THE EFFECT OF A SINGLE EXPOSURE TO A LOW DOSE BISPHOSPHONATE ON HUMAN MESENCHYMAL STEM CELLS ON A TITANIUM SURFACE (AN IN VITRO STUDY)

Mehta A, Owji N, Morden N, Alqhtani N, Buti J, D'Aiuto F, Knowles J.

University College London, Eastman Dental Institute, London. Great Britain

Abstract

Bisphosphonates have been shown to improve osseointegration however the effect of a single exposure versus a prolonged exposure to the drug remains unclear. This project aimed to investigate the effect of a single exposure to alendronate on the morphology and ultrastructure of human mesenchymal stem cells (hMSCs) compared to cells that had a continuous exposure for seven days.

Titanium discs with hMSCs were exposed to alendronate. The first group were exposed for seven days. The second group were exposed for 24-hours only, following which, the bisphosphonate-containing media was replaced with plain growth media. Morphological and ultrastructural changes were observed after 24-hours, three days and seven days using fluorescence imaging and scanning electron micrographs.

Following 24-hours' exposure to alendronate, cells demonstrated increased morphological and ultrastructural changes compared to control cells. Over the seven- day period, these cells exhibited similar or even more morphological changes compared to cells that were exposed to the drug for up to seven days.

A single exposure to alendronate may have similar, if not enhanced effects on the morphological and ultrastructural changes of hMSCs on a titanium surface compared to cells that have been exposed to the drug for up seven continuous days.

Introduction

The global prevalence of tooth loss in 2010 was 2.3% representing 158 million people worldwide (Kassebaum et al., 2014). In the United Kingdom, the proportion of adults with twenty or less teeth were 13% in 2009 with the mean number of missing teeth found to be three (excluding third molars) (Steele et al., 2000, Bernabe and Sheiham, 2014). Tooth loss has been shown to affect patients' quality of life (Nuttall et al., 2001) with negative effects on daily activities including patients' ability to carry out basic functions such as eating and smiling (Adulyanon et al.,1997).

The challenge of restoring these spaces, improve masticatory function and aesthetic requirements whilst providing a prosthesis with long-term successful outcomes can be resolved by dental implants. Implant therapy relies on successful osseointegration, that is, "a direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant" (Branemark 1959, Branemark, 1983). This process involves a series of cellular and extracellular biologic events which can be separated into three distinct stages; Osteogenesis, osteoconduction and osteoasaptation (Davies, 1998).

Many factors have been identified to enhance or inhibit osseointegration which are outlined in table 1 (Goutam et al., 2013).

Enhancement of osseointegration	Inhibition of osseointegration
Implant design, shape and diameter	Excessive implant mobility and micromotion
Titanium coating on Co-Cr metal implant	Nonsteroidal anti-inflammatory drugs especially selective COX-2 inhibitors
Laser treatment of Implant Surface	Warfarin and low molecular weight heparins
Human parathormone (1-34)	Inappropriate porosity of the porous coating of the implant
Bone source augment to socket	Osteoporosis, rheumatoid arthritis
Mechanical stability and loading conditions applied on the implant	Radiation therapy, smoking, advanced age, nutritional deficiency and renal insufficiency
Pharmacological agents such as simvastatin and bisphosphonates	Pharmacological agents such as cyclosporin A, methotrexate

Table 1: Factors affecting osseointegration of dental implants.

Extensive research has been conducted to enhance osseointegration and emerging evidence has begun to assess the use of bisphosphonates and mesenchymal stem cells to improve and accelerate the process. Mesenchymal stem cells are essential due to their ability to differentiate into cells that aid in regeneration (Hass et al., 2011, Kobolak et al., 2016). The quantity of mesenchymal stem cells is critical as it directly effects the number that can differentiate into osteoblasts for bone formation. Bisphosphonates have extensively been used to treat osteoporosis to reduce bone resorption, an essential aspect for bone healing (Drake et al., 2008b). Studies have shown significantly more bone formation around implants coated with bisphosphonates. Kajiwara and co-workers found a significant amount of new bone had formed around the bisphosphonate coated implants compared to the calcium coated and pure titanium implants in a rat model. Abtahi and co-workers investigated the effect of bisphosphonate coated titanium implants in a split-mouth study in sixteen patients (Abtahi et al., 2012). One implant was coated with a thin layer of two bisphosphonates (60% pamidronate and 40% ibandronate) and the other implant served as a control. A resonance frequency analyser measured implant stability to assess fixation. The results showed that bisphosphonate-coated implants had a larger increase in implant stability quotient (ISQ) values from baseline to six months compared to their paired controls, indicating that this local administration of bisphosphonates may improve the osseointegration process.The effect of low dose bisphosphonates on human mesenchymal stem cells and their osteogenic response to a

