The use of a novel bone allograft wash process to generate a biocompatible, mechanically stable and osteoinductive biological scaffold for use in bone tissue engineering

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Abstract

Fresh-frozen biological allograft remains the most effective substitute for the ‘gold standard’ autograft, sharing many of its osteogenic properties but, conversely, lacking viable osteogenic cells. Tissue engineering offers the opportunity to improve the osseointegration of this material through the addition of mesenchymal stem cells (MSCs). However, the presence of dead, immunogenic and potentially harmful bone marrow could hinder cell adhesion and differentiation, graft augmentation and incorporation, and wash procedures are therefore being utilized to remove the marrow, thereby improving the material’s safety. To this end, we assessed the efficiency of a novel wash technique to produce a biocompatible, biological scaffold void of cellular material that was mechanically stable and had osteoinductive potential. The outcomes of our investigations demonstrated the efficient removal of marrow components (~99.6%), resulting in a biocompatible material with conserved biomechanical stability. Additionally, the scaffold was able to induce osteogenic differentiation of MSCs, with increases in osteogenic gene expression observed following extended culture. This study demonstrates the efficiency of the novel wash process and the potential of the resultant biological material to serve as a scaffold in bone allograft tissue engineering. © 2014 The Authors. Journal of Tissue Engineering and Regenerative Medicine published by John Wiley & Sons Ltd.

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1. Introduction

An ever-increasing need for bone graft material is placing increased pressure on its availability, quality and integration capabilities (National Joint Register, 2012). Biocompatibility, osteogenic capacity, biomechanical strength and architecture are all important factors in the successful incorporation of graft bone and can determine the speed of recovery (Marcos-Campos et al., 2012). As such, for clinical use it is important to use a material which encompasses these qualities.

The ‘gold standard’ grafting material is currently autograft bone. Sourced from the patient, this mix of mineralized extracellular matrix (ECM), bone marrow and osteogenic cells is the most osteogenic material available, with both osteoinductive and osteoconductive properties. However, the quality and volume of the material acquired is often not able to meet the demands of surgical procedures requiring large volumes, may be of substandard biomechanical stability and may contain few viable pre-osteogenic cells, e.g. mesenchymal stem cells (MSCs) (Hernigou et al., 2005).
Synthetic and biological alternatives are available to autografts (Kolk et al., 2012; Seebach et al., 2010); however, whilst synthetic materials aim to duplicate many of the same features found in autografts (Beswick and Blom, 2011), with post-production modifications and addition of growth factors, biological materials are still considered the most effective and are the most widely-used alternative (Zimmermann and Moghaddam, 2011).

A commonly used biological alternative to autograft is allograft bone, incorporating many of the same qualities as autograft and displaying good healing potential (Miller and Block, 2011). However, it does not include a source of viable osteogenic cells and is therefore associated with a lack of osteogenic and angiogenic stimulatory properties (prerequisite properties for clinical success). Consequently, bone tissue-engineering approaches aim to incorporate osteogenic cells into allograft scaffolds (Schubert et al., 2011; Aarvold et al., 2012; Xie et al., 2007) and improve the properties of this biological material. However, while allograft contains no viable cells, it does contain residual dead cell matter, which reduces the ability of progenitor or osteogenic cells to adhere to bone, may act to induce an immune response and could potentially harbour transmissible diseases (Varettas and Taylor, 2011), all of which could profoundly affect its use as a scaffold in bone tissue engineering. This potential for harmful disease transmission, albeit somewhat alleviated by stringent donor selection, virus and microbiological testing, means that it is advised that allograft material is sterilized by γ-irradiation before clinical use (American Association of Tissue Banks, 2006). γ-Irradiation involves subjecting the target material to a dose of 25 kGy whilst deep-frozen. However, this sterilization technique fails to remove the dead cell matter and marrow containing the antigenic cell types (Czitrom et al., 1985) and thus it can still elicit an immune response (Bos et al., 1983). Furthermore, this method has been demonstrated to decrease osteogenic potential by reducing biocompatibility through the production of peroxidized lipids (Moreau et al., 2000), as well as diminishing the biomechanical stability of the bone (Dux et al., 2010; Cornu et al., 2011).

