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Preliminary investigation of novel bone graft substitutes based on strontium–calcium–zinc–silicate glasses

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Abstract Bone graft procedures typically require surgeons to harvest bone from a second site on a given patient (Autograft) before repairing a bone defect. However, this results in increased surgical time, excessive blood loss and a significant increase in pain. In this context a synthetic bone graft with excellent histocompatibility, built in antibacterial efficacy and the ability to regenerate healthy tissue in place of diseased tissue would be a significant step forward relative to current state of the art philosophies. We developed a range of calcium–strontium–zinc–silicate glass based bone grafts and characterised their structure and physical properties, then evaluated their in vitro cytotoxicity and in vivo biocompatibility using standardised models from the literature. A graft (designated BT109) of composition 0.28SrO/0.32ZnO/0.40 SiO_2 (mol fraction) was the best performing formulation in vitro shown to induce extremely mild cytopathic effects (cell viability up to 95%) in comparison with the commercially available bone graft Novabone® (cell viability of up to 72%). Supplementary to this, the grafts were examined using the standard rat femur healing model on healthy Wister rats. All grafts were shown to be equally well tolerated in bone tissue and new bone was seen in close apposition to implanted particles with no evidence of an inflammatory response within bone. Complimentary to this BT109 was implanted into the femurs of ovariectomized rats to monitor the response of osteoporotic tissue to the bone grafts. The results from this experiment indicate that the novel grafts perform equally well in osteoporotic tissue as in healthy tissue, which is encouraging given that bone response to implants is usually diminished in ovariectomized rats. In conclusion these materials exhibit significant potential as synthetic bone grafts to warrant further investigation and optimisation.

1 Introduction

At present, 90% of bone graft procedures employ either autograft or allograft bone [1, 2]. Whilst these materials are suited for the repair of bone tissue, they have inherent limitations associated with their use. Autografts (harvested from a second site on the patient) provide a source of tissue that is biologically active, and carries no risk of rejection upon implantation [3]. However, harvesting bone from a second site on a patient results in increased operative time, blood loss, and post operative pain; with major complications reported at a rate of 9% and minor complications reported at a rate of 21% [4]. The preferred alternative to autograft bone is to use allograft bone (banked human bone). Allografts offer similar characteristics to autograft bone, but do not contain osteogenic cells [5]. However, allografts also have a number of significant disadvantages.

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Primary amongst these is their limited supply [6], lack the osteoinductive potential, variable bone quality [7] and the risk of disease transmission; in 2002 the centres for disease control reported 26 cases of bacterial infection associated with allografts [8]. In addition to bacterial infection, viral contamination can also occur; and despite the risk of such infections being low transmission of HIV 1 from sero-negative cadaver donors has occurred [9].

In order to overcome the disadvantages associated with autograft and allograft bone a variety of artificial materials have been developed for use as synthetic bone grafts. Ideally such materials should be biocompatible, show minimal fibrotic reaction, undergo remodelling, support new bone formation [5], be intrinsically antibacterial and offer therapeutic effects for the treatment of diseased or damaged tissue. Current state of the art synthetic materials for bone grafting include calcium phosphate materials like synthetic hydroxyapatite (HA), β-Tricalcium phosphate (β-TCP), as well as calcium sulphates and bioactive glasses. However, none of these grafts currently meet the requirements of synthetic bone graft substitute. For example calcium phosphates such as β-TCP grafts undergo rapid resorption via dissolution and fragmentation in as little as 12 weeks [10]. Consequently there is insufficient persistence of the scaffold to encourage bone apposition, and/or destabilization of the newly formed bone due to scaffold disintegration. Furthermore, the rapid dissolution can trigger an inflammatory response due to elevated levels of degradation products from the scaffold [11]. Alternatively, calcium sulphate grafts can be used, but again these resorb in as little as 5–7 weeks [12], thus experience similar problems to β-TCPs. In addition neither genre of the above materials have intrinsic antibacterial efficacy, and offer no therapeutic effect on host tissue.