titanium surface has been investigated by Alqhtani and co- workers. In an *in-vitro* model, continuous systemic application of low dose bisphosphonates (alendronate and pamidronate, 100nM and 10nM), for up to 14 days, improved the activity and differentiation of human mesenchymal stem cells (Alqhtani et al., 2017). The data showed that bisphosphonates exerted a stimulatory effect on human mesenchymal stem cell osteogenic differentiation. The authors suggested that this increased osteogenic activity may enhance wound healing and accelerate the osseointegration process (Alqhtani et al., 2017). The study concluded that the application of low dose bisphosphonates, 1000 times less than a clinical dose, had a beneficial effect on hMSC proliferation and osteogenic differentiation on titanium surfaces.

The current literature shows that the systemic administration of low dose bisphosphonates has demonstrated an enhancement of hMSC activity, however, it is unclear if a single exposure to the drug will result in the same effect as a continuous exposure over a number of days. It is also unknown how long the effects can last for when the cells receive a single exposure to the drug for 24 hours only.

Therefore, the aim of this study was to determine the effect of a systemically administered low dose bisphosphonate, alendronate, on the morphology and ultrastructure of mesenchymal stems cells on titanium discs when cells are exposed to alendronate for 24 hours only compared to a continuous exposure for up to seven days.

Materials and Methods

This study observed the activity of human mesenchymal stem cells (hMSCs) on a titanium surface following the administration of different concentrations of a low dose bisphosphonate (alendronate) and comprised three variables: time, drug concentration and length of exposure to the drug. Observations were recorded at three intervals:

- One day (24 hours), which served as the baseline measurement
- Three days
- Seven days

Two different concentrations of alendronate (Sigma-Aldrich, Dorset, UK) were used: 100nM (T1) and 10nM (T2).

These were run in two parallel groups:

- **Group 1: hMSCs exposed to the drug continuously for up to seven days**
- **Group 2: hMSCs exposed to the drug for the first 24 hours only.**
  After this, the alendronate containing media was replaced with plain growth media
- For each experimental condition there was also a control (C)

Samples stained for fluorescence imaging (using phalloidin or anti-vinculin antibody) were run in triplicate to preclude experimental bias and allow for sample failure. The hMSCs were tested for cell proliferation and examined with a fluorescence and scanning electron microscope.

<u>Cell culture:</u>

This study used hMSCs obtained from the Institute for Regenerative Medicine at the Texas A&M Health Science Centre College of Medicine, Texas, USA. The cells were isolated from the bone marrow (iliac crest) of a 22-year-old male donor. The hMSCs were isolated using density centrifugation, plated and then harvested at 60%-80% confluence.

Flow cytometry analysis demonstrated the cells ability to grow and differentiate into osteoblasts, adipocytes and chondrocytes. Cells as passage one (P1) were purchased and on arrival at the UCL Eastman Dental institute, London, UK, the cells were expanded and harvested as passage four (P4) for the use of this study.

Titanium disc preparation:
75 titanium discs (Strauman LTD, Switzerland), 15mm in diameter and 1mm in thickness were first polished to remove surface irregularities and roughness. This was followed by a sterilisation process which involved sonification in isopropanol, immersion in 0.1-N Nitric acid and exposure to ultra-violet light.

Cell sample preparation:
Cells were seeded onto the titanium discs in 24-well plates with the addition of either plain growth media (control), media containing 100nM alendronate (T1) or media containing 10nM alendronate (T2). The cells were placed in the incubator and observations were recorded after 24 hours, three and seven days. During the seven-day period, the media was replaced twice following several PBS washes. Group one received new media containing the appropriate concentration of alendronate and group two received plain media only. Summaries of the experimental protocol are explained in figures 1 and 2.

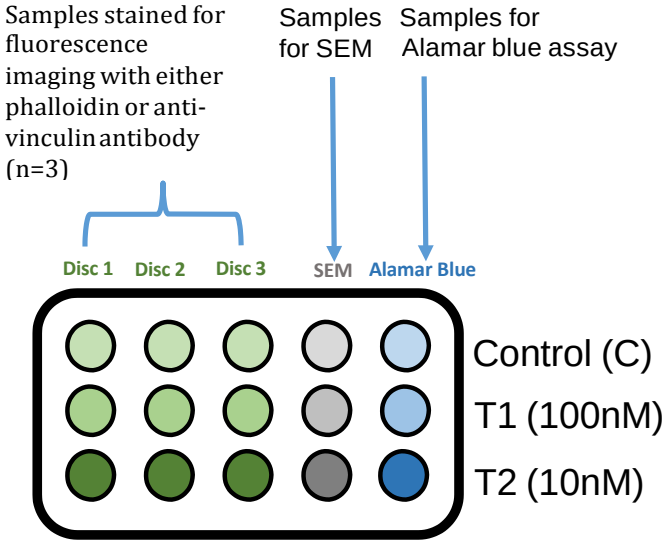

Figure 1: Set up of a well plate at the start of the experiment.

