according to the manufacturer's instructions. Briefly, 100 μl of each sample was added to each well of a 96-well plate in duplicate. A further 100 μl of picogreen dye was then added to each well. Samples were analysed using a fluorescent plate reader (Victor 1420 multi-label counter) at 485/535 nm wavelength.

DNA from three different locations on each HA disc sample was measured (Fig. 1). These three locations were: Location 1, cells suspended in culture medium; Location 2, cells adhered to the HA disc (cells were lysed *in situ* on the disc to obtain the DNA value of cells adhered to HA surface); and Location 3, cells attached to the tissue culture plastic control (TCP CTL) well surface (cells were also lysed

Table 1
Nomenclature for HA samples.

HA sample	Non-polarized (control)	Polarized (+ve surface)	Polarized (–ve surface)
Sintered in air	AIR CTL	AIR+	AIR–
Sintered in H ₂ O	H ₂ O CTL	H ₂ O+	H ₂ O–

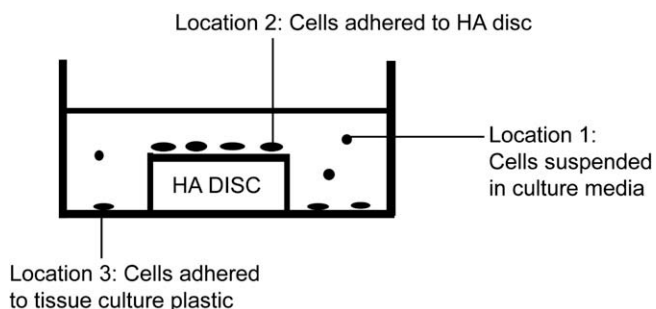


Fig. 1. Schematic diagram demonstrating the different locations of the DNA measured from cells lysed either suspended in medium (1), adhered to actual HA scaffold (2) or adhered to interior of tissue culture plastic well (3).

in situ in this sample group). These three regions were analysed by removing the medium from each sample well and transferring to 1.5 ml vials. These supernatants were then microcentrifuged to pellet any suspended cells. The excess medium was removed and discarded. One millilitre of lysis TE buffer (provided in picogreen kit) was added to each cell pellet. This process analysed Location 1 as identified in Fig. 1.

After removal of the medium, the HA discs (plus any adhered cells) were transferred to new, individual wells of a 24-well plate (to analyse Location 2 as seen in Fig. 1). One millilitre of TE buffer was also added to these samples. The last cell adherence location (Location 3, as seen in Fig. 1) was analysed by adding 1 ml of TE lysis buffer to each well. This allowed any cells adhered to the inside of tissue culture plastic wells to be detected using the picogreen assay. All samples for analysis underwent a freeze/defrost cycle in order to ensure full cell lysis. Prior to analysis with picogreen, the samples were homogenized further by vortexing the TE buffer plus sample and scraping any surfaces to which cells had adhered using individual sterile pipette tips. For each sample group, six samples were taken at each time point.

2.5. AlamarBlue

At 7 days, the HA discs were removed from the incubator and rinsed with phosphate-buffered saline. One millilitre of alamarBlue working solution was added to each sample prior to incubation for 90 min at 37 °C (protected from light). After incubation, 100 μ l of the alamarBlue solution was transferred to a 96-well plate (Falcon) in triplicate for each of the samples and read on the fluorescence plate reader (Cytofluor, Perceptive Biosystems) at 530 nm excitation, 590 nm emission. Six measurements were performed for each sample group. The following seven sample groups were analysed:

- AIR+ = % viability measured from cells seeded onto positive surface of air-treated hydroxyapatite disc.
- AIR- = % viability measured from cells seeded onto negative surface of air-treated hydroxyapatite disc.
- AIR CTL = % viability measured from cells seeded onto non-charged surface of air-treated hydroxyapatite disc.
- H₂O+ = % viability measured from cells seeded onto positive surface of water vapour treated hydroxyapatite disc.
- H₂O- = % viability measured from cells seeded onto negative surface of water vapour treated hydroxyapatite disc.
- H₂O CTL = % viability measured from cells seeded onto non-charged surface of water vapour treated hydroxyapatite disc.
- TCP CTL = % viability measured from cells seeded onto tissue culture plastic surface (seeded into well of 96 well plate).

