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Soft tissue response to glycerol-suspended controlled-release glass particulate

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Vesicoureteral reflux and urinary incontinence have previously been treated by various means including the endoscopic delivery of injectable bulking materials such as silicone micro-implants, PTFE implants, glass particles, fat and bovine collagen. These first three materials do not degrade and collagen requires frequently repeated injections in order to sustain the restored continence provided. Vesicoureteric reflux in children usually resolves independently before the age of five. Correction is required before this, because treatment by prophylactic antibiotics is frequently unsuccessful in preventing breakthrough infection. The ideal material for injection should have large particles to avoid migration, inject easily and controllably, be non-toxic and dissolve over the period of time by which time the kidney will be mature. Three different controlled-release glass (CRG) granule compositions have been prepared by Giltech Ltd, and suspended in a suitable carrier medium (in this case glycerol). The degradable glasses, which have two different size ranges of 200–300 and < 53 μm, and three different solution rates, were injected intramuscularly into the dorso-lumbar region of rats. Histological analysis of cryostat cut section after time periods of 2 d, 4 and 9 wk, and 6 mon has been performed. Histology sections were stained for neutrophils and macrophages using enzyme histochemistry. ED1 (monocytes and immature macrophages), ED2 (mature tissue macrophages), CD4 (helper/inducer T-lymphocytes and macrophages), CD8 (suppresser/cytotoxic T-lymphocytes), Interleukin-1β, IL-2 (activated T-lymphocytes), Major Histocompatibility Complex (MHC) class II (activated macrophages and activated B-lymphocytes), α–β (T-lymphocytes) and CD45RA (B lymphocytes) antibodies have beed used to stain immunohistochemically each sample. This study demonstrates that particulate, degrading glass is stimulating an inflammatory response in soft tissue at time periods up to 6 mon. It should be noted that very small particulate, fast degrading glass is leading to tissue necrosis and should not be considered further for these applications. However, larger particulate, slower degrading materials are demonstrating effective potential for stress incontinence applications.

1. Introduction

Controlled-release glass (CRG) was first developed in the 1970s primarily for use in food production industries [1–4]. These degradable glasses contain no silica, and incorporation of various metal ions into the CaO/P_2O_5 matrix allows a constant source supply of essential metal ions to be delivered when the glass is placed into an aqueous environment. Hence it has been possible to use pellets of CRG containing metal ions such as copper and cobalt, as pesticides, fungicides and in animal feeds. More recently, CRG has been used clinically, where incorporation of silver into the basic degradable network provides a silver ion release vehicle which reduces infection as a result of the bacteriostatic nature of silver ions. Effective infection control has been observed when these CRGs have been used in long-term indwelling catheters [5] and in wound dressings [6]. Furthermore, research into using these glasses as a bone graft substitute [7, 8] and as a material with blood contacting applications [9], has been undertaken.

CRG is manufactured in a similar way to conventional soda–lime glass in that the constituents are heated to temperatures above 1000 °C, then cast into various forms such as solid blocks, powder, granules, tubes, fibre or wool. The solution rate of the CRG is dependent on the chemical composition of the glasses and also on the physical form, as this determines the

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surface area in contact with the aqueous environment. Hence, the rate at which the CRG degrades can be determined and adjusted prior to manufacture. For this study three different granular samples of CRG have been provided by Giltech Ltd for analysis to determine the effective potential of this material as a bladder augmentation material intended to relieve temporary stress incontinence. Implantation of bulking materials at appropriate sites in the bladder has been shown to be effective in relieving the symptoms of stress incontinence and vesicoureteral reflux. Urinary incontinence in individuals of all ages can be a devastating problem with social and personal consequences often far more distressing than the urological conditions. Implanting bulking materials into the bladder neck to create a smaller orifice is currently an accepted clinical procedure [10–12]. In children, urinary tract infections in the presence of vesicourteral reflux allow backflow of infected urine into the renal parenchyma causing serious damage, and lead to renal scouring. There is an urgent need to correct this as it may lead to renal nephropathy and end-stage renal disease and renal failure in bilateral cases. Vesicoureteric reflux in children usually resolves independently before the age of five. Correction is required before this, however, as treatment by prophylactic antibiotics is frequently unsuccessful in preventing breakthrough infection. With a biomaterial in place, less pressure is needed by the sphincter muscle to close the bladder opening and the body can again control the urine flow.