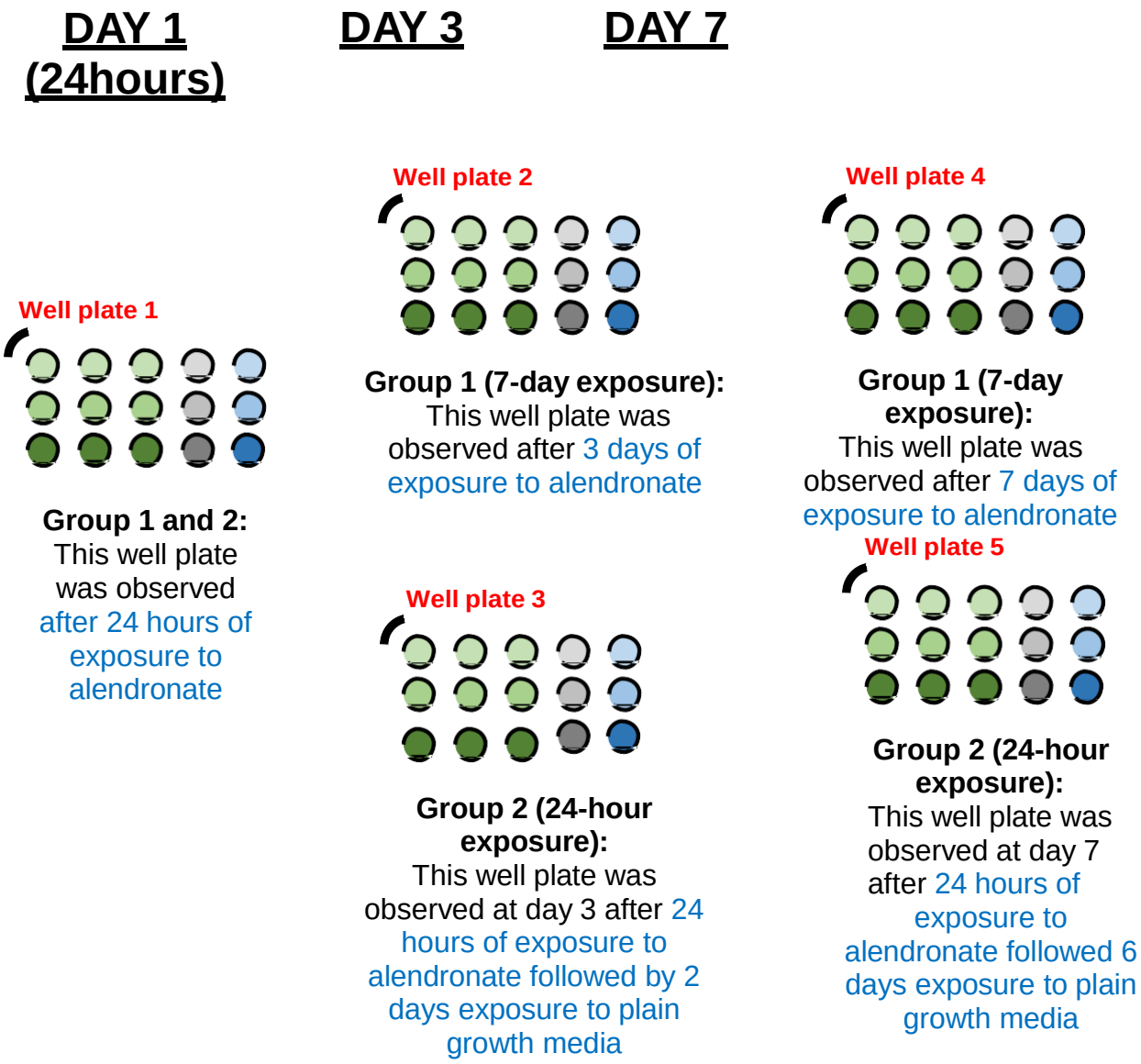

Figure 2: Illustration of experimental protocol.

This experimental protocol was repeated twice; samples in 'run one' were stained with phalloidin and samples in 'run two' were stained with anti-vinculin antibody. Phalloidin, with a fluorescent marker, binds directly to the cytoskeleton allowing visualisation using fluorescence microscopy and staining with anti-vinculin antibody allowed visualisation of the adhesin vinculin. This assessed adhesion and attachment of the cells to the titanium surface.

