Comparative Evaluation of Three Different Collagen-Ceramic Composite Scaffolds for Use as Alternatives to Bone Grafts

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CLINICAL POINTS

Large bone defects often require reconstructive surgical procedures involving transplantation of tissue.

The emergence of tissue engineering and the development of scaffolds is likely to circumvent some of the problems associated with traditional transplantation methods.

Biocompatibility, ease of sterilization and mechanical strength are important considerations in determining the suitability of a scaffold for a particular site.

Interconnected porosity is important also, as it affects the extent of vascularisation and the migration of proliferating cells within the scaffold.

Scaffold properties can be influenced by the properties of the materials it is composed of and the methods used in its preparation.

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Abstract

Aim of Study

This paper reports a comparative study conducted on novel Collagen-Ceramic composite scaffolds. Three scaffolds were compared based on pore size, porosity, and thickness of the collagen network. Characterisation of these properties will facilitate cell-specific optimisation of these scaffolds for use as successful clinical alternatives to auto- and allo-graft procedures.

Methods

Collagen-based scaffolds were fabricated using different forms of calcium phosphate and freezedrying methods. Scaffold A, the control, was made with calcium phosphate R powder and lyophilised using a stainless steel freezing tray. Scaffold B was made with calcium phosphate S and lyophilised using a stainless steel freezing tray. However scaffold C was made with calcium phosphate S and lyophilised using a polysulphone freezing tray.

Results

The results indicated that scaffold A had the minimum pore diameter for cell penetration and proper vascularisation of the ingrown tissue, but is less than ideal for optimum bone growth. Scaffold B showed a pore diameter that is almost three times larger and collagen network struts that were three times thicker than that of scaffold A. Scaffold C showed marked increase in porosity compared with scaffold B, but a thinner collagen frame.

Conclusion

study has helped to compare and contrast some of the different combinations of freezedrying treatments and calcium phosphate types, showing that numerous types of collagen scaffolds can be developed and customised to help in the treatment of various types of bone abnormalities. The large pore size and increased collagen frame thickness of Scaffold B appear to make it the most promising of the three scaffolds to be used in future bone graft procedures.

Introduction

Tissue engineering involves the application of principles of materials science, cell biology, and engineering to the development of ideal tissue substitute materials that can mimic and maintain normal tissue function[1]. Large bone defects often require reconstructive procedures for anatomical restoration and to achieve stability. This can be achieved by many different means (ranging from the use of supportive casts to a series of invasive techniques) depending on the fracture pattern and relevant patient factors.

Most clinical procedures involving large bone defects use autograft tissue transplantation (from the patient himself) as the source of the repair tissue. This autograft bone tissue is harvested from the patient and re-implanted at the site of bone tissue damage. Other sources of bone tissue include allografts (from a different individual), xenografts (from a different species, mainly bovine) and synthetic materials[2]. At present, autografts are the clinically preferred treatment since they allow earlier removal of external fixation devices, and improved anatomical and functional outcome[3]. However, there are a significant number of disadvantages with autograft tissue, specifically limited supply of donor material (particularly in older patients with degenerative bone diseases), donor-site morbidity, increased blood loss and postoperative pain[4].

Allografts, although currently in clinical use, are often complicated by fracture, resorption, and non-union between the graft and recipient bone[5]. Synthetic materials are slowly resorbed and replaced and therefore lead to longer healing and recuperation times. Little information is available regarding their long term effects and issues with toxicity are also of major concern[6,7].

However, the emergence of tissue engineering, through the development and application of scaffolds, promises to upgrade bone graft procedures and give physicians access to numerous therapies that allow healing of fractures and deformities. In 1970 W.T. Green, an orthopaedic surgeon, was responsible for conducting some of the first research in the area of tissue engineering. He stipulated that by implanting chondrocyte cells into the spicule of a bone (the area of bone matrix where cell multiplication and bone growth occurs) he would be able to cause cartilage to form. Although he was unsuccessful, this opened the doorway to tissue engineering. In the Mid-1980's, Dr. Vacanti and Dr. Langer devised a method to generate functional tissue by using bio-degradable polymers, called scaffolds, seeded with viable cells[8].

