Development of soluble glasses for biomedical use Part II: The biological response of human osteoblast cell lines to phosphate-based soluble glasses

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Soluble glasses are considered to be of potential clinical value in orthopaedic and dental surgery. However, the biological response to these materials is not well understood. To determine the effects of these glasses, two human osteoblast cell lines, MG63 and HOS (TE85), were incubated *in vitro* in the presence of increasing concentrations of extracts of the glasses. The effects of the extracts on cell growth was measured using the MTT assay and an ELISA assay was used to measure the expression of bone sialoprotein (BSP), osteonectin (ON) and fibronectin (FN), antigens which play a fundamental part in the integrity and function of hard connective tissue. The results showed that the proliferation of the cells was adversely affected only by the more soluble glasses, which also down-regulated the expression of the bone-associated proteins. In contrast, the extract of the glass with the lowest dissolution rate, which contains relatively elevated levels of Ca^{2+}, was found to enhance bone cell growth and antigen expression. These findings suggest that the compositions of these glasses at least partly determine the response of cells and thus, that the glasses could be modified to elicit a more optimal biological response and clinical efficacy.

1. Introduction

A number of different types of ceramic materials have been developed that have osteoconductive and osteoinductive properties, which are effective in the repair and regeneration of bone. These include hydroxyapatite (HA), Bioglass®, apatite and wollastonite containing glass ceramics (A-W GC) and other inorganic phosphates, including glasses. The development of phosphate glasses for use in orthopaedic implants has attracted much interest because their chemical and physical properties make them suitable for use as bone-bonding materials [1, 2]. For example, one type of bioactive glass, Bioglass®, has been found to exhibit particularly beneficial characteristics *in vivo* [3, 4]. However, although bioglasses induce rapid healing, they have SiO_2 as a constituent and are rarely absorbed, raising uncertainty about the long-term effects of Si *in vivo*.

Many of these materials have the capacity to form a hydroxycarbonate apatite layer on their surfaces *in vitro* and *in vivo* [5], compatible with the mineral phase of bone. Although some of the materials exhibit favorable physical and mechanical properties, they are often very hard, brittle and difficult to machine. Their fragile nature thus limits the production of suitable shapes for implantation to treat various bone defects in orthopaedic and dental applications. In addition, these materials frequently have very slow resorption rates *in vivo*. [6]. For example, HA blocks implanted in dogs did not change their volume fraction or mean width after four years *in vivo* [7]. Resorbable materials would offer a distinct advantage, particularly in certain dental applications, where such materials for bone regeneration could be used to prepare a site prior to implantation as in alveolar ridge augmentation [8]. In addition, the regeneration of periodontal tissues has been demonstrated using a type of degradable membrane placed after surgical exposure of periodontal defects [9].

A group of phosphate-based glasses using the components P_2O_5-CaO-Na_2O [10] have recently been produced which have a variable degree of solubility [11]. These glasses consist of a polymer-like, regular tetrahedral structure based on [PO_4] groups [12, 13]. The addition of network modifiers such as CaO and Na_2O interrupt the physical structure of the glasses, while the proportion of CaO determines the dissolution times, which can vary from a few hours to several months

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[14]. As their constituent atoms are found in the inorganic mineral phase of bone, they have a chemical affinity with natural bone. The ease of manufacture and the ability to control the solubility of these phosphate glasses makes them potentially clinically useful for promoting the regeneration of soft as well as hard connective tissues as occurs, for example, in the repair of periodontal defects and bone [6]. Moreover, since the solubility of these glasses can readily be pre-determined by modification of their chemical composition, they have additional clinical potential as resorbable materials, for which a second surgical procedure to remove the implanted material is unnecessary. Furthermore, soluble phosphate glasses can be produced at relatively low temperatures compared with Bioglass®, thus allowing the incorporation of bioactive macromolecules, which could have potent modulating effects. However, little is known about the influence of these glasses on the growth and functions of target cells in implanted tissues.

The MG63 and HOS TE85 human cell lines have osteoblast-like characteristics and are often used as experimental models for investigating aspects of osteoblast function [7]. In the present study we have measured the effects of extracts of four different formulations of soluble glasses on the proliferation of these cells and on the expression of bone sialoprotein (BSP), osteonectin (ON) and fibronectin (FN), antigens which have a major role in the structure and integrity of bone.