Nuclear staining was performed using 4', 6- diamidino-2-phenylindole (DAPI) (Thermo Fisher, Hertfordshire, UK), a fluorescent stain that binds strongly to the DNA of the cells. All samples labelled for F-actin and vinculin were stained with DAPI.
The cell proliferation assay, Alamar blue, was used to measure proliferation of the hMSCs indicating cell viability. One sample in each group of the second run were assessed using the alamar blue protocol.

Imaging:
All discs were assessed using an inverted fluorescence microscope at 10 x magnification and 20 x magnification (Leica DM IRB Fluorescence Time Lapse Facility, Milton Keynes, UK). Image analysis was performed using ImageJ. Nuclei counts were performed on the fluorescence images, using Image-Pro Premium 9.2 (Media Cybernetics, Marlow, Buckinghamshire, UK). A stage micrometre of 0.1mm divisions was used to calibrate and calculate the area of each image. The nuclei were isolated from the background using the automated software. Where the software missed a nucleus, it was added manually and where two nuclei were counted as one, they were split manually. This provided a count of the number of cells per image. As this technique could be considered subjective, inter- and intra- examiner assessment were carried out on 10% of samples. There was consistent agreement between all the measurements taken for this assessment.
Cell morphology was analyzed using scanning electron microscopy (SEM) and examined using an SEM FEI XL30 FEG Scanning Electron Microscope (FEI, Eindhoven, Netherlands).

Statistical analysis:
Descriptive statistics were expressed as scatter graphs to display the nuclei counts for each image. The area of each image was 1.20mm (1.27mm x 0.95mm). Bar graphs were used to show the fluorescence intensity levels detected for the alamar blue assay. Inferential analysis was conducted using a factorial experimental model to determine the impact of different factors on the percentage change in nuclei count over time compared to counts present at 24 hours (baseline). These factors included time, drug concentration and length of exposure to the drug. Possible interactions among terms were also explored. Factorial experiment analysis was performed using R Software.

Results

Cell morphology was assessed using two different staining methods and the nuclei were visualized with a fluorescent nuclear stain. SEM images were taken to further evaluate morphology and topography of the cells.

Day 1:

Control samples:
At 24 hours, fluorescence imaging showed cells had attached to the titanium surface and were beginning to spread with some evidence of elongation and alignment. The SEM samples also showed separate cells which were starting to elongate and align. The cell processes observed were extending from the cell body to the surface and were usually single and straight.

Test samples:
Cells exposed to 100nM alendronate in the growth medium (T1) and 10nM alendronate in the growth medium (T2) appeared more numerous on many of the discs compared to control discs. Samples stained with phalloidin showed multi-layers of cells, both disorganised and organised. The SEM images of the T1 and T2 samples shows ultrastructural differences when compared to the control. These cells showed an increase in cell processes in both test groups, in both runs. Numerous long cell processes that were notably undulating could be observed (figure 3).