2.6. Statistical analysis

Data obtained from the picogreen assay was analysed using a three-factor analysis of variance (ANOVA; between three groups of charged surface (positive (+) vs. negative (-) vs. no charge (CTL), air/water-treated (air vs. H₂O) and time point (4 h vs. 7 days)). Data obtained from the alamarBlue assay was analysed using a two-way ANOVA (i) within (tissue culture plastic sample group as this sample was run against every factor) and (ii) between (charged surface (positive (+), negative (-), no charge (CTL) vs. air/water-treated (AIR vs. H₂O)) using ezANOVA software. Statistical significance was taken to be $p < 0.05$.

3. Results

Fig. 2 shows the typical TSDC traces from polarized HA sintered in both air and water vapour. During depolarization a peak current density of 26 nA cm⁻² was measured when heating to 600 °C for the water-treated scaffolds in comparison to approximately 4 nA cm⁻² current density from the HA discs sintered in air alone. Such peak current densities are typical of polarized HA materials and are dependent on polarization temperature, polarization electric field and sintering conditions [4]. The difference in current density for the air- and water-sintered samples was not sufficiently high to anticipate that there would be any influence on cell response.

The results from the picogreen assay can be seen in Figs. 3 and 4. The bar charts in Fig. 3 show the amount of DNA (as measured in picogreen assay) in the different sample locations. The symbol * represents DNA measurements from hydroxyapatite samples that were significantly higher in relation to the respective 4 h and 7 day samples. It also represents DNA measurements from HA samples that were significantly higher in relation to the corresponding medium and well groups ($p < 0.05$). The results show that the majority of cells seeded onto the HA discs were retained on the surface of the disc – between 95% and 99% of the total cell number per sample were located on the HA rather than in the medium or on the well interior that housed the disc. Fig. 3 also demonstrates the significant increase in cell number from 4 h to 7 days.

Fig. 4 shows the picogreen results from the cells adhered to the HA discs only. The bar chart shows the amount of DNA (as measured in picogreen assay) from cells seeded onto the positively charged surfaces, negatively charged surfaces and non-charged hydroxyapatite discs. The symbol * represents DNA measurements from HA samples that were significantly higher in relation to the

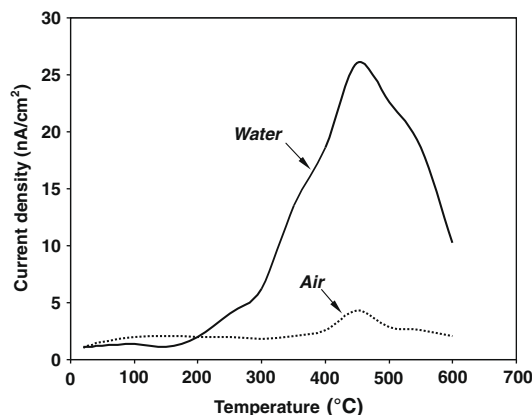


Fig. 2. Graph demonstrating typical charge retained by air- or water vapour-treated HA discs after polarization.

