The effect of Nrf2 activators on mesenchymal stem cell viability in response to light curable dimethacrylate resins

3D architectural influences on mesenchymal stem cells co-cultured with H400 epithelial oral cell line using hydrogels

By

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Abstract

Introduction. Dental restorative materials are useful in the repair of diseased or damaged teeth. These materials can cause cell damage through oxidative stress and the generation of free radicals which can be limited by Nrf2 activators including oleanolic acid and tBHQ (tert-Butylhydroquinone).

Objective. The main purpose of this study was to determine whether Nrf2 activators could protect mesenchymal stem cells (MSCs) from dental resin cytotoxicity.

*Methods.*MSC viability following exposure to resins over a 4 day period was determined by trypan blue counting and analysis of gene expression of NQO1 (Nad(P)H dehydrogenase, quinone 1), Gclc (glutamate-cysteine ligase, catalytic subunit), and Hmox1 (haemoxygenase (decycling) 1) using the semi-quantitative reverse transcriptase polymerase chain reaction (sqRT-PCR). Resistance to stress was examined by Instron and Vickers Hardness testing.

Results. The addition of 50-μM tBHQ to culture medium increased viable MSC numbers, compared with controls; however cell death occurred in the presence of supplemented resins. Highest Nrf2 activated gene expression occurred in50-μM tBHQ containing resin. The addition of tBHQ or oleanolic acid to resins affected their resistance to stress.

Conclusion. In the presence of dental resins, supplementation of culture medium with Nrf2 activators demonstrates decreased cytotoxicity.

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1. Introduction

1.1 Restorative materials

Over a billion dental resins are placed annually to replace a diseased portion of a tooth that may have occurred following trauma or as a result of caries. For a dental material to be considered for teeth restorations, it has to be able to withstand the chewing processes found in the oral environment and to also be aesthetically pleasing (Palin et al., 2005).There are 2 types of dental restorative materials: direct and indirect ones. For direct restorations, the filling material is inserted directly into the injured tooth, whereas for the indirect restorations the materials are made outside of the oral environment and then placed within the diseased tooth (Bouillaguet, 2004). Amalgam and composite resins are examples of direct dental restorative materials, while an example of an indirect dental material is porcelain. The placement of these materials provides a treatment approach to enable restoration of a more normal appearance and function of a tooth.

1.2 Photo-cured dental resins

Photo-cured dental resins constitute one of the materials that visibly resemble natural teeth. However, to be considered for teeth restoration these resins should be able to bond to the tooth, withstand temperature and pH changes and have increased hardness. Dental resin composites were developed in response to an increase in the request for dental restorations that mimic tooth appearance. Due to their low cost, tooth bonding, aesthetics and decreased toxicity compared with dental amalgam, these dental composite resins have been used for tooth restorations over the last 50 years (Chen et al., 2011; Hosseinalipour et al., 2010; Leprince et al., 2011; Lovell et al., 2001). However, dental

resins have two major disadvantages compared with dental amalgam: a decreased mechanical strength and an increase in polymerisation shrinkage, which makes them more suitable for anterior not posterior teeth restorations, because posterior teeth are subjected to increased wear due to mastication processes (Emami and Soderholm 2009; Palin et al., 2003). Anterior teeth restorations require the use of a material that better mimics the appearance of teeth such as resin composites, whereas posterior teeth restorations need a material with increased wear resistance because of the greater chewing forces in this region of the oral cavity, characteristics not completely achieved by current dental resin composites (Hosseinalipour et al., 2010; Zimmerli et al., 2010).

1.3 Structure of the organic matrix of dental resin composites:

Dental resins have an organic matrix usually made of dimethacrylate monomers, such as BisGMA, which is a bulky aromatic monomer developed by Bowen (1963). However, the high molecular weight and rigidity of BisGMA makes its conversion to polymer difficult. The hydrogen bond network and aromatic constituents of BisGMA result in a stiff molecule. The TEGDMA molecule is considerably shorter than BisGMA, more flexible and less viscous so is used as a solvent to increase the conversion rate and decrease the viscosity of the dental resin composite, although its inclusion leads to decreased strength of the dental composite. TEGDMA increases the degree of conversion of monomer into polymer because of its low molecular weight when used in combination with BisGMA. However the increase in polymerisation rate due to the usage of BisGMA leads to an increase in polymerisation shrinkage (Goncalves et al., 2009; Hosseinalipour et al., 2010; Tian et al., 2008). Therefore, the final concentrations of both BisGMA and TEGDMA in a dental resin directly affect its degree of conversion, rate of polymerisation

and mechanical properties (Atai et al., 2005). When these materials are used to make the organic matrix of the dental resin, TEGDMA increases the double bond conversion, whereas BisGMA leads to an increase in the reactivity of the resin (Hosseinalipour et al., 2010; Tian et al., 2008). The conversion of a monomer to a polymer is known as the polymerisation reaction, which is started by camphorquinone, a photoinitiator, when subjected to a curing light with a wavelength of 470 nm. Photo-curing results in the decomposition of camphoroquinone, which leads to the formation of free radicals. This leads to the activation of the polymerisation reaction in association with a tertiary amine photoreductant such as ethyl dimethylaminobenzoate. The latter acts as an accelerator of this reaction through rapid transfer of electron/protons, thus initiating the conversion of the monomers into polymers (Shi et al., 2010). Due to the dimethacrylate resins ability to formpolymer networks that have intermediate temperatures higher than their curing temperature, photo-curing results in a polymer with about 70% conversion rate (Shi et al., 2010).

1.4 Antioxidant protection of cells

The usage of a BisGMA/TEGDMA resin composite for dental restorations may result in toxicity in cells present within the oral environment. Due to its elution from the polymerised dental resin, TEGDMA has been shown to result in significant cell death and decreased cell growth though mechanisms which involve reactive oxygen species (ROS) and free radical release(Hansel et al., 1998). One agent that is able to provide antioxidant protection is N-acetyl cysteine, which acts as a free radical scavenger and protects pulp cells *in vitro* and *in vivo* (Kerksick and Willoughby, 2005). This molecule plays a vital role in the maintenance and metabolism of an antioxidant found in all cells of the body,

glutathione. Glutathione protects cells against oxidative stress and has a role in the maintenance of normal cellular processes such as proliferation (Kerksick and Willoughby, 2005).

1.5 Nrf-2 pathway

Activation of the cells' own protective pathways however may provide a novel and more efficient route to reduce the cytotoxic effect of dental resins. Signalling involved in detoxification and antioxidant cell responses utilises the nuclear factor erythroid 2-related factor 2 pathway (Nrf2).The Nrf2 transcription factor along with its repressor, Keap1 (kelch-like ECH associated protein 1) regulates the cells defence mechanism against oxidative stress. This factor acts as an activator of antioxidant genes, whose action leads to restoration of cell redox homeostasis, following cellular exposure to oxidative stress. Subsequently, cellular homeostasis is restored via the detoxification of the oxidative agent or reparation of injured molecules (McMahon et al., 2004).Under normal cellular conditions Nrf2 is located in storage molecules in cell cytoplasm. Accumulation of ROS within a cell generates an oxidative stress response, which can trigger Nrf2 to undergo translocation from cell cytoplasm to nucleus, where it generates an antioxidant response. Once activated, Nrf2 binds to antioxidant response elements (AREs), which are upstream DNA promoter sequences present in a range of cytoprotective genes, which induces their transcription (Imhoff and Hansen 2010; Reisman et al., 2009). NQO1, Gclc, and Hmox1 are examples of genes transcriptionally activated by this molecule. NQO1 plays a role in defending cells from oxidative stress and redox cycling by catalysing the reduction of quinones (involved in cellular cytotoxicity). Gclc has a role in cell homeostasis by acting as the rate-limiting enzyme in the production of glutathione, which conjugates ROS.

Hmox1 defends cells against oxidative stress by catalysing the breakdown of haem into bilirubin, a critical cell antioxidant (Reisman et al., 2009). Therefore, the activation of Nrf2/ARE –regulated genes results in activation of the cells own innate antioxidant defence mechanism (Wang and Jaiswal, 2006). The Nrf2 pathway can be activated by several chemical activators including tBHQ, sulphoraphane, oridonin, oltipraz and oleanolic acid (Wang and Jaiswal, 2006).

1.6 Nrf-2 activators: oleanolic acid and tBHQ

Oleanolic acid (pentacyclictriterpenoid) can protect cells from oxidative stress by boosting antioxidant defence mechanisms (Zhang et al., 2012). Oleanolic acid was also shown to play a significant role in protecting hepatocytes from carbon tetrachloride (a hepatotoxicant that causes oxidative stress) (Liu et al., 1998; Reisman et al., 2009).This acid is a known activator of the Nrf2 pathway, providing a mechanism for antioxidant cell protection (Reisman et al., 2009). It also activates MAP kinases such as JNK (c-Jun N-terminal kinases) and ERK (extracellular signal-regulated kinases), molecular pathways involved in antioxidant protection (Shi et al., 2010).

Another molecule that plays a role in cell protection against oxidative stress is tBHQ. Cellular exposure to tBHQ results in activation of the Nrf2 pathway by modifying the redox status in mitochondria as has been shown in HeLa cells. Thus, mitochondrial oxidative stress is induced by tBHQ, which leads to activation of the Nrf2 pathway, regulated by the Trx2 (mitochondria specific antioxidant, thioredoxin-2) (Imhoff & Hansen, 2010).

1.7 Effect of Nrf-2 activators on cells antioxidant protection

Oleanolic acid or tBHQ can be incorporated into dental resins, or supplemented in the cell culture medium to potentially protect cells against resin-induced ROS. Thus, in the present study, the cytotoxic effects of a 60/40% BisGMA/TEGDMA dental resin were analysed, by subjecting bone marrow stromal cells to discs made of this resin, when cultured in normal, oleanolic acid or tBHQ supplemented medium. The viability of cells was determined after 4 days and expression of Nrf2 activated genes (NQO1, Gclc and Hmox1) was analysed. This work was undertaken to identify a less toxic restorative material that does not result in marked cell death when placed clinically. The mechanical properties of BisGMA/TEGDMA resins in combination with a range of concentrations of tBHQ and oleanolic acid when compared with control resin (not containing tBHQ or oleanolic acid) including the rate of polymerisation, degree of conversion of the monomer into a polymer, cure rate, flexural modulus, flexural strength and hardness of each resin composite were investigated. This work was undertaken to demonstrate that the less toxic materials developed could survive the conditions found in the oral cavity.

2. Hypothesis

Cells can be protected from the toxic effect of dental resins through the incorporation of antioxidant molecules such as oleanolic acid and tBHQ into the resin composite itself or into the cell culture medium without adversely affecting the material properties.

3. Aims

The main purpose of this work was to investigate whether dimethacrylate resins exhibit a toxic effect on MSCs and if the addition of Nrf2 activators (tBHQ or oleanolic acid) to the resin itself or supplemented in the cell culture medium may protect the cells from such an effect. Additionally, the effect of the incorporation of tBHQ or oleanolic acid in the cell culture medium on the proliferation of cells and on the expression of NQO1, Gclc and Hmox1 in mesenchymal stem cells was also investigated. The effect of tBHQ or oleanolic acid on the mechanical properties of the dimethacrylate resin was also analysed.

4.Materials and Methods

2.2 Dental resins:

Control resin:

All materials were sourced from Sigma Aldrich, UK and used as standard unless otherwise stated. Control resins were made, containing BISGMA,TEGDMA,CQ (Camphorquinone) and Co-initiator (2 dimethylaminoethylmethacrylate; DMAEMA), using the percentages provided in Table 1. These were also used as stock solutions for the basis of tBHQ and oleanolic acid supplemented resins. These were mixed using a magnetic stirrer at 60^{0}C.The resin was stored in a brown bottle at 4^{0}C to avoid premature photo-curing. Similarly during mixing of the dimethacrylate resin constituents, the beaker containing each resin was covered in aluminium foil to avoid photo-curing.

Material	wt%
BisGMA	60
TEGDMA	40
CQ	0.2
DMAEMA	0.8

Table 1The percentages used to make the control resin

tBHQ resin:

Resins with concentrations of 1000 µM, 1500 µM, and 2000, 2500 and 3000µM tBHQ (Fluka Analytical, UK) were synthesised using stock solutions made using the same constituents as shown in Table 1.The tBHQ solutions were made and stored as for control resins. The amount of tBHQ required to make a resin with a concentration of 1000 µM tBHQ resin was calculated: 0.00302 g of tBHQ solution in 20 g of control resin.

Oleanolic acid containing resin:

Resins with concentrations of 2000, 2500, 3000, and 4000μM oleanolic acid were made, as described for the tBHQ resin above.

2.3 Measurement of degree of conversion and polymerisation rate:

The degree of conversion of each resin was determined following analysis using Fourier transform infrared spectrophotometer (FTIR)(Nicolet 6700, Thermoscientific), which passes infrared radiation through each resin disc. FTIR analysis provides a useful tool in the comparison of both the degree of conversion and rate of polymerisation of dental restorative materials which contain different concentrations of resin composites. This is achieved through the continuous detection of the decrease of monomer= CH2 at 6164 cm^{-1}, as it is converted to polymer. The degree of conversion of both the control and the experimental resins (those containing either tBHQ or oleanolic acid) was analysed using a FT-IR spectrometer. For 180 seconds the degree of conversion and polymerisation rate of each resin sample were recorded at an acquisition rate of 10 s^{-1} and spectra taken at an 8 wave number resolution. The rate of polymerisation was calculated from the degree of conversion of the dental resin as a function of time (Dewaele et al., 2006). The degree of conversion of each resin sample was calculated after these resin samples were exposed to a curing light for 40s. A disc shaped mould was placed on a glass slide. The uncured resin sample was poured inside the mould and covered with a glass slide. The uncured resin was exposed to a curing light for 40 sec, to allow polymerisation of the resin monomer as it is shown in Fig.1 (Curtis et al. 2009; Dewaele et al., 2006; Hadis et al., 2011; Palin et al., 2003).

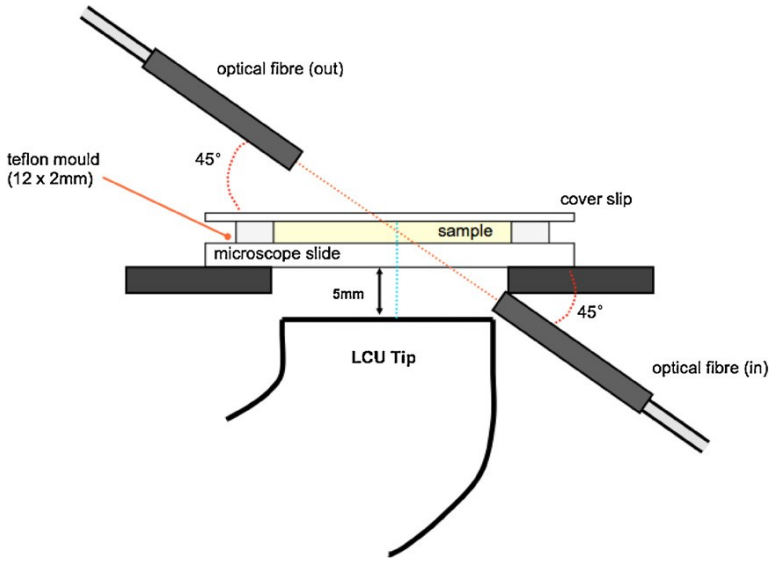

Figure 1 Real-time FT-IR measurements (Hadis et al., 2011).

2.4 The production of resin discs and rectangular bars:

20 discs and 10 rectangular bars were made from each resin as described in section 2.3; however the resin monomer was covered with acetate film, to allow easier removal of the resin disc, and flattened using another glass slide. A thin layer of Vaseline (release agent) was used to cover the surfaces that come in contact with the resin. This allowed easier removal of the resin from the mould and decreased radical interaction with oxygen. Each resin bar had dimensions of 25*2*2 mm. A curing light was used to irradiate the resin for 40 s. The bars and discs were placed in petri dishes covered in aluminium foil and stored overnight at room temperature to ensure completion of the polymerisation reaction prior to downstream testing.

2.5 Mechanical testing of resin bars:

The analysis of the flexural modulus and flexural strength of each resin sample involved the formation of rectangular resin bars for three point bending test. Failure was initiated by tensile stresses acting on the “lower convex surface of the specimen”, when the resin

bars were subjected to loading (Hosseinalipour et al., 2010; Palin et al.,2005; Zimmerli et al., 2010). Each rectangular resin bar sample was mechanically tested, using a universal testing machine (Instron 5544, UK) to determine the strain and stress levels within each sample. By producing a stress-strain graph, the fracture stress, the yield stress, and the modulus of elasticity were determined for each resin sample. The elasticity value provides information regarding the rigidity of a particular sample and the steeper the slope of the stress-strain graph, the more rigid the resin composite is. Ten resin bars were subjected to loading with the use of Instron universal machine in a 3 point bend test. Each resin bar was placed centrally on a support span of 20 mm, and a blunt point tip was used to perform a 3 point bend test. Each resin bar was subjected to load using the Instron machine at a cross head speed of 1 mm/min and 1 kN load (Fig. 2).