There is currently a variety of injectable implants used to relieve incontinence in this manner. Polytetraflouroethyulene (PTFE) [13] granules have been used as a bulking agent as have silicone rubber particulates [10]. These materials are suspended in a carrier fluid such as glycerol, saline or a hydrogel. The carrier performs as a lubricant and assists in particle flow as the implant is transported from syringe to the implant site via an endoscopic needle. After a short period of time the carrier medium disperses from the implant site and is eradicated from the body. The remaining granules become encapsulated with a fibrous tissue. PTFE and silicone implants however, are non-degradable and for temporary relief of stress incontinence may not be considered to be suitable. Concern has been expresed as to the long-term fate of these particles. Collagen has also been used endoscopically to treat stress incontinence and vesicoureteral reflux [14]. Collagen, however, requires frequent repeated injections to the implant site. The ideal material for injection should have particles large enough to avoid migration, inject easily and controllably, be non-toxic and dissolve over a period of time during which the kidney will mature. CRG has the potential to fulfill all of these requirements. In this study, an evaluation is made of the soft tissue response to implant particulate material using a tissue model with which this group has considerable experience. Three different controlled-release glass (CRG) granule compositions have been prepared by Giltech Ltd and suspended in a suitable carrier medium (in this case glycerol). The degradable glasses, which have two dfferent size ranges of 200–300 and <53 μm, and three different solution rates, were injected intramuscularly into the dorso-lumbar region of adult rats. Histological analysis of 7 μm thick serial cryostat cut sections after four different time periods of 2 d, 4 and 9 wk and 6 mon was performed.

2. Materials and methods

The CRG was examined in particulate form using three different compositions and two different particulate sizes as detailed in Table I. The solution rates for these glasses were: X, 200–300 μm, 0.02 mg cm^{-2} h^{-1}; Y, 200–300 μm, 0.12 mg cm^{-2} h^{-1}; Z, <53 μm, 0.34 mg cm^{-2} h^{-1}. All implants were suspended in glycerol. All solution rates have been determined *in vitro* in aqueous conditions. A significant objective of these studies will be to ascertain the effect the cellular environment has on these solution rates. A control sample of glycerol only was also included in the experiment and was labeled sample W. Sample aliquots weighing 0.1 g each of the CRGs in glycerol and glycerol only were sterilized by gamma irradiation before implantation intramuscularly into Wistar rats. Two sample sites were employed in each animal. Four animals were employed at each time period of 2 d, 4 and 9 wk, and 6 mon. The implants were placed bilaterally into a pocket created by surgical intervention in the dorso-lumbar muscle of the animal. All animal studies were carried out according to procedures approved by home office project licence 40/1099. At the time of explantation, the implant site and surrounding tissue was removed from the sacrificed animal and snap frozen. A microtome cryostat was used to cut 7 μm thick serial sections. Analysis of the implant/tissue site was performed by specific staining of the sample sections for various inflammatory cell types. Neutrophils and macrophages were stained using enzyme histochemistry kits (neutrophils: naphtol As-d chloroacetate esterase kit, macrophages: α-naphthyl acetate esterase, both from Sigma, Poole, England). ED1 (monocytes and immature macrophages), ED2 (mature tissue macrophages), CD4 (helper/inducer T-lymphocytes and macrophages), CD8 (suppresser/cytotoxic T-lymphocytes), interleukin-1β, IL-2 (activated T-lymphocytes), major histocompatibility complex (MHC) class II (activated macrophages and activated B-lymphocytes), α–β (T-lymphocytes) and CD45RA (B-lymphocytes) antibodies have been used to immunohistochemically stain sections from each sample.