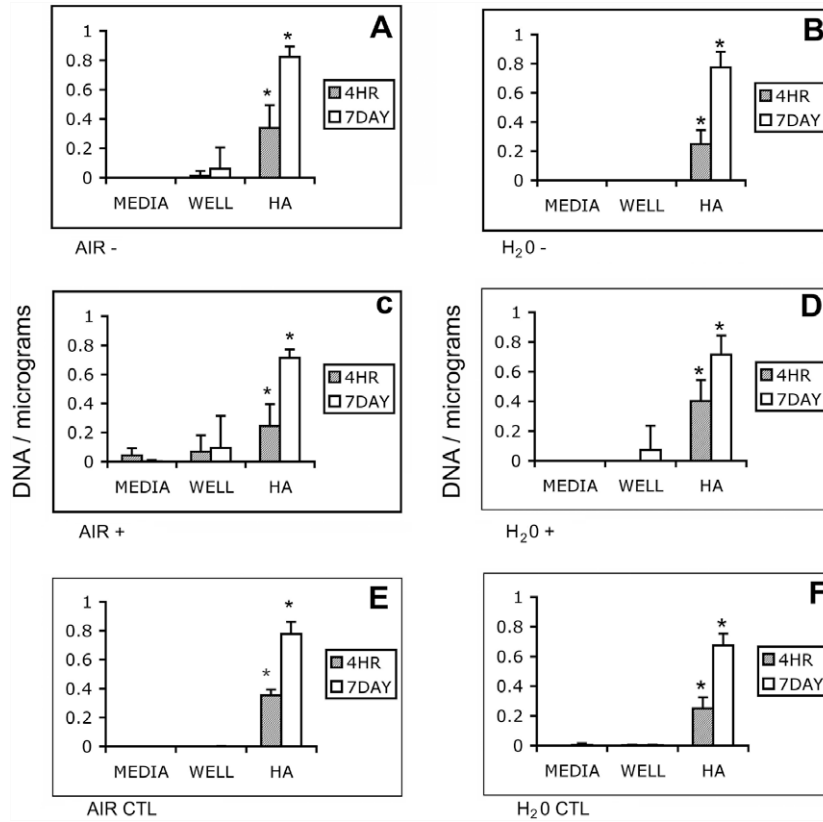


Fig. 3. A comparison of DNA levels measured at 4 h and 7 days from the medium, well and surfaces of positive, negative and non-polarized control HA discs sintered in air (A, C, E) or water (B, D, F). The symbol * represents DNA measurements from hydroxyapatite samples that were significantly higher in relation to the respective 4 h and 7 day samples.

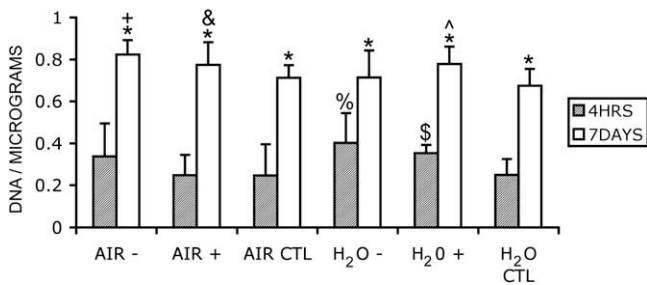


Fig. 4. A comparison of DNA levels measured at 4 h and 7 days from the surfaces HA discs polarized after sintering in air (AIR–, AIR+) or water (H₂O–, H₂O+) and unpolarized controls (AIR CTL, H₂O CTL). The symbol * represents DNA measurements from hydroxyapatite samples that were significantly higher in relation to the 4 h and 7 day samples. The symbol % represents DNA measurements from the 4 h H₂O– samples that were significantly higher in comparison to the 4 h AIR+, AIR CTL and H₂O CTL groups. The symbol \$ represents DNA measurements from the 4 h H₂O+ samples that were significantly higher in comparison to the 4 h AIR+, AIR CTL and H₂O CTL groups. The symbol + represents DNA measurements from the 7 day AIR– samples that were significantly higher in comparison to the 7 day H₂O– and H₂O CTL groups. The symbol ^ represents DNA measurements from the 7 day H₂O+ samples that were significantly higher in comparison to the AIR CTL and H₂O CTL groups. The symbol & represents DNA measurements from the 7 day AIR+ samples that were significantly higher in comparison to the H₂O CTL group. All significances are *p* < 0.05.

