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The effect of sterilization methods on the osteoconductivity of allograft bone in a critical-sized bilateral tibial defect model in rabbits

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ABSTRACT

Clinically, allogeneic bone graft is used extensively because it avoids the donor site morbidity associated with autograft. However, there are concerns over the optimal sterilization method to eliminate immunological risks whilst maintaining the biological efficacy of the graft. This study compared the effect of Supercritical fluid (SCF) treatment and gamma irradiation at 25 kGy on the osteoconductivity of allograft bone in a bilateral critical sized defect rabbit model. Osteoconductivity was evaluated at 2 and 4 weeks using X-ray, CT, histology (qualitative and quantitative) and immunohistochemistry (Alkaline Phosphatase and Cathepsin-K). Both grafts were well tolerated and osteoconductive. At 2 weeks, there was decreased bone volume and density in the gamma irradiated graft compared to the SCF treated graft, corresponding with a greater inflammatory response histologically and increased Cathepsin-K expression. Catabolic activity predominated at 4 weeks, with both grafts undergoing significant resorption and remodeling inside the defect. Alkaline Phosphatase expression was greater in the SCF group at both time points indicative of a more anabolic response. Allograft bone sterilized with either gamma irradiation or SCF treatment was osteoconductive and capable of healing a critical sized tibial defect in a rabbit. Gamma irradiated allografts elicited an acute inflammatory reaction when implanted which may increase the amount of graft resorption compared to the SCF treated bone.

1. Introduction

Despite the emergence of synthetic bone graft substitutes as a viable option for the treatment of a range of bony defects, allograft bone remains the most commonly used graft behind autograft. Bone allografts have been used in a range of clinical situations to fill bone defects [1–3], gaps in multifragmentary fractures [4], non-unions [5,6], cysts and voids resulting from tumor removal due to osteosarcoma [7–9]. Despite their widespread use, concerns relating to disease transmission or host immune response result in all allograft tissues undergoing processing and terminal sterilization prior to implantation. Current techniques used to process and sterilize allograft bone compromise the inherent mechanical and biological properties of the graft which hinder their clinical performance.

Gamma irradiation is currently the most common method to terminally sterilize bone allograft due to its efficacy against bacteria and viruses, and good tissue penetration characteristics [10]. However, it has been clearly established that gamma irradiation damages bone in a dose dependent manner through either direct [11,12] or indirect mechanisms [13,14]. These changes have been shown to disrupt the attachment, viability and differentiation of osteogenic cells *in vitro* [15–18]; while *in vivo* studies have reported significant dose dependent reductions in graft incorporation using different animal models [19–21]. Despite these results, retrospective meta-analyses comparing the clinical outcomes of gamma irradiated vs. non-gamma irradiated allografts report conflicting findings. Buckley et al. [22] reported no difference in the effectiveness of gamma irradiated vs. fresh-frozen allografts used in acetabular revisions at 5 years, with an approximate 88% survivorship in both. Conversely, in another study comparing complications in tumor reconstruction surgery, the incidence of fracture was more than double with gamma irradiated (39%) vs. non-irradiated bone (18%) [23]. Such reports are difficult to interpret given the different repair environments and thus the range of other confounding variables which can affect the clinical outcome in addition to the properties of the graft. Regardless, these reports highlight the need for more controlled research investigating the effects of gamma irradiation under relevant treatment conditions and in models analogous to clinical bone grafting situations.

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Recently, supercritical fluid (SCF) technology has been investigated as a potential alternative to gamma irradiation. A SCF is any a substance that exists above its critical temperature and pressure, resulting in unique physicochemical properties intermediate between a liquid and a gas [24]. Supercritical carbon dioxide ($SCCO_2$) is the most commonly used SCF due to its low critical parameters (T_c = 31.1 °C, P_c = 73.4 bar), which allows it to be used to treat thermally labile biological tissues without deleterious effects on proteins and enzymes [25]. Furthermore, under these conditions $SCCO_2$ has been used to terminally sterilize a range of bacteria and viruses [26–32] and facilitate delipidation of bone [33]. Additionally, we have reported that the mechanical properties of cortical bone under different loading modalities are also preserved following SCF sterilization [34]. Given these promising results, the aim of the present study was to investigate the effect of SCF sterilization on the osteoconductive properties of allograft bone. Osteoconductivity describes the grafts ability to act as a scaffold for the attachment and proliferation of host mesenchymal stems cells to begin osseointegration [35,36], and is thus central to the clinical efficacy of a graft. In this study, cortical-cancellous allograft was prepared and sterilized using either SCF sterilization or gamma irradiation and then implanted into an established bilateral tibial defect model in rabbits [37–41]. The osteoconductivity of the grafts was then evaluated at 2 and 4 weeks to evaluate the effect of these treatments on the early inflammatory and early bone formation stages of defect healing respectively.