These stains allow the tissue response to the presence of the implant to be analyzed in considerable

TABLE I Materials provided for *in vivo* implantation

Sample code	Description	Granular diameter range (μm)
W	Control sample glycerol only	N/A
X	Na_2O, CaO, P_2O_5, B_2O_3	200–300
Y	Na_2O, CaO, P_2O_5	200–300
Z	Na_2O, CaO, P_2O_5, Ag_2O	<53

TABLE II Immunohistochemical stains performed on histological sections of received implant/tissue interface

Haemotoxylin and eosin	Haemotoxylin component stains cell nuclei blue–black, with good intra-nuclear detail, whilst eosin stains cell cytoplasm and most connective tissue fibers.
ED1	Recognizes immature macrophages, monocytes and dendritic cells. Granulocytes are negative. The recognized antigen is predominantly located intracellularly, although some membrane expression occurs.
ED2	Recognizes a membrane antigen on resident mature tissue macrophages. Monocytes, dendritic cells and granulocytes are negative. No other cell types but macrophages are positive for ED2
CD4	Expressed on most thymocytes and approximately two-thirds of peripheral blood T cells, In humans and rats, CD4 is expressed on monocytes and macrophages. CD4 is an accessory molecule in the recognition of foreign antigens in association with MHC class II antigens by T cells.
CD8	Expressed on most thymocytes and approximately one-third of peripheral blood T cells, which constitute the CD4 negative cells. CD8α is in all natural killer (NK) cells in the rat.
IL-1β	Recognizes recombinant rat IL-1β at concentrations of >10 nm on activated β cells, macrophages and monocytes.
IL-2	Recognizes a glycoprotein found on activated rat T-cells but not resting lymphocytes.
MHC Class II	Expressed by activated B cells, activated monocytes, activated macrophages and some epithelial cells. Expression is increased by interferon γ which also induces expression on fibroblsts, epithelial and endothelial cells.
α-β	Detects an α–β T cell receptor found on 90-99% of all peripheral blood T cells.
Anti-B	Labels B cells among thoracic duct lymphocytes with little labeling in bone marrow and none on thymocytes. Acts as an isotype control.

detail. Table II describes the information that can be determined using these staining techniques.

3. Results

Following staining, it was clear that neutrophil and macrophage were the dominant cell types present in the implant sites. Positive staining for neutrophils was observed after 2 d implantation with all materials. The neutrophils were found in localized clusters near the implant site as can be seen with the control sample of glycerol and glass X at 2 d in Figs 1 and 2. However, neutrophils were not seen in any of the tissue sections in the remaining time periods. Mast cells were present in all tissue samples, but it was noted that an increased number of these cells was present in clusters near glass X at 6 mon, glass Y at 2 d and 6 mon, and glass Z at 4 wk and 6 mon. Examples of mast cells are detailed in Figs 3 and 4. Fig. 3 demonstrates an acceptable population of mast cells in soft tissue and Fig. 4 demonstrates an excessive number of mast cells in the fibrous tissue near an implant site of glass Y at 6 mon.

Enzyme and immunohistochemical staining confirmed the presence of macrophages in all sections at all time periods except with glass X at 6 mon. The macrophage presence, however, was always localized to the implant site. This is demonstrated in Figs 5 and 6, where macrophage enzyme staining has been performed on glasses X and Y at 9 and 4 wk time periods, respectively.