measurements from negatively charged 7 day air sintered hydroxyapatite (AIR–) samples that were significantly higher in comparison to 7 day H₂O– and H₂O CTL groups. The symbol + represents DNA measurements from the 7 day AIR– samples that were significantly higher in comparison to the 7 day H₂O– and H₂O CTL groups. The symbol ^ represents DNA measurements from the 7 day H₂O+ samples that were significantly higher in comparison to the AIR CTL and H₂O CTL groups. The symbol & represents DNA measurements from the 7 day AIR+ samples that were significantly higher in comparison to the H₂O CTL group. It can be seen from Fig. 4 that there is a significant increase in cell number from 4 h to 7 days for discs in all conditions.

Fig. 5 shows the 7 day alamarBlue assay results of cells grown on charged/uncharged hydroxyapatite discs. The symbol * represents % viability measured from samples that were significantly higher in comparison to the control (AIR CTL, H₂O CTL and TCP CTL) samples analysed. Significantly higher numbers of cells were seen to be present on both the positively and negatively charged surfaces of the HA discs in comparison to the HA uncharged controls and the standard tissue culture plastic controls. There was no significant difference seen between the air- and water-treated negatively and positively charged surfaces with regards to cell proliferation.

4. Discussion

Fig. 2 demonstrates the increase in charge retention of water vapour-treated HA scaffolds in comparison to air-treated HA scaffolds. The HA is polarized by a mechanism known as protonic migration which relates to the transportation of protons in the OH[–] columnar structure [14]. During this process the hydroxide

4 h and 7 day samples respectively. The symbol % represents DNA measurements from the 4 h H₂O– samples that were significantly higher in comparison to the 4 h AIR+, AIR CTL and H₂O CTL groups. The symbol \$ represents DNA measurements from the 4 h H₂O+ samples that were significantly higher in comparison to the 4 h AIR+, AIR CTL and H₂O CTL groups. The symbol + represents DNA

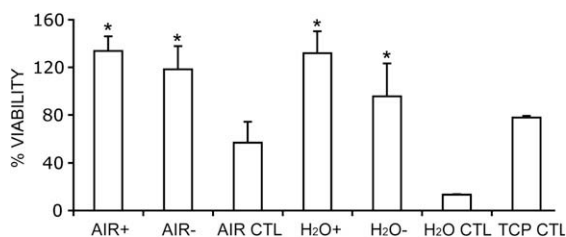


Fig. 5. Relative % viability of cells grown on positive, negative and non-polarized control HA discs sintered in air or water and the TCP control.

ions are forced to rotate and align themselves in the direction of the applied DC electric field. A current discharge during subsequent reheating of samples demonstrates that they have an associated surface charge. The water vapour-sintered discs retain a higher charge than the air-sintered samples due to the greater conductivity of the water vapour-treated discs. The presence of water vapour in the atmosphere during sintering prevents the dehydration of hydroxide ions and thus increases protonic conductivity. In contrast, in air, partial dehydration of the hydroxide ions leaves vacancies within the structure, which predominantly determine the conductivity. No information is currently available in the literature regarding the effects of how a charge of either 4 nA cm^{-2} , in the case of the air-sintered samples, as opposed to 26 nA cm^{-2} for the water-sintered samples, relates to cell activity. The magnitude of the difference appears to be relatively large but, on the overall scale, the significance of the difference can only be speculated upon; further work would be necessary to derive any conclusions in this respect.