2. Materials and methods

2.1. Glass formulations

Ternary glasses were prepared using the precursors calcium carbonate ($CaCO_3$), calcium hydrogen phosphate ($CaHPO_4$), sodium dihydrogen orthophosphate (NaH_2PO_4) and phosphorous pentoxide (P_2O_5) as starting materials. The amount of P_2O_5 was kept fixed at 45 mol %, the remainder consisting of CaO and Na_2O, as shown in Table I. The solubility of the glasses is related to the proportion of CaO, glasses containing less than approximately 20% CaO being very soluble (glasses designated as numbers 44 and 47), whereas those containing more than approximately 20% are much less soluble (glasses numbered 51 and 54).

The glass precursors were weighed and placed in a platinium/10% rhenium crucible, then fired in a Carbolite furnace, model RHF 1500 (Carbolite, Sheffield, UK) at 1100–1200 °C for 1 h. The molten mixture was cast in a graphite mold preheated to 350–400 °C and then furnace cooled to remove residual stresses. Three glass rods approximately 70 mm long × 15 mm wide were produced per composition, then melted together and cast to give one final glass rod of the same dimensions. Individual discs of approximate total surface area 5 cm^2 were cut from each rod using a diamond rotary saw, Model 660 (Testbourne, Basingstoke, UK).

TABLE I Composition of phosphate glasses

Glass	P_2O_5 mol %	CaO mol %	Na_2O mol %
44	45	8	47
47	45	16	39
51	45	28	27
54	45	40	15

2.2. Preparation of glass extracts

The glass discs were sterilized by dry heat at 180 °C for 2 h. They were cooled and placed into 50 ml Falcon tubes (Becton Dickinson, Cowley, UK) containing 25 ml of the cell culture medium described below. This latter volume was chosen in accordance with Shand [15], who recommended that the ratio between the surface area of the test material and the volume of the extraction liquid to be 5 ml/cm^2. The culture media containing the glasses were collected after 24 h of incubation at 37 °C, with occasional agitation. Because of the possible formation of precipitated glass material, the extracts were aseptically passed through a 0.22 μm Millipore filter and stored at − 20 °C.

2.3. Cell culture

Two human osteosarcoma cell lines, MG63 and HOS (TE85), were used in this study. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air, in flasks containing 10 ml of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 2 mM L–glutamine, 50 IU/ml of penicillin and 50 μg/ml of streptomycin (Gibco, Glasgow, UK). The culture media were changed twice weekly and for sub-culture the cell monolayers were washed with phosphate-buffered saline (PBS) and incubated with trypsin-EDTA for 5 min at 37 °C to detach the cells, prior to reculture in 96-well microtitre plates.

The following dilutions of the glass extracts were prepared using HEPES-buffered DMEM culture medium: none (undiluted extract); 1 : 4; 1 : 16; and 1 : 64. MG63 and HOS cells were incubated with these dilutions of the extracts in 96-well microtitre plates as described below.

2.4. MTT assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a substrate that is converted by a mitochondrial enzyme, which is active in living cells, to yield a dark blue/purple formazan product. The intensity of the color produced is directly related to the number of viable cells, and thus to their proliferation *in vitro*. The MG63 and HOS cells were plated at a density of 500 cells/well in replicate 96-well culture plates and incubated at 37 °C. After 48 h, media were removed and replaced by the extracts. Cells receiving fresh culture medium, with no added extracts, were used as controls. The cells were then incubated for a further 2 and 5 days and cell proliferation measured at these time periods using the MTT test (Chemicon, Temecula, CA, USA). Absorbance was measured at 570 nm (A_{570}) using a Titertek Multiskan Plus spectrophotometer (Labsystems, Helsinki, Finland). The results are expressed as the average absorbance of six replicate wells.