Figs 7–9 show haemotoxylin and eosin staining performed on samples W at 2 d, X at 9 wk and Y at 4 wk. At 2 d, sample W can be seen to be spread throughout the tissue, breaking up the muscle cells with a matrix consisting of fibroblasts, neutrophils and macrophages as confirmed by enzyme and immunohistochemical staining. Sample X at 9 wk has no neutrophil presence but in the matrix forming a capsule around the implant are localized macrophages

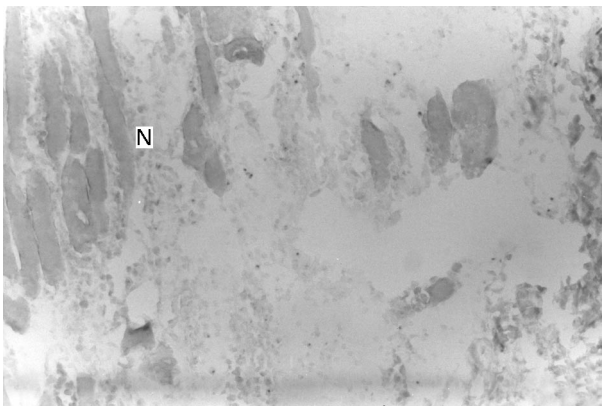

Figure 1 Neutrophil enzyme stain of control sample W after 2 d. N, neutrophils. × 40

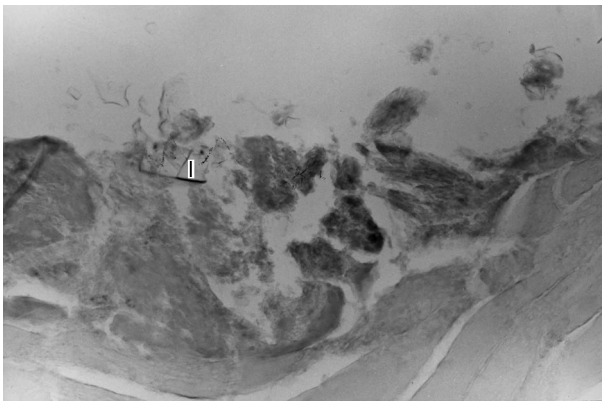

Figure 2 Neutrophil enzyme stain of sample X after 2 d. I, the implant. × 40

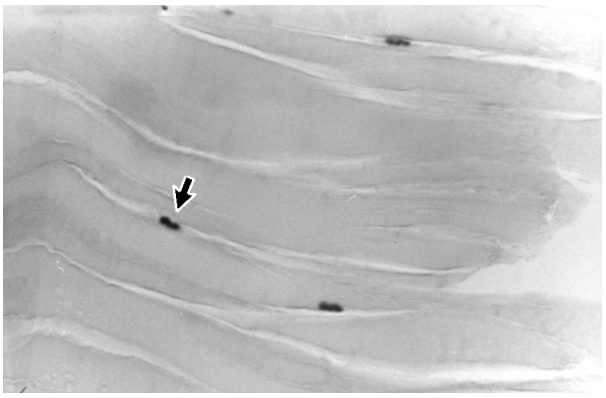

Figure 3 Acceptable number of mast cells in soft tissue. Arrow indicates a mast cell. × 40

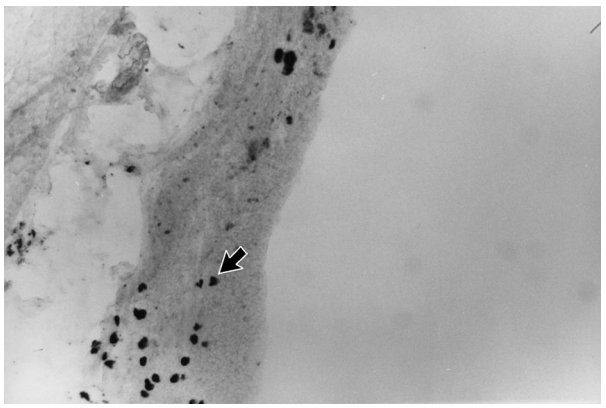

Figure 4 Excessive number of mast cells seen in fibrous tissue near sample Y implant after 6 mon. Arrow indicates a mast cell. × 40