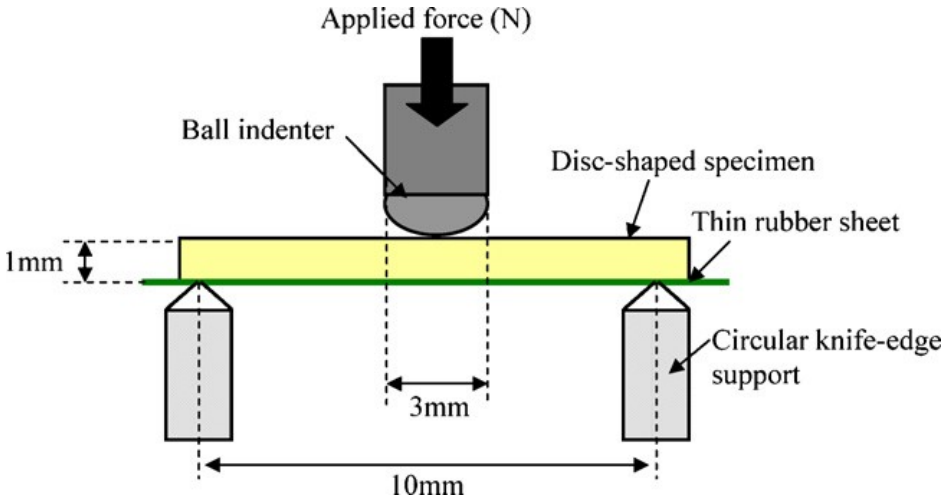

Figure 2 Experimental set-up of mechanical testing of rectangular resin bars (Curtis et al., 2009).

The width and the thickness of each resin bar were also recorded using an outside micrometre. The following equation was employed for the calculation of the stress of each resin sample condition:

$$\sigma_{max} = 3PL/2bd$$

The strain of each dental resin sample was calculated using this equation:

$$\epsilon = (6Dd/L^2 * 100,$$

where P=load at fracture, L=support span, b=specimen width, d=specimen thickness, D=midspan deflection.

The calculation of the flexural modulus of each resin condition was calculated using the following equation:

$$E = \sigma/\epsilon,$$

Where σ=stress and ϵ=strain(Palin et al., 2005).10 samples were tested for each resin and an average taken.

2.6 Hardness testing:

Posterior teeth restorations are subjected to wear and erosion due to high mastication forces. The resistance of the dental resins to such forces can be evaluated by microhardness testing (Goncalves et al.,2009; Hosseinalipour et al., 2010; Palin et al., 2005), where a high value for a sample implies an increased resistance to chewing forces. Hardness testing refers to the property of a given material to resist plastic deformation. There are 2 different types of hardness tests that can be used to analyse hardness of a specimen: microindentation and macroindentation. Types of macroindentation tests include the Vickers hardness test, the Brinell hardness test and the

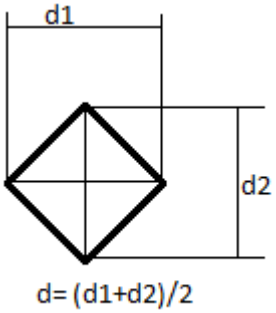

Figure 3Vicker hardness indentation appearance on the surface of a material

Barcol hardness test, which are used to apply test loads of more than 1 kgf to the specimen. When the test load applied is lower than 1 kgf, a microhardness test is used such as the Vickers hardness test or Knoop hardness test. The test employed in this study was the microindentation Vickers hardness test, which typically applies forces of approximately 2 N to create 50 μm indentations. 3 resin discs were tested for each resin condition, and an average taken by using a hardness tester (Struers Duramin) to perform 3 Vickers indentations on each resin disc. This test measures the hardness of a sample by calculating the size of these indentations left on the surface of the specimen using a pyramid-shaped diamond indenter, which has a square base and a 136^0 apex angle. The required material is put on an anvil, which has a screw threaded base. The screw threads are used to place the anvil in close proximity to the indenter. The release of the load was done automatically. The specimen was subjected to a load of 100 kgf for 15 seconds. The application of the load to the specimen lead to the formation of indentations that appeared like dark squares on a lighter background, when visualised with a microscope. The 2 diagonals of each square were measured and an average calculated as shown in Fig. 3.The hardness Vickers number was then calculated using the following equation:

$$HV = 1.854\, F/d^2,$$

where HV is the hardness Vickers number, F is the force applied to the specimen and d^2 represents the surface area determined by taking the average of the 2 diagonals of the square left by the indenter (Della Bona et al., 2007).

2.7 Culture media:

All media were made under aseptic conditions within a laminar flow hood and stored under sterile conditions at 4^0C.

a-MEM:

α-MEM (Minimum essential medium Eagle, α-modification) was made according to the manufacturer's instructions to a final pH of 7.3 prior to filter sterilising through a nitrocellulose 0.2 µm filter into 500 ml sterile bottles.

Transport medium:

Transport medium was made according to the proportions shown in Table 2.

Material	Amount
αMEM	20 ml
Pen/Strep	2 ml
HEPES (1M)	500 µl
Amphotericin (250 µg/ml)	200 µl

Table 2. Chemical components used to make the transport medium, Pen/Strep refers to Penicillin/ Streptomycin (penicillin/100 units/ml, streptomycin/100 units/ml) and HEPES refers to 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethansulfonic acid.

Basal culture medium:

Basal culture medium for BMSCs was made according to the proportions listed in Table 3, before storage in a sterile bottle.

Material	Amount
αMEM	60 ml
Pen/Strep	600 µl
HEPES (1M)	1500 µl
Amphotericin(250 µg/ml)	72 µl
L-Glutamine (200 mM)	600 µl
Foetal Calf Serum (10%)	9 ml

Table 3.The constituents used to make the BMSCs culture medium, where Pen/Strep refers to Penicillin/ Streptomycin (penicilin/100 units/ml, streptomycin/100 units/ml) and HEPES refers to 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethansulfonic acid.

tBHQ supplemented medium:

tBHQ was dissolved in DMSO (dimethyl sulphoxide) (Sigma Aldrich) to achieve a final concentration of 0.1 M tBHQ. The tBHQ dissolved in DMSO was then sterilised through a nitrocellulose 0.2 µm filter. This solution was used to make the tBHQ supplemented medium at various concentrations by employing the following equation:

$$0.1y = \frac{a}{10^6} * \frac{b}{10^3},$$

where 0.1 is the concentration of tBHQ dissolved in DMSO, $\frac{a}{10^6}$ is the tBHQ concentration required, and $\frac{b}{10^3}$ is the amount of culture medium used. For example, 50 µM tBHQ concentration was made by the addition of 30 µl tBHQ dissolved in DMSO to 60 ml cell culture medium.

Oleanolic acid supplemented medium:

Oleanolic acid was used to supplement culture medium at a range of concentrations as described for tBHQ supplemented medium. As oleanolic acid was found to precipitate in ethanol, methanol, and culture medium, it was dissolved in DMSO using the same calculations as provided for tBHQ supplemented medium.

2.9 Isolation of cells:

Bone marrow stromal cells were extracted from the femora of 250 g male, Albino Wistar rats (supplied by Aston University, UK). An incision running from the angle of the knee and a lateral, relieving incision were made, to expose the soft tissue. The femur was cleaned of external soft tissues, using a scalpel, and subsequently placed in the transport medium. Femora were then placed in a petri dish, and the distal and proximal ends removed using bone cutters. A syringe containing 10 ml of supplemented αMEM culture medium was used to irrigate the femur at both ends, and the resulting liquid containing cells was poured into a fresh universal container. This container was centrifuged (Durafuge 100, Thermo electron corporation) for 3 min at 1200 rpm to pellet the cells. The supernatant was removed, and the cells were resuspended in 2 ml culture medium prior to seeding in a T75 flask containing 10 ml culture medium. The BMSC were allowed to adhere to the bottom of the flask, by placing the flask in the incubator for 48 hours before changing the medium every 2 days.

2.10 Cell culture and arrangements in well plates:

All feeding of cells was performed in the laminar flow hood. The culture medium was removed using a pipette, and replaced with fresh; 15 ml αMEM supplemented culture medium. The cells were also checked visually for bacterial or fungal contamination using a phase contrast microscope.

5,000 cells per well were transferred to 6 well plates, for viability analysis and 4 ml of culture medium was added to each well. Culture plates were then placed in the incubator for 24 h to allow cell adherence. 15 resin discs were used from each of control resin, control resin supplemented with 2000 μM tBHQ, or 2000 μM oleanolic acid. The discs were disinfected by immersion in 70% ethanol for 10 minutes and allowed to air dry in the laminar flow hood before transferring to culture wells. The resins were placed with the cured side on top of the cells. Two time-points of 2 and 4 days were used for experimental analysis. Analyses were performed in triplicate.

2.10 Cell counting using Trypan blue stain:

Viable cells were counted using the Trypan blue staining procedure. Cultured cells were trypsinised using 1ml of 0.25% Trypsin/EDTA for each well and after cell detachment the trypsin was neutralised with 1 ml of culture medium. The number of viable cells was determined by staining them with 0.4% Trypan blue added to an equal volume of cell suspension. Viable cells were counted using a Neubauer haemocytometer and a phase contrast microscope. The haemocytometer and the cover slips were cleaned using 70% ethanol. The cleaned cover slip was moistened with exhaled breath prior to placing it on the haemocytometer. Approximately 10 µl of cell suspension/trypan blue stain was pipetted at one edge of the cover slip, so that it ran under the cover-slip. The haemocytometer grid was then visualised with a phase contrast microscope where the viable cells appeared bright and colourless, whereas the dead cells appeared blue. Both viable and dead cells were counted in the 4 large, corner squares of the grid and an average taken (Fig. 4). Using the following equation the total number of viable cells in 1 ml was calculated:

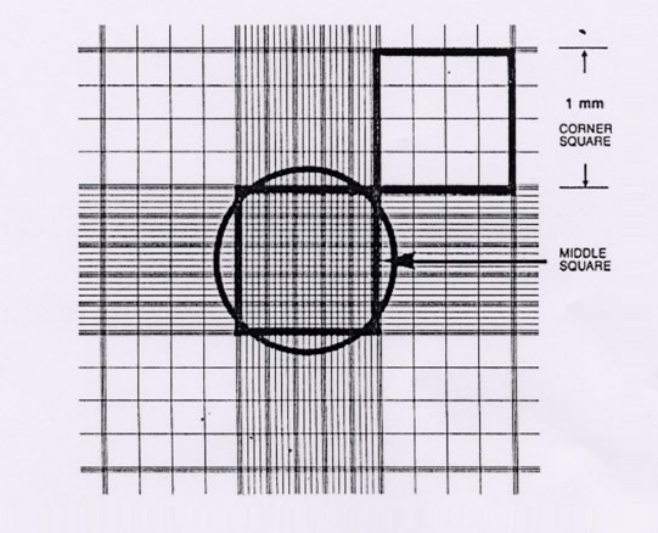

Figure 4 Haemocytometer grid seen under the phase contrast microscope(Wong et al. 2001)

$$TC = x/4 * 2 * 10^4,$$

Where TC was the number of total viable cells in 1 ml; x was the average of the cell counts from the squares of the haemocytometer grid, 2 was the dilution factor (1:1

dilution of cell suspension and trypan blue stain) and 10^4 represented the conversion factor.

2.11 Semi-quantitative RT-PCR analysis:

Cell lysis and RNA isolation:

RNA was isolated using the QiagenRneasy Kit (Qiagen, UK) and all reagents were kept on ice prior to use. The effect of tBHQ and oleanolic acid on the expression of genes: NQO1, Gclc, and Hmox1 was investigated. 5×10^5 bone marrow stromal cells were seeded in each well of a 6 well plate. Gene expression was analysed on day 1 and 2 following the exposure to 50 µM and 100 µM tBHQ and oleanolic acid supplemented medium, respectively and levels were compared with unsupplemented controls. At appropriate time points, cells were trypsinised using 2 ml trypsin/well, transferred to universal containers, centrifuged at 1200 rpm for 3 minutes, the supernatant was discarded and cell pellets were washed in 1 ml PBS. Cells were repelleted by centrifugation, the supernatant was removed and 350 µl RLT lysis buffer was used to resuspend the cell pellet, which was then placed in a 1.5 ml Eppendorf tube and stored at -80^{0}C prior to further downstream processing. 350 µl of 70% ethanol was added to the pelleted cells after the cells were homogenised. This was transferred to a RNeasy spin column in 2 ml collection tubes. The sample was centrifuged at 10,000 rpm for 30 sec. 700 µl of Buffer RW1 was added to the column, and spun again at 10,000 rpm for 30 sec. The flow through was discarded and 500 µl added to the spin column. The spin column was then centrifuged at 10,000 rpm for 2 min. The effluent was discarded and the column placed in a new collection tube and centrifuged at full speed for 1 min. The column was then placed in a 1.5 ml collection tube and 30 µl of RNase free water added to the

membrane. This tube was centrifuged at 10,000 rpm for 1 min to elute the RNA. Following isolation, the RNA was quantified using a Biophotometer (Eppendorf, UK). 2 µl RNA and 68 µl RNase-free water were added to a cuvette and a reading obtained which represented the concentration of RNA in µg/ml for each sample.

RNA visualisation:

All substances were kept on ice for the duration of the experiment set-up. A 1% agarose gel was made by mixing 0.7 g of agarose in 70 ml 1XTAE buffer (Qiagen, UK) in a large conical flask. The mixture was heated until boiling within a microwave oven and subsequently cooled under running tap water. 3 µl of SYBR Gold (used to stain RNA) was added to the agarose solution and mixed prior to pouring into the casting tray. A well forming comb was inserted and the gel allowed setting at room temperature. An electrophoresis tank was filled with 1XTAE, and the cast gel was placed in the tank. The comb was carefully removed. Each RNA sample was made up to a total volume of 6 µl, by mixing 1 µl of RNA from each sample with 5 µl of RNA loading buffer (Invitrogen, UK). The samples thus made were loaded in the wells of the agarose gel. Samples were electrophoresed at a voltage of 120V for 30 min. Gel images of RNA samples were captured using the Genesnap software (SynGene, UK).

Reverse Transcription:

All the samples and reagents were kept on ice prior to use. Reverse transcription was performed using the Omniscript Kit (Qiagen, UK). Each conversion required the use of 2µg of RNA; however, the maximum volume that could be used was 12 µl. Twelve microliters of RNA or RNA with RNase-free water (to make up a volume of 12 µl) was

added to a clean tube. Two microliters of Oligo-dT of 10 µM stock was added to each sample to make a final concentration of 1 µM. The samples were then heated at 80°C for 10 min in a heating block. The reverse transcription mastermix was made by mixing the following: 2 µl of 10X buffer RT, 2 µl of 5 mM stock of dNTP, 1 µl of 10 µl stock of RNase inhibitor and 1 µl of omniscript reverse transcriptase. The samples/oligodT tubes were removed from the heating block and quenched on ice for 5 min. Six microliters of mastermix was added to each sample tube, which was mixed briefly by vortexing and centrifuged to collect the residual liquid. All the samples were then incubated at 37°C for 60 min, and at 95°C for 5 min. The samples were then placed on ice prior to cDNA concentration using Microcon filters (Millipore, UK). Water was added to the cDNA to make a total volume of 500 µl. This was transferred to a spin column, and spun at 10,000 rpm for 2 min. The level of the liquid was visually checked, and the sample was re-spun at 8,000 rpm for 1 min. At this point, there should have been a volume of less than 70 µl within the tube. The column was inverted in a collection tube and spun at 800 rpm for 1 min. cDNA was stored at -20^{0}C prior to further use.

PCR analysis:

cDNA levels were normalised against the housekeeping gene GAPDH using REDTaqReadyMix PCR Mix. A forward and reverse primer mastermix was prepared by adding 10 µl of each reverse and forward primers (25 µM) (Invitrogen, UK) (Table 4) to 60 µl RNase free water, to make a final dilution of 1:4 of the forward and reverse primer. The REDTaq master mix was made by adding 12.5µlREDTaq (Sigma, UK) and 2 µlForward and Reverse Primer Mastermix to 9.5 µl RNase free water. 24 µl of REDTaq master mix was added to 1 µl cDNA in a PCR tube, for each cDNA sample. These were

then transferred to a PCRmachine. The cycles used were as follows: 94°C for 5 min, 94 °C for 20 sec (1), 60°C for 20 sec (2), 68°C for 20 sec (3), then cycles (1) to (3) were repeated 27 times followed by 72°C for 10 min. A 1.5% agarose gel was made by mixing 0.9gagarose with 60 ml 1XTAE buffer. Four µl of GelRed (this was used to stain the DNA) was added to the gel before it was poured in the tray as described above. The samples (6µl/well) were loaded together with a DNA ladder (Invitrogen, UK) (3 µl) in one well as control. Amplified products were electrophoresed for 30 min at 120V. Gel images of DNA samples were captured using the Genesnap software. Genetool (SynGene) software was used to assign relative values for each amplified product based on pixel density.

Gene name	**Symbol**	**Sequence (5"-3")**	**AT**	**PS**	**Gene ID**
NAD(P)H dehydrogenase, quinone 1	NQO1	F ACGACATCACAGGGGAGCCGAA R CGGGTGGGGTGTGGCCAATG	57°C	190bp	24314
glutamate-cysteine ligase, catalyticsubunit	Gclc	F GACCACGTCCGGAGACACGG R CAGGGCAGCCTAGCCTGGGAA	57°C	176bp	25283
hemeoxygenase (decycling) 1	Hmox1	F CACAGCTCGACAGCATGTCCCA R TATGCGTGGGCCACCAGCAG	60°C	288bp	24451

Table 4 Nucleotide sequences of primers used for the RT-PCR analysis, AT=annealing temperature; PS=product size, Gene ID= gene accession number.