2. Materials and methods

2.1. Graft preparation

Cortical-cancellous allograft chips were prepared from the tibias of 6 month old female New Zealand white rabbits used in other studies conducted within this laboratory. The tibias were denuded of residual soft tissue and then progressively milled down to a 1–2 mm particle size using a rotary drum mill (Noviomagus Bone Mill, Spierings Orthopaedics, The Netherlands). Following milling, the bone chips were cleaned by lavaging with sterile saline and then soaking in 6% hydrogen peroxide solution for two 15 min periods. This was followed by a further 30 min of soaking in 70% isopropanol alcohol solution in an ultrasonic bath, then rinsing again with sterile saline. During all stages of cleaning aseptic practices were maintained.

2.2. Sterilization

Prior to sterilization the bone chips were weighed and divided into two equal groups for gamma irradiation at 25 kGy or supercritical fluid (SCF) sterilization. The equal portions were subsequently divided into ten smaller portions and packaged into Tyvec® pouches (DuPont, Paris, France) for sterilization.

The gamma irradiated grafts were sterilized at a dose of 25 kGy using a $cobalt^{60}$ irradiation source (Steritech, Wetherill Park, Australia), with dosage confirmed using perspex dosimeters. The grafts were packaged on dry ice to maintain low temperatures during treatment. This has been shown to minimize collagen damage [42] and free radical generation [14] which impair the properties of the graft. Following treatment, the bone was removed from the dry ice and kept at room temperature until surgery.

The SCF group was sterilized using a *Nova2200* supercritical carbon dioxide sterilizer (NovaSterilis, NY, USA). Briefly, the grafts were sealed inside a stainless steel pressure vessel. Carbon dioxide gas was then heated and pressurized past its critical point to 35 °C and 1450 psi where it becomes supercritical. *Novakill*© additive, containing peracetic acid (14.1%) and hydrogen peroxide (4.9%), was introduced at between 25 and 100 ppm and mixed inside the vessel for 1 h. The system was then depressurized and the grafts removed and stored at room temperature until surgery.

Three samples of graft material from each group were imaged using a tabletop scanning electron microscope (Hitachi Tabletop Microscope TM-1000, Hitachi, Tokyo, Japan) for time zero analysis. Low to moderate magnification (50–1000× objectives) images were taken looking for any noticeable changes in surface characteristics between groups.

2.3. Surgery

The experimental model used for this study was an established bilateral metaphyseal tibial critical-sized defect model in rabbits [37–39,41]. Ethical approval was obtained from the Animal Care and Ethics Committee of the University of New South Wales prior to commencement. Ten skeletally mature 6 month old (3.5 kg) New Zealand White rabbits were used in this study, providing twenty defect sites. Under general anesthetic, the skin and periosteum over the proximal tibia were incised and reflected using a periosteal elevator. A 5 mm wide by 15 mm long defect spanning the metaphyseal and diaphyseal region was then created 3 mm below the joint line in the anteromedial cortex. The defect was created using a microburr with a 3 mm diameter tip under saline irrigation to minimize thermal damage. The defects were flushed with sterile saline to remove any residual bone particles or other debris. Graft material was then packed into the defects up to the height of the original cortex with approximately 0.75 cc of either the gamma irradiated or supercritical fluid sterilized graft (n = 5 per group per time point). A spatula was used to ensure tight packing of the defect. The periosteum was then reflected back over the defect being careful to maintain packing, and the skin was closed in layers using 3-0 Vicryl suture (Ethicon, Somerville, NJ). Animals were given post-operative analgesia (Temgesic 1 mL subcutaneously) and returned to their holding cells. The animals were free to weight-bear immediately post-operatively as tolerated.