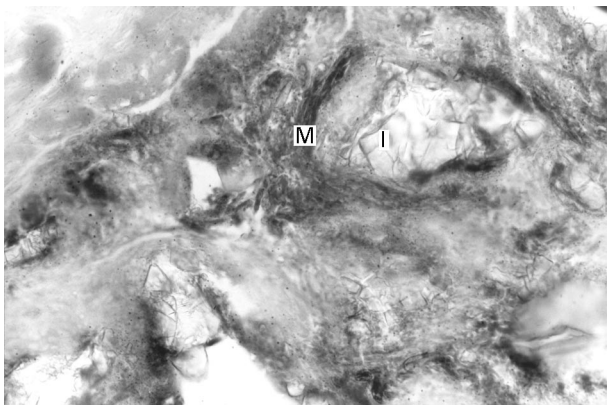

Figure 5 Macrophage enzyme stain of sample X after 9 wk. I, the implant; M, macrophages. × 40

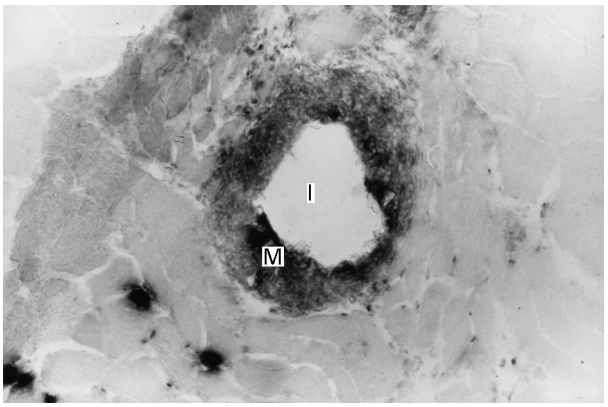

Figure 6 Macrophage enzyme stain of sample Y after 4 wk. I, the implant, M, macrophages. × 40

Figure 7 Haemotoxylin and eosin stain of control sample W after 2 d. × 40.

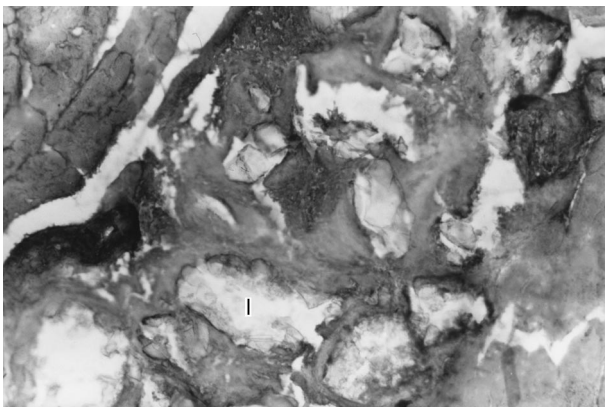

Figure 8 Haemotoxylin and eosin stain of sample X after 9 wk. I, the implant. × 40

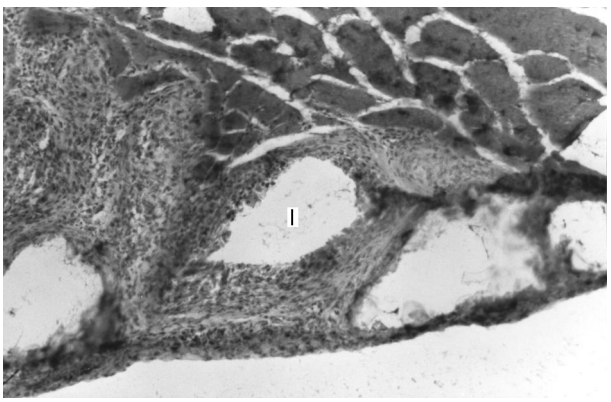

Figure 9 Haemotoxylin and eosin stain of sample Y after 4 wk. I, the implant. × 40

(as confirmed by enzyme and immunohistochemical staining) and fibrous tissue. Sample Y at 4 wk and 6 mon has a similar soft tissue response, in that no neutrophils are present but localized macrophages are present in the surrounding capsule around the implant.