2.13 Statistical analysis

Data analysis was performed by one-way Anova using statistical software (Minitab, UK). A difference of $P<0.05$ was considered statistically significant. Anderson-Daarling test

was used to determine whether the data followed a normal distribution. Tukey's post hoc tests was used for pairwise comparison at a significant value of $P<0.05$.

5. Results

3.1 Degree of conversion of dental resin discs:

The control resin exhibited a degree of conversion of 70.03%; whereas resin supplemented with 1500 µM tBHQ had the highest degree of conversion, and 1000 µM tBHQ containing resins exhibited the lowest degree of conversion. The graph presented below shows that the addition of 1500 µM tBHQ, 2000 µM tBHQ, and 2000 µM oleanolic acid increased the degree of conversion, whereas, 1000 µM tBHQ, and 4000 µM oleanolic acid significantly decreased the maximum degree of cure when compared with the control resin (Fig.5, Fig.6.).

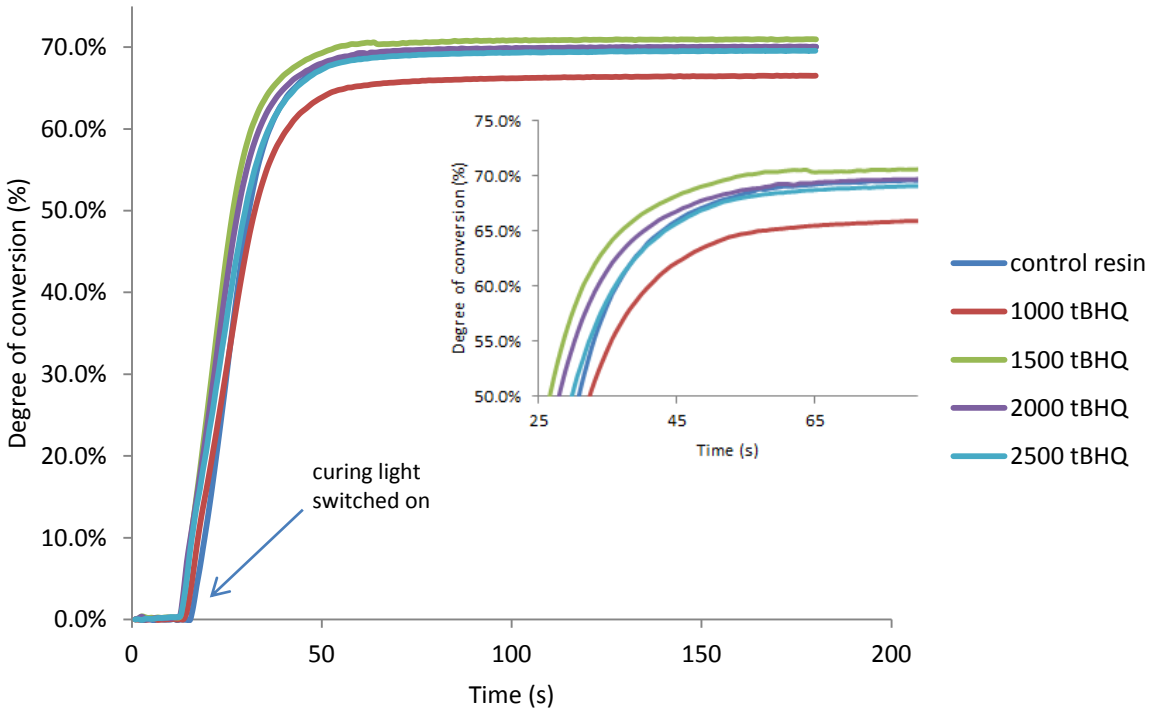

Figure 5 Graph showing a comparison of the degree of conversion of resins containing various concentrations of tBHQ with control resin. The curing light was applied 10 seconds after the recording started. 1000, 1500, 2000 and 2500 tBHQ represent the concentrations of the tBHQ added to stock solutions used to make up control resins.

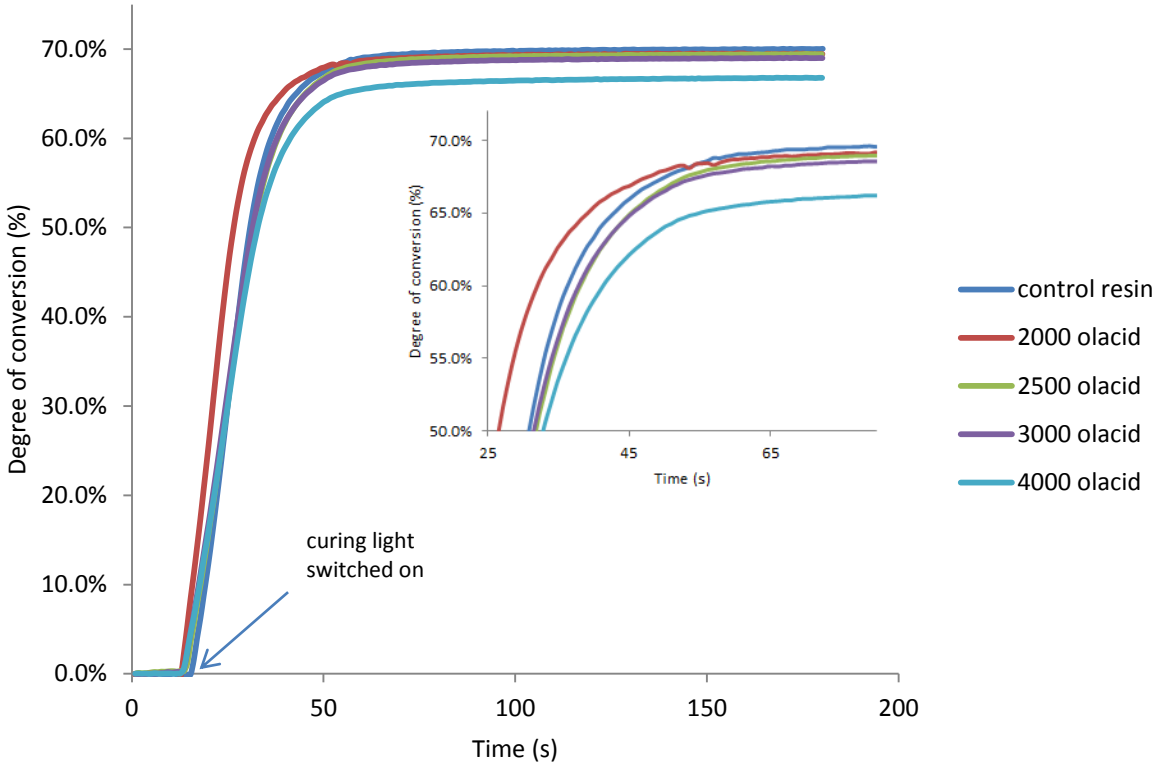

Figure 6 A graph showing the comparison of the degree of conversion of resins containing various concentrations of oleanolic acid with the control resin. The curing light was applied 10 seconds after the recording started. 2000, 2500, 3000 and 4000 olacid represent the concentrations of the oleanolic acid added to stock solutions used to make up control resins.

3.2 Rate of polymerisation of dental resin discs:

Fig. 7 and Fig 8 show the average rate of polymerisation of the control and experimental resins. The highest polymerisation rate occurred in the presence of resin supplemented with 1500 µM tBHQ, whereas the lowest polymerisation rate was found at resin supplemented with 1000 µM tBHQ. The addition of 1500 µM tBHQ, 2000 µM tBHQ and 2000 µM oleanolic acid to control resin increased the rate of polymerisation, whereas the addition of 1000 µM tBHQ and 4000 µM oleanolic acid to the control resin significantly decreased the maximum polymerisation rate compared with the control resin.

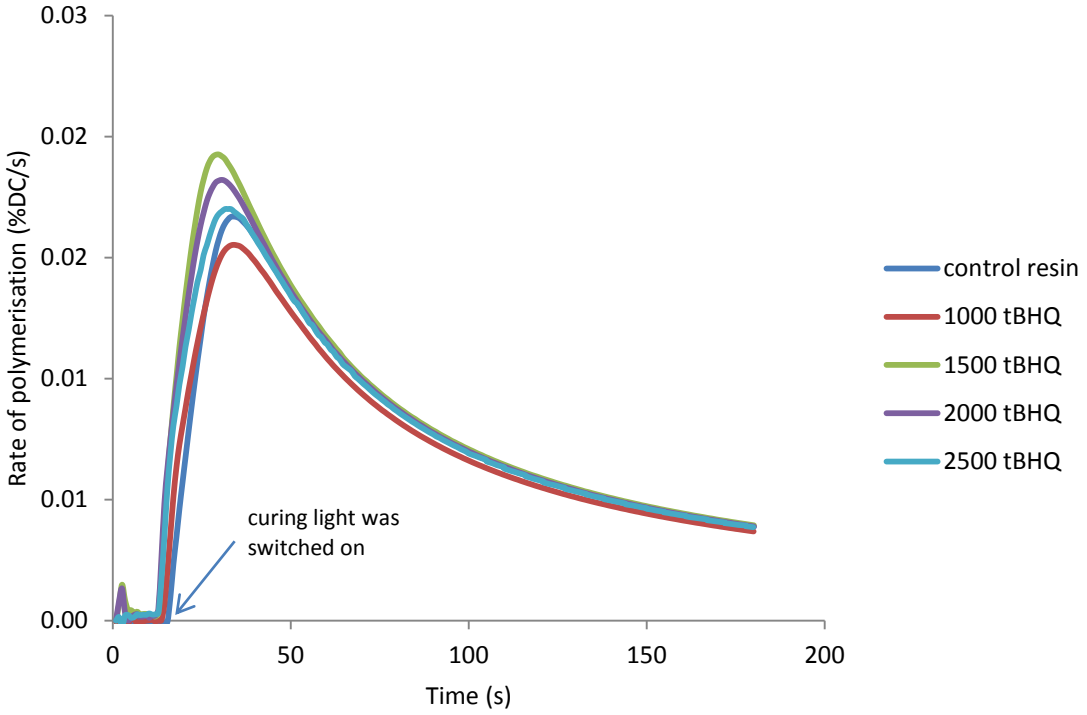

Figure 7 The comparison of the polymerisation rate of a range of concentrations of tBHQ containing resins with control resin. The curing light was applied 10 seconds after the recording started. 1000, 1500, 2000 and 2500 tBHQ represent the concentrations of the tBHQ added to stock solutions used to make up control resins. 1500 µM tBHQ had the highest rate of polymerisation.

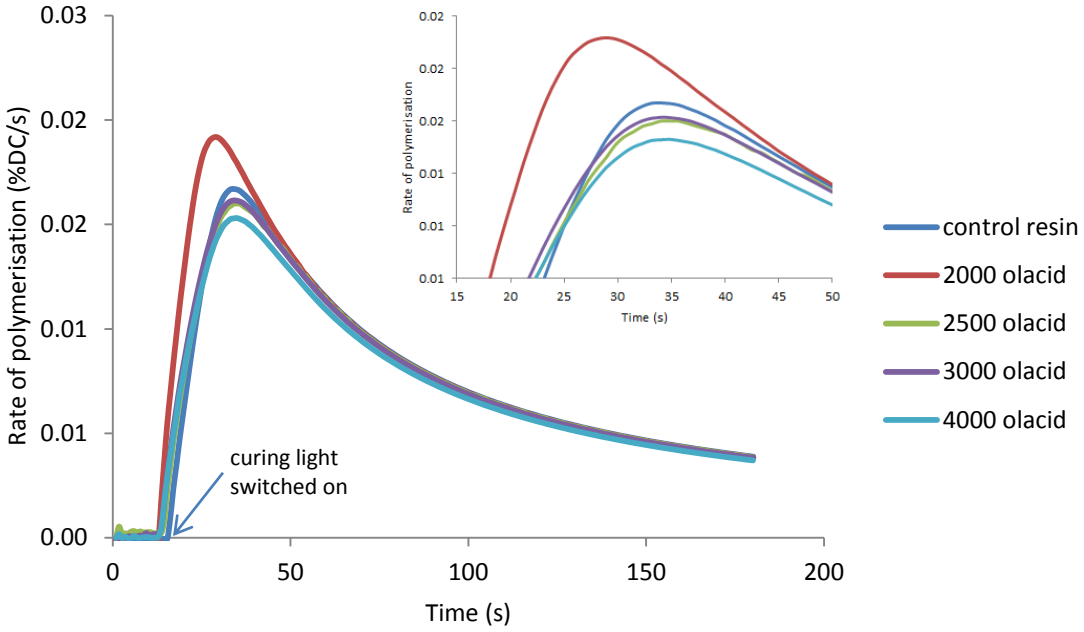

Figure 8 The comparison of the rate of polymerisation of a range of concentrations of oleanolic acid containing resins with control resin. The curing light was applied 10 seconds after the recording started. 2000, 2500, 3000 and 4000 olacid represent the concentrations of the oleanolic acid added to stock solutions used to make up control resins. 2000 µm oleanolic acid exhibited the highest rate of polymerisation.

3.3 Mechanical testing of rectangular resin bars:

When the flexural strength was compared across resins, it was highest in the control, resins supplemented with 1000 µM tBHQ or 2000 µM oleanolic acid; and lowest in resins supplemented with 1500 µM tBHQ or 2500µM tBHQ (Fig 9).

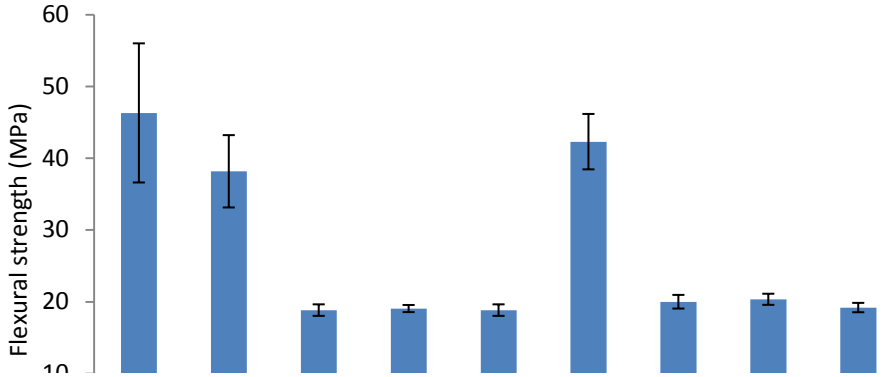

Figure 9The flexural strength of control resin and resins containing either tBHQ or oleanolicacid was analysed. Control resin had the highest flexural strength, whereas 2000 µm oleanolic acid containing resin exhibited a slightly lower value.Error bars indicate standard deviation over a mean average of 10 samples.

Control resin compared with all resins containing various tBHQ concentrations ($p<0.005$). However, there was no significant difference between the resins containing 1500 µM, 2000 µM and 2500 µM tBHQ ($p>0.005$). There was no difference in the flexural strength of the control resin and 2000 µM oleanolic acid containing resin. When these two resins were compared with 2500 µM, 3000 µM and 4000 µM oleanolic acid, there was a significant difference in the flexural strength.

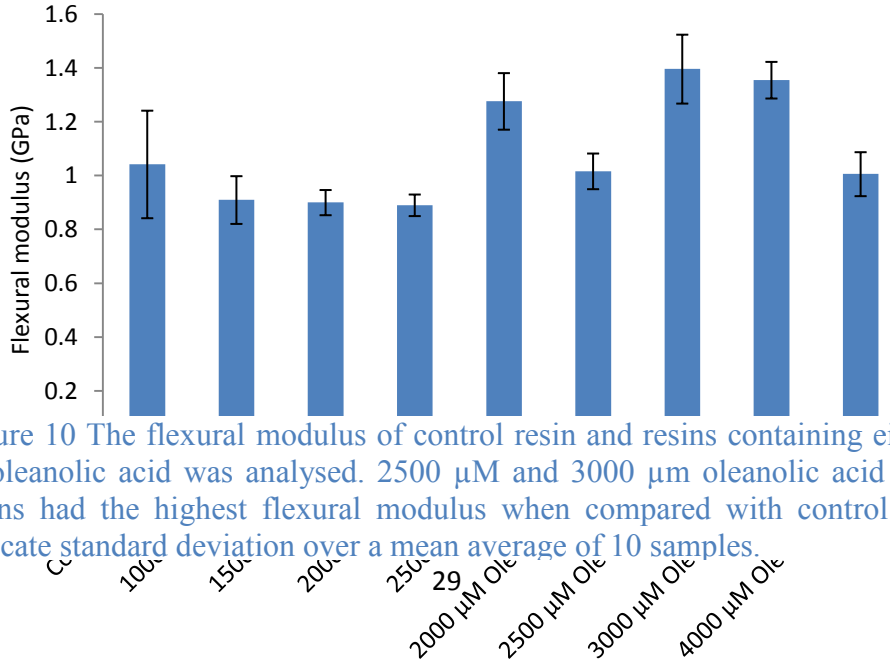

Figure 10 The flexural modulus of control resin and resins containing either tBHQ or oleanolic acid was analysed. 2500 µM and 3000 µm oleanolic acid containing resins had the highest flexural modulus when compared with control.Error bars indicate standard deviation over a mean average of 10 samples.