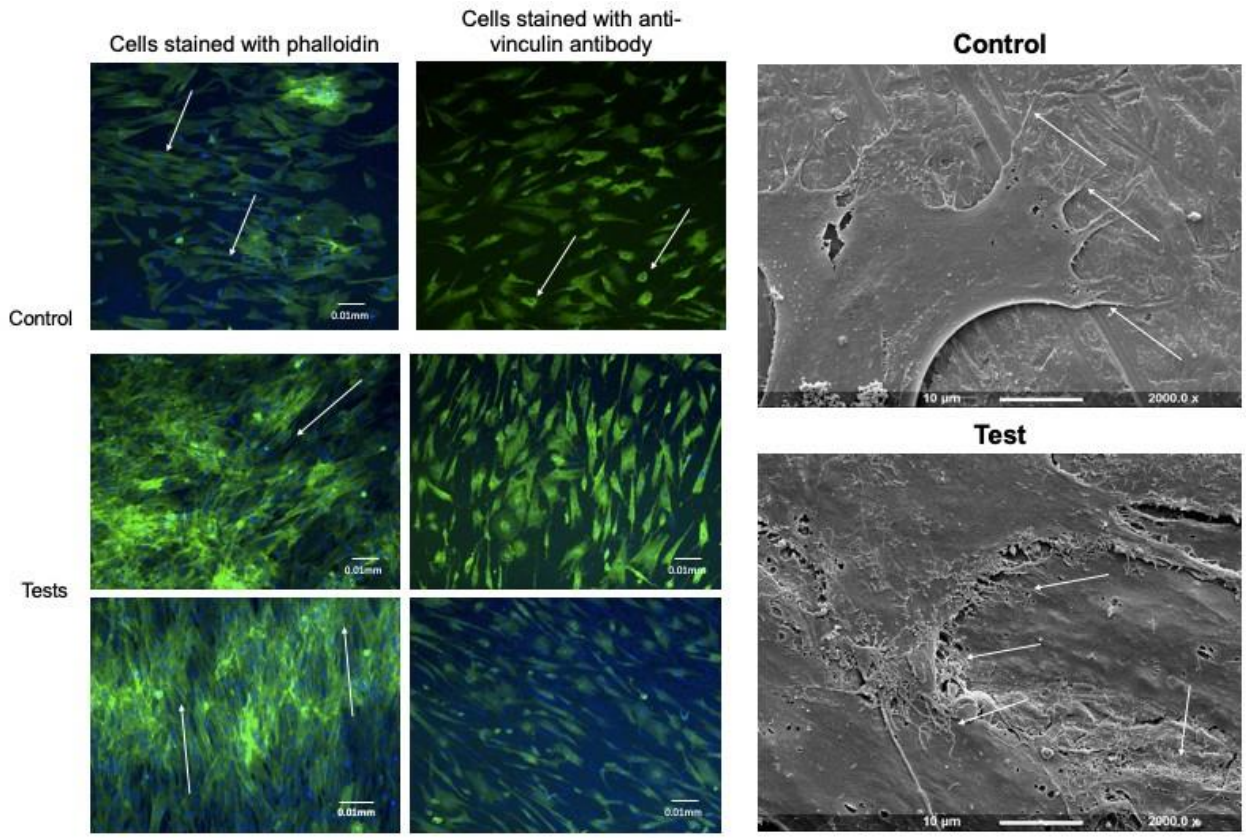

Figure 3: Fluorescence images show cells in the test group to be more numerous with evidence of elongation and alignment. The SEM images show cells in the test group to have an increase in cell processes that are notably undulating.

Day 3:

Control samples:
The cells of the controls could be seen to be more aligned in regions where they were numerous. No differences were observed in the images between group one and group two for both experimental runs within the control discs. The SEM assessment of control cells showed an increase in quantity of cells present on the titanium surface. Morphological features were similar to those observed on the control discs at 24 hours including cell processes appearing mostly straight.

Test samples:
All test samples stained with phalloidin demonstrated clear alignment with multi layers. Few differences could be observed between cells that were exposed to alendronate for 24 hours (group two) compared to cells that had been exposed to alendronate for three days (group one). Images from both groups displayed similar characteristics; elongation, alignment and spreading. SEM inspection of the test discs confirmed there were large areas of multi-layered, elongated and aligned cells. Multiple branching cell processes were also observed along the advancing front of many cells in both T1 and T2 groups. Cells exposed to the drug for 24 hours only were wider with more cell processes and more layers occupying the titanium surface (figure 4).

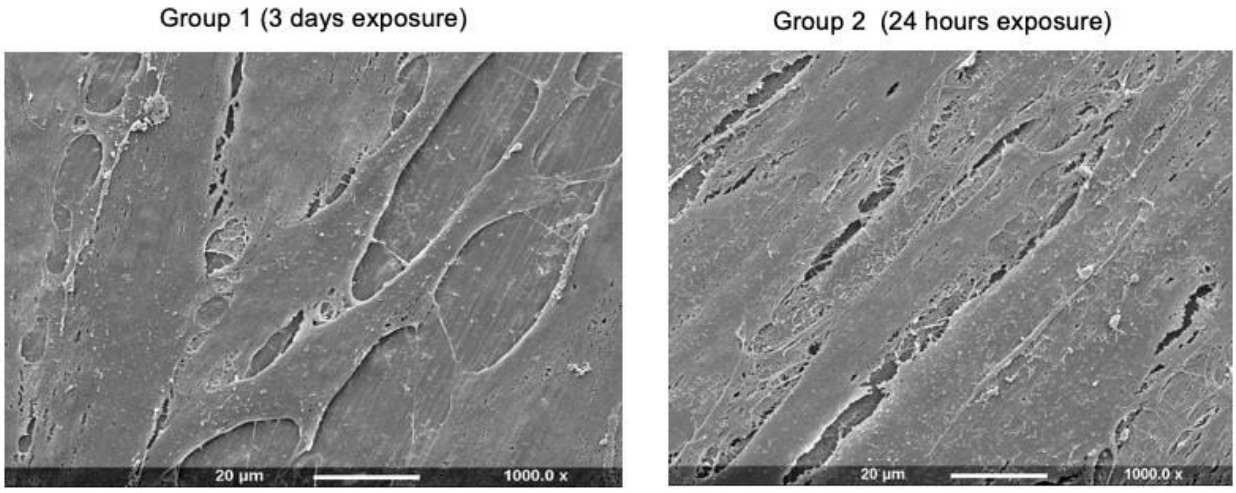

Figure 4: Results at Day 3. SEM images show cells exposed to the drug for 24 hours to be wider with more cell processes. More layers of cells are occupying the titanium surface.