Tissue necrosis can be observed in relation to the presence of sample Z in the soft tissue at the 4 and 9 wk time periods. Fig. 10 shows a 9 wk Z sample of an immunohistochemical stain of ED1. Fig. 11 also shows the ED1 stain of sample Y at 2 d.

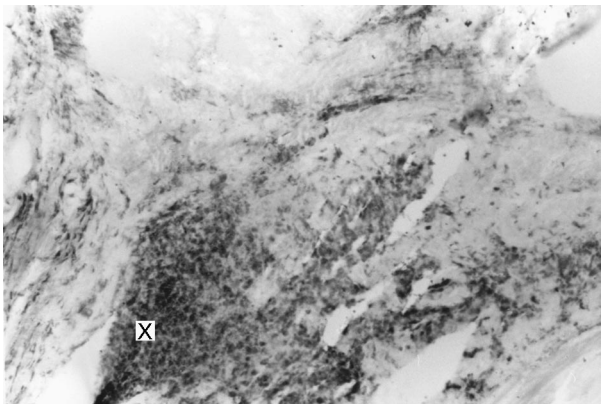

Figure 10 ED1 immunohistochemical staining of sample Z after 9 wk. X, tissue necrosis. × 40

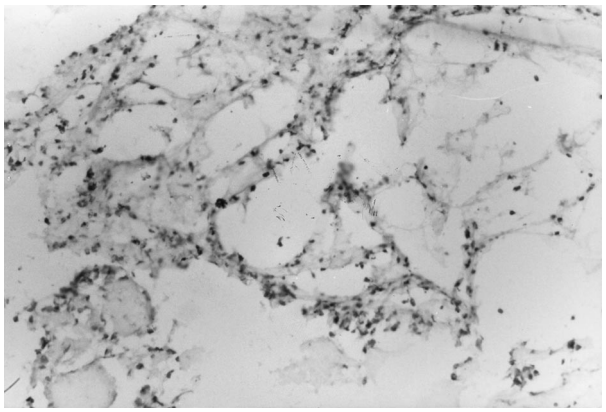

Figure 11 ED1 immunohistochemical staining of sample Y after 2 d. ED1 is stained black. × 40

4. Discussion and conclusion

The neutrophil presence at 2 d in all sections suggests an acute inflammatory response. The absence of these cells, however, in the remaining time periods indicate that this acute inflammation is quickly resolved. However, the presence of macrophages in all samples at all time periods (except X at 6 mon) indicate an on-going chronic inflammatory response to the presence of the implanted material. This study demonstrates that particulate, degrading glass is stimulating an inflammatory response in soft tissue at time periods up to 6 mon. The subsequent tissue response is clearly associated with the rate of degradation. Glass X, the slowest release rate, shows little evidence of any chronic inflammatory response by 6 mon, whereas glass Z with the greatest surface area and fastest solution rate, is clearly degrading at a rate which is leading to tissue damage and necrosis. The excessive presence of mast cells in the fibrous tissue local to the implant site in some cases may suggest a link with an allergic reaction to the glass presence. *In vitro* isolations of mast cell lines such as HMC-1 [14] will be carried out to determine the validity of this link.

This novel biomaterial has an exciting potential in a wide range of biomaterial applications. Its ability to dissolve within the physiological environment leaving no residue opens up an enormous number of varied applications. This study has as its objective an evaluation of the potential use of the particulate glass in stress incontinence applications. However, these data will serve to highlight the many other roles the CRG may play. It is, as yet, unclear how degrdation rates determined *in vitro* are modified *in vivo* and how the tissue will respond to release products. However, control of *in vivo* degradation remains a viable objective and will bring with it exciting possibilities in the field of space fillers, inorganic ion-release and tissue regeneration.

It should be noted that the very small particulate, fast degrading glass (glass Z) is leading to tissue necrosis and should not be considered further for this specific application. However, large particulate, slower degrading materials are demonstrating an effective and sustained inflammatory response and may prove useful for stress incontinence applications.

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