One-way Anova showed a significant difference in the flexural modulus between control resins and 2500 μM tBHQ and 2000 μM tBHQ containing resins ($p<0.05$). However, there was no difference in flexural modulus between control resin and 1500 μM tBHQ containing resin ($p>0.05$). There was a significant difference in the flexural modulus between control resin and 2500 μM, 3000 μM oleanolic acid containing resins ($p<0.05$). There was no significant difference between control resin and 2000 μM, 4000 μM oleanolic acid containing resins ($p>0.05$) (Fig. 10).

3.4 Hardness testing of resin discs:

The higher the value of the hardness test, the better the resin was cured. The results of hardness testing for each resin are shown in Fig.11. A 3000 μM oleanolic acid containing resin exhibited the highest hardness value, whereas 4000 μM oleanolic acid containing resin had the lowest value compared to control resin

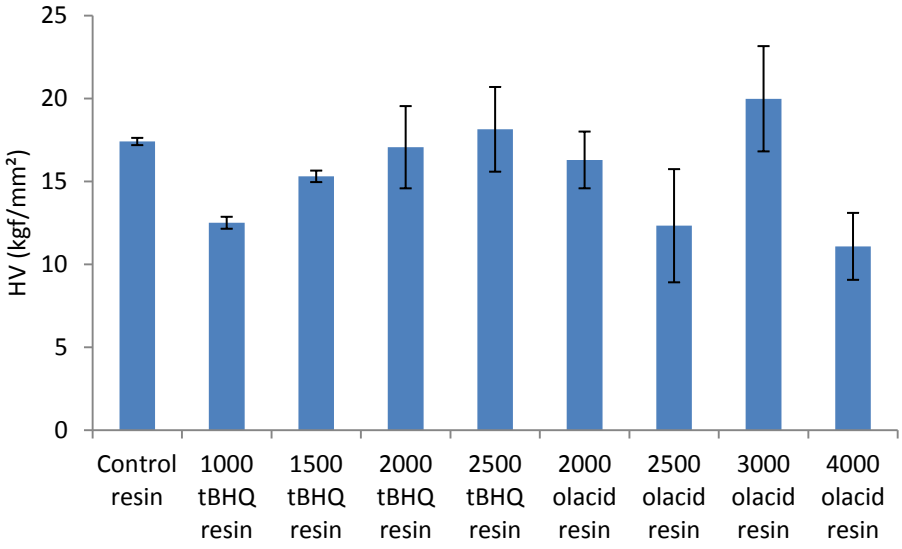

Figure 11The hardness value of control resin and resins containing tBHQ or oleanolic acid was analysed. The highest hardness value was exhibited by 3000 µm oleanolic acid containing resin. Error bars indicate standard deviation over a mean average of 3 samples.

One-way Anova showed a significant difference in the hardness value of control resin when compared to 1000 µM, 1500 µM tBHQ containing resins and 2500 µM, 4000 µM oleanolic acid containing resins ($p<0.05$).

3.5 Cell viability in relation to tBHQ or oleanolic acid supplemented medium:

The response of cells to culture medium supplemented with various concentrations of tBHQ or oleanolic acid was analysed. 5000 cells/well were initially seeded in 6 well plates. Identical concentrations were used for both tBHQ and oleanolic acid supplemented medium: 50, 75, 100, 200, and 1000 µM. Cells grown in these supplemented media were compared with cells grown in normal culture media by cell viability counting and subsequently a growth curve was plotted for the 4 day experimental period. The highest number of viable cells was detected in the presence of 50 µM tBHQ supplemented medium, whereas there was a significant decrease in the viable cells/ml when cells were exposed to 75, 100, 200, and 1000µM tBHQ

supplemented media. When cells were subjected to oleanolic acid supplemented medium similar results were observed as for tBHQ supplemented media, although the number of viable cells was significantly lower compared with cells grown in tBHQ supplemented medium (Figs. 12,&13). 50 µM tBHQ supplemented medium resulted in a considerable increase in viable cell numbers compared with controls (Fig. 12).

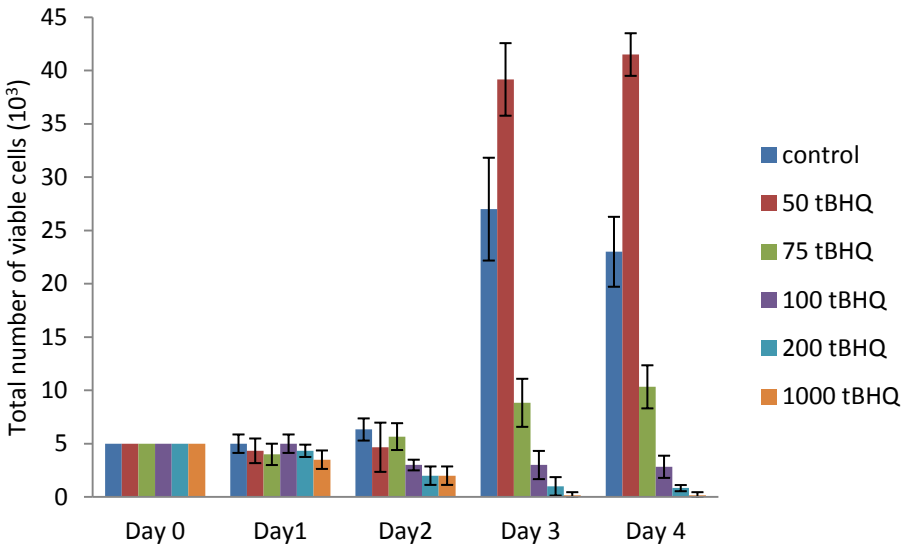

Figure 12 A graph showing cell viability when the cell culture medium was supplemented with various concentrations of tBHQ over 4 days. Cell culture medium supplemented with 50 µM tBHQ significantly increased the number of viable cells. Error bars indicate standard deviation over a mean average of 3 samples.

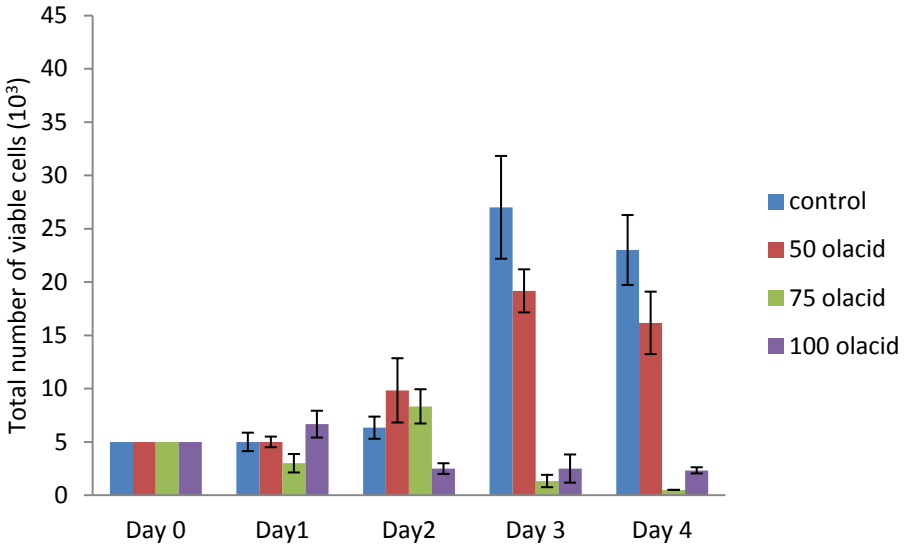

Figure 13 A graph showing cell viability when the cell culture medium was supplemented with various concentrations of oleanolic acid over 4 days. Cell culture medium supplemented with 50 µM oleanolic acid exhibited a slightly lower number of viable cells compared with control. Error bars indicate standard deviation over a mean average of 3 samples.

One-way Anova showed a significant difference in the number of viable cells grown in 75 µM oleanolic acid supplemented medium when compared with cells grown in 100 µM oleanolic acid supplemented medium on day 1. There was no significant difference in the number of viable cells grown in control culture medium and cells grown in medium supplemented with the various tBHQ concentrations on day 1. There was a significant difference in the number of viable cells grown in control culture medium and medium supplemented with 200 µM, 1000 µM tBHQ on day 2. There was a significant difference in the number of viable cells grown in control culture medium and cells grown in medium supplemented with 50 µM, 75 µM, 100 µM, 200 µ, 1000 µM tBHQ and 50 µM, 75µM, 100 µM oleanolic acid on both day 3 and day 4. On day 4, there was no significant difference in the number of viable cells grown in control culture medium and medium

supplemented with 100 µM, 200 µM, 1000 µM tBHQ and 75 µM, 100 µM oleanolic acid.

Subsequently, control resin discs were added to cells grown in both 50 µM tBHQ and 50 µM oleanolic acid supplemented medium. This experiment was performed in triplicate and cells counted using trypan blue staining at 4 time points. The addition of control resin discs to the supplemented medium resulted in a marked decrease in the number of viable cells (Fig.14).

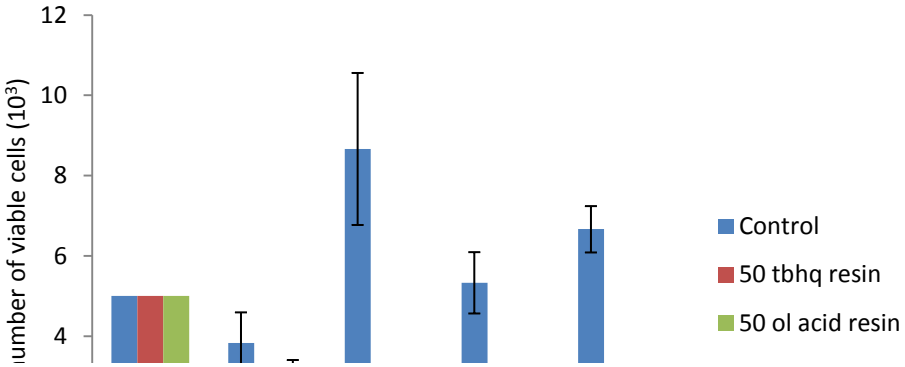

Figure 14The number of viable cells in the presence of discs made from control resin when cultured in supplemented media was analysed. Cell viability decreased significantly with time, even in the presence of tBHQ and oleanolic acid supplemented medium. Error bars indicate standard deviation over a mean average of 3 samples.

3.6 Gene expression analysis:

The expression of selected genes that are regulated by the Nrf2 transcription factor (NQO1, Gclc, and Hmox1) was examined for cells cultured in the presence of either 50 µM tBHQ, or 50 µM oleanolic acid on day 1 and day 2. Expression levels were compared with control cells grown in unsupplemented culture medium. The highest relative expression of NQO1 and Gclc occurred in the presence of 50 µM tBHQ at day 1. However, at day 2, their expression slightly decreased. Control cells exhibited relatively

lower level of expression of Gclc on day 1, which was increased slightly by day 2. Oleanolic acid resulted in a higher expression of Gclc, compared to NQO1 at day 2. At day 1, the level of GAPDH was too low to allow the analysis of gene expression. The levels of Hmox1 gene were too low even after 40 PCR cycles, which may indicate that neither tBHQ nor oleanolic acid activate its expression at this concentration in this cell type (data not shown) (Fig. 15).

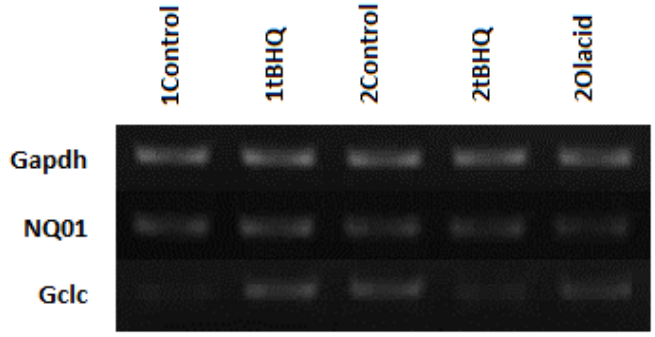

Figure 15 Relative expression levels of NQO1 and Gclc in cells cultured in 50 µM tBHQ or 50 µM oleanolic acid supplemented medium compared to control. The highest expression of both NQO1 and Gclc was found in cells grown in 50 µM tBHQ supplemented medium, at day 1.Samples were normalised against the housekeeping gene, GAPDH, where DNA was obtained from the following samples: 1Control= control BMSCs at day 1; 1tBHQ= BMSCs cultured in 50 µM tBHQ supplemented medium at day 1; 2Control= Control BMSCs cells at day 2; 2tBHQ= BMSCs cultured in 50 µM tBHQ supplemented medium at day 2; 2Olacid= BMSCs cultured in 50 µM oleanolic acid supplemented medium at day 2.

6. Discussion

Dental restorative materials that contain BisGMA/TEGDMA in proportion of 60:40 were examined in this experiment were previously shown to have a highly toxic effect on cells. Incorporation of Nrf2 activators within resins may enable its subsequent release and protective effects. Alternatively the pre-treatment of BMSCs with Nrf2 activators may

provide a route for cell protection. The experiments undertaken in this study were designed to test this reasoning.

4.1 Mechanical properties of dental resin composites:

The polymerisation reaction of the dimethacrylate resins leads to the formation of a stiff material from a highly viscous one, with the formation of a highly crosslinked polymer network. Dental resin composites that are photo-cured usually exhibit low conversion values, due the structure of the polymer network which results in stiffness of reacting species. The degree of conversion of a material such as BisGMA can be improved by the addition of a diluent comonomer such as TEGDMA, which results in easier handling and mixing of the final dental composite (Emami & Soderholm 2009; Palin et al., 2003). Due to lower molecular weight compared with BisGMA, TEGDMA increases the number of covalent bonds formed during the polymerisation reaction. This polymerisation reaction is associated with a tight packing of the monomer molecules (Dewaele et al., 2006) which results in bulk contraction of the resin monomer, which in turn leads to polymerisation shrinkage. This shrinkage can have a negative impact on the mechanical properties of the composite and on the bonding of the composite to the tooth. Polymerisation shrinkage occurs after the addition of TEGDMA due to its higher number of carbon double bonds, which also increases the degree of conversion. After photo-curing, higher viscosity results in a stiffer material. This viscosity results in decreased rate of polymerisation of carbon double bonds.

Due to masticatory forces dental composites are at an increased risk of fracture. This leads to formation of cracks, which negatively impact on their survival rate. A resin composite that has a high hardness value may be able to withstand crack formation or at

least crack dissemination (Curtis et al., 2009).However, crack formation in combination with incomplete polymerisation is associated with erosion of the resin composite and leakage into the nearby tissues, which may lead to pulpal inflammation (Zimmerli et al., 2010).

The findings of this study suggest that the addition of tBHQ or oleanolic acid to the control resin did not significantly affect the mechanical properties of the resins. The FTIR measurements supplied a mechanism for the quantitative analysis of the conversion of dimethacrylate based monomers. A number of factors affect the degree of conversion of a resin composite including the intensity and duration of the curing light and the rate of polymerisation. The flexural modulus and the flexural strength are a result of the degree of conversion of each resin (Palin et al., 2003).

4.2 Viability of cells when grown in tBHQ and oleanolic acid supplemented medium:

Gronthos et al. (2000) showed that dental pulp stem cells formed calcified nodules and have a slow growth rate. BMSCs, however, can form osteoblast-like cells (cells that are required for bone formation) and osteocytes (cells found in bone tissue) which more closely resembles the morphology and function of dentine, and have an increased growth rate. Thus, BMSCs were chosen as the cells to be used in this experiment. The findings of the present study suggest that there is a significant increase in cell viability when BMSCs were grown in culture medium supplemented with 50 μm tBHQ. Cells cultured in supplemented medium containing the higher concentrations of tBHQ and oleanolic acid exhibited significantly decreased viability over the 4 day culture period, with a marked decrease on day 4. As 50 μM tBHQ was shown to increase cell viability, a further experiment examined the effect of the addition of discs made of control resin to cells

grown in this medium. When cells were counted on a 4 day period it was found that the addition of control resin significantly decreased the number of viable cells. This highlights the toxic effect resin discs have on cells, even when the culture medium was supplemented with tBHQ, or oleanolic acid. This may provide a background for the study of the concentrations of these Nrf2 activators to be added to induce cellular protection from the toxicity of dental resin composites.

4.3 Nrf2 pathway and cell viability:

Previous research has demonstrated that oleanolic acid and tBHQ play a role in the activation of the Nrf2 pathway, thus reducing the oxidative stress on cells (Imhoff & Hansen 2010; Reisman et al., 2009). The addition of 50 μM tBHQ to culture medium for BMSCs increased the amount of viable cells, which was previously not reported. The analysis of NQO1, and Gclc gene expression showed that 50 μM tBHQ or oleanolic acid supplementation was able to activate the Nrf2 pathway. Thus, by supplementing the culture medium with these Nrf2 activators may lead to increased protection of cells against dental resin composites. The medium supplemented with 50 μM tBHQ resulted in the highest increase in the expression of NQO1 and Gclc genes, which may provide protection against cytotoxicity produced by dental resins by possibly limiting the production of quinones and reactive oxygen species. A slightly lower expression of both genes occurred in the presence of 50 μM oleanolic acid, thus 50 μM tBHQ may provide better protection against oxidative stress and free radical formation. However, there was either low or no expression of the Hmox1 gene, which shows that this gene is not activated by Nrf2 activators.