Scaffolds are artificial structures capable of supporting three-dimensional tissue formations, onto which cells can be implanted. One approach is to implant scaffolds for tissue growth in vivo to direct tissue formation in situ. Biocompatibility of the substrate materials is imperative – the material must not elicit an unresolved inflammatory response nor demonstrate immunogenicity or cytotoxicity[9]. The mechanical properties of the scaffold must be sufficient to facilitate the desired tissue healing and must not collapse when handled by the surgeon during the procedure. Tissue scaffolds must be easy to sterilise to prevent infection. A requirement of particular importance in bone tissue engineering is a controllable interconnected porosity to allow cells to migrate into the scaffold centre and to facilitate vascularisation of ingrown tissue. Porosity is a ➔

measure of void spaces in a material, where the void may contain gas or liquid. It is defined by the ratio:

$$\frac{\text{volume of void space}}{\text{total volume of the object}}$$

and can be expressed as a percentage. A typical porosity of 90% as well as a pore diameter of at least 100 μm is known to be a prerequisite for cell penetration and complete vascularisation of the implanted scaffold in bone tissue engineering[6].

This paper reports a study conducted on novel collagen-ceramic composite scaffolds and discusses their potential use in the treatment of various ailments involving bone deformities and abnormalities.

Background

In previous experimental work, calcium phosphate and collagen have been combined to make bone graft scaffolds, as they mimic the natural composition of bone. Collagen is the most abundant and ubiquitous structural protein in the body, and may be readily purified from both animal and human tissues[10]. Around 60% of bone weight is made of calcium phosphate (Ca10(PO4)6(OH)2), thus explaining why calcium phosphates have been intensively investigated as a major component of scaffold materials for bone tissue engineering[6]. Calcium phosphates have an excellent biocompatibility due to their close chemical and crystal resemblance to bone mineral. They also have osteoconductive properties and can bind directly to bone under specific conditions[6].

While the excellent biological performance of calcium phosphates has been well documented, their relatively slow biodegradation and their low mechanical strength limit their application in engineering of new bone tissue, especially at load bearing sites. The properties of synthetic calcium phosphates vary significantly with their crystallinity, pore diameter, porosity and composition. More extensive interconnected porosity of a substance permits faster bone ingrowth but weakens the material; the ideal pore size is thought to be between 150-500 μm[11]. Increased crystallinity, low porosity and small pore diameter tend to give higher stiffness, higher compressive and tensile strengths, and greater fracture toughness[6].

This study investigated three different types of scaffolds made with different forms of calcium phosphate (CP) and treated with different freezedrying methods. Scaffold A, the control, was made with calcium phosphate R powder (CP R) and lyophilised using a stainless steel freeze drying tray. This was chosen as the control because previous testing found it to have the qualities needed for sufficient bone growth[12]. Scaffold B was made with calcium phosphate S (CP S) and lyophilised using a stainless steel freeze drying tray. Scaffold C was also made with CP S but was lyophilised using a polysulphone freeze drying tray. The exact compositions of the two different types of calcium phosphate preparations can not be disclosed in this paper, as they are awaiting patency for future distribution and marketing. However, it can be mentioned that the molecular make up of CP S is subjectively weaker than CP R as it has a lower crystallinity and density.

The three scaffolds were compared based on the thickness of the collagen frame, porosity and pore size. To achieve a more complete comparison of the three scaffolds, mechanical testing should be carried out and their tensile and compressive strengths should be compared with various types of bones within the human body. Use of Calcium phosphate S rather than Calcium phosphate R and freezedrying with the polysulphone tray rather than the stainless steel tray are two areas that are expected to have an important role in the future of tissue engineering practices. Characterisation of these properties will facilitate cell-specific optimisation of these scaffolds for use as successful clinical alternatives to auto- and allo-graft procedures.

Methods

Scaffolds are produced using a lyophilisation (Freeze-drying) technique. Firstly, a collagen slurry is fabricated by blending the collagen in a mild acetic acid solution. Maintaining a constant low temperature during this step ensures that the collagen protein does not denature and gelatinise. The suspension is then Freeze-dried using rigidly controlled temperature and pressure parameters results in a highly porous collagen-calcium phosphate composite scaffold. Briefly freezing the suspension results in the nucleation of ice crystals, which are surrounded by the collagen-calcium phosphate composite material. Essentially, the collagen-calcium phoshpate co-precipitate is forced into the spaces between the growing ice crystals to form a continuous interpenetrating network of ice and co-precipitate. By sublimating the ice crystals out over a long period of time, a sponge like three dimensional construct, comprised of a biocompatible collagen-CP composite, is produced. The use of a freezedryer allows extremely precise control of the shelf (and consequently the slurry), temperature. This controls the size of the resulting pores in the fabricated collagen scaffold.