Alternative sterilization or processing methods have been described for human bone material, either in addition to, or as a replacement for, γ-irradiation. These methods attempt to remove all soft tissue from the allograft, leaving behind a scaffold devoid of cellular material whilst retaining biocompatibility and biomechanical stability (Hassabella et al., 2009).

Although the addition of chemical agents, such as peracetic acid and ethanol, may reduce the osteoinductive potential of the material (Bormann et al., 2010), their controlled use has been shown to have a limited effect on the biomechanical stability and biocompatibility of the material (DePaula et al., 2005; Haimi et al., 2009), whilst still reducing infectious viral particles and killing harmful bacteria (Pruss et al., 2003). This potential for complete removal of immunogenic cellular components has led to marrow removal techniques becoming more prevalent in the production of structures required as scaffolds in tissue-engineering applications (Ma et al., 2013; Djouad et al., 2012; Hashimoto et al., 2011; Dutra and French, 2010).

Although tissue-engineered bone allografts offer huge potential for the improvement of bone regeneration, a method of producing a biomechanically stable and biocompatible bone allograft material that retains the bone’s innate structure and osteogenic properties is still required. Furthermore, for clinical translation, the methods to produce such allografts should be rapid and require minimal processing, whilst allowing the generation of sufficiently large structures for bespoke bone void filling. Thus, this study was designed to assess the application and efficacy of a novel bone allograft wash process on human bone from whole femoral heads. This wash process was modified from that published by Yates et al. (2005) to be more rapid and include the chemical sterilants peracetic acid–hydrogen peroxide and ethanol and an increased number of wash/centrifugation steps. Specifically, it aimed to ascertain whether the resulting structure had any alterations to its biocompatibility, immunogenicity, osteoinductive ability or biomechanical stability, which will ultimately determine its potential as a scaffold in bone tissue-engineering applications.

2. Materials and methods

2.1. Preparation of washed bone material

2.1.1. Sample procurement

Fresh-frozen femoral heads were obtained, through ethical approval from the National Health Service Blood and Transplant Tissue Services, from consenting live donors undergoing hip replacement surgery: male, n = 16; aged 38–82 (mean 53) years; and female (n = 12; aged 42–78 (mean 41) years. Upon removal, the samples were stored at –80 °C until required.

2.1.2. Wash process

Whole femoral heads were defrosted overnight at 5 °C, then submerged in 300 ml distilled water preheated to 60 °C and sonicated for 15 min at 60 °C (F5300b; Decon, UK). The femoral heads were then drained and rinsed in 300 ml distilled water preheated to 60 °C whilst agitated on an orbital shaker at 200 rpm at 60 °C for 5 min (IOC400; Weiss-Gallenkamp, Loughborough, UK). The femoral heads underwent a wash–centrifugation combination three times, in which they were submerged in 300 ml distilled water preheated to 60 °C and agitated in an orbital shaker at 200 rpm at 60 °C for 10 min, with an initial wash of 30 min and subsequent washes 10 min. The femoral heads were then centrifuged at 1850 × g for 15 min at ambient temperature ( Sorvall, using rotor RC3BP; Thermo-Scientific, Hemel Hempstead, UK). After three wash–centrifugation steps the femoral heads were sonicated for 10 min at 60 °C in 300 ml prewarmed (60°C) sterilant solution containing 3% v/v...
hydrogen peroxide (H3410, Sigma-Aldrich, Gillingham, UK) and 0.02% v/v peroxo-acetic acid (PAA; 77240, Sigma-Aldrich). Samples were transferred to 300 ml 70% v/v ethanol and sonicated for 10 min at 21 °C. Finally, the femoral heads underwent two washes in 300 ml distilled water and agitated at 200 rpm for 10 min at 60 °C before being centrifuged at 1850 × g for 15 min at ambient temperature to remove any remaining liquids. The washed, disinfected femoral heads were then dissected into 1 cm³ bone cubes, using an oscillating saw (NS3A, De Soutter, Aston Clinton, Buckinghamshire, UK), snap-frozen and stored dry at –80° until use.