2.5. ELISA assay

The enzyme-linked immunosorbent assay (ELISA) requires few cells and can measure antigen expression in large numbers of samples simultaneously. Cell suspensions were prepared and 500 cells/well were seeded into 96-well microtitre plates and incubated for two days at 37 °C, after which the medium was removed and replaced by either 100 µl of fresh DMEM (controls) or the respective extracts. After a further five days of incubation the medium was removed and the cells fixed with 3% paraformaldehyde in PBS containing 0.1% saponin, in order to permeabilize the cells to allow entry of the antibodies. The cells were then washed with PBS containing 0.05% Tween (BDH). Non-specific antibody binding was blocked by adding 200 µl of PBS-Tween containing 1% w/v skimmed milk to each well and incubating for 1 h. After washing, the cells were reacted for 1 h with rabbit polyclonal antibodies against bone sialoprotein (BSP), osteonectin (ON) (courtesy of Dr Larry Fisher, NIDCR, Bethesda, USA) and fibronectin (FN) (Dako Ltd, High Wycombe, UK), all at 1 : 1000 dilution. After washing, horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1 : 5000 dilution) was applied for 30 min. After further washing, the substrate, o-phenylenediamine dihydrochloride, was added and the plate incubated for 10 min at 37 °C. The reaction was stopped by the addition of 25 µl of 2M H_2SO_4. Absorbance was measured at 450 nm (A_{450}) using the Titertek Multiskan Plus spectrophotometer.

3. Results

3.1. Effects of glass extracts on cell proliferation

Under the phase contrast microscope, both the MG63 and HOS cells cultured in the presence of the less soluble glass extracts (numbers 51 and 54) appeared to be indistinguishable from control cells cultured in full medium only. Similarly, cells incubated with the 1 : 16 and 1 : 64 dilutions of the more soluble glasses (extracts 44 and 47) also appeared to be unaffected. However, both cell lines incubated with the neat and 1 : 4 dilution of these glasses for two days appeared to have fewer and more rounded cells, while after five days these cultures clearly contained less adherent cells and more cells which had detached and were floating in the culture medium.

In order to examine whether the extracts of the glasses affected the proliferation of the human osteoblast-like cells, the MTT assay was performed after two and five days of incubation of the cells in the presence of increasing concentrations of the extracts, as described in the Materials and Methods section. The results in Fig. 1 show that there was a marked difference in the growth of the cells, which depended on the glass and dilution of the extract used. Thus, while none of the extracts of the less soluble glasses (numbers 51 and 54) had any effect on the proliferation of MG63 cells at two or five days, the presence of the 1 : 4 and neat concentrations of the more soluble glass extracts (44 and 47) markedly reduced the number of cells (Fig. 1a). Moreover, the inhibition exerted by the 1 : 4 dilution of glass 47 became more severe by day five, only 10% of the control level compared with 25% at day two. Similarly, the 1 : 16 dilution of glass 44 also inhibited growth by day five, by 65%, whereas this concentration had no effect on the number of MG63 cells at day two. The response of the HOS cells was nearly identical, as shown in Fig. 1b, extract numbers 51 and 54 again having no effect on cell proliferation while increasing concentrations of extracts 44 and 47 progressively inhibited cell growth and, as with the MG63 cells, more strongly on day five than on day two.

3.2. Effects of glass extracts on antigen expression

The ELISA technique was used to measure whether the extracts of the glass affected the expression of BSP, ON and FN by the MG63 and HOS cells, as described in the Materials and Methods section. The results in Fig. 2 show that after five days of incubation, increasing concentrations of all of the extracts except the least soluble (number 54) generally resulted in the down-regulation of the expression of BSP. This inhibition was particularly apparent for the MG63 cells cultured with the neat and 1 : 4 dilution of the most soluble glass (extract 44), which reduced antigen expression to only 63% and 56% of the control level, respectively (Fig. 2a). The two highest concentrations of the other soluble glass extract, number 47, also inhibited the expression of this antigen, although to a lesser extent (to 68% and 75% of the control MG63 cells). Fig. 2b also shows that extracts 44 and 47 had a much less pronounced effect on BSP expression by HOS cells.

The extracts of the most soluble glasses also down-regulated ON expression in both cell lines, as shown in Fig. 2. Moreover, while extract 44 potently inhibited ON in the MG63 cells (Fig. 2A), extract 47 reduced the level of this antigen more strongly in the HOS cells (Fig. 2B). In contrast, although the same extract had an inhibitory effect on FN expression in the HOS cells, it more markedly down-regulated this antigen in the MG63 cells, to approximately 75% of the control level at a 1 : 4 dilution, as shown in Fig. 2A. Extracts of the less soluble glass, number 51, also down-regulated FN expression in the MG63 cells but not the HOS cells.