Of interest in this regard are the bioactive glasses. These materials are composed of sodium oxide, calcium oxide, phosphorus pentoxide, and silicon dioxide [13] and have resorption rates that can be tailored by varying the glass composition. Bioactive glasses can also form a chemical bond with bone through the formation of a calcium phosphate layer at their surface [14]. These materials exert control over osteoblast genes and as such regulate osteogenesis and the production of growth factors [15]. However, whilst they have been successfully used as a graft expander in dental and maxillofacial surgery [16] they are perceived by some surgeons to offer no advantage over other synthetic bone graft substitutes [5], with lack of antibacterial efficacy being cited as a significant area of concern [17]. It would be beneficial if a synthetic bone graft substitute was available to surgeons that would have built in antibacterial properties, and was based on elements that could illicit a therapeutic effect on surrounding host tissue.

With these features in mind we have developed a new range of calcium–strontium–zinc–silicate (CaO–SrO–ZnO–SiO_2) glasses for implantation in the human skeleton. Previous publications by the authors have highlighted the antibacterial efficacy of zinc ion release (Zn^{2+}) from hard tissue biomaterials [18] which, when coupled with evidence that zinc can increase DNA in osteoblasts and decrease bone resorption activity [19] indicate that zinc (Zn) release from Zn-based glass grafts will likely provide beneficial therapeutic effects in vivo. In addition to the inclusion of Zn in the glass grafts, strontium (Sr) has also been incorporated into the glass structure. Sr has an affinity for bone and is incorporated into it by surface exchange and ionic substitution. Recently, Sr-based drugs have been used in the treatment of osteoporosis where Sr^{2+} has been shown to inhibit bone resorption and stimulate bone formation. In-vitro studies have shown that Sr based drugs inhibits osteoclast activity whilst stimulating osteoblast proliferation [20]. It is perceived that the combined release of Zn^{2+} and Sr^{2+} from silicon based glasses will provide synergistic therapeutic effects to improve bone health at the implant site for all patients including those suffering from diseases like osteoporosis, whilst minimising the risk of primary deep infection at the implant site due to the established antibacterial nature of the Zn^{2+} and Sr^{2+} ions [18, 21].

2 Materials and methods

2.1 Glass synthesis

Three CaO–SrO–ZnO–SiO_2 glasses were synthesized (Table 1). Appropriate amounts of analytical grade calcium carbonate, strontium carbonate, zinc oxide and silicon dioxide (Sigma Aldrich, Dublin, Ireland), were weighed out in a plastic tub and mixed in a ball mill for one hour, then dried (100°C, 1 h). The pre-fired glass batches were then transferred to platinum crucibles for firing (1480°C, 1 h). Glass melts were subsequently quenched into water and the resulting frits were dried, ground and sieved to retrieve a <45 μm glass powder for subsequent analysis in vitro. Additionally, a particle size distribution of 90–350 μm was prepared for the in vivo analysis. Novabone® (Novabone Products, Alachua, Florida, USA) bone graft was used (as supplied) as a control material for the in vitro assessment of the novel glasses prior to implantation in the small animal trial. Novabone® is a bioglass comprising CaO, Na_2O, P_2O_5 and SiO_2 and is used clinically for bone repair in dentistry, oral and maxillofacial surgery and orthopaedic surgery and was considered to be an appropriate control in terms of biological acceptability and form (particulate).

Table 1 Glass compositions (mol fraction)

Glass designation	SiO_2	ZnO	CaO	SrO
BT 107	0.40	0.32	0.28	0
BT 108	0.40	0.32	0.14	0.14
BT 109	0.40	0.32	0	0.28

2.2 Thermal characterisation of glasses

A combined differential thermal analyser-thermal gravimetric analyser (DTA-TGA, Stanton Redcroft STA 1640, Rheometric Scientific, Epsom, England) was used to measure the glass transition temperature (T_g) of each glass. A heating rate of 10°C min^{-1} (up to 1000°C) was used in an air atmosphere with alumina as a reference in a matched platinum crucible.

2.3 Structural characterisation of glasses

The network connectivity (NC) of the glasses was calculated with Eq. 1 using the molar compositions of the glass.

$$NC = \frac{\text{No. BOs} - \text{No. NBOs}}{\text{Total No. Bridging species}} \quad (1)$$

where NC = Network Connectivity, BO = Bridging Oxygens, NBO = Non-Bridging Oxygens

2.4 X-ray diffraction

Powdered samples of each glass were pressed to form discs (32 mm ∅ × 3 mm). Ethyl cellulose was used as a backing material. Samples were analysed using $CuK_{\alpha 1}$ radiation emitted from an Xpert MPD Pro 3040/60 X-ray diffraction unit (Philips, Eindhoven, Netherlands).