2.2. Assessment of wash efficiency

2.2.1. Biochemical assays

To assess the removal of marrow contaminants from the whole femoral head, samples of wash solutions were collected after each step of the protocol (n = 14). The soluble components, protein, DNA and haemoglobin, were assessed using Bradford reagent (B6916, Sigma-Aldrich); PicoGreen assay (P7589, Life Technologies, Paisley, UK) and Drabkin’s reagent (D594, Sigma-Aldrich), prepared with Brij 35 solution (B4184, Sigma-Aldrich), respectively, using 50 µl aliquots and conducted according to the manufacturers’ protocols.

2.2.2. Residual DNA quantification within washed bone samples

Bone cubes from three fresh-frozen and three washed femoral heads (1 cube/femoral head) were snap-frozen, impacted and ground to produce a coarse powder. Samples (100 mg) were predigested (56 °C for 48 h) in proteinase k solution (19131, Qiagen, Manchester, UK) before DNA was extracted using the DNeasy Blood and Tissue Kit (69506, Qiagen). Extracted DNA was concentrated using ethanol precipitation and total DNA quantified using a Nanodrop spectrophotometer (ND1000, Thermo-Scientific, Hemel Hempstead, UK).

2.2.3. Histological analysis

Cubed bone samples from the fresh-frozen (n = 2) and washed bone (n = 2) were fixed in 50 ml formal saline (4% v/v formaldehyde, 0.9% w/v sodium chloride) at room temperature for 48 h, then decalcified in 20% v/v EDTA, pH7.4. Decalcified bone samples were embedded in paraffin wax and 5 µm sections cut and mounted. The sections were stained with haema-toxylin and eosin (H&E) and Masson’s trichrome (RRSP131-c, Biostain, Manchester, UK).

2.2.4. Immunohistochemical analysis

Sections of fresh-frozen (n = 3) and washed bone (n = 3) were dewaxed and rehydrated before antigen retrieval using chemotrypsin, and incubated overnight at 4 °C with primary HLA class 1 ABC (EMR8-5) antibody (ab70328; Abcam, Cambridge, UK). The samples were incubated for 30 min with the secondary antibody (sc-3795; Santa Cruz Biotechnology, Heidelberg, Germany) and antibody binding disclosed using the avidin–biotin interaction (PK-7100; Vector Laboratories, Peterborough, UK) and DAB (3,3-diaminobenzidine) staining (H-2200, Vector Laboratories). All images were acquired on a Leitz DMRB microscope (Leica Microsystems, Milton Keynes, UK), using Deltapix Infinity X and supporting software (Deltapix, Maalov Beyvej, Denmark).

2.3. Biocompatibility of washed bone

2.3.1. Production of bone extract-conditioned medium

Powder samples of bone cubes from fresh-frozen, unwashed (n = 3) and washed (n = 3) bone samples were soaked in five times their weight of standard culture medium for 72 h at 37 °C under constant agitation (ISO-10993-5, 1993E). The solution was centrifuged at 400 × g for 5 min and at 10875 × g for 10 min to remove fine bone particles. The resulting supernatant was used as extract-conditioned medium in cytotoxicity assays on MSCs.

2.3.2. Effect of extract-conditioned medium on MSC viability

Human MSCs were isolated from the bone marrow of a 55 year-old female, with ethical approval using established methodology (Strassburg et al., 2010). The cells were expanded in standard culture medium containing α-modified Eagle’s medium (M4526, Sigma-Aldrich), 10% fetal calf serum (PCS; 10270, Sigma-Aldrich), 10 µM ascorbate-2-phosphate (A8960-5G, Sigma-Aldrich), 2 mM GlutaMAX (35050-038, Life Technologies, Paisley, UK) and combined antibiotic–antimycotic solution (50 000 U penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B; A5955, Sigma-Aldrich). At passage 2, MSCs were seeded into 96-well plates at 3.3 × 10⁴ cells/cm² and incubated for 24 h. The culture medium was replaced with 100 µl extract-conditioned medium. After 24 h of incubation, cell viability was assessed by adding 5 µl WST-1 (05015944001, Roche, UK) to each well. The culture plates were incubated for 4 h at 37 °C and read at absorbance 450/620 nm (Multiscan FC, Thermo Scientific, UK). Cells remaining in the 96-well culture plate were rinsed with phosphate-buffered saline (PBS) and lysed with 100 µl 2% Triton X-100. To each well, 100 µl lactate dehydrogenase (LDH) reagent (MAK066, Sigma-Aldrich) was added and incubated at room temperature for 30 min. The plates were read at absorbance 485/620 nm.