4.4 Limitations:

Counting cells with trypan blue has several disadvantages. The number of blue cells increases with time thus cells have to preferably be counted at the same time after addition of the trypan blue (Hannan and Reilly 1988).

4.5 Future work:

This study examined the effect of dental restoration materials and Nrf2 activators on BMSCs. This effect should also be investigated on other types of cells found in the oral cavity such as dental pulp stem cells, which are highly likely to come in direct contact with the dental resin (Gronthos et al. 2000). The marked decrease in cell viability when cultured with resin discs may be due: leakage of monomers in the culture medium, low concentration of the Nrf2 activators used or inability of the Nrf2 activators to be released from the resin discs and thus protect the cells. Future work, should investigate the addition of higher concentrations of Nrf2 activators in the medium of cells exposed to resin discs.

The effect of other Nrf2 activators including sulforaphane, oridonin and oltipraz on BMSCs and cells present within the mouth could also be analysed to find a suitable supplement that may protect cells from the oxidative effects of dental resins. Sulforaphane and oridonin were shown to protect cells against oxidative stress by inducing the activation of the Nrf2 pathway (Du et al., 2008; Shinkai et al., 2006; Thimmulappa et al., 2002). Oltipraz was also shown to induce antioxidant responses in cells (Konwinski et al., 2004; Miao et al., 2003).

There is a potential for biomedical research and clinical application of growing cells in medium supplemented with Nrf2 activators, activators which were found in this study to

greatly increase the number of viable cells *in vitro*. As previously mentioned, the addition of 50 μM tBHQ to the supplemented culture medium for BMSCs significantly increased cell numbers. This may have an impact on clinical application by the development of a supplement containing Nrf2 activators that boost the antioxidant defence mechanisms of cells resulting in increased viability and growth. By being able to isolate the cells a patient requires, rapidly multiply them in culture conditions and transplant them back into the patient this may provide new techniques for stem cell and cell therapy. By growing the cells faster results in shorter times the cells spend outside the body. This shorter period will decrease the risk of these cells becoming contaminated, or changing their phenotype/genotype. It may therefore be beneficial to determine the effects of Nrf2 activators on different cell types.

4.6 Conclusion:

Supplementing the culture medium with Nrf2 activators, tBHQ and oleanlic acid, activated the expression of NQ01 and Gclc in BMSCs which potentially resulted in protection of these cells against oxidative stress. There was no discernible difference between tBHQ and oleanolic acid on the mechanical properties of resins when compared with controls. However, the addition of 50 μm tBHQ to the BMSCs culture medium greatly increased the number of viable cells compared with cells grown in control culture medium. This means that the highest relative expression of NQO1 and Gclc occurred in the presence of 50 μM tBHQ supplemented medium at day 1. 50 μM tBHQ supplemented cell culture medium resulted in the highest number of viable cells when these cells were subjected to control resin at day 4. Therefore, tBHQ provides better

protection against oxidative stress when compared with oleanolic acid, without having a negative impact on the mechanical properties of the resin in a clinical setting.

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3D architectural influences on mesenchymal stem cells co-cultured with H400 epithelial oral cell line using hydrogels

This project is submitted in partial fulfilment of the requirements for the award of the MRes'

Abstract

Introduction: Cells cultured in 2D environments behave differently compared with 3D cultures possibly due to less complex interactions/signalling.

Objective: The main purpose of this study was to determine how different 3D culture environments influenced the behaviour and differentiation of bone marrow stromal cells (BMSCs) when co-cultured with the H400 oral epithelial cell line.

Methods: The viability of BMSCs encapsulated in collagen or alginate gels digested with collagenase or 4% sodium citrate respectively was determined using trypan blue cell counting. Gene expression analysis for osteogenic markers in BMSCs and cytokeratin markers in H400 cells were examined using the semi-quantitative reverse transcriptase polymerase chain reaction (sqRT-PCR).

Results: Fewer viable cells were present in 3D compared with 2D environment. BMSCs encapsulated in alginate and collagen gels exhibited increased expression of osteopontin. There was no difference in the expression of cytokeratin markers for encapsulated H400 cells compared with 2D cultured H400 cells.

Conclusion: BMSC encapsulation in 3D matrices may influence these cells to differentiate along particular lineages providing information for future research for clinical purposes.

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1. Introduction

1.1 3D scaffolds and mesenchymal stem cells

Development of biomaterials for replacing or regenerating injured or diseased tissues has resulted in the emergence of tissue engineering (Ortinau *et al*., 2010), which involves the encapsulation of cells in 3D scaffolds that mimic the extracellular matrix of cells (ECM). Such scaffolds that have been successfully used for cell encapsulation and immobilisation are typically hydrogels including collagen and alginate gels. These 3D hydrogels are similar to the ECM in that they provide physical support and in the case of collagen constitute a major component of soft tissues(Hunt *et al*., 2009;Hunt *et al.,* 2010), whereas alginate is a major component of brown seaweed (Funami *et al.,* 2009). Encapsulation of cells in alginate and collagen gels also impacts on their proliferative capacity, function, oxygen accessibility and metabolic waste removal and this has to be taken into consideration when designing 3D matrices for particular applications. For clinical purposes, vascularisation and angiogenesis should occur in line with replacement of the hydrogel by the host tissue to ensure good oxygen supply in the newly formed tissue, while degradation of the hydrogel should have a minimal toxic effect on cells. Moreover, hydrogels should be able to withstand mechanical forces associated with the *in vivo* environment and have a structure that encourages viability and proliferation of encapsulated cells. By co-culturing different cell types in 3D hydrogels, cellular interaction can occur which results in increased cell growth compared with cells grown in 2D environments that exhibited decreased proliferation (Hunt *et al.,*2009). With this approach, 3D scaffolds have been created from polymers such as alginate and collagen to

provide a suitable environment for stem cell growth and differentiation along desired lineages (Hunt *et al.,*2009; Yan *et al.,* 2010).

Although cells grow more slowly in a 3D environment, these cells are more morphologically similar to cells found inside a given organ (Campbell and Watson 2009). Cell types that have reportedly been cultured in such scaffolds include BMSCs, cardiac, adipocytes and chondrocytes (Lund *et al.,*2008; Wong *et al.,* 2000). BMSCs are a fibroblast-like population of cells found in a variety of tissue types including muscle, bone marrow and blood (Paul *et al.,* 2009) and have been shown to be able to differentiate along many lineages including those of osteogenic, cardiogenic or adipogenic. This differentiation is regulated by a variety of cytokines, growth factors and the extracellular matrix which makes these cells suitable candidates for tissue engineering, regeneration and cell therapy. 3D networks composed of substances like collagen (a major component of the extracellular matrix) and alginate (which provides a favourable environment for cell encapsulation and immobilisation) were found to provide stem cells with the necessary cues to differentiate down certain lineages (Ortinau *et al.,* 2010). Collagen was recently discovered to have a significant impact on the osteogenic differentiation of stem cells (Bidarra *et al.,* 2011;Paul*et al.,* 2009). Thus, stem cells may be encapsulated in these scaffolds with a goal to generating bone tissue repair *in vivo* (Lund *et al.,* 2008; Nishimura *et al.,* 2012).

1.2 Alginate and collagen hydrogels

Unbranched copolymeric polysaccharides such as alginate can be used as scaffolds for tissue engineering to create structures demonstrating biocompatibility for use in bone substitutes, vascular grafts or contact lens. Alginate is made of (1-4) glycosidic linked β-D-mannuronic (M) and α-L-guluronic (G) acid residues (Fu *et al.,* 2011b; Funami *et al.,* 2009; Fundueanu *et al.,*1999; Kakita and Kamishima 2008; Li *et al.,* 2007; Simpson *et al.,* 2004; Wong *et al.,*2001). Alginate interacts with divalent cations to form a 3D structure in three steps: mono-complexation where individual guluronic acid residues form bonds with single calcium ions; dimerisation where two of these units join together; followed by lateral associations of these dimer units in cavities (Fu *et al.,* 2011b). This 3D structure is known as the egg-box model, as the ions are found in cavities analogous to eggs in an egg-box (Fig. 16).

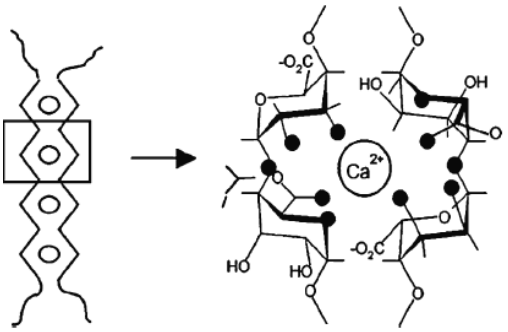

Figure 16The egg-box model of calcium alginate gels (Fu *et al.* 2011a)

These bonds occur in cavities made from the pairing of these guluronic acid residues. This model was first proposed by Grant *et al* (1973) and its structural analysis was performed by X-ray diffraction however the calcium alginate fibres were reduced to the point the crystal structure could not be resolved. To address this problem a new model was required – the parallel and antiparallel model and was proposed by Donati *et al.,* 2005. It was discovered that blocks formed of guluronic-mannuronic acid residues contributed to the formation of the cavity structure together with guluronic acid residues (Li *et al.,* 2007; Simpson *et al.,* 2004). The 3D structure of the alginate gel is affected by the proportion and length of these acid residues, and by the amount of alginate compared

with the number of calcium ions (Kakita & Kamishima 2008; Wong *et al.,* 2001). Cells are encapsulated during the binding of the divalent calcium ions to the guluronic acid residues in the alginate, which results in the formation of a 3D network, suitable for tissue engineering and regeneration (Fu *et al.,*2011b; Funami *et al.,* 2009; Fundueanu *et al.,* 1999; Kakita & Kamishima 2008; Li *et al.,*2007). The molecular composition of the alginate has an impact on the formation of a 3D network because a high amount of guluronic acid residues in the alginate results in the formation of hard gels prone to breakage; whereas a high amount of mannuronic acid results in the formation of a less hard, more elastic gel (Kakita& Kamishima 2008). Additionally, these formed have an impact on cell growth, since increased guluronic monomers in the alginate result in decreased cell viability; whereas increased mannuronic monomers result in increased cell proliferation (Banerjee et al. 2009).

Another polymer that can be used in tissue engineering for cell entrapment is collagen. Collagen is composed of three collagen strands that consist of repeating amino acid units of glycine, proline and hydroxyproline that together form a triple helix structure. This triple helix combines to form fibrous elements that constitute a large part of the extracellular matrix (ECM). Not only does the collagen form a vital structural component of the ECM, but also ensures cell binding, thus making it a suitable material for tissue engineering (Lund *et al.,* 2008; Stahl *et al.,* 2010). The rate of cell proliferation has an impact on gel integrity, since increased cell proliferation may result in the weakening of the structure of these gels.

1.3 Mesenchymal stem cell encapsulation in hydrogels

Designing 3D scaffolds that mimic the *in vivo* environment in which cells reside, requires identification of the interactions and signals that occur between different cell types (Campbell & Watson 2009). This study attempts to generate different 3D architectures using hydrogels comprised of alginate and collagen to analyse the reciprocal influence of different cell populations. Thus, cells were encapsulated in different scaffolds in differentiation medium and also co-cultured with other cell types. Beads of alginate containing cells were incorporated in collagen and alginate gels to study the effect these have on the morphology of both bone marrow stromal cells and also epithelial cells. In addition, cell behaviour was compared between 3D and 2D cultured environments. This study may provide information on 3D cellular arrangements and how these influence cells encapsulated in hydrogels.

2. Hypothesis

The influences of 3D architecture/encapsulation on BMSCs and H400 epithelial cells and co-cultures of BMSCs withH400 epithelial cells within alginate or collagen gels may influence differentiation of these cells compared with a 2D environment. Cellular encapsulation may not significantly adversely affect the mechanical and chemical properties of these gels.

3. Aims

The main purpose of this work was to determine whether 3D cellular arrangements, encapsulation and co-culture of BMSCs with H400 oral epithelial and cardiac cells influence the differentiation potential of stem cells as determined by gene expression analysis. Additionally, the effect of cells on the mechanical properties of these hydrogels was analysed.

4. Materials and Methods

Unless otherwise specified, all materials and reagents were purchased from Sigma Aldrich, UK.

4.1 Culture media:

All media were made under aseptic conditions within a laminar flow hood and stored under sterile conditions at 4^0C.

a-MEM:

α-MEM (Minimum essential medium Eagle, α-modification) was made according to the manufacturer's instructions to a final pH of 7.3 prior to filter sterilising through a nitrocellulose 0.2 µm filter into 500 ml sterile bottles.

DMEM:

DMEM (Dulbecco's Modified Essential Medium Eagle) was made according to the manufacturer's instructions to a final pH of 7.3 prior to filter sterilising through a nitrocellulose 0.2 µm filter into 500 ml bottles.

Transport medium:

Transport medium was made according to the proportions shown in Table 5.

Material	Amount
αMEM	20 ml
Pen/Strep	2 ml
HEPES (1M)	500 µl
Amphotericin (250 µg/ml)	200 µl

Table 5 Chemical components used to make the transport medium, Pen/Strep refers to Penicillin/ Streptomycin(penicilin/100 units/ml, streptomycin/100 units/ml) and HEPES refers to 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethansulfonic acid.

Basal culture medium:

Basal culture medium for BMSCs was made according to the proportions listed in Table 6, before storage in a sterile bottle at 4°C.

Material	Amount
αMEM	60 ml
Pen/Strep	600 µl
HEPES (1M)	1500 µl
Amphotericin(250 µg/ml)	72 µl
L-Glutamine (200 mM)	600 µl
Foetal Calf Serum (10%)	9 ml

Table 6 The constituents used to make the BMSCs culture medium, where Pen/Strep refers to Penicillin/ Streptomycin (penicilin/100 units/ml, streptomycin/100 units/ml) and HEPES refers to 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethansulfonic acid .

Basal culture medium for H400 cells was also made according to the proportions listed in Table 7, before storage in a sterile container at 4°C.

Material	Amount
DMEM	20 ml
FCS (10%)	2 ml
L-glutamine (200 mM)	200 µl
HEPES (1M)	0.5 ml
Hydrocortisone (0.4 mg/ml)	200 µl

Table 7Theconstituents used to make the H400 epithelial cells culture medium

4.2 Isolation of cells

Bone marrow stromal cells were extracted from the femora of 250 g male, Albino Wistar rats (supplied by Aston University, UK). An incision running from the angle of the knee and a lateral, relieving incision were made, to expose the soft tissue. The femur was cleaned of external soft tissues, using a scalpel and subsequently placed in transport medium(Section 4.1). Femora were then placed in a petri dish, and the distal and proximal ends removed using bone cutters. A syringe containing 10 ml of supplemented αMEM culture medium (Section 4.1) was used to irrigate the femur from either end and the resulting liquid containing cells was poured into a fresh universal container. This container was centrifuged (Durafuge 100, Thermo electron corporation) for 3 min at 1200 rpm to pellet the cells. The supernatant was removed and the cell pellets were resuspended in 2 ml culture medium prior to seeding in a T75 flask containing 10 ml culture medium. The BMSCs were allowed to adhere to the flask, by incubating at 37^0C and 5% CO_2(Sanyo, CO_2 incubator)for 24 hours prior to medium changes every 2 days (Yan *et al.,* 2010).

Immortalised H400 oral keratinocytes, a human oral squamous cell carcinoma were also used (Prime *et al.,* 1990).

4.3 Gel synthesis:

- Collagen gels:

During the synthesis of collagen gels, all the reagents and materials were kept on ice to prevent thermal gelation of collagen. Prior to collagen addition, 10DMEM and reconstitution buffer were made as follows: 10XDMEM was made using 10.20g DMEM, 3.70g sodium bicarbonate and 100 ml dH_2O and the reconstitution buffer was made using 2.20g sodium bicarbonate, 4.77g HEPES and 0.05M sodium hydroxide. The reagents were mixed as per proportions shown in Table 8, with collagen being the last added to avoid premature gelation. Bubble formation was minimised by slowly pipetting the collagen along the wall of the reaction vessel.

Material	Amount
10XDMEM	0.15 ml
Reconstitution buffer	0.15 ml
3mg/ml Collagen	1.20 ml

Table 8Theconstituents used to make the collagen gels.

For the water equilibrium content, gene expression analysis, confocal microscopy and histology experiments, the cell suspension was added at a concentration of 5×10^5 cells per ml of this collagen solution. As sodium hydroxide is toxic to cells, BMSCs were added to the collagen solution after collagen addition (Lund *et al.,* 2008). The cell/collagen solution was immediately transferred to 24 well plates, with 1 ml collagen/cell suspension solution per well. For gel formation these well plates were

transferred to the incubator for 40 min. For the co-culture experiments, H400 cells were seeded on the top surface of these gels and culture medium was added prior to incubation.

- Alginate gels

Two types of alginate were used: a non-medical and a medical grade alginate. The concentrations used for both types of alginate were 3% and 5% (w/v). Both of these solutions were made by mixing the required concentrations of alginate sodium powder in distilled water on a magnetic stirrer at 55°C (Kakita & Kamishima 2008).