Day 7:

Control samples:
By day 7 there was an increase in cells in all groups compared to their day one baseline images. Cells appeared greater in quantity, represented by higher numbers of blue stained nuclei and the arrangement and morphology of the cells were consistently aligned and elongated. These images were similar to the appearance of test samples that had been achieved by 24 hours and day three. The scanning electron micrograph supported these findings with evidence of multi-layer, aligned cells and a clear increase in cell processes.

Test samples:
For all treatments, the fluorescence images showed that the cells were numerous, confluent, elongated and aligned. Little differences could be observed between images from day seven cells that had been exposed to alendronate for only 24 hours (group two) and cells that had been exposed to alendronate for all seven days (group one). SEM images support the fluorescence images demonstrating multi-layered, elongated cells

with numerous cell processes. SEM images show the control samples to now have similar features to test samples (figure 5).

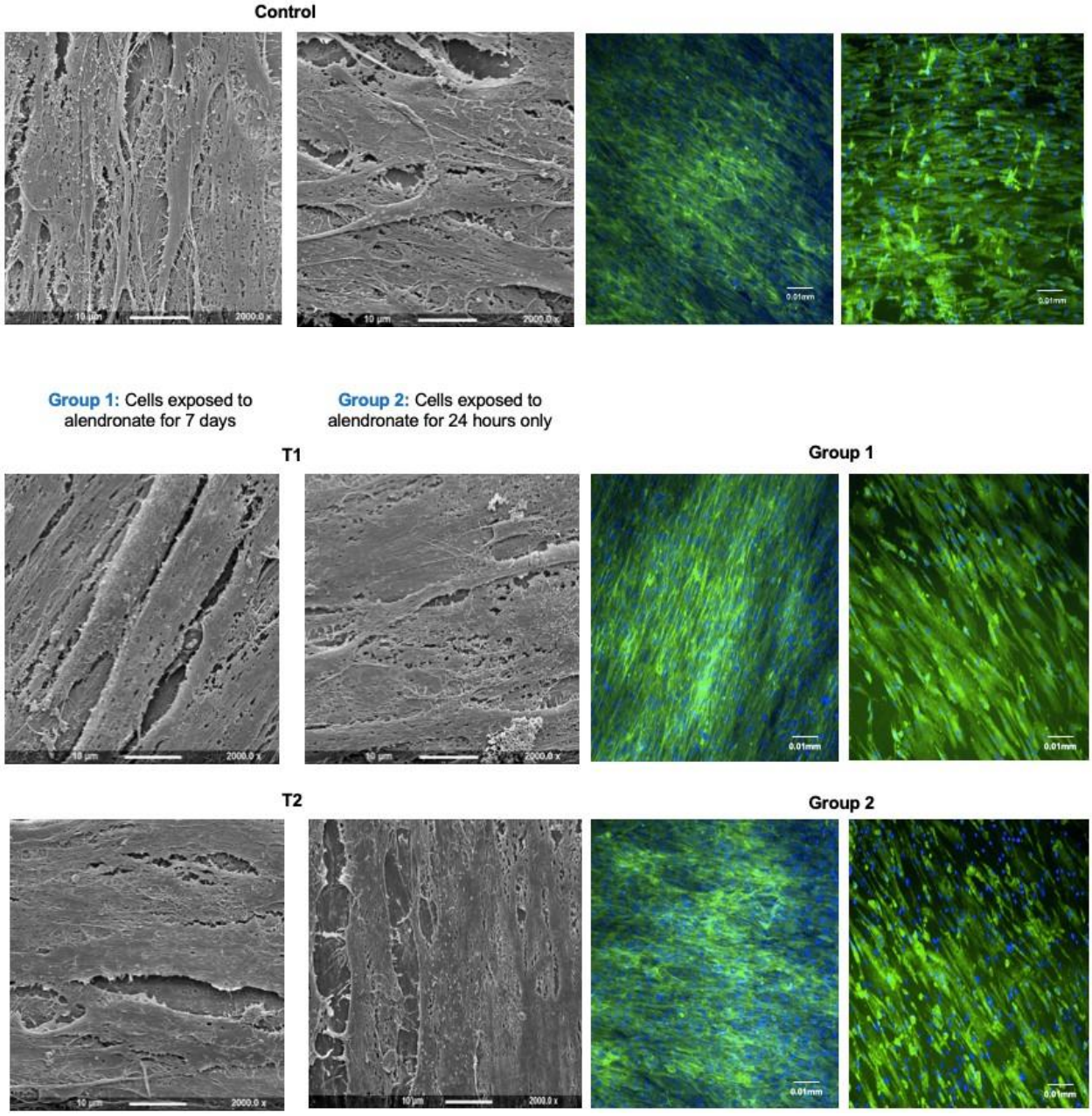

Figure 5: Results at Day 7. SEM and fluorescence images show control and test cells exhibiting similar morphological and ultrastructural features. All cells appear confluent, elongated and aligned. Cells exposed to alendronate for 24 hours show similar features to those exposed for seven days.