2.4. Osteoinductive capacity of washed bone

Mesenchymal stem cell samples from two donors (a female aged 67 and a male aged 72 years) were used to seed 1 cm³
cubes of washed bone material with $5 \times 10^5$ MSCs in 250 μl standard medium. Each MSC sample was cultured on bone cubes from three different donors (all aged >70 years). After seeding, the cubes were incubated for 1 h at 37°C, washed three times in PBS and centrifuged at 500 × g for 5 min to remove non-adherent cells. These cells were combined with residual cells trypsinized from the tissue-culture wells and counted using a haemocytometer to assess seeding efficiency. A non-cell-seeded control was run simultaneously.

MSC-seeded bone cubes were either cultured in standard or osteogenic differentiation medium (standard medium containing 10 mM β-glycerophosphate (G9891, Sigma) and $1 \times 10^{-7} \text{m}$ dexamethasone (D8893, Sigma) and cultured for 28 days. At 0, 14 and 28 day time points, cell viability was assessed using an alamarBlue® assay (DAL1025, Life Technologies). Briefly, the medium was replaced with 5% alamarBlue® in relevant medium. After 2 h of incubation a 100 μl sample was measured using a fluorescence plate reader (FLx800, Biotek, UK) at 540 nm and excitation 600 nm.

### 2.4.1. qRT–PCR analysis of osteogenic differentiation

At time points 0, 14 and 28 days, medium was removed from bone cubes by centrifugation at 500 × g for 5 min. The bone cubes were transferred to clean tubes and 1 ml TRIzol reagent (AM9738, Ambion, Life Technologies) added, then incubated for 5 min with constant agitation. After incubation, the bone cubes were centrifuged at 500 × g for 5 min to remove all TRIzol reagent. RNA was extracted as previously described (Minogue et al., 2010), quantified using a Nanodrop and reverse-transcribed to cDNA, using a High Capacity cDNA Reverse Transcription Kit (4368814, Life Technologies). Real-time quantitative polymerase chain reaction (PCR) was performed on a StepOnePlus real-time PCR system (4376600, Life Technologies), using Lumino-Ct qPCR ReadyMix (i6669, Sigma-Aldrich). Assays were prepared using FAM-BHQ1 assays (all primers and probes from Sigma-Aldrich) for the following genes: runt-related transcription factor 2 (RUNX2; NM_001024630), forward CGCTGCAACAAGACC, reverse CGCCATGACAGTAACC; osteopontin (OP; NM_000582), forward CTGACATCCAGTAGCTCG, reverse CAGCTGACTGTTTCATA; and osteocalcin (OC; NM_199173), forward CGCAGCTTTGCATCG, reverse GCCATGATACAGGTAGC. Data were normalized to the house-keeping gene mitochondrial ribosome protein 19 (MRPL19; NM_014763), forward CCACATCCAGAGTTCTA, reverse CGCAGAGGATTATAAAGTTCAAA) and displayed as $2^{-ΔΔCt}$ (Minogue et al., 2010; Livak and Schmittgen, 2001) relative to day 0 controls.

### 2.5. Biomechanical stability of washed bone

#### 2.5.1. Compression testing

For assessment of biomechanical stability, fresh-frozen femoral heads from male [$n = 10$, aged 72–90 (mean 78.2) years] and female [$n = 5$, aged 70–85 (mean 77.4) years] donors were laterally bisected along the coronal plane to produce two equal halves. One half was retained, while the other was washed according to the method described earlier. The two halves were grid-marked along the axis of normal compression, cut into 1 cm$^3$ cubes ($n = 242$) and labelled with orientation and designated coordinates.

The cubes were loaded onto a compression-testing machine (LRXPlus, Lloyd Instruments, Sussex, UK) and subjected to one round of compression to failure, with the settings: preload 0.2 N, maximum deflection 3 mm, and load of 5 kN at a speed of 5 mm/min. Data were recorded for parameters of yield, elasticity and failure, using NEXYGENplus software.