The picogreen data (Figs. 3 and 4) show that osteoblast cells adhere and proliferate on all the different types of HA discs tested. The seeding technique demonstrated a good continuity, with approximately 99% of the cells seeded having attached to the HA discs in all conditions (Fig. 3). The results show that there was a significant difference between DNA measurements for the positive and negatively charged HA samples in comparison to the HA controls, sintered in both air and water. In particular, at the 4 h time point, a significant increase was seen in the amount of DNA (thus number of adhered cells) on the HA charged samples (both negative and positive) in comparison to the H₂O control. There was a significant increase seen in the amount of DNA on the H₂O + HA sample at 4 h in comparison to the AIR+ sample at 4 h. The same increase was seen for H₂O- vs. AIR+ HA sample at the same time point. An explanation for this may be that the negatively charged surface could be attracting positively charged ions, such as Ca^{2+} , to the cells seeded onto the surface [23], thus affecting the cell adhesion proteins initially produced by the cells attaching to the surface. Negatively charged ions, such as Cl^- , may also affect the positively charged surface of the HA, improving cell adhesion. At 7 days, there was a significant increase in cells grown on the negatively charged surface of the HA that was sintered in air when compared to the H₂O- and H₂O CTL groups. There was also a significant increase in the number of cells on the positively charged water-sintered HA samples at 7 days in comparison to the H₂O control and air control HA samples. The effect of the charge on the cell proliferation is clearly demonstrated here, but the mechanism as yet remains unknown.

The 7 day alamarBlue assay showed a significant increase in cell activity on the surfaces of both the positively and negatively charged air-sintered HA samples when compared to the cell response to both the uncharged HA controls and the tissue culture plastic controls (Fig. 5). A similar response was observed for the samples sintered in water vapour. It can be seen from Fig. 5 that the % viability of cells measured for the two controls differs signif-

icantly in that the air control sample has an initial cell viability measurement that is four times that of the water control sample. If this is taken as the starting point, comparison of the relative increase in % viability for the air and water samples is interesting. It can be seen that the cell viability of the polarized water vapour-sintered samples, relative to the control, has increased more rapidly than the equivalent air-sintered samples. This may warrant further investigation.

The alamarBlue assay detects cell viability via metabolic activity. As has been seen, in this assay, no overall difference was seen between the air- or water vapour-treated negatively charged HA discs after 7 days. This is in contrast to the picogreen assay, which measures the quantity of DNA present. It appears that, even though an increase in cell number was seen (Fig. 4), no difference in the metabolic activity from the cell populations cultured on the negatively charged air-treated HA disc in comparison to the cells cultured on the negatively charged water vapour-treated disc was observed (Fig. 5). These aspects should be taken into account when considering the final application intended for the HA material. For example, it may be more important to increase the cell number on a tissue-engineered construct prior to patient implantation. Alternatively, it may be more important to be aware of the cell's metabolic activity if, for example, implanting a treated HA-based construct directly into the patient.

Overall, in terms of actual cell numbers recorded, there was a measurable increase in the number of cells attaching to charged HA surfaces as opposed to non-charged HA, as well as differences in the rate of proliferation on charged HA samples. Other significant observations were apparent in relation to the % viability results measured with the alamarBlue assay. An increase in metabolic activity of the osteoblast cells in contact with charged HA was seen in all cases relative to the controls. No differences in the final measures of metabolic activity were observed for either positively or negatively charged samples, although the initial values for the two control samples differed by a factor of four.

5. Conclusions

The results demonstrate that polarizing HA discs and creating both positive and negative charges on the surfaces can influence the *in vitro* response and activity of osteoblastic cells. It has been shown that all the HA samples involved in this study (both charged and uncharged) supported osteoblast growth and attachment. A charge (either positive or negative) on the HA surface produced an increase in cell attachment at 4 h and cell proliferation over a 7 day period. It can also be seen that metabolic activity was significantly increased as a result of adding a charge to the HA surfaces. Both negative and positive charges present on HA surfaces demonstrated a significant increase in cell metabolic activity in comparison to the uncharged HA and tissue culture plastic controls. Thus, modifying the HA surface to increase cell proliferation and metabolic activity using the described manufacturing techniques could have great potential in improving HA interaction in terms of encouraging cellular activity and promoting faster healing.

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