Results of the quantitative analysis:
All experimental models did not find a significant association between control or either test groups (T1 or T2) on the percentage change in number of nuclei compared to baseline counts.

Alamar blue assay:
The alamar blue assay was performed on one disc in group one and group two of the cells stained with anti-vinculin antibody (run two). Fluorescence intensities were recorded at 24 hours, at day three and at day seven. For cells that were exposed to the bisphosphonate for 24 hours only, fluorescence intensity decreased as the number of days increased. For cells that were exposed for seven continuous days, there was an initial reduction in fluorescence intensity between day one and day three. Following this, the fluorescence intensity increased by day seven.

Conclusions

Results from this study demonstrated positive morphological and ultrastructural changes on the hMSCs following exposure to alendronate. This was consistent with current evidence as described by Alqhtani and co-workers (Alqhtani et al., 2017).
At day three, the control samples increased in number and there was evidence of morphological changes within the cells, similar to those observed in test samples that had been established at 24 hours. These changes included multi-layering of cells, cell elongation and an extension of cell processes. The SEM images appeared to be similar, however cells which received 24 hour exposure, were greater in width and more extensively spread over the disc. The cells also exhibited more cell processes.
At day seven, similar observations were seen in the fluorescence images and scanning electron micrographs; there were no differences in morphological features from group one or group two experiments and if a difference was observed, it was in favour of cells exposed to alendronate for 24 hours only (group two). These results provide new information on how long structural changes can be observed following exposure to low doses of alendronate for up to seven days.
Nuclei counts varied over the seven days however there was an overall increase in cell count. By day seven, there were more cells compared to day one, and group two (cells exposed to alendronate for 24 hours only) had more cells than group one (cells exposed to alendronate for up to seven days). This data supports the morphological changes observed in the fluorescence and SEM images. The alamar blue proliferation assay indicated that as the number of days increased, cell viability decreased for most samples.
In this study, the beneficial effect of alendronate was predominantly observed during the first three days following exposure to the drug. This is an important finding which has advantageous clinical applications with regards to bone healing and osseointegration. Studies show that new bone formation can start as early as one week following implant placement (Berglundh et al., 2003a) and the most important time period in the process of osseointegration for hMSCs is approximately day four (Salvi et al., 2015a). Therefore, if activity of hMSCs can be enhanced or promoted during this crucial time period, it could lead to an acceleration in osseointegration. The findings of this research add to the limited knowledge base on bisphosphonates and osseointegration but highlight the benefit for their potential use.

Limitations:
Due to clustering of the cells on the disc, it was not possible to standardize selection of the area to be imaged per disc. Instead, the most densely populated area of the disc was selected. In order to reduce future bias, computer generated random co-ordinates could be formulated and these areas assessed with the use of a grid overlying the sample. Multiple co- ordinates can be selected to get a better representation of the disc which could improve the validity of the existing methodology for assessing cell morphology as well as nuclei counts.

Although assessment of morphologic features gives an indication of cell behaviour, imaging alone does not give information regarding changes in cell metabolic activity. The main limitation of the data collected regarding the Alamar blue proliferation assays was the limited sample size and hence no conclusions can be drawn. Future studies should assess cell viability, proliferation and ability to differentiate. The alamar blue assay should be used with a larger sample size, or alternative assays could be employed such as adenosine triphosphate (ATP) assay. Dead or dying cells contain little to no ATP and therefore this assay provides an accurate representation of live cells. The ability of cells to differentiate into mature osteoblasts is essential for successful osseointegration. Osteogenic factors could be introduced directly into the culture medium to encourage the hMSCs toward an osteogenic differentiation. The cells might then be assessed by testing for various differentiation markers such as intracellular alkaline phosphatase (ALP) and calcium deposition.

Within the limitations of this *in-vitro* study, the results suggest that a single exposure to alendronate for 24 hours may have a similar or more positive effect than cells exposed to the drug for seven days. Furthermore, these positive effects can last up to seven days.

Implications for research

Further *in vitro* studies are required to assess additional study outcomes including cell proliferation and differentiation measurements. Following identification of the ideal drug, dose and follow up period, an *in vivo* model can be tested.