### 2.6. Statistical analysis

Statistical analysis was conducted using Origin Pro v. 8.5 (Silverdale Scientific, Stoke Mandeville, UK). Mann–Whitney non-parametric statistical analysis was performed on the WST-1, LDH, PCR and alamarBlue® results, with a paired t-test used on mechanics data. Significance was set at $p \leq 0.05$.

### 3. Results

#### 3.1. Wash process removes marrow components

The removal of marrow components from the femoral heads was assessed by comparing soluble factors present in the spent wash solutions to samples taken from washed bone and soaked for 1 h in deionized water at 60°C with agitation. This indicated a total removal of 99.2 ± 1.8% recorded DNA, 98.9 ± 1.5% soluble protein and 100% haemoglobin from the trabecular material, resulting in a DNA content of 16.9 ng DNA/mg dry bone material.

#### 3.2. Histology

H&E staining of unwashed bone showed large quantities of soft marrow and cells deposited in the trabecular structure (Figure 1a). Encapsulated osteocytes and the endosteum were also evident. A fine meshwork was still present in the washed bone material (Figure 1b); however, this was almost completely untethered from the trabeculae. Additionally, there was a diminished cell presence with no obvious endosteum, although a few osteocytes were still present encapsulated in lacunae.

In addition, Masson’s trichrome staining only showed changes to soft tissue histology (Figure 1c, d), with similar morphological staining of osteoid (red) and mineralized (ossified) (blue) matrix between washed and unwashed bone.
3.3. Immunogenicity of washed bone

As MHC antigens are used to recognize foreign material, samples were immunohistochemically stained for HLA serotypes A, B and C. The washed bone displayed little or no immunopositivity (Figure 1f). In comparison, unwashed material was heavily stained, including endosteum and soft marrow tissue containing fat cells, stromal cells and osteocytes (Figure 1e).

3.4. Biocompatibility of washed bone

MSC cultures subjected to extract-conditioned medium from washed bone material showed a significant 10.6 ± 4.4% (p = 0.05) increase in cellular metabolic activity (Figure 2a), with LDH assays showing a small but not significant increase in cell number (Figure 2b).

In contrast, fresh-frozen bone extract-conditioned medium caused a significant, 32 ± 13.78% (p = 0.03) decrease in cellular metabolic activity (Figure 2a) and a significant (p = 0.02) 20 ± 9.03% decrease in total cell number compared to standard culture medium.

3.5. Washed bone is osteoinductive and supports MSC osteogenic differentiation

AlamarBlue® results for MSCs seeded on washed bone displayed significant fold increases in metabolic activity at days 14 and 28 in both standard and osteogenic medium (p < 0.001) (Figure 3a). There was a significant increase in activity between days 14 and 28 (p < 0.01); however, there was no significant difference between medium types at any time point.

Osteogenic markers for initial differentiation (RUNX2), immature osteoblast gene (OPN) and mature osteoblast gene (OCN) were used to determine both osteogenesis and the extent of cell maturation. Non-seeded, washed bone material displayed no detectable amplification.

Expression of the early osteogenic gene RUNX2 by MSCs seeded on washed bone (Figure 3b) was significantly increased at day 14 compared to day 0 in both
standard \( (p < 0.001) \) and osteogenic \( (p < 0.001) \) media, with significantly larger increases noted in osteogenic medium \( (p = 0.005) \). By day 28, \textit{RUNX2} expression by MSCs in standard medium had returned to day 0 control levels, whereas expression in osteogenic medium remained significantly higher than both day 0 controls and standard medium at day 28 \( (p < 0.001) \).

Expression of the immature osteoblast marker gene \textit{OPN} (Figure 3c) was also significantly increased by day 14 in both standard \( (p < 0.001) \) and osteogenic media \( (p < 0.001) \) compared to day 0 controls. However, levels were significantly lower in osteogenic medium compared to standard medium at day 14 \( (p = 0.003) \). By day 28, expression of \textit{OPN} remained significantly higher than day 0 controls in both medium types \( (p < 0.001) \), with no significant difference noted between standard and osteogenic media.