These solutions were then placed in a fridge prior to downstream testing. A concentration of 0.1 M $CaCl_2$was used to cross link the alginate (Fundueanu *et al.,* 1999; Simpson *et al.,* 2004). The non-medical grade alginate was used only for mechanical testing to identify whether there were any differences in its mechanical properties when compared with the medical grade alginate. For the water equilibrium content, mechanical testing, gene expression analysis and confocal microscopy, the cell suspension was added at a concentration of 5×10^5 cells per ml of each concentration of medical grade alginate solution. Filter paper soaked in $CaCl_2$ was placed on the bottom of each 24 well plate, prior to addition of the cell/alginate solution, with 1 ml alginate/cell suspension solution per well. Filter paper soaked in $CaCl_2$was also added on top of the alginate/cell solution. $CaCl_2$ was added to these solutions to cross-link the alginate in order to encapsulate cells in a 3D matrix. The cross-linked gels containing cells were then transferred to 12 well plates and culture medium added. For the co-culture experiments, H400 cells were seeded on top of these solutions after transfer of gels to 12 well plates and culture medium was added prior to incubation.

- Alginate beads:

Alginate beads were synthesised by dropwise addition of alginate into a 0.1M aqueous calcium chloride bath. The space used between the alginate and the calcium chloride beads was about 6 cm, because beads containing short tails occurred at a smaller distance, whereas beads distortion was noticed with longer distances (Fundueanu *et al.,* 1999). These beads were made of an alginate-cell mixture by mixing 5×10^5 cell suspension with 3% alginate, which were subsequently dropped in 0.4% aqueous calcium chloride (Martinsen *et al.,* 1989). These resulted in the instantaneous formation of cells encapsulated in a 3D network (Fundueanu *et al.,* 1999). Beads were then transferred to each alginate or collagen gel solution. The beads were transferred to the alginate gel before cross linking it by adding 1 ml of 0.4 % calcium chloride solution. These beads were then incubated at 37^0C in a 5% CO_2 atmosphere.

4.4Digestion of collagen and alginate gels:

The collagen and alginate gels were digested with collagenase (Invitrogen, UK)and 4% sodium citrate respectively for cell release. Released cells were mixed with culture medium to dilute and inactivate the collagenase or 4% sodium citrate. Cells were collected by centrifugation at 3000 rpm for 3 min, prior to downstream analysis.

4.5 Cell culture

All feeding of cells was performed in the laminar flow hood. The culture medium was removed using a pipette, and replaced with fresh culture medium. The cells were also checked visually for bacterial or fungal contamination using a phase contrast microscope (Fluovert, Leitz).

4.6 Trypan blue staining

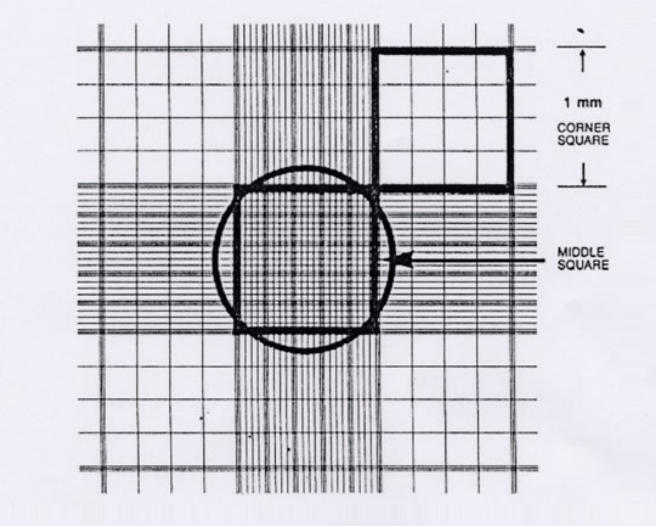

Figure 17Haemocytometer grid seen using the phase contrast microscope (Wong et al., 2001).

Viable cells were counted using the Trypan blue staining procedure, a dye that is excluded from cells that have intact membranes (McGuigan *et al.,* 2008). Cells were released from collagen and 3% alginate gels through either collagenase or sodium citrate treatment. The number of viable cells was determined by staining these cells with 0.4% Trypan blue added to an equal volume of cell suspension. Viable cells were counted using a Neubauer haemocytometer and the phase contrast microscope. The haemocytometer and the cover slips were cleaned using 70% ethanol. The cleaned cover slip was moistened with exhaled breath prior to placing it on the haemocytometer. Approximately 10 µl of cell suspension/trypan blue stain was pipetted at one edge of the cover slip, so that it ran under the cover slip. The haemocytometer grid was then visualised with a phase contrast microscope where the viable cells appeared bright and colourless, whereas the dead cells appeared blue. Both viable and dead cells were counted in the 4 large, corner squares of the grid and an average taken (Fig.17). Using the following equation the total number of viable cells in 1 ml was calculated:

$$TC = x/4 * 2 * 10^4,$$

where TC was the number of total viable cells in 1 ml; x was the average of the cell counts from the squares of the haemocytometer grid, 2 was the dilution factor (1:1

dilution of cell suspension and trypan blue stain) and 10^4 represented the conversion factor (Wong*et al.,* 2001).

4.7 Live/dead staining and confocal microscopy:

The culture medium was removed from alginate and collagen gels containing cells and these gels were washed with 10% PBS (phosphate buffered saline). The washed gels were then placed in cell stain double staining kit (Fluka Analytical, UK) containing 10-calcein acetoxymethylester (calcin-AM), 5-propidium iodide and 5 ml PBS for 15 minutes at 37^0C. This had the effect that live cells appeared green and dead cells were red, when visualised using a Zeiss LSM 700 Meta Confocal Microscope. Then, ImageJ software (National Institutes of Health, USA) was used to determine the proportion of viable cells compared with dead cells in these hydrogels.

4.8Rheometry

Due to gels being impossible to immobilise on the bottom plate of the rheometry was undertaken on liquids. The rheological properties of collagen and alginate solutions (with and without cell addition) were determined using the Bohlin Gemini Rheometer. The shear stress and shear rate were determined under a controlled stress of 11 Pa at 1Hz frequency, for the calculation of viscosity of each cellular and acellular medical alginate and non-medical alginate. The rheology testing of each alginate sample was performed using 60 mm cone plate geometry of a 2^0angle at a steady temperature of 25^0C. The cone was lowered onto the surface of the alginate solution placed on the bottom plate (Yan *et al.,* 2010).

4.9Mechanical testing:

The analysis of the gels resistance to load involved the formation of alginate and collagen gels as described above (Section 4.1). Failure was initiated by compressive stress, when the gels were subjected to loading on a universal testing machine (Instron 5544, UK). By producing a load-deformation plot, the resistance to load was compared for each alginate sample prepared as per alginate gels (section 3.1) without addition of cell suspension. The failure point indicated the flexibility of each sample, which depended on the amount of cross-linking of the alginate. Each alginate sample was placed centrally on a platen, covered with sandpaper to prevent slippage, and another platen was used to provide the compressive force at a cross head speed of 1 mm/min (Fig. 18). The width of each alginate disk was approximately 6.5 cm (measured with a digital meter). Ten samples were tested for each concentration of both medical and non-medical grade alginate and an average taken. Five samples were tested for each concentration of medical grade alginate gel containing cells.

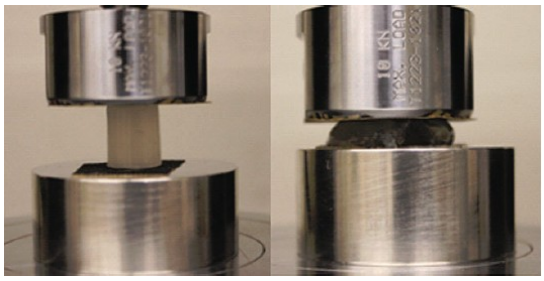

Figure 18 Experimental set-up for mechanical testing of gels (Fu *et al.*,2011a).

4.10 Equilibrium water content:

Three samples were tested for each alginate concentration, for both medical and non-medical grade alginate, for alginate gels containing cells, for acellular and cellular collagen gels and an average determined. One ml of alginate or collagen was used for

each sample. Prior to freeze drying the samples were washed in 10% PBS three times to remove any uncross linked solution. The wet weight was recorded using a digital balance (Mettler AE163) to obtain the swollen weight (W_S). The samples were then freeze-dried for 3 days, prior to weighing to obtain the dry weight (W_d). The swollen and dry weight were then used to calculate the equilibrium water content, using the following equation:

$$EWC\ (\%) = [\frac{W_s - W_d}{W_s}]$$

(Banerjee *et al.,* 2009;Fundueanu *et al.,* 1999).

4.11Semi-quantitative RT-PCR analysis

Celllysis and RNA isolation:

For gene expression analysis, 5×10^5 bone marrow stromal cells were seeded inside collagen or alginate gels, with some gels having 3×10^5 H400 cells seeded on the surface. These were compared with the expression of genes in 5×10^5 BMSCs and 5×10^5 H400 cells cultured in a 2D environment (T25 flask). A further experiment analysed the expression of cardiac differentiation genes in BMSCs cultured in 5-Azacitidine supplemented medium compared with BMSCs cultured in normal culture medium in a 2D environment. RNA was isolated using the Rneasy Kit (Qiagen, UK) and all reagents were kept on ice prior to use. The effect of encapsulation of BMSCs or H400 cells in collagen or alginate gels on the expression of stem cell genes, osteogenic and cytokeratin differentiation genes, respectively was investigated. Gene expression was analysed on day 11, when cells were released from the collagen or alginate gel, centrifuged at 1200 rpm for 3 minutes, the supernatant discarded and cell pellets washed in 1 ml PBS. Cells were repelleted by centrifugation, the supernatant was removed, and 350 µl RLT lysis

buffer was used to resuspend the cell pellet, which was then placed in a 1.5 ml Eppendorf tube and stored at -80^{0}C prior to further downstream processing. 350 µl of 70% ethanol was added to the pelleted cells after the cells were homogenised. This was transferred to RNeasy spin column in 2 ml collection tubes. The sample was centrifuged at 10,000 rpm for 30 sec, the flow through was discarded; then 700 µl of Buffer RW1 was added to the column, and spun again at 10,000 rpm for 30 sec. The flow through was discarded, 500 µl added to the spin column, followed by centrifugation at 10,000 rpm for 2 min. The effluent was discarded and the column placed in a new collection tube and centrifuged at full speed for 1 min. The column was then placed in a 1.5 ml collection tube and 30 µl of RNase free water added to the membrane. This tube was centrifuged at 10,000 rpm for 1 min to elute the RNA. Following isolation, the RNA was quantified using a Biophotometer (Eppendorf, UK). 2 µl RNA and 68 µl RNase-free water were added to a cuvette and a reading obtained which represented the concentration of RNA in µg/ml for each sample.

RNA visualisation:

All substances were kept on ice for the duration of the experimental set-up. A 1% agarose gel was made by mixing 0.7 g of agarose in 70 ml 1XTAE buffer (Qiagen, UK) in a conical flask. The mixture was heated until boiling within a microwave oven and subsequently cooled under running tap water. 3 µl of SYBR Gold (used to stain RNA) was added to the agarose solution and mixed prior to pouring into the casting tray. A well forming comb was inserted and the gel allowed solidifying at room temperature. An electrophoresis tank was filled with 1XTAE, and the cast gel was placed in the tank. The well forming comb was carefully removed. Each RNA sample was made up to a total

volume of 6 µl, by mixing 1 µl of RNA from each sample with 5 µl of RNA loading buffer (Invitrogen, UK). The samples were loaded into the wells of the agarose gel. Samples were electrophoresed at a voltage of 120V for approximately 30 min. Gel images of RNA samples were captured using the Genesnap software (SynGene, UK).

Reverse Transcription:

All the samples and reagents were kept on ice prior to use. Reverse transcription was performed using the Omniscript Kit (Qiagen, UK). Each conversion required the use of 2 µg of RNA; however, the maximum volume that could be used was 12 µl. Twelve microliters of RNA or RNA plus RNase-free water (to make up a volume of 12 µl) was added to a clean Eppendorf tube. Two microliters of Oligo-dT of 10 µM stock was added to each sample to make a final concentration of 1 µM. The samples were then heated at 80°C for 10 min in a heating block. The reverse transcription mastermix was made by mixing the following: 2 µl of 10X buffer RT, 2 µl of 5 mM stock of dNTP, 1 µl of 10 µl stock of RNase inhibitor and 1 µl of omniscript reverse transcriptase. The samples/oligodT tubes were removed from the heating block and quenched on ice for 5 min. Six microliters of mastermix was added to each sample tube, which was mixed briefly by vortexing and centrifuged to collect the residual liquid. All the samples were then incubated at 37°C for 60 min, and at 95°C for 5 min. The samples were then placed on ice prior to cDNA concentration using Microcon filters (Millipore, UK). Water was added to the cDNA to make a total volume of 500 µl. This was transferred to a spin column, and spun at 10,000 rpm for 2 min. The level of the liquid was visually checked, and the sample was re-spun at 8,000 rpm for 1 min. At this point, there should have been

a volume of less than 50 µl within the tube. The column was inverted in a collection tube and spun at 800 rpm for 1 min. cDNA was stored at -20^{0}C prior to further use.

PCR analysis:

cDNA levels were normalised against the housekeeping gene GAPDH using the REDTaq Ready PCR Mix. A forward and reverse primer mastermix was prepared by adding 10 µl of each reverse and forward primers(25 µM)(Invitrogen, UK) to 60 µl RNase free water, to make a final dilution of 1:4 of the forward and reverse primer. The REDTaq master mix was made by adding 12.5µl REDTaq (Sigma, UK) and 2 µl Forward and Reverse Primer Mastermix to 9.5 µl RNase free water. 24 µl of REDTaq master mix was added to 1 µl cDNA in a PCR tube, for each cDNA sample. These were then transferred to a Mastercycler gradient (Eppendorf) for DNA amplification prior to visualisation using an agarose gel. The cycles used were as follows: 94°C for 5 min, 94 °C for 20 sec (1), 60°C for 20 sec (2), 68°C for 20 sec (3), then cycles (1) to (3) were repeated 27 times followed by 72°C for 10 min. The agarose gel was made by mixing 0.9gagarose with 60 ml 1XTAE buffer to give a final concentration of 1.5% agarose. Four µl of GelRed (this was used to stain the DNA) was added to the gel before it was poured in the tray as described above. The samples (6µl/well) were loaded together with a DNA ladder (Invitrogen, UK) (3 µl) in one well as control. Amplified products were electrophoresed for approximately 30 min at 120V. Gel images of DNA samples were captured using the GeneSnap software. GeneTool (SynGene) software was used to assign relative values for each amplified product based on pixel density.

4.12 Statistical analysis

Data analysis was performed by one-way Anova using statistical software (Minitab, UK). A difference of $P<0.05$ was considered statistically significant. Anderson-Daarling test was used to determine whether the data followed a normal distribution. Tukey's post hoc test was used for pair wise comparison at a significance value of $P<0.05$.

5. Results

5.1 Cell viability:

An increase in the digestion time of both alginate and collagen gels resulted in a decrease in the number of viable cells, with the highest decrease in cell viability occurring when gels were subjected to a 30 min digestion time. This trend was observed throughout the cell culture counting period on days 2, 6 and 11 as shown in Fig.19.

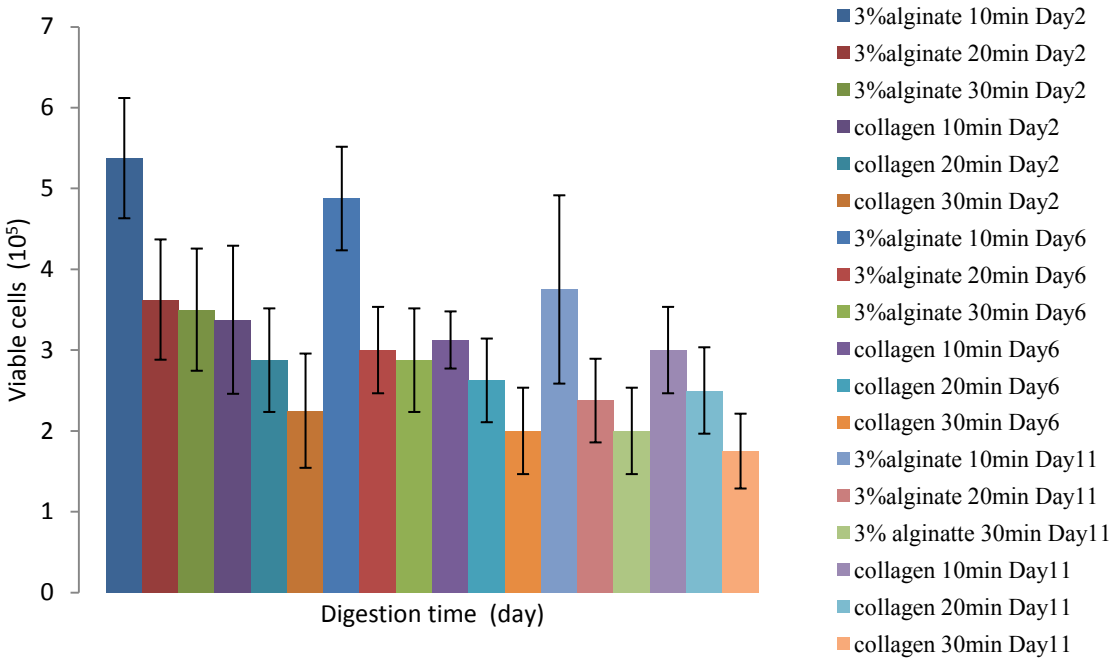

Figure 19 Total number of viable cells when seeded within collagen or alginate gels at days 2, 6 and 11. Collagen was digested by collagenase and alginate was digested by sodium citrate (4%) for 10 min, 20 min, and 30 min. A decrease in total number of cells was observed with increased digestion time. Data shown are the result of triplicate measurements ± SD.