It is notable, however, that in contrast to the effects of the extracts on cell proliferation, some of the diluted extracts up-regulated antigen expression substantially above the level of the control culture. This was evident for extract 47, which elicited a higher level of BSP (in both cell lines), ON (in the HOS cells) and FN (in the MG63 cells), and particularly for extract 54, which appeared to up-regulate all three antigens in both cells.

4. Discussion

The present *in vitro* study was carried out to determine the effects of phosphate-based soluble glasses on certain key activities of bone cells *in vitro*, in order to assess their potential value as biomaterials in orthopaedic and dental bone repair and regeneration. This was carried out by using ''extracts'' of the glasses as the test materials, since these extracts would contain similar substances to

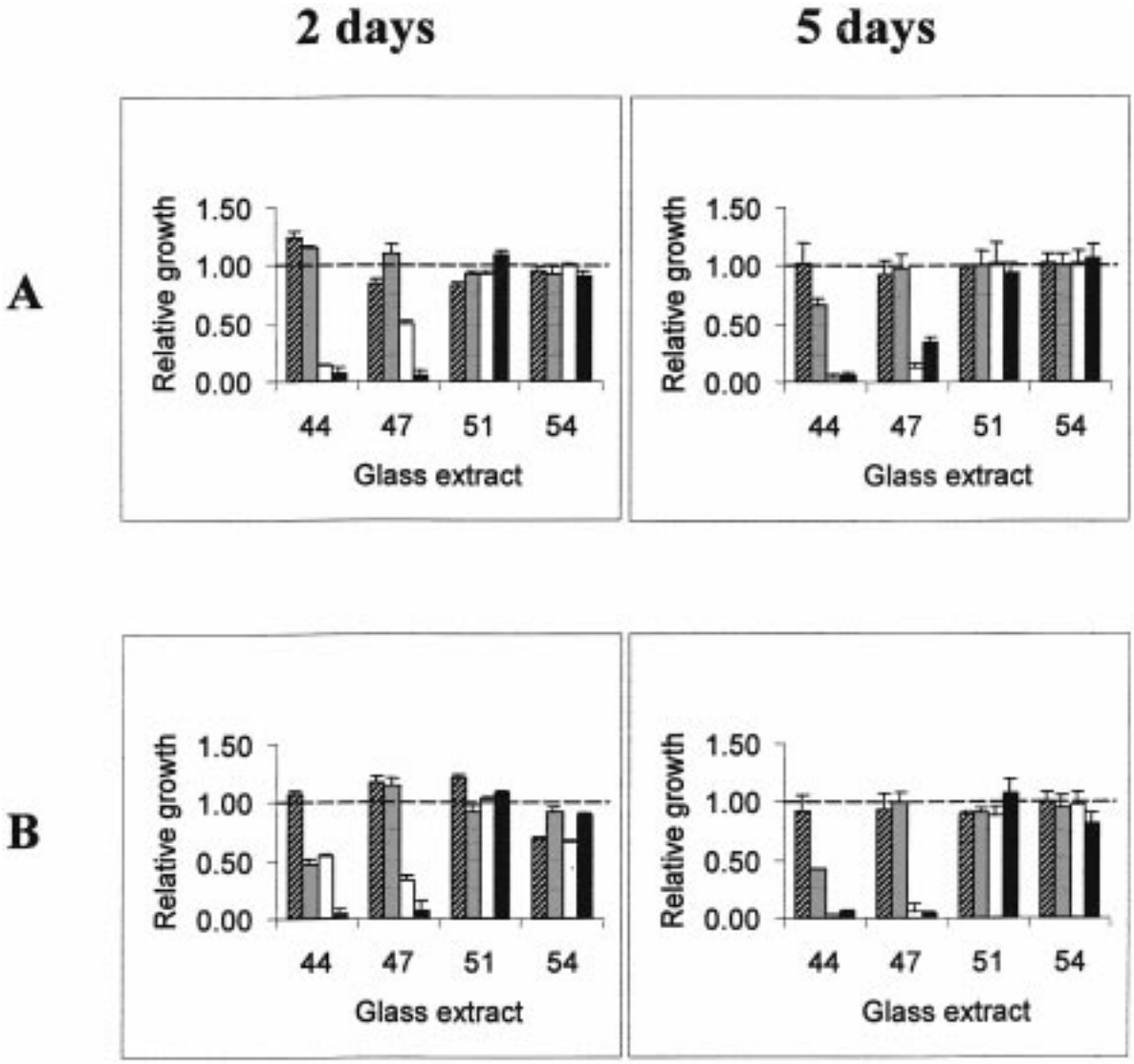

Figure 1 MTT assay of the effects of glass extracts on the proliferation of MG63 cells **(A)** and HOS cells **(B)** during 2 and 5 days of culture. The black dashed lines represent the mean absorbance of control cells incubated with medium only and no glass extract. The ratios of the mean absorbance levels of cells incubated with the extracts compared with the mean absorbance levels of the control cells were calculated and are shown as the relative growth. Key: 1 : 64 (striped bar); 1 : 16 (gray bar); 1 : 4 (white bar); neat extract (black bar). Vertical lines represent $\pm$ SD ($n = 6$).

those which would leach out of the glasses after implantation *in vivo*.

Microscopic examination suggested that while the extracts of the less soluble glasses (high Ca^{2+}, low Na^{+}) did not apparently affect the cells morphologically, progressively higher concentrations of the most soluble glasses (low Ca^{2+}, high Na^{+}) caused the cells to become more rounded and to detach from the plastic surface of the culture dish. Measurements of the actual growth of the