2.5 Surface area determination

In order to control the amount of surface area exposed to extracts during the in vitro biological evaluation the specific surface area of each glass (<45 μm) was determined using the advanced surface area and porosimetry, ASAP 2010 System analyser (Micrometrics Instrument Corporation, Norcross, USA). 300 mg of each <45 μm glass was used to calculate the specific surface areas using the Brunauer-Emmett-Teller (BET) method.

2.6 Preparation of extracts

10 g of each glass were autoclaved to ensure sterility. Tissue culture water (Sigma Aldrich, Dublin, Ireland) was selected as the solvent to prepare extracts. An equivalent surface area of 1 m^2 of each glass ($n = 3$) was aseptically immersed in 10 ml of sterile tissue culture water and incubated (37°C ± 2°C). ISO10993 part 5 [22] recommends an incubation period of at least 24 h (37°C ± 2°C); however, to simulate longer periods in vivo and to examine the effect of extraction time on cytotoxicity, specimens were stored for 1, 5 and 30 days. After each storage period, for each specimen, 100 μl aliquots of extract were removed and used for cytotoxicity analysis.

2.7 In vitro assessment of extracts

The established cell line L-929 (American Type Culture collection CCL 1 fibroblast, NCTC clone 929) was used in this study as required by ISO10993 part 5 [22]. Cells were maintained on a regular feeding regime in a cell culture incubator at 37°C/5% CO_2/95% air atmosphere. The culture media used was M199 media (Sigma Aldrich, Ireland) supplemented with 10% foetal bovine serum (Sigma Aldrich, Ireland) and 1% (2 mM) L-glutamine (Sigma Aldrich, Ireland). The cytotoxicity of glass extracts was evaluated using the Methyl Tetrazolium (MTT) assay in 6 well plates. 100 μl aliquots of undiluted extract were added into wells containing L929 cells in culture medium in triplicate. The prepared plates were incubated for 24 h at 37°C/5% CO_2. Next, the cells were subjected to the MTT assay by replacing the supernatant with 500 μl/well of fresh tissue culture medium. The MTT assay was then added in an amount equal to 10% of the culture medium volume/well. The cultures were then re-incubated for a further 2 h (37°C/5% CO_2). Next, the cultures were removed from the incubator and the resultant formazan crystals were dissolved by adding an amount of MTT Solubilization Solution (10% Triton x-100 in Acidic Isopropanol. (0.1 n HCl)) equal to the original culture medium volume. Once the crystals were fully dissolved, the absorbance was measured at a wavelength of 570 nm. 100 μl aliquots of tissue culture water were used as controls, and cells were assumed to have metabolic activities of 100%.

2.8 In vivo assessment of glass grafts

The in vivo assessment of the grafts took part in two stages in order to asses:

1. The healing response of bone to each of the glasses in healthy juvenile animals. A poor response to a material in this model would preclude further study.
2. The healing response in osteoporotic rats where it has been shown that the bone response to healing is compromised. The best performing material (*identified in stage one and/or the* in vitro *experiments*) would be used in order to ascertain whether or not these glasses

are worthy of further development as bone substitutes in osteoporotic or other compromised bone.

2.9 Stage one: assessment in femurs of healthy juvenile rats (healing model of bone repair)

Twenty-four immature female Wistar rats aged 4–6 weeks were used for this study (under license from the UK home office) and were housed in groups under standard laboratory conditions. Samples of each material were implanted into the right femur under aseptic conditions. Animals were anaesthetised using Isoflurane in oxygen; the right femur was exposed using sharp and blunt dissection and a 1 mm diameter defect created in the mid-shaft using a number 5 round stainless steel dental bur kept cool with sterile saline. Implant material were autoclaved (for sterilisation) and prior to implantation the test materials were washed in 70% alcohol and then washed 3 times in phosphate buffered saline; this had the effect of removing dust and moistening the materials made them easier to manipulate; granules (90–350 μm in diameter) of a test material or Novabone® were placed into the bone defect and gently packed prior to closing the wound with resorbable sutures; a single dose of carprofen was administered via intra-peritoneal injection at a dose and volume appropriate to the size of the animals. Animals were allowed to recover and maintained and monitored daily for 4 weeks prior to sacrificing using a schedule one method. The right femur of each animal was dissected free and placed in 4% formaldehyde. The femurs were demineralised using formic acid and cut into blocks prior to processing to paraffin embedded sections stained with haematoxylin and eosin.