One-way Anova showed a significant difference in cell viability when cells were encapsulated in alginate gels which were digested with 4% sodium citrate for either 10 or 30 min at day 2 and day 6 compared with day 11 ($p<0.005$). One-way Anova showed a significant difference in cell viability when cells were encapsulated in alginate digested

with 4% sodium citrate for 20 min between day 2 and day 11 ($p=0.002$). However for 20 min digestion time, there was no difference in cell viability between day 6 and day 11 ($p>0.05$). There was no significant difference observed when cells were encapsulated in collagen and digested with collagenase for either 10, 20 or 30 min at day 2,6 or 11 ($p>0.05$).

5.2 Confocal microscopy

Alginate and collagen gels containing cells were stained for viability and visualised using confocal microscopy which revealed a more round morphology of encapsulated cells compared with cells seeded on the top surface of the hydrogels. This may be due to the 3D environment and higher mechanical forces that take place inside the hydrogel which may immobilise cells in the 3D matrix compared with cells seeded on the top surface which were more spread and flattened. When the proportion of viable and non-viable cells was analysed using the ImageJ software on the bottom and top surface of the gels (as shown in Fig. 20), it was found that there was an increased number of viable cells on the top surface of the gels, with a significant decrease in the number of viable cells on the bottom surface of these gels (Fig. 20). This pattern was observed throughout the gels tested.

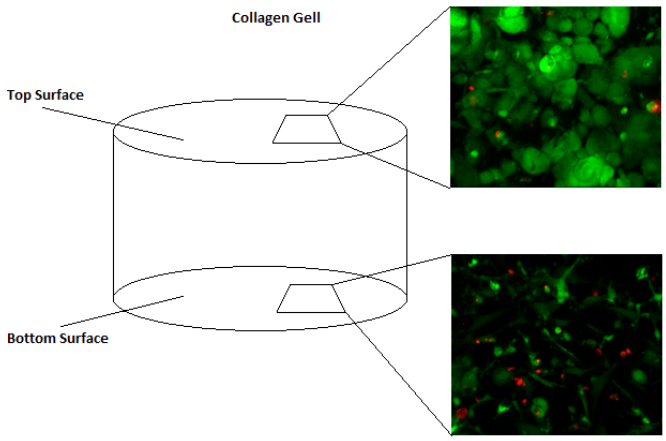

Figure 20 Graph showing an example of cell viability on a section of the top and bottom surface of a collagen gel, where green=viable cells and red=non-viable cells.

5.3 Rheology:

The 3% medical and non-medical grade alginate solutions exhibited typical Newtonian behaviour because the viscosity was independent of shear rate as shown in Fig. 21. However, the addition of cells to the medical grade alginate gave this material a pseudoplastic behaviour because its resistance to shear decreased with an increase in shear rate. As the shear rate increased, the viscosity decreased. This is typical for materials exhibiting pseudoplastic behaviour like cellular 3% medical grade alginate as shown in Fig.21.

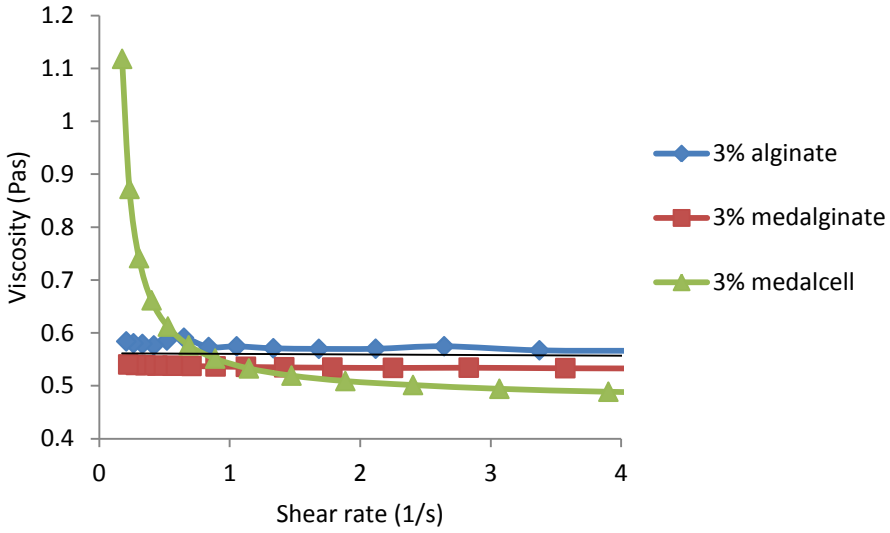

Figure 21 Comparison of the rheology of 3% alginate, 3% medical grade alginate (3% medalginate) and 3% medical grade alginate containing cells (3% medalcell). The 3% medical and non-medical grade alginate solutions exhibited Newtonian behaviour whereas 3% cellular medical grade alginate solution exhibited pseudoplastic behaviour.

As shown in Fig. 22, 5% alginate exhibited Newtonian behaviour, because the viscosity was independent of time under shear rate, whereas both cellular and acellular medical grade alginate solutions exhibited pseudoplastic behaviour (also known as thixotropic behaviour) as viscosity decreased as a function of time.

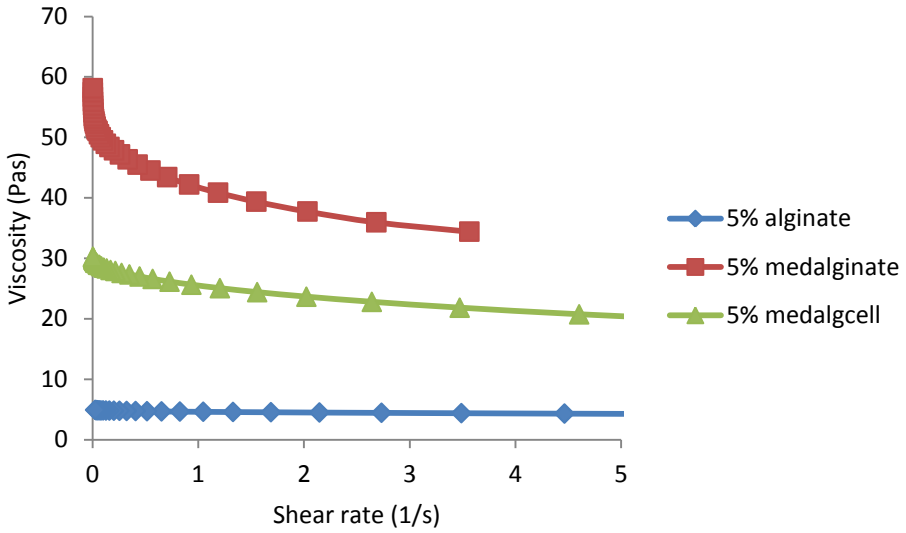

Figure 22 A comparison of viscosity of 5% medical grade alginate (5% medalginate) and 5% medical grade alginate containing cells (5% medalginate cell). The addition of cells had no effect on the behaviour of the medical grade alginate as both cellular and acellular alginate solutions exhibited a pseudoplastic behaviour, whereas the non-medical grade alginate solution exhibited a Newtonian behaviour.

As shown in Fig. 23&24, the plot of shear stress vs. shear rate of both non-medical and medical grade alginate was a straight line, which represented typical behaviour of a Newtonian fluid. As shown in Fig. 23, the medical grade alginate had a higher viscosity compared with the non-medical grade alginate. The addition of cells to 3% medical grade alginate led to a decrease in the viscosity of this material, however the decrease in viscosity was less apparent than with the 3% non-medical grade alginate.

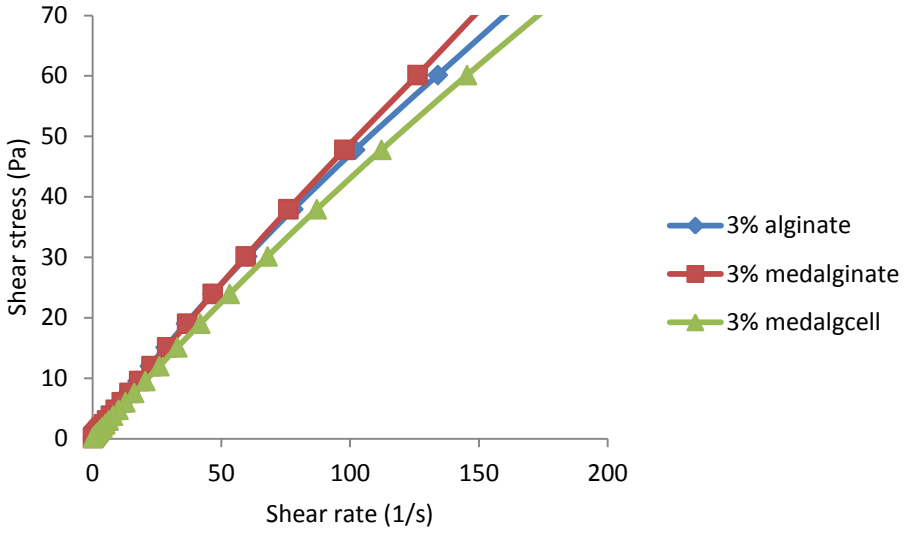

Figure 23 Comparison of viscosity of 3% alginate, 3% medical grade alginate (3% medalginate) and 3% medical grade alginate containing cells (3% medalgcell). The addition of cells to the medical grade alginate resulted in this solution exhibiting the lowest resistance to shear stress, which means that cell addition lowers the viscosity of the medical grade alginate.

As shown in Fig.24, the medical grade alginate had a higher viscosity compared with the non-medical grade alginate. The addition of cells to 5% medical grade alginate led to a decrease in the viscosity of this material, however the decrease in viscosity was less apparent than with the 5% non-medical grade alginate.

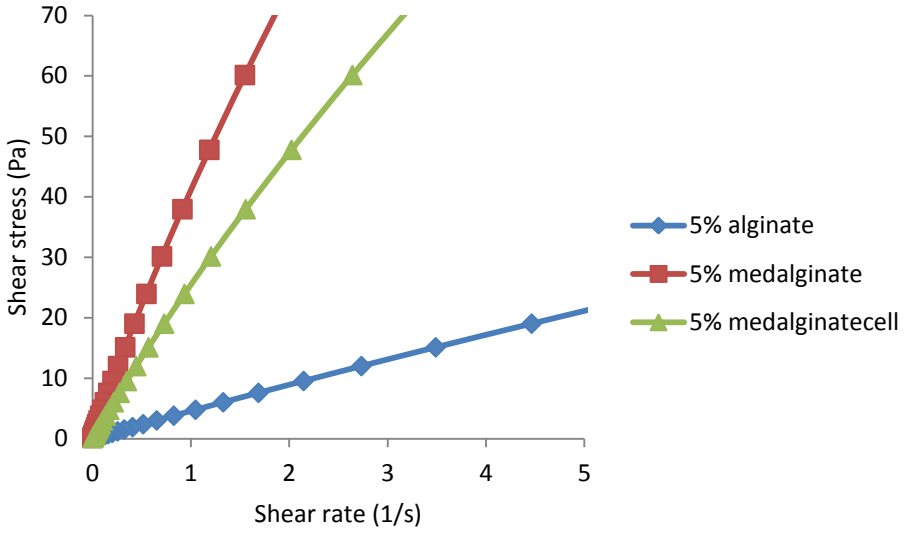

Figure 24 A comparison of viscosity of 5% alginate, 5% medical grade alginate (5% medalginate) and 5% medical grade alginate containing cells (5% medalginatecell). The addition of cells to the medical grade alginate resulted in this solution exhibiting the lower resistance to shear stress, which means that cellular medical grade alginate was less viscous than acellular medical grade alginate, with the non-medical grade alginate exhibiting the lowest resistance to stress thus the lowest viscosity.

5.4Mechanical testing:

When the deformation was compared across the concentrations of medical grade alginate it was found that 5% medical grade alginate was more resistant to deformation compared with the 3% medical alginate. The addition of cells to both concentrations of medical grade alginate resulted in the formation of a weaker gel which was more prone to breakage as shown in Fig. 25. One way Anova showed a significant difference between acellular and cellular medical grade alginate and between the 5% and 3% medical grade alginate gels ($p<0.005$).

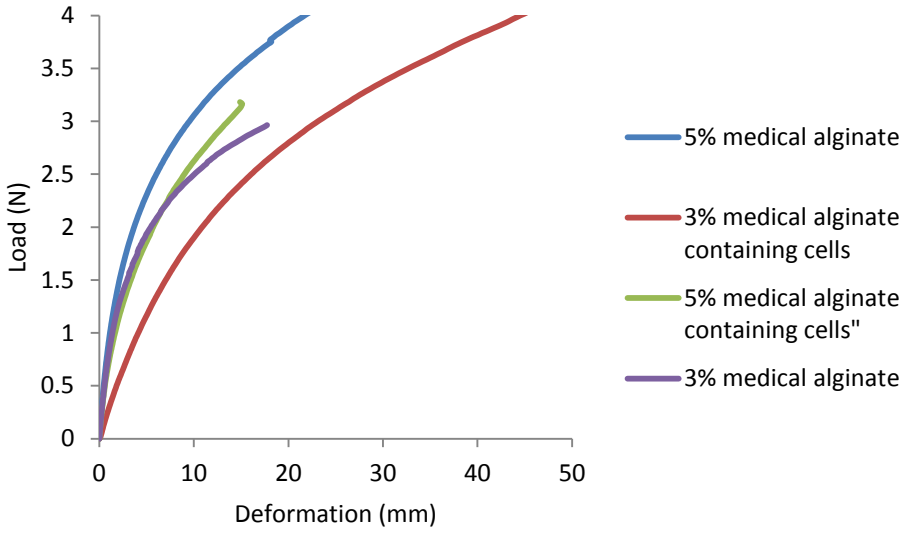

Figure 25 A comparison of mechanical strength of medical grade alginate and medical grade alginate containing BMSCs at alginate concentrations of 3% and 5%, when subjected to load. The gels containing cells exhibited a lower compression modulus (less resistant to deformation under applied load) when compared with gels containing no cells.

When the deformation was compared between both concentrations of the medical and non-medical grade alginate, the medical grade alginate was shown to be more resistant to deformation compared with the non-medical grade alginate as shown in Fig. 26. One way Anova showed a significant difference between medical and non-medical grade alginate ($p<0.005$).

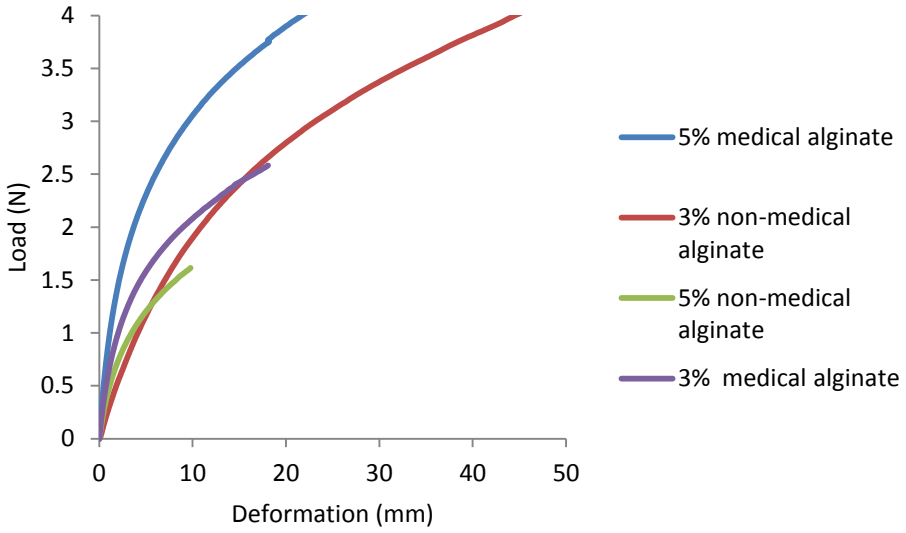

Figure 26 Comparison of the compressive modulus of medical grade alginate and non-medical grade alginate at concentrations of 3% and 5%. The 5% medical grade alginate exhibited a higher compressive modulus (more resistant to deformation under applied load) compared with the 3% medical grade alginate.

5.5Water equilibrium content:

When the equilibrium water content (EWC) was analysed across gels, the medical grade alginate exhibited a higher EWC compared with the non-medical grade alginate. The addition of cells resulted in hydrogels which exhibited a lower EWC when the medical grade alginate was compared with the non-medical grade alginate as shown in Table 9. Addition of cells to the collagen gels led to a decrease in the EWC, as shown in Table 10. The appearance of swollen and dry gels is depicted in Fig. 27.

Figure 27 Photographs of swollen and dry gels, with dry gels exhibiting shrinkage due to loss of liquid following freeze drying.

Weight	3% MA		3% MAC		5% MA		5% MAC		3% NMA		5% NMA	
	SW	DW	SW	DW	SW	DW	SW	DW	SW	DW	SW	DW
g	0.71g	0.02g	0.88g	0.07g	0.70g	0.03g	0.80g	0.09g	0.70g	0.02g	0.43g	0.02g
EWC		96.35		91.14		95.12		88.55		96.44		93.45

Table 9The equilibrium water content of cellular and acellular medical grade alginate compared with non-medical grade alginate, where MA=medical grade alginate, MAC=medical grade alginate containing cells, NMA=non-medical grade alginate, SW=swollen weight, DW=dry weight, EWC=equilibrium water content. The weight of swollen and dry gels was obtained in grams. Data shown are the result of triplicate measurements ± SD.