cells using the MTT assay confirmed these findings, demonstrating that cell proliferation was unaffected by the less soluble glasses but was markedly reduced by the extracts of glasses 44 and 47. This inhibitory effect was found to be more pronounced with extract 44, occurring with this glass even in the presence of highly diluted extracts and also at an earlier period of incubation than was observed with glass 47.

The two most soluble glasses were similarly found to strongly down-regulate the expression of BSP, ON and FN, proteins which have a major role in bone metabolism and integrity. Although the higher concentration of the extract of glass 51 also had some inhibitory effect on the levels of these bone-associated antigens, it was notable that the extract of the least soluble glass, number 54, was found to up-regulate all three antigens in both the MG63 and HOS cell lines.

The reasons for these effects of the extracts on cell proliferation and bone protein expression are not clear, but they are undoubtedly associated with the differential influence of the various ion species released from the glasses into the culture media and perhaps also with material-induced pH differences between the extracts [10]. The present results demonstrate that these factors, arising from differences in the chemical composition and differential dissolution of the glasses, have an important impact on cell growth and function. Thus, the soluble glasses 44 and 47 have a relatively high rate of dissolution, releasing far more Na^{+} and $(PO_4)^{2-}$ and resulting in a substantial increase in pH compared with glasses 51 and 54 (unpublished data). Although an alkaline environment has been shown to be favored by osteoblasts [16], the high pH produced by the more soluble glasses may nevertheless be detrimental to optimal osteoblast metabolism and may at least partly explain the apparently deleterious effects of these extracts on cell proliferation and bone protein expres-