2.10 Stage two: assessment in femurs of adult ovariectomised rats

The experimental material comprised granules of glass designated BT109 which had been selected on the basis of the results from the in vitro assessment. Twelve female rats were used for this part of the study, 6 animals were ovariectomised and 6 sham operated, (without the removal of the ovaries) at 3 months of age 6–7 weeks prior to implantation of BT109. The surgical protocol and tissue processing were the same as described in stage one.

3 Results

3.1 Glass characterization

The glasses synthesized in this work maintained a constant SiO_2 to ZnO ratio, whilst the CaO content was incrementally replaced by SrO; since CaO and SrO are considered as network modifiers [23, 24] the CaO/SrO substitutions ensure that the NC remains fixed at a value of 1 for each glass (Table 2).

Table 2 Glass transition temperature (T_g), network connectivity (NC), and specific surface area for each glass

Glass	T_g (°C)	NC	Surface area ($S_{BET}/m^2\ g^{-1}$)
BT 107	680	1.00	1.27
BT 108	680	1.00	1.64
BT 109	698	1.00	1.19

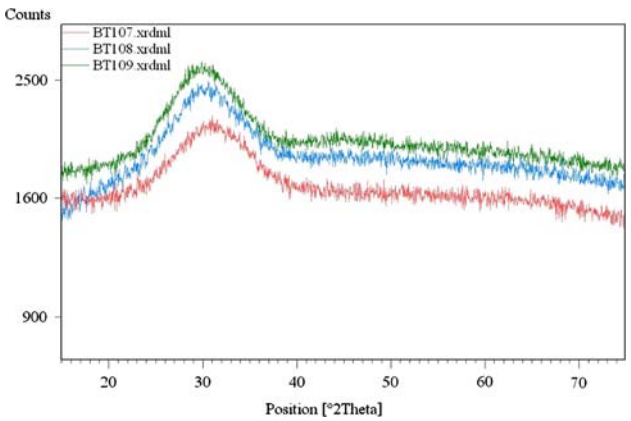

Fig. 1 X-ray diffraction patterns for glasses BT107–109

Separately, the structure (as investigated using XRD; see Fig. 1) of each glass showed no evidence of residual crystallinity, indicating each glass is completely amorphous. However, whilst the T_g values for BT107 and BT108 are identical (Table 1), there is a marked difference (+18°C) in the T_g of BT109.

3.2 In vitro assessment of glasses

The cytotoxicity of each experimental graft examined herein was determined using the ISO approved MTT assay on extracts ($n = 3$) obtained after 1, 5 and 30 days incubation in tissue culture water at 37°C (±2°C). The controls for this assay were tissue culture water (100% cell viability) and Novabone (commercial glass graft). The results obtained indicate each glass, including Novabone, induce a mild to moderate cytotoxic response (Fig. 2) when derived extracts from each material are exposed to L929 mouse fibroblasts.

For BT109 and Novabone the mean cell viability remained constant over the each time period, 85% and 68% respectively. From the MTT assay it is clear that BT109 exhibits a better in vitro response than all of the glasses examined including the Novabone.

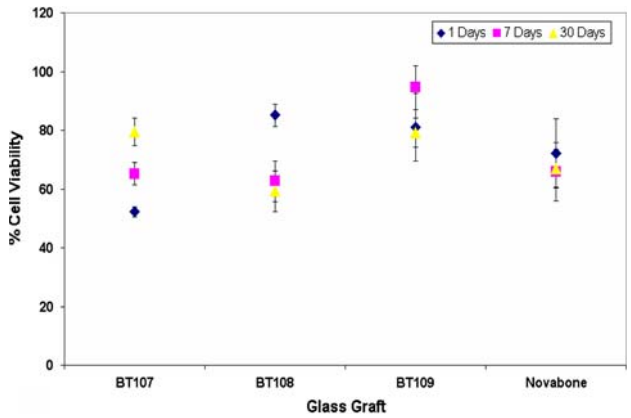

Fig. 2 Cell viability as a function of time for each glass graft

3.3 In vivo assessment of glasses

3.3.1 Stage one: assessment in femurs of healthy juvenile rats (healing model of bone repair)

All animals survived surgery with no clinical evidence of adverse reaction although 2 femurs appeared to have developed a callus at the surgical site; this was not obvious clinically, but only became apparent on radiographic examination carried out as part of the monitoring of the demineralisation process.