Weight	Col		ColC	
	SW	DW	SW	DW
g	0.89	0.01	0.99	0.02
EWC		97.84		97.05

Table 90The equilibrium water content of cellular and acellular collagen, where Col=collagen gel, ColC=collagen gel containing cells, SW=swollen weight, DW=dry weight, EWC=equilibrium water content. The weight of swollen and dry gels was obtained in grams. Data shown are the result of triplicate measurements ± SD.

One-way Anova showed a significant difference between both concentrations of medical and non-medical grade alginate in both the swollen and dry state ($p<0.001$). There was also a significant difference between cellular and acellular medical grade alginate in swollen and dry state ($p<0.001$). There was a significant difference between cellular and acellular collagen in the swollen state ($p<0.001$), with no significant difference between cellular and acellular collagen in the dry state ($p=0.095$).

5.6 Gene-expression analysis:

The impact of hydrogel 3D architecture on BMSC behaviour and osteogenic differentiation was assessed by RT-PCR. As shown in Fig. 28 (A), osteopontin was expressed in all samples. The relative expression of collagen-1α was also analysed as collagen was not only used in the formation of hydrogels but also has an important function in bone formation, with BMSCs exhibiting low or minimal levels of collagen-1α. When these samples were analysed for mesenchymal stem cell marker expression, it was found that Sox2 or Oct3 were not detected. The relative expression of PCNA was detected throughout all samples, with the lowest expression occurring in BMSCs encapsulated in 3% alginate gel. When the relative expression of the same genes were analysed in BMSCs cultured in a 2D environment, there was no expression of osteopontin detected, relatively low expression of collagen-1α, Sox2 and Oct3, with very

low expression of PCNA. The co-culture of BMSCs with H400 epithelial cells in collagen and 3% medical grade alginate resulted in BMSCs exhibiting a minimal expression of both osteogenic and proliferating markers compared with BMSCs encapsulated in 3% medical grade alginate, which exhibited the highest up-regulation of these marker types.

The H400 cells encapsulated in alginate and collagen gels were analysed for expression of cytokeratin marker gene expression and levels were compared with those in H400 cells cultured in a 2D environment. H400 cells encapsulated in collagen gel and H400 cells seeded on the top surface of 3% alginate gel, co-cultured with BMSCs encapsulated in this gel exhibited an up-regulated expression of cytokeratins-5, -6, -10 and had a lower expression of cytokeratin-13, with low or no expression of desmoglein 3 being detected compared withH400 cells cultured in a 2D environment. When the expression of such genes was analysed in H400 cells cultured in a 2D environment it was found that these cells expressed cytokeratins -8, -9 and desmoglein-3, with relatively low expression levels of cytokeratin-10 and no expression of cytokeratin-5 detected (Fig. 28 (B)). Therefore, relative expression of cytokeratin markers was up-regulated in H400 oral epithelial cells encapsulated in collagen or co-cultured with BMSCs in 3% medical grade alginate gel compared with H400 cells cultured in a 2D environment.

Thus, RT-PCR data showed a decrease in the relative expression of osteogenic, proliferating and cytokeratin markers when BMSCs were co-cultured with H400 cells in a 3D environment compared with BMSCs or H400 cells encapsulated in a 3D matrix.

BMSCs were also cultured in culture medium supplemented with 5-Azacitidine and analysed for cardiac differentiation, which showed that these BMSCs expressed high

levels of myogenin D and low levels of myogenic differentiation D markers. The expression of these cardiac differentiation genes were compared with expression of same genes in BMSCs cultured in culture medium, where no expression of either myogenin D, or myogenic differentiation D was detected (Fig.28 (D).

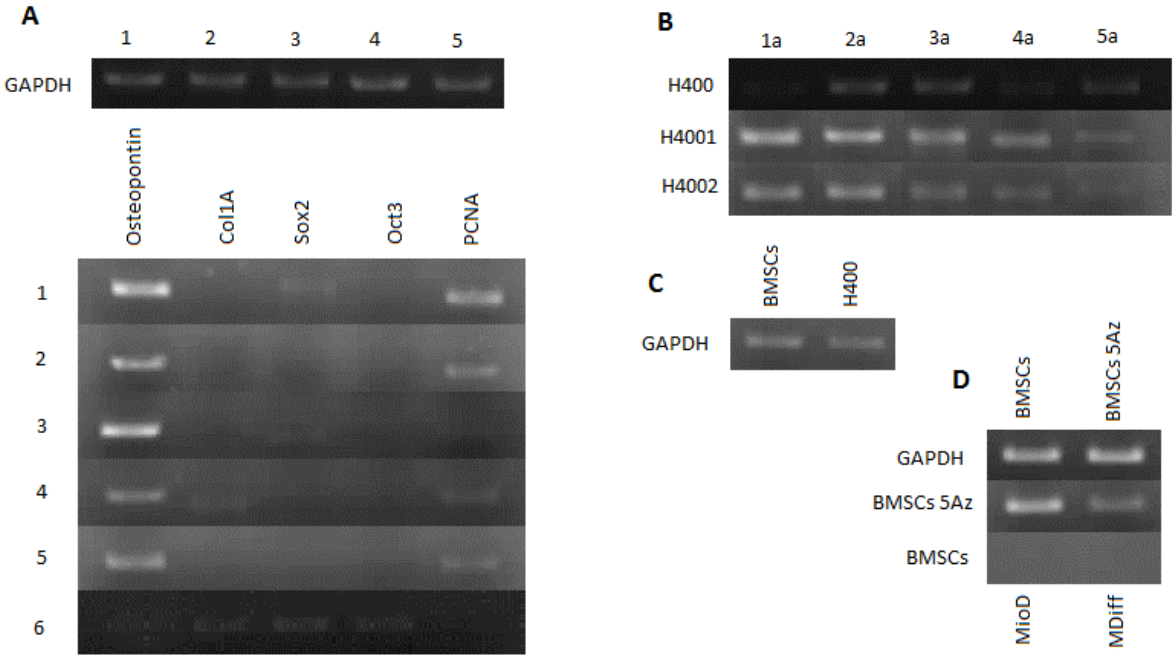

Figure 28 Gene expression analysis. (A) Samples were normalised against the housekeeping gene, GAPDH, where cDNA was obtained for the following samples: 1= BMSCs encapsulated in a collagen gel; 2= BMSCs encapsulated in a collagen gel, co-cultured with H400 on top of the gel; 3= BMSCs encapsulated in 3% alginate gel; 4= BMSCs encapsulated in 3% alginate gel, co-cultured with H400 on top of the gel; 5= BMSCs encapsulated in 5% alginate gel; 6= BMSCs cultured in the 2D environment. The relative expression of osteogenic markers (of osteopontin, collagen-1α (col1A)), mesenchymal stem cell markers (Sox2 and Oct3) and proliferating cell nuclear antigen (PCNA) was investigated.(B)Samples were normalised against the housekeeping gene, GAPDH, where DNA was obtained from the following samples: H400= H400 cells cultured in a 2D environment; H4001= H400 cells encapsulated in a collagen gel; H4002= H400 cells seeded on top of a 3% alginate gel, co-cultured with BMSCs encapsulated in this gel. The relative expression of cytokeratin differentiation markers including cytokeratin 5 (1a); cytokeratin 6 (2a); cytokeratin 10 (3a); cytokeratin 13 (4a); and desmoglein 3 (5a) was analysed.(C) Samples were normalised against the housekeeping gene, GAPDH, where DNA was obtained from BMSCs and H400 cells cultured in a 2D environment.(D) Samples were normalised against the housekeeping gene, GAPDH, where DNA was obtained from the following samples: BMSCs cultured in culture medium (BMSCs),BMSCs cultured in 5-Azacitidine (BMSCs 5Az) supplemented culture medium. The relative expression of cardiac differentiation markers, myogenin D (MioD) and myogenic differentiation D (MDiff) were analysed.

6. Discussion

The experiments performed demonstrated that the structure of the alginate gel used (medical or non-medical grade) as well as the concentration (3% or 5%) had an impact on not only the mechanical characteristics but also on the cell viability and gene expression of BMSCs and H400 cells.

6.1 Cell viability

The findings of the present study suggest that there is a decrease in cell viability when alginate and collagen gels are subjected to digestion for increased periods of time (20, 30 min when compared with 10 min digestion time). Cells encapsulated in alginate had a higher viability rate compared with cells encapsulated in collagen gels. There was a significant decrease in viable cells when alginate gels were digested with sodium citrate for 10 minutes compared with alginate gel digestion for 20 and 30 minutes (the 20 and 30 minute digestion time had a similar but small decrease in cell viability). Cell viability when cells were encapsulated in collagen showed a steady decrease as a function of digestion time. The decrease in the number of viable cells with the increase in digestion time of collagen and alginate gels may imply that the solutions used to digest these matrices might have a toxic effect on cells. The alginate digestion by sodium citrate had an overall less toxic effect on cells than collagen digestion by collagenase. Confocal microscopy analysis showed that there was a decrease in the number of viable cells in the bottom surface of the gels which may be due to poor nutrient, oxygen supply and inefficient metabolic waste removal.

6.2 Rheology:

Both the concentration of alginate and the amount of guluronic or mannuronic acid residues have an impact on the viscosity of these materials. There was no significant difference between the 3% medical grade and non-medical grade alginate, both exhibiting a typical Newtonian behaviour, where viscosity is independent of the shear rate. However, the addition of cells to the 3% medical grade alginate resulted in this material moving from a Newtonian to a pseudoplastic behaviour because an increase in the shear rate was observed with an increase in the shear stress. The 3% cellular medical grade alginate exhibited a significant increase in viscosity at low shear rate, whereas a slight increase in shear rate resulted in a massive decrease in viscosity. This shows that the addition of cells negatively impacted on the viscosity of the material. The viscosity of acellular medical grade alginate solutions was independent of the shear rate, exhibiting a constant viscosity of 0.6 Pas. An increase in fluidity was observed with a decrease in viscosity for 5% alginate when force was applied, a behaviour typical for a tixotropic material. The decrease in resistance with a decrease in viscosity may be due to the different ratios of guluronic or mannuronic acid residues found in medical and non-medical grade alginate. Thus, 3% alginate was a Newtonian fluid, whereas 5% alginate was a pseudoplastic fluid.

6.3Mechanical testing and water equilibrium content

The high content of mannuronic acid residues in the medical alginate resulted in the formation of gels that had a higher compressive modulus compared with the non-medical alginate, which had a higher content of guluronic acid residues. The 5% medical alginate was more resistant to deformation than the 5% non-medical alginate due to a higher

cross-linkage, nonetheless after 2.5 Newton load in the 3% medical grade alginate, this behaviour was reversed and the non-medical grade alginate became more resistant to deformation than the 3% medical grade alginate. Further research could identify the nature of this behaviour, by testing alginate concentrations in between the ones tested in this experiment and performing X-ray crystallography to check whether the cross-linking of alginate has a unique behaviour at this concentration. A slight decrease in compression modulus was observed when cells were added to the medical grade alginate; however the decrease was less than for the non-medical grade alginate. Up to 2.5 Newton load the addition of cells to the 5% medical grade alginate gave it an increased flexibility comparable with the 3 % medical grade alginate. The addition of cells had no impact on the relative rate of deformation of the gels, when the same concentration of cellular and acellular medical alginate were analysed. This means that cells affected the flexibility, not the failure rate of gels (Fu *et al.,*2011; Funami *et al.,* 2009; Fundueanu *et al.,*1999; Kakita & Kamishima 2008). Thus, by performing mechanical and rheological tests on medical grade alginate gels containing various densities of encapsulated and co-cultured cells, may lead to development of hydrogels for specific implantations in host tissues.

6.4 Gene expression analysis:

The differentiating ability of BMSCs is regulated through the external environment and cellular interaction. This study analysed the osteogenic potential of BMSCs encapsulated and co-cultured with H400 epithelial cells in collagen or alginate gels. The ability of BMSCs to differentiate along the osteogenic lineage may imply a decreased level of expression of stem cell markers as suggested by results in Fig. 28, where an up-regulation in the expression of osteopontin (normally up-regulated in the mineralisation stage of

bone formation (Tataria *et al.,* 2006)) was related with a minimal expression of Sox-2 and OCT3 stem cell markers. There was also a low expression of collagen-1α in BMSCs co-cultured with H400 epithelial cells in 3% medical grade alginate, and a minimal expression of collagen-1α in the other hydrogels which implies that the expression of collagen-1α had a negative impact on the bone formation ability of BMSCs. There was also an increase in the relative expression of PCNA in the encapsulated BMSCs compared with the cultured BMSCs which showed the greater proliferative potential of these cells. Nonetheless, co-culturing BMSCs with H400 epithelial cells had a negative impact on the ability of BMSCs to differentiate down the osteogenic lineage as shown in Fig.13. However encapsulating BMSCs in a 3D environment is not enough for a full lineage differentiation as the interaction of BMSCs with the hydrogel also has an impact on the BMSCs differentiation ability. For example, BMSCs encapsulated in 3% medical grade alginate showed increased up-regulation of osteopontin compared with BMSCs encapsulated in 5% medical grade alginate which showed that an increased cross-linking of alginate by calcium ions negatively impacted on the differentiation ability of these BMSCs. These 3D hydrogels have to allow encapsulated cells to interact with host cells *in vivo* for a successful implantation in clinical applications.

The way BMSCs attached to hydrogels had an impact on their behaviour and on how they interacted with other cells. BMSCs encapsulated in 3% medical grade alginate exhibited the highest level of gene expression of osteopontin and minimal expression of SOX2 and OCT3 which may suggest that the structure of these hydrogels had an impact on the osteogenic differentiation of BMSCs compared with control BMSCS cultured in a 2D environment. This trend was also observed in H400 oral epithelial cells encapsulated in

collagen gel which exhibited an up-regulation of cytokeratins -5,-6,-10,-13 and desmoglein-3 compared with control cells cultured in a 2D environment. Keratin-5 is expressed in early epithelial differentiation, thus, an up-regulation of Keratin-5 in H400 epithelial cells encapsulated in collagen, or co-cultured with BMSCs in 3% medical grade alginate (as in Fig. 28), demonstrated the ability of H400 cells to express early cytokeratin markers (Alam *et al.,* 2011). The cultured cells exhibited relatively minimal cytokeratin-10 expression, whereas the encapsulated cells had a higher expression of this gene. This demonstrated potential epithelial differentiation of the encapsulated H400 cells because cytokeratin-10 is expressed in early epithelial differentiation. Thus, encapsulated BMSCs and H400 cells show differentiation potential compared with BMSCs and H400 cells cultured in a 2D environment.

The supplementation of BMSC culture media with 5-Azicitidine led to expression of cardiac specific genes within these BMSCs including myogenin and myogenic differentiation D, which showed the ability of BMSCs to become cardiomyocite-like cells. Future studies could be undertaken to confirm the differentiation of BMSCs down the cardiomyogenic lineage and to attempt hydrogel encapsulation of BMSCS supplemented in a 5-Azacitidine culture medium followed by determination of gene expression for cardiomyogenic markers at different time periods. Possibly a western blot analysis can be conducted with antibodies against specific cardiogenic markers such as desmin α-actinin, troponin T and β-myosin heavy chain (Haghani *et al.,* 2012).

Conclusively, the design of a suitable 3D environment for BMSCs encapsulation should take into consideration the viscosity, resistance to deformation, architecture, chemical

properties, the effect of degradation of the hydrogel *in vivo* and how these properties affect cellular response.

6.5 Limitations

Counting cells following trypan blue staining has one main disadvantage. The number of blue cells increases with time thus cells have to preferably be counted at the same time after addition of the trypan blue (Hannan and Reilly 1988).

6.6 Future work

Other mature cell types could be co-cultured with BMSCs encapsulated in such hydrogels in an attempt to identify whether this encapsulation will stimulate the BMSCs to differentiate along a certain lineage. As osteopontin expression may be induced by organic phosphates including β-glycerophosphate (Tuan *et al.,* 2003), future work could determine the effect these phosphates play on the osteogenic differentiation of BMSCs. Further studies could also determine the optimal co-culture conditions and structure of the hydrogels needed to promote BMSC differentiation for clinical applications. The ability of BMSCs to secrete growth factors and cytokines and to suppress the immune system could be analysed to determine the impact on the repair of the host tissue when these BMSCs are engrafted *in vivo*. Different concentrations of sodium citrate and collagenase solutions could be tested to identify a method to digest the alginate and collagen gels respectively to release the highest number of viable cells (Hunt *et al.,* 2010).

6.7Conclusion

Encapsulating BMSCs in alginate and collagen 3D matrices may influence these cells to differentiate along particular lineages. This differentiation ability of BMSCs was indicated by cells expressing osteogenic differentiation markers when encapsulated in collagen or alginate gels with or without co-culture with H400 cells and no expression of mesenchymal stem cell markers. The addition of these cells appeared to only exert a small impact on the mechanical properties by slightly decreasing the resistance to load of such gels. Therefore, a suitable alginate matrix for cell immobilisation in a clinical context is one that can withstand the mechanical forces encountered *in vivo* and whose viscosity allows the optimum interaction between encapsulated cells and host cells. BMSCs encapsulation in such networks may lead to the development of novel materials that can be used in the field of tissue engineering and regenerative medicine.

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