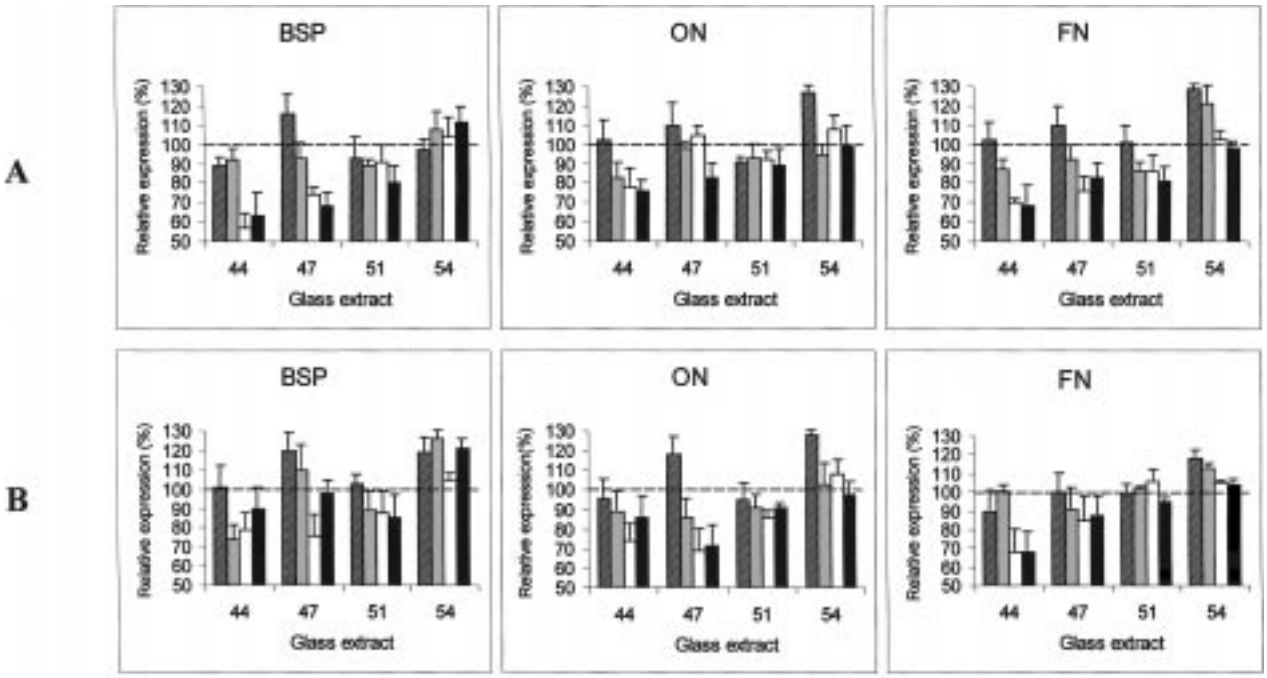

Figure 2 ELISA assay of the effects of glass extracts on the expression of BSP, ON and FN by MG63 cells (A) and HOS cells (B). The cells were incubated for 5 days in the presence of glass extracts. Antigen levels were measured by ELISA, as described in the Materials and Methods Section, and compared with the level present in control cultures containing no glass extracts (defined as 100%). Key: 1 : 64 (striped bar); 1 : 16 (gray bar); 1 : 4 (white bar); neat extract (black bar). Vertical lines represent $\pm$ SD ($n = 6$).

sion. In marked contrast, the up-regulation of the three bone-associated antigens
by the extract of the least soluble glass, number 54, may be related to the high Ca^{2+} content of this glass which, despite having the lowest dissolution rate, nevertheless releases more Ca^{2+} than any of the other glasses [10].

Divalent cations, and Ca^{2+} in particular, are known to play a key part in cell activation mechanisms, thereby controlling many growth-associated processes and functional activities of cells [17, 18]. In the present experiments it is thus possible that the elevated levels of Ca^{2+} leached from glass 54 may have enhanced the response of the bone-like cells. It is not yet clear, however, whether this type of material would have similar ''beneficial'' effects *in vivo*, where the circulation of tissue fluids could ablate the influence of such eluted substances more effectively than in the closed environment which exists *in vitro*. Moreover, in the experimental protocol of this study, an ELISA assay was used which measured only cell-associated antigens, whereas a substantial proportion of BSP, ON and FN would be expected to be secreted into the ECM.

Since the extracellular levels of these antigens were not measured, it is possible that the effects of the extracts could also have been mediated by modulation of protein secretion, as has previously been reported for Ca^{2+}, for example [17]. Experiments are therefore in progress to delineate the relative effects of these materials on the level of protein production (i.e. transcription of bone-specific genes) compared with post-translational processing events.

Irrespective of the precise mechanism, however, the suitability of these soluble glasses as implant materials requires careful consideration in view of the observed inhibition of growth and down-regulation of bone proteins by some of the extracts. Nevertheless, their apparent failure to elicit an adverse inflammatory response *in vivo* [19], and the present finding that certain chemical formulations could be developed to promote tissue regrowth, warrants further evaluation of these glasses as potentially valuable clinical materials.

5. Conclusion

This study has shown that extracts of two highly soluble phosphate-based glasses caused some inhibition of growth and bone antigen expression by two human osteoblast cell lines. However, extracts of the less soluble glasses, which contain high levels of Ca^{2+}, up-regulate the proliferation of the cells and the expression of BSP, ON and FN. Chemical modifications of these glasses are now in progress in order to develop improved formulations which enhance key biological responses, and hence the clinical value of these materials in orthopaedic and dental surgery.

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