3.3.2 Histological examination

Particles of test material could be visualised optimally by altering the sub-stage condenser of the microscope. They were viewed at a variety of magnifications and appeared as small angular, pale glassy particles usually slightly out of plane of the section. The particles themselves were aggregated and occupied angular spaces in the medullary space and cortical bone (Fig. 3). In all but one animal particles had escaped into the surrounding soft tissue and there was evidence of a localised foreign body reaction with macrophages, scattered giant cells and fibrosis No evidence of inflammation in terms of increased vascularity, chronic inflammatory cells or neutrophils was seen. The cortex of the femurs healed in all animals except one in which BT107 was implanted.

Particles of implanted material within the central medullary space were surrounded by either new bone without an intervening fibrous tissue layer or fibrous tissue in which bone was laid down. Glass BT109 appeared to induce bone formation directly in all six animals (Fig. 4) whereas BT108, BT107 & Novabone had a more mixed response with bone and fibrous tissue present. There was no evidence of inflammation or a foreign body giant cell/osteoclast response in the medullary space and residual marrow tissue was histologically normal.

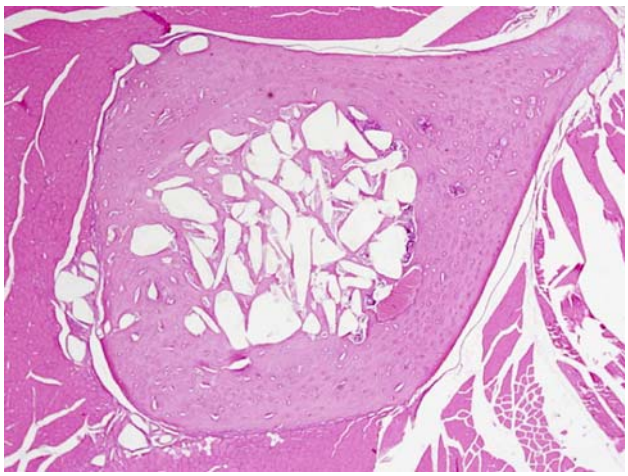

Fig. 3 Cross section of femur of rat implanted with BT107 (×2). The particles are present as angular spaces both in the medullary cavity of the bone as well as in the surrounding soft tissue

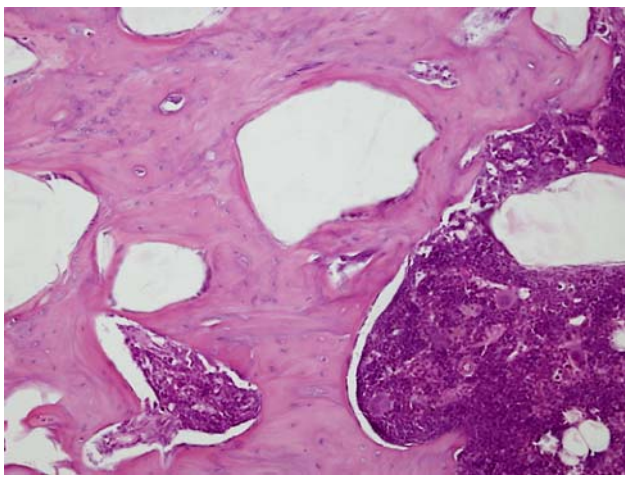

Fig. 4 Formation of bone directly surrounding particles of BT109. Magnification ×20

3.3.3 Stage two: ovariectomized female wistar rats

All animals survived surgery with no apparent ill effects but the ovariectomised animals were larger than their sham operated counterparts. Particles of test material could be visualised in the bone of four out of six ovariectomised animals and four out of six control animals and were present in the medullary space and cortical regions (Fig. 5). In all animals particles had escaped into the surrounding soft tissue and there was evidence of a localised foreign body reaction with macrophages, scattered giant cells and fibrosis. Very limited inflammation was observed in terms of increased vascularity, the presence of chronic inflammatory cells and neutrophils. There was no difference in the soft tissue response between the control and ovariectomised groups. The original cortical bone defect did not