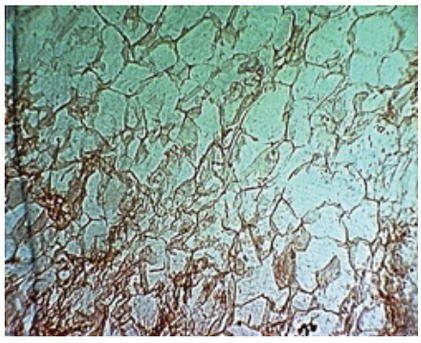
Synthesis of Scaffolds

3.6 grams of type 1 microfibrillar bovine tendon collagen was added to a 0.05M acetic acid solution. This suspension was blended in a cooled reaction vessel at 4°C for 90 minutes. Fifty percent by weight of calcium phosphate powder was added to the slurry and the slurry was blended for a further 90 minutes. The slurry was then degassed. Once degassed, 67 ml of slurry were placed in a suitable freeze drying tray (stainless steel or polysulphone), and placed in the freezedryer. Once the freeze drying process had been completed, samples of the scaffold were removed using a sharp circular punch (9.5 mm diameter) for analysis of pore structure.

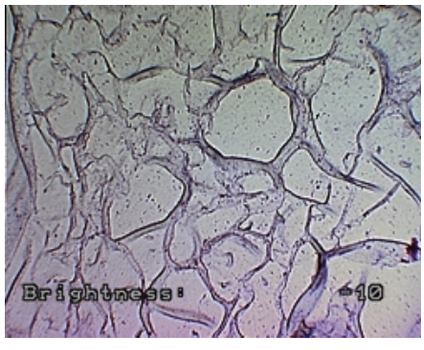
Preparation of scaffolds for microscopy

The samples were embedded in JB-4 glycol methacrylate in order to make them suitable for slicing, staining and observation under a light microscope. Firstly, the collagen-CP samples were fixed using 10% neutral buffered formalin for 24 hours at room temperature to ensure sample morphology was not affected by the embedding process. Samples were then dehydrated in a series of alcohol solutions over 24 hours: The distilled H20 and the 100% EtOH steps were repeated multiple times to allow→

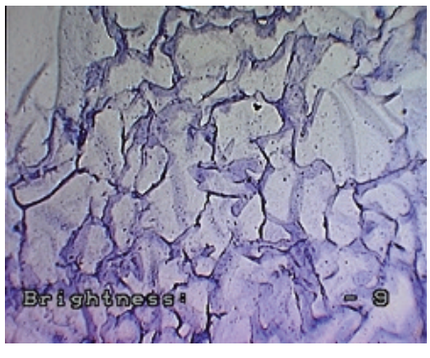
Solution	Time	Temperature
distilled H_20	30 mins - 1 hour	room temp.
distilled H_20	30 mins - 1 hour	room temp.
50% EtOH	30 mins - 1 hour	room temp.
70% EtOH	30 mins - 1 hour	room temp.
80% EtOH	30 mins - 1 hour	room temp.
95% EtOH	30 mins - 1 hour	room temp.
95% EtOH	30 mins - 1 hour	room temp.
100% EtOH	30 mins - 1 hour	room temp.
100% EtOH	30 mins - 1 hour	room temp.
100% EtOH	30 mins - 1 hour	room temp.



▲ ***Fig. 1. Scaffold A (control).***
at 10X magnification.



▲ ***Fig. 2. Scaffold B.***
at 10X magnification. Note enlarged pore diameter.



▲ ***Fig. 3. Scaffold C.***
at 10X magnification. Note increased porosity.

extended time for proper hydration and dehydration respectively. Samples were equilibrated for 12 hours at 4°C in 1:1 ethanol:catalysed JB4 solution A. Samples were then infiltrated with 100% JB4 A and the solution was catalysed using JB4 B to polymerise the embedding solution. The samples were sliced 10 micrometers thick using the microtome and every fifth slice was stained using either Alizarin Red or Toluidine Blue and mounted upon a slide for microscopy.