Expression of the mature osteoblast marker gene \textit{OCN} (Figure 3d) was upregulated to similar extents in both standard and osteogenic media compared to day 0 controls \( (p < 0.001 \text{ in both standard and osteogenic media}) \). Expression was further increased in both media by day 28 \( (p < 0.001 \text{ in standard and osteogenic media relative to day 0}; p < 0.001 \text{ and } p = 0.04 \text{ in standard and osteogenic media, respectively, relative to the same medium type at day 14}) \), with a small but significant increase noted in osteogenic medium compared to standard medium at day 28 \( (p = 0.04) \).
3.6. Washed bone is biomechanically stable

At the point of yield there were no significant differences to load at yield, stress or work at yield (Figure 4a, c, d). The washed material was compressed less at the point it lost its elasticity, with a significant decrease in the deflection at yield for washed bone compared to unwashed, from 1.49 ± 0.04 mm to 1.35 ± 0.04 mm \( (p = 0.002) \) (Figure 4c).

The parameters of elasticity indicated a significant increase in Young’s modulus of washed bone over unwashed bone from 75.9 ± 3.4 MPa to 84.6 ± 3.6 MPa \( (p = 0.02) \) (Figure 5a), with a small but insignificant increase in stiffness. The results of parameters at failure indicated no significant differences between the washed and unwashed material in any parameter (Figure 6a–d).

4. Discussion

Allograft bone material is essential in surgical procedures aimed at replacing large areas of bone loss, sharing many of the characteristics of ‘gold standard’ autograft but with greater availability. However, whilst the use of allograft material has shown good long-term healing potential, its marrow material is comprised of dead cell matter containing no viable osteogenic cell source, diminishing its ability to osseointegrate (through immunogenicity and infection) and detrimentally affecting cell adherence and activity \( (\text{Bonsignore et al., 2013}) \). Therefore, wash techniques are being developed to remove the marrow material. Importantly these wash methods aim not to detrimentally effect the latent abilities of the ECM in supporting the proliferation and regulation of cell activity \( (\text{Dutra and French, 2010; Yates et al., 2005; Declercq et al., 2013; Choi et al.,}) \).
In addition, the material and its properties may be exploited further as a scaffold in tissue engineering, with replication of the bone microenvironmental niche (Hammoudi et al., 2012; Thebaud et al., 2012), having been demonstrated to result in better osseointegration compared to standard allograft material (Coquelin et al., 2012). Thus, this study used a novel rapid wash process to remove marrow material from fresh-frozen allograft femoral heads, assessing for marrow removal, biocompatibility, osteoinductive potential and biomechanical stability, and determining its potential as a scaffold for use in future bone tissue engineering.

The biochemical results provide evidence that the wash process removes a large proportion of soluble protein, DNA and haemoglobin trapped in the trabecular structure of the whole femoral head. This removal was equivalent to soluble protein removal reported by Yates et al. (2005) and superior in the removal of both soluble protein and DNA to Ibrahim et al. (2012); however, these processes did not include a chemical sterilant step. Our study resulted in a material with a low DNA value (16.9 ng DNA/mg dry material) and which, histologically, displayed a washed matrix devoid of a marrow component, which may otherwise affect osseointegration. These characteristics are in accordance with other studies producing decellularized bone (Hashimoto et al., 2011) and other tissue structures (Dutra and French, 2010; Cornejo et al., 2012) and the standards proposed by Crapo et al. (2011) for the evaluation of a decellularized soft tissue.

The cytotoxicity assays confirmed the biocompatibility of the washed bone material while, interestingly, highlighting the detrimental properties of the unwashed fresh-frozen bone in this in vitro study. The washed material caused no decrease in cell number or metabolic activity, with the alamarBlue® assays demonstrating the sustained viability of cells seeded into the materials’ structure. Conversely fresh-frozen bone caused a significant reduction in both the total cell number and metabolic activity of the cultured cell population, suggesting a detrimental effect of non-washed, fresh-frozen bone. The cytotoxic effect of fresh-frozen material in vitro has previously been noted by Board et al. (2009), although there are no reports of trials comparing the clinical outcomes of fresh-frozen vs washed allograft. Despite the fact that fresh-frozen material displays good long-term healing, the leaching of cytotoxic factors from this material into the surrounding tissue could be detrimental to host-derived osseointegration, or affect the health of surrounding tissues in vivo, and thus requires investigation utilizing appropriate in vivo studies. It is of interest that through washing and the removal of the marrow, the material was able to maintain cell metabolic activity and viability, showing good biocompatibility and, importantly, not inducing any cytotoxic effects. This is in contrast to γ-irradiation, in which the technique itself is thought to affect cell viability through the production of cytotoxic peroxidized lipids (Moreau et al., 2000).