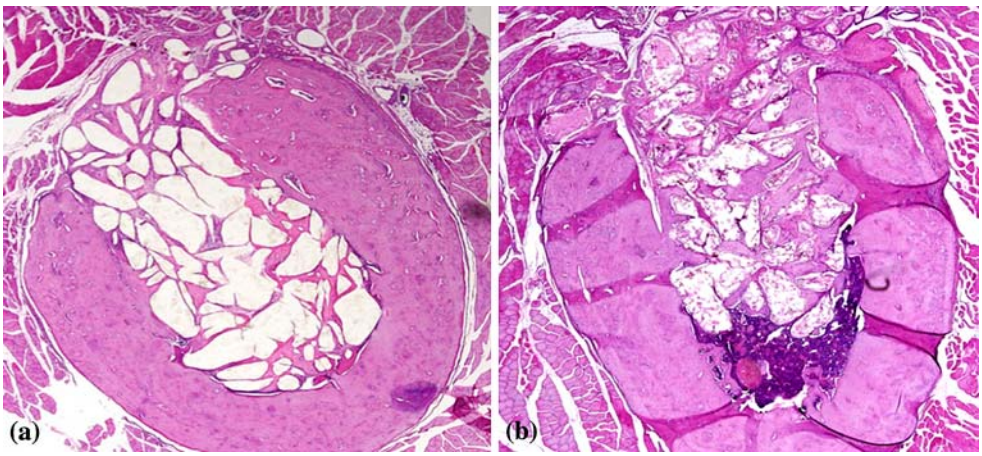

Fig. 5 Cross section of a non-healing rat femur in ovariectomised (**a**) and sham operated (**b**) rats (×4). The cortical bone is incomplete and the particles are present both in the medullary cavity and surrounding soft tissue. Bone formation is present on the medullary cavity of both animals

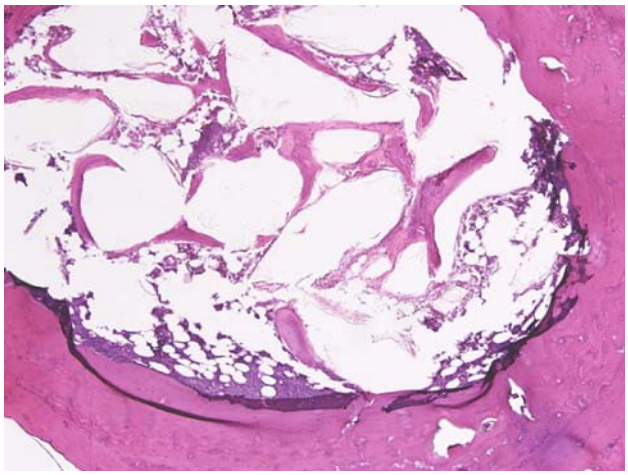

Fig. 6 Medullary cavity of femur in an ovariectomised rat (×10). The angular spaces were filled with particles which are surrounded by well formed spicules of bone

heal fully in three out of six ovarectomised animals or in four out of six control animals. In these animals particles induced the formation of fibrous tissue in the region of the cortical defect but in the marrow spaces induced bone (Fig. 6). This was seen in both the control and ovarectomised animals. In the remaining animals in which the cortical plate had healed there was a varied picture. In one animal in the control and ovarectomised groups there was little evidence of bone formation in the marrow space and in the remaining animals bone formation occurred in fibrous tissue.

4 Discussion

As part of this work each glass was characterised such that only the intended variable; Sr content of each graft, could be monitored in terms of biological response. However, the results indicate that while both BT107 and 108 are structurally identical, BT109 is structurally dissimilar. This difference is illustrated by a significant increase in T_g (18°C) for BT109. Previous work by the authors has noted that Sr can be substituted into similar glasses up to 0.12 mol fraction of the glass without significantly affecting the glass structures [23]. However, BT109 contains 0.28 mol fraction SrO and this increased loading may facilitate charge compensation of Zn^{2+} in the network to form stable Zn/Sr tetrahedron, which isomorphically replace SiO_2; thus leading to an increased proportion of bridging oxygen's in the glass network and subsequently a glass with increased stability and consequently the higher T_g.

