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Radiation demineralised bone enhanced osteoinductive capacity after transplantation

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Abstract

Using a mediating alkyne gas during the radiation treatment prevents the degradation of natural and synthetic polysaccharides and proteins. The product has higher viscosity and is more elastic than the original material and, therefore, gives enhanced functionality. Protein, within demineralised bone, too can be modified to give enhanced osteoinductive capacity after transplantation. Thus new functionalities can be achieved from the new products produced in food and medical products.

Keywords: Radiation; Polysaccharides; Cross-linking; Demineralised bone; Collagen; Molecular weight

1. Introduction

Previously a radiation process was described which in the presence of an acetylenic gas enables the cross-linking of polysaccharides, protein and interactive blends [1,2]. The process was shown to allow the production of a tailor-made and reproducible products and so be offered as a unique material capable of being offered to enhance the functionality for their existing application or for new applications which were not possible to achieve.

Human bone, when treated by a process for the differential removal of bone mineral, yields demineralised bone matrix (DBM) which has been shown to have the capacity to actively induce new bone [3].

The sequence of events that follow implantation of a DBM graft in vivo, has been well described [4]. Induction of new bone is due to bone morphogenetic proteins (BMP) within the matrix, while the non-BMP fraction of the matrix may have a passive role in retaining the BMP at the site of implantation, although it is not essential for the formation of new bone. Demineralisation leads to easier release of the BMP. The first step in the action of DBM is the differentiation of the proliferated mesenchymal cells at the implant site into chondroblasts, evidenced by the synthesis of type II collagen and cartilage proteoglycans [5]. This period of chondrogenesis takes approximately four days. Over the next three days (8–10 days post implantation) the cartilage begins to mineralise and vascularisation of the graft begins. At this point osteoblasts and osteoclasts are seen at the graft site. Alkaline phosphatase activity and calcium uptake increase, indicating the beginning of osteogenesis. The osteoblasts start making new mineralised bone, whilst the osteoclasts resorb the mineralised cartilage. Over the next weeks an ossicle of new bone is formed. Consequently, such DBM material has wide application in oral and maxillofacial surgery, since the osteoinductive capacity of such allogeneic bone has the ability to form new bone by transforming the primitive

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mesenchymal precursor cells into chondroblasts or osteoblasts [5,6]. The radiation process described here enables DBM to have greatly enhanced osteoinductive activity, which accelerates and improves the quality of new bone formation. In view of the results found with collagen, it is probable that these radiation induced changes can be attributed to cross-linking of the bone morphogenic protein.

2. Procedures

The radiation processing and animal experiments have been described [1]. DBM was subjected to the gas-mediated radiation processing treatment as described [2]. The samples are first evacuated of air and replaced with acetylene gas. A first γ-irradiation treatment with 10 kGy induces the cross-linking [2]. Thereafter, after triple packaging, the collagen carrier was added and γ-irradiated all samples of control DBM and radiation process DBM* were sterilized by γ-irradiation (15 kGy).

Ninety healthy male white rats (250–300 g) were used for this experimental study. The animals were divided in the three groups: negative control, control (using DBM), and experimental (using new processed bone DBM*)). The rats were housed (5 rats in each cage) in a standard Experimental Animal Room. They were fed a solid diet during the experimental periods. After 2 weeks adaptation periods, the animal experiments were started.

Healthy male rats were sacrificed by cervical dislocation in order to obtain the femurs and tibias of each rat. After procuring the bones, they were kept in a deep freezer at −70 °C) for 24 h and thereafter crushed to fine particles using a bone mill. The bones were then processed as follows: The particles were stirred in distilled water 6 times repeatedly every 30 min, demineralized in 0.5 N hydrochloric acid for 5 h, followed by four washings in sterile water for 2 h, soaked in absolute ethanol for 1 h at room temperature (25 °C), further washed in distilled water for 3 h, soaked in diethylether for 30 min and dried *in vacuo* overnight.

After these procedures the DBM particles were divided into various sizes using appropriate sieves. Only 350–600 μm sized DBM particles were used in this study. The DBM was subjected to the gas-mediated radiation processing treatment described above, after which they are as were designated New Processed Bone (DMB*). After triple packaging, all samples of DBM and DMB* were sterilized by γ-irradiation (1.5 Mrad).

Animals were injected preoperatively with Oxytetracycline and then anesthetized withKetamine Hydrochloride (intra-muscularly, 10 mg/kg) and Xylazine Hydrochloride (0.15 ml/kg).