Comparative analysis of scaffolds

A quantitative pore analysis was then carried out in which stained samples were imaged using light microscopy at a magnification of 10 times (10X) and representative images of the pore structure were digitally captured. The definitive porosity and collagen thickness were not calculated, but a subjective comparison of the images obtained was done to get an indication of the differences between the scaffold samples. To measure strength and rigidity, mechanical testing on the different scaffolds was scheduled. However, due to time constraints, the results are not available for inclusion in this paper.

Results

Scaffold A (the control) was made with calcium phosphate "R" powder and placed with the standard metal tray in the freezedryer. It displayed a pore size of approximately 100 μm in diameter. Figure 1 shows a Scaffold A sample stained with Alizarin Red.

Scaffold B was made with calcium phosphate "S" powder and placed with the standard metal tray in the freezedryer. The pore diameter was approximately 250- 300 μm with a thick collagen frame. Figure 2 shows a Scaffold B sample stained with Toluidine Blue.

Scaffold C was made with calcium phosphate "S" powder and placed with the polysulphone plastic tray. The pore diameter was approximately 200 μm. Using subjective comparison, a marked increase in porosity compared with scaffold B was noted, with a markedly thinner collagen frame. Figure 3 shows a Scaffold C sample stained with Toluidine Blue.

Discussion

For the successful transfer of extracorporal bone tissue engineering in clinical practice, clear and precise definition of the clinical demand or problem should be addressed. The severity and the location of the bone defect needs to be precisely assessed before planning tissue engineering. Clinical aspects that should be considered in bone tissue engineering include the evaluation of the biomechanical aspects of the skeletal implantation site, the immunological reactions towards the scaffold construct and the biodegradability of the scaffolds[13].

One can infer from comparison of Scaffolds A, B and C that their different porosities and pore sizes may result in different bone healing responses and thus could be used to develop different types of bone grafts for use in various body sites. Scaffold A displayed the minimum pore diameter (100 μm) for cell penetration and sufficient vascularisation of the ingrown tissue, but it was less than ideal (150-500 μm) for optimum bone growth6. In scaffold B, the freezedrying method remained the same, but the form of calcium phosphate used was switched from CP R to the subjectively inferior CP S. This led to a pore diameter that was almost three times larger and a collagen frame that was markedly thicker due to the increase in amount of collagen available per pore. Although the results from mechanical testing are not available at present, it can be postulated that scaffold B would be weaker than scaffold A due to the greater pore diameter of scaffold B.

From a clinical perspective, bone grafts made with CP S scaffolds would be reserved for areas requiring weaker, non weight bearing bones such as those in the maxillary facial areas. Considering the fact that materials of low heat conductance allowed greater nucleation of ice crystals in the freezedryer, the study expected that treating Scaffold C with the polysulphone plastic trays should result in a larger pore diameter compared to those treated with the stainless steel tray (i.e. Scaffolds A and B). Its reason for having a smaller pore diameter than scaffold B remains unclear and should be studied extensively.

Conclusion

The current objective in tissue engineering is to design reproducible bioactive and bio-resorbable three dimensional scaffolds with customized porosity and pore structure. These should also retain their structure and integrity for predetermined times in the presence or absence of load bearing conditions(6). Use of Calcium phosphate S rather than Calcium phosphate R and freezedrying with the polysulphone tray rather than the stainless steel tray are two areas that are expected to have an important role in the future of tissue engineering practices.

Although all three scaffolds had the minimum pore diameter for cell penetration and proper vascularisation of the ingrown tissue, scaffold B seemed to have struck the best balance in terms of large pore size for fast vascularisation and thick collagen frames for support. Given these characteristics, scaffold B seems to be the most promising scaffold for use in future bone graft procedures. Further evaluation of its strength, toxicity and impact on human healing must be carried out before recommending its use in a clinical setting.

This study has helped to compare and contrast some of the different combinations of freezedrying treatments and calcium phosphate types, showing that numerous types of collagen scaffolds can be developed and customised to help in the treatment of various types of bone abnormalities. However, before being deemed safe and clinically applicable, in vitro and in vivo studies are imperative in order to integrate this technology into the human biological system and to reduce unwanted side effects and toxicity.

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