Implications for clinical practice

A single exposure to the drug may be more beneficial in enhancing osseointegration than a 7-day course as it not only reduces the overall cost of treatment, but increases the likelihood of patient compliance to this adjunctive therapy.

Bibliography

1. ABTAHI, J., TENGVALL, P. & ASPENBERG, P. 2012a. A bisphosphonate-coating improves the fixation of metal implants in human bone. A randomized trial of dental implants. *Bone,* 50**,** 1148-1151.
2. ABTAHI, J., TENGVALL, P. & ASPENBERG, P. 2012b. A bisphosphonate-coating improves the fixation of metal implants in human bone. A randomized trial of dental implants. *Bone,* 50**,** 1148-51.
3. ADULYANON, S., VOURAPUKJARU, J. & SHEIHAM, A. 1996. Oral impacts affecting daily performance in a low dental disease Thai population. *Community Dent Oral Epidemiol,* 24**,** 385-9.
4. ALQHTANI, N. R., LOGAN, N. J., MEGHJI, S., LEESON, R. & BRETT, P. M. 2017. Lowdose effect of bisphosphonates on hMSCs osteogenic response to titanium surface in vitro. *Bone Rep,* 6**,** 64-69.

5. BERGLUNDH, T., ABRAHAMSSON, I., LANG, N. P. & LINDHE, J. 2003a. De novo alveolar bone formation adjacent to endosseous implants. *Clin Oral Implants Res,* 14**,** 251-62.
6. BERNABE, E. & SHEIHAM, A. 2014. Tooth loss in the United Kingdom--trends in social inequalities: an age-period-and-cohort analysis. *PLoS One,* 9**,** e104808.
7. BRANEMARK, P. I. 1959. Vital microscopy of bone marrow in rabbit. *Scand J Clin Lab Invest,* 11 Supp 38**,** 1-82.
8. BRANEMARK, P. I. 1983. Osseointegration and its experimental background. *J Prosthet Dent,* 50**,** 399-410.
9. DAVIES, J. E. 1998. Mechanisms of endosseous integration. *Int J Prosthodont,* 11**,** 391- 401.
10. DRAKE, M. T., CLARKE, B. L. & KHOSLA, S. 2008a. Bisphosphonates: Mechanism of action and role in clinical practice. *Mayo Clinic Proceedings,* 83**,** 1032-1045.
11. GOUTAM, M., CHANDU, G., MISHRA, S. K., SINGH, M. & TOMAR, B. S. 2013. Factors affecting Osseointegration: A Literature Review. *Journal of Orofacial Research,* 3**,** 197.
12. HASS, R., KASPER, C., BOHM, S. & JACOBS, R. 2011. Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. *Cell Commun Signal,* 9**,** 12.
13. KAJIWARA, H., YAMAZA, T., YOSHINARI, M., GOTO, T., IYAMA, S., ATSUTA, I., KIDO,M. A. & TANAKA, T. 2005. The bisphosphonate pamidronate on the surface of titanium stimulates bone formation around tibial implants in rats. *Biomaterials,* 26**,** 581-7.
14. KASSEBAUM, N. J., BERNABE, E., DAHIYA, M., BHANDARI, B., MURRAY, C. J. &
15. MARCENES, W. 2014. Global Burden of Severe Tooth Loss: A Systematic Review and Meta-analysis. *J Dent Res,* 93**,** 20S-28S.
16. KOBOLAK, J., DINNYES, A., MEMIC, A., KHADEMHOSSEINI, A. & MOBASHERI, A. 2016. Mesenchymal stem cells: Identification, phenotypic characterization, biological properties and potential for regenerative medicine through biomaterial micro-engineering of their niche. *Methods,* 99**,** 62-8.
17. NUTTALL, N., STEELE, J., PINE, C., WHITE, D. & PITTS, N. 2001. Adult dental healthsurvey: The impact of oral health on people in the UK in 1998. *British Dental Journal,* 190**,** 121.
18. SALVI, G. E., BOSSHARDT, D. D., LANG, N. P., ABRAHAMSSON, I., BERGLUNDH, T., LINDHE, J., IVANOVSKI, S. & DONOS, N. 2015a. Temporal sequence of hard and soft tissue healing around titanium dental implants. *Periodontology 2000,* 68**,** 135- 152.
19. STEELE, J. G., TREASURE, E., PITTS, N. B., MORRIS, J. & BRADNOCK, G. 2000. Total tooth loss in the United Kingdom in 1998 and implications for the future. *Br Dent J,* 189**,** 598-603.

Corresponding Author:
Dr Anjali Metha
E-mail: anjalibhimjiyani@gmail.com