In the usual manner, after calvarial flaps were reflected, bone defects were created around the middle calvarium with an ∅6 mm trephine-burr and a low-speed dental drill. During formation of defects with the burr, cooling water (sterilized saline) prevented the operating site from overheating. Then the three groups of animals were subjected to the following procedure:

- Negative control group: the wounds were closed without any graft materials.
- Control group: these were grafted with pure DBM (15 mg/each).
- Experimental group: these were grafted with DBM* (15 mg/each).

For all groups closure was performed using 4-0 Vicryl, layer by layer.

All the above procedures were performed aseptically and ethical approval was obtained prior to the experimental stages.

After surgery five animals from each group were sacrificed within 1, 2 and 3 weeks respectively, and the defect site in the calvarium removed with full thickness flap, including periosteum. The sections of the calvaria were fixed in 10% neutralized formalin.

After fixation of the calvarium in 10% neutralized formalin, the bone defects were decalcifying for 3 days with 5% nitric acid. In the usual manner, dehydration and cleaning were performed, after which the calvarium samples were embedded in paraffin. The paraffin sections were stained and examined under a microscope for bone formation.

Control group: these were grafted with pure DBM (15 mg/each).

Experimental group: these were grafted with DBM* (15 mg/each).

Full-thickness of calvarium, including the defect site, were obtained from the animals at each interval and tested for the strength of the inter-bony-union after varying periods of implantation (see Fig. 1).

3. Result and discussion

The results are shown in Table 1 for the control group and those implanted with the specially radiation processed bone. The processed demineralised bone (DBM*) is more effective than the control in creating new bone throughout the healing time of the bone, and after three weeks, the bone is more than three times stronger than is the control.

A major problem to be overcome in the application of DBM in human surgical transplantation is that currently both the DBM and any associated biopolymer carrier to facilitate the delivery of the powder DBM must be prepared under aseptic conditions and dispensed from a customised hypodermic syringe to ensure the sterile presentation of the osteoinductive agent during the surgical procedure. Moreover, these are usually dispensed as a wet putty or gel in a modified hypodermic syringe which must be stored as low as −40 °C to prevent any deterioration of the system. Such dispensers are, therefore, difficult to store and handle at such low temperatures and the integrity of the system can be harmed if the low temperatures cannot be