2.4. Tissue processing and endpoints

The tibias were harvested at 2 and 4 weeks post-operatively. Experimental sample size was determined by a prospective power analysis of published data in the same experimental model [37–39]. Expected values and effect size were estimated based on positive (autograft) and negative control (empty defect) data, as well as data from synthetic osteoconductive materials. An ANOVA a priori power analysis with an alpha value of 0.05 and beta value of 0.2 was then used to determine a sample size of n = 5 per group per time point (Table 1).

Anteroposterior and lateral radiographs of all harvested tibias were taken using a high resolution Faxitron X-Ray machine (MX20, Faxitron X-Ray Corporation, IL, USA). The X-rays were then examined in a blinded fashion by two investigators to assess implant resorption and healing using semi-quantitative grading scales [37–39]. Implant resorption was graded on a scale of 0–4: 0 = no resorption, 1 = softening or rounding of implant edges, 2 = 33% resorption, 3 = 67% resorption, 4 = complete resorption. Radiographic healing was graded on a scale of 0–4: 0 = no healing, 1 = 25% of cortex closed, 2 = 50% of cortex closed, 3 = 75% of cortex closed, 4 = complete cortical closure. Semi-quantitative scores were analysed using a Mann–Whitney U test in IBM SPSS Statistics 20 (20.0, SPSS Inc., NY, USA).

Computed tomography (CT) was performed on each tibia using a Siemens Somatom Sensation 4 CT scanner (Siemens Medical Solutions, TN, USA). Bone volume and density were calculated for a region of interest extending from the border of the defect sagittally to the posterior tibial cortex using Inveon Research Workplace software package (Siemens Medical Solutions, TN, USA). Following CT, all specimens were fixed in 10% neutral buffered formalin for a minimum of 48 h, decalcified in 10% formic acid and embedded in paraffin for qualitative histology and histomorphometry. The tibias were sectioned parallel to the long axis of the tibia through the anteromedial aspect of the defect. Five micron sections were cut and stained using routine Hematoxylin and Eosin (H&E). Two sections per defect were used for both qualitative and quantitative analysis (n = 10 per group per time point). Histomorphometric assessment was performed on all H&E slides using a custom-written Matlab script (Matlab 7.11.0, The Mathworks Inc., MA, USA). 1.25× objective images of the whole defect were taken and then preprocessed to convert the color space from red/green/blue (RGB) to Commission Internationale d'Eclairage L*a*b. The region of interest of the defect was then selected, and areas of residual graft material, new bone and fibrous tissue were assigned by thresholding the color intensity of the image. The resultant number of pixels was then used to express the amount of each material as a percentage of the whole defect.

Immunohistochemistry was performed on three animals per group per time point. Paraffin sections were processed using a procedure derived from techniques previously reported [43,44]. Alkaline Phosphatase (ALP) (sc-137213 Santa Cruz Biotechnology Inc.) at a concentration of 0.125 μg/mL was chosen as a generalized anabolic indicator of bone activity. Similarly, Cathepsin-K (Cat-K) (sc-48353, Santa Cruz Biotechnology Inc.) at a concentration of 0.05 μg/mL was selected as a generalized marker of catabolic activity [45,46]. Furthermore, nonimmunized mouse immunoglobulin (IgG) (DakoCytomation, Glostrup, Denmark) at a concentration of 2 μg/mL was used as a negative control to rule out nonspecific staining. Sections were assessed qualitatively for the staining intensity and the distribution of expression in different tissue components (residual graft material, new bone and fibrous tissue) and cell populations (osteoblastic-like cells, osteoclastic-like cells, osteocytes, mesenchymal stem cells and fibroblasts) of interest. Histomorphometry

Table 1
Summary of the study design; n = 5 per group per time point for the SCF sterilization and 25 kGy gamma irradiation groups.

Group	Time point	
	2 weeks	4 weeks
SCF	5	5
Gamma	5	5
Sample size	10	10