In order to evaluate possible cytopathic effects caused by the graft materials, the ISO approved MTT assay was employed [22]. As such this assay is widely accepted as a precise and a reproducible method for quantitatively measuring cell viability [25]. The results of the assay indicate that the observed divergence in T_g resulted in an affiliated variation in the in vitro results. For BT107 and 108 the minimum cell viability recorded was 55 and 60% respectively. Conversely, for BT109 the minimum cell viability recorded was 80% increasing to a maximum of 95%. This result correlates well with those taken from the glass characterisation work, where it was shown that BT109, by virtue of its significantly higher T_g, was a glass with a far more stable network, *ergo,* less susceptible to degradation under the conditions examined. Consequently, over the period of 30 days accumulation of ions in the extract medium were such that they never approached concentrations capable of eliciting a cytotoxic response in vitro. For BT107 and BT108 however there is varied cell viability with incubation times for the extracts. For BT108 there are a decreasing percentage of viable L929 cells at each time period. This indicates dissolution of the glass and

gradual accumulation of Zn and Sr ions in the extract medium, resulting in increased cytotoxicity with time. Conversely, for BT107 the opposite is true; with the highest percentage of viable cells being recorded cells exposed to the 30 day extracts. The basis for this trend is unclear at present; however, ion release studies are in progress to help explicate these observations. Ancillary to these observation and individual performances of the grafts it is of particular importance to highlight that the mean cell viabilities recorded for each of the grafts were similar, in the case of BT107 and 108, while BT109 exhibited a higher mean cell viability that the commercial control material Novabone; indicating significant potential for the experimental materials as synthetic bone graft substitutes.

The model used for in vivo assessment is a well established healing model and it is known that small defects in the rat femur will heal spontaneously within 4 weeks. In our study all cortical defects apart from one (BT107) healed irrespective of which material was implanted. However a difference was seen in the response in the cortical region and marrow spaces between materials. In all cases where BT109 was implanted, bone was present, directly surrounding the particles. In contrast there was a more variable response with the other materials with bone forming in fibrous tissue. This favourable in vivo response to BT109 is consistent with our in vitro findings of higher cell viability and therefore this material was chosen for further study in ovarectomised animals. Due to the particulate nature of the material some granules escaped into the soft tissues in all cases. This invoked a foreign body giant cell response and may be associated with infiltrates of chronic inflammatory cells including lymphocytes and plasma cells. However these were not present indicating the particles are not immunogenic and pro-inflammatory.

BT109 showed a similar response in the femurs of ovariectomised and normal rats and this is encouraging since it has been shown that the bone response to implants is diminished in ovariectomised rats [26–28]. In these studies [26–28] solid cylinders of test material were implanted into rat femurs/tibia and image analysis performed to compare the response between ovariectomised and normal rats to hydroxyapatite coated implants [26, 27]. In our study, the granular form of the implant material precluded a similar analysis and so we cannot be certain there are no small differences between ovariectomised and normal rats.

The healing in the mature animals was much poorer than in the immature animals used in stage one and this may be related to the age of the animals. Only three out of six ovarectomised animals and two out of the six control animals healed. A number of studies have shown a poorer bone response to implanted material in the bones of older rats [29, 30]. However, in this preliminary study we have shown that BT109 appears to behave similarly in normal and ovariectomised animals in terms of bone production. This material may therefore have potential as a therapeutic bone substitutes in patients with compromised bone healing, for example; those with osteoporosis. Further studies are planned to test this material more rigorously in vivo.

5 Conclusion

The objective of this work was to evaluate the potential of particulate CaO–SrO–ZnO–SiO_2 glasses for bone grafting applications. The results of this preliminary investigation indicate that in cell culture (MTT assay) these materials exhibit mild to moderate cytopathic effects on L929 mouse fibroblast cells; comparable to the cytotoxicity of the commercial material Novabone, which was used as a benchmark material for the in vitro screening. Complimentary to this, the results obtained through the rat femur healing model indicate that the novel glass grafts are capable of inducing bone growth in close apposition to the implanted particles. On that basis, the materials examined herein exhibit sufficient potential as a bone graft substitutes to warrant further investigation.

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