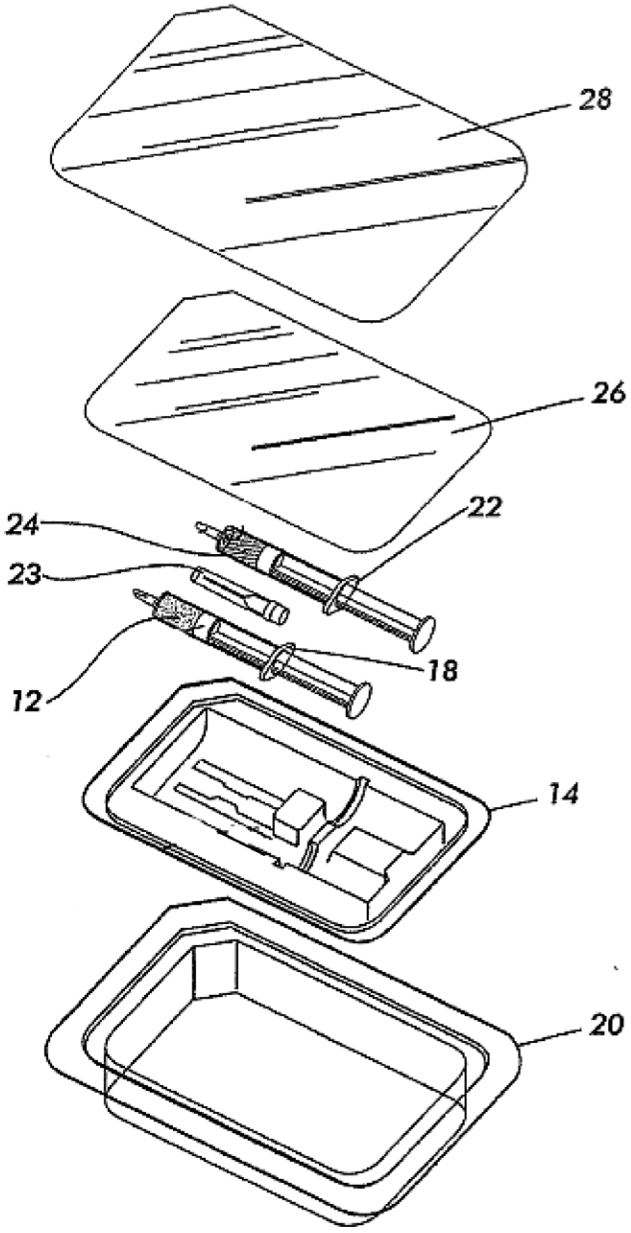

Fig. 1. A delivery system for demineralised bone – collagen surgical implant. (12) Crosslinked osteoniductive agent; (14) primary blister; (18) syringe for osteoinductive agent; (20) secondary blister; (22) syringe for sterile water; (23) needle; (24) sterile water; (26) primary peelable lidding; (28) secondary peelable lidding.

Table 1
Strength to inter-bony union (in MPa)

Time	DBM	DBM*
1 Week	2.7324	2.8731
2 Weeks	2.8231	4.3721
3 Weeks	2.8481	8.9940

strictly maintained. Without such a system the fine DBM powder must be handled and delivered to the surgical site in an arbitrary manner, with a valuable proportion ending up in the wrong location, even on the theatre floor!

The process reported here has the ability to transform DBM into a material with greatly enhanced osteoinductive activity (DBM*) reflected in a marked improvement in the strength of the newly formed bone. Furthermore, it can be terminally radiation sterilised, and using the novel delivery system which has been developed, which can effectively and safely deliver the DBM* to the surgical lesion [7]. Taken together, the processed DBM* packaged in the newly-developed delivery system can accelerate and improve the quality of new bone formation, and also be produced in ready-to -use administration sets which can be stored safely at room temperature for years. This system has already been extensively used in clinical practice in South Africa for more than two years and more than 1300 sets have been used with excellent results.

This process entails the filling of the hypodermic syringe with both the dry DBM and carrier mixture (which is collagen obtained from human cortical bone), and employing a hub with a small hole to close the syringe. The filled syringes can subsequently be evacuated to remove all air from the mixture, blanketed with the mediating gas, and subsequently exposed to the desired dose of gamma irradiation.

Following the radiation process, the syringe containing a mixture of DBM* and collagen is placed in a customised primary PET blister pack, together with a syringe containing an appropriate volume of sterile pyrogen-free water, as well as a needle. The primary blister is subsequently evacuated to remove all air and traces of the mediating acetylenic gas in the syringe with the crosslinked mixture, blanketed with high-purity nitrogen and hermetically sealed with a customised laminate sealing material to prevent any entry of air into the primary blister. The primary blister is subsequently placed into the secondary PET blister and sealed in air with the same lidding material. The double hermetically sealed set is then placed into a tertiary cardboard container and terminally gamma sterilised to a minimum absorbed dose of 25 kGy.

To use the set, the secondary and primary lid materials are peeled off in theatre and the water is injected with the aid of the needle into the syringe containing the crosslinked osteoinductive mixture in order to hydrate the latter. Following the hydration, the resultant pliable viscous putty is then applied to the operation site. In practice, on the election of the surgeon, the sterile pyrogen-free water has been replaced by blood of the patient to perform the hydration of the DBM*. Further biological methods have been used to confirm the effectiveness of the radiation processed bone, which are outside the scope of this paper.

4. Conclusion

DBM* shows a marked enhancement in osteogenic potential, providing a safe and easy-to-use demineralised allograft and xenograft as compared to non-processed demineralised bone. The new bone growth resulting from the use of DBM* shows an almost 300% increase in

strength compared to that of non-processed demineralised bone (Table 1). A further advantage of the of the DBM* is its increased mechanical strength and its relative insolubility, ensuring it does not wash away upon implantation, factors which have previously limited the use of DBM in surgical settings. The formation of the putty or gel reconstituted from the dry DBM* takes place much faster than its non-processed equivalent – a property that is advantageous during implantation. The faster solidifying gels are also better retained at the implantation site, an important factor therapeutically under conditions of bleeding. This allows for the benefits of the full dose of DBM to be delivered to the surgical site. The dry DBM* can be stored under inert nitrogen gas, eliminating oxidative degradation. This obviates the need to transport and store the product under refrigerated conditions, resulting in a shelf-life of at least five years. The product is reconstituted in fresh form in theatre directly before use.

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