Gene expression analysis demonstrated the osteoinductive capabilities of the washed bone and its ability to support differentiated cells. Interestingly, increased osteogenic gene expression was seen with standard medium alone, with increases in early marker RUNX2, as well as the osteoblast marker osteopontin and mature osteoblast gene osteocalcin, suggestive of differentiation and cell maturation. These results suggest that the induction and maturation of the MSCs is due to the bone ECM itself and may be due to the integrin–ECM binding (Frith et al., 2012; Sun et al., 2011) or encapsulated...
bone-specific growth factors, such as bone morphogenetic proteins (BMPs), which elicit an osteogenic response upon release (Mauney et al., 2005). The induction of MSC osteogenesis by the washed bone without the addition of an osteogenic medium suggests that the ECM proteins and entrapped growth factors are relatively unaffected by the chemicals in the novel wash process, and still remain functional.

The material produced by this study demonstrates comparable biomechanical stability at both yield and failure to a fresh-frozen control. Whilst there were changes to Young’s modulus and yield displacement, the small increase in rigidity did not negatively affect the overall structural stability. Additionally, the increase in Young’s modulus seen after the removal of marrow is similar to that documented by Halgrin et al. (2011), who concluded that the marrow had caused increased transverse pressure and local stress on trabeculae, leading to its premature failure. Our results would also suggest that the material was not demineralized by the wash process, as this would have compromised the biomechanical stability of the bone (Chen and McKittrick, 2011). Utilization of halved femoral heads reduced interpatient variability, which may arise through changes in sex, age, weight or even disease state (Green et al., 2011; Homminga et al., 2002), and intrahead variability due to the differing compressive and tensile structures of the trabeculae (Martens et al., 1983).

Accurate determination of the biomechanical strength of the washed bone material is important in its eventual use, either directly as a surgical allograft, or as a scaffold for MSC-based tissue engineering. The three-dimensional (3D) structure itself is thought to influence the activity of osteogenic cells through mechanotransduction (Kilian et al., 2010; Shih et al., 2011) or simply through shear force (Yourek et al., 2010). Maintenance of rigidity and an appropriate porous 3D architecture is essential for osteoconductivity and new bone infiltration (Tagil et al., 2000), whilst preventing recoil of the impacted bone allograft that may negatively influence cementation (Kligman et al., 2003). The biomechanical compression results therefore suggest that washed bone is mechanically comparable to commonly used fresh-frozen allograft material and, together with improved biocompatibility and sustained osteoinductive potential, may thus offer an attractive alternative to existing unwashed bone allografts.

5. Conclusion

The results of this study depict a novel wash process for fresh-frozen allograft, able to remove ~99.5% of marrow components from whole femoral heads, leaving a biocompatible, mechanically stable, biological material, which, importantly, is still able to support cell proliferation and induce osteogenic differentiation. Additionally, the wash process removed the MHC class 1-positive marrow material present in fresh-frozen bone, which demonstrated detrimental cytotoxic effects on cultured cells in vitro. This large-scale removal of immunogenic marrow and the biocompatibility of the structure, together with the preservation of the materials’ osteoinductive and mechanical properties, illustrate the potential of this washed bone material as a scaffold for bone tissue-engineering applications. Importantly, the novel wash process described in this study offers a number of potential benefits, including the rapid removal of marrow components from multiple whole femoral heads simultaneously, with minimal processing. The use of femoral heads in this process also allows for the production of large biological scaffolds for application in large-scale bone tissue engineering.

Conflict of interest

The authors have declared that there is no conflict of interest.

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