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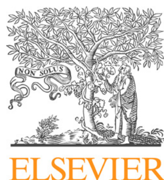

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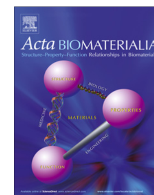

Full length article

Recovery of low volumes of wear debris from rat stifle joint tissues using a novel particle isolation method

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ABSTRACT

Less than optimal particle isolation techniques have impeded analysis of orthopaedic wear debris *in vivo*. The purpose of this research was to develop and test an improved method for particle isolation from tissue. A volume of 0.018 mm^3 of clinically relevant CoCrMo, Ti-6Al-4V or Si_3N_4 particles was injected into rat stifle joints for seven days of *in vivo* exposure. Following sacrifice, particles were located within tissues using histology. The particles were recovered by enzymatic digestion of periarticular tissue with papain and proteinase K, followed by ultracentrifugation using a sodium polytungstate density gradient. Particles were recovered from all samples, observed using SEM and the particle composition was verified using EDX, which demonstrated that all isolated particles were free from contamination. Particle size, aspect ratio and circularity were measured using image analysis software. There were no significant changes to the measured parameters of CoCrMo or Si_3N_4 particles before and after the recovery process (KS tests, $p > 0.05$). Titanium particles were too few before and after isolation to analyse statistically, though size and morphologies were similar. Overall the method demonstrated a significant improvement to current particle isolation methods from tissue in terms of sensitivity and efficacy at removal of protein, and has the potential to be used for the isolation of ultra-low wearing total joint replacement materials from periprosthetic tissues.

Statement of Significance

This research presents a novel method for the isolation of wear particles from tissue. Methodology outlined in this work would be a valuable resource for future researchers wishing to isolate particles from tissues, either as part of preclinical testing, or from explants from patients for diagnostic purposes. It is increasingly recognised that analysis of wear particles is critical to evaluating the safety of an orthopaedic device.
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1. Introduction

Adverse tissue reactions to wear debris, which may lead to aseptic loosening, are one of the most common causes of total hip replacement (THR) revision [1]. Aseptic loosening remains the most cited reason for THR revision, accounting for 51% of single-stage revisions, while adverse reactions to particulate wear debris account for 10.8% of single-stage revisions [1]. However, the extent of adverse reactions to wear debris is likely to be underestimated, as this was not an option on the revision report forms in the early phase of the National Joint Registry. Several reasons for revision are often given and categories are not mutually exclusive; aseptic loosening and other cited reasons including pain (22.0% of single-stage THR revisions) and lysis (15.6%) are often the result of reactions to wear debris [2,3]. Device failure may be due to a combination of mechanical failure, resulting in high wear, and adverse responses to the wear particles, due to use of potentially unsafe materials. Assessing the biocompatibility of wear debris is therefore crucial to preventing unsafe devices, such as several recalled metal-on-metal THRs, from reaching the market [4]. This is reflected in the publication of recent FDA guidelines [5] for the

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biological evaluation of medical devices, which requests the pre-clinical assessment of wear debris, including geometric and/or physicochemical properties, specifically in relation to potential biological hazards due to mechanical failure, such as excess wear debris produced as a result of coating delamination. It is thus likely that there will be an increased interest in analysis of wear particles, which may require the use of particle isolation techniques from serum, synovial fluid or tissue.

Particle isolation and subsequent characterisation requires removal of protein and other contaminants such as lipids [6]. Treatment using strong acids or bases has been effectively used to solubilise tissue and facilitate particle extractions. Such protocols have included sodium hydroxide [7–11], potassium hydroxide [12–14] and nitric acid [15–17]. The acids or bases were used at high concentrations from 4 M to 12 M and for one to five days for formalin-fixed tissue, most often at 65 °C. Other studies have isolated metal and ceramic particles using mixed enzymatic and acid/base digestion [18–20]. Although chemical treatment using acids or bases is more efficient than enzymatic tissue digestion [21], acids and bases damage metal particles [22], and thus have been predominantly used to isolate polyethylene particles. Previous studies have used papain and proteinase K to digest periprosthetic tissue to isolate metal particles, with subsequent centrifugation to remove proteins [23], a method which was used and adapted in several later studies for either serum or tissue digestion [22,24,25]. However, such methods involved high temperature stages such as boiling samples in sodium dodecyl sulphate (SDS), which may be overly destructive to recent orthopaedic materials such as silicon nitride (Si_3N_4), which has dissolution properties. Furthermore, the method detailed in the ISO standard for metal particle isolation from tissue [26] requires the use of a tissue homogeniser which could affect large particles and employs multiple washes at relatively low centrifugation speeds of 16,000 RCF (relative centrifugal force) for only ten minutes, which could be insufficient to sediment certain particles. The method could therefore lead to particle loss, making it unsuitable for the recovery of low particle volumes. Due to such disadvantages, particle isolation is not always performed in practice. Further drawbacks of previous methods have included the need for expensive equipment and the length of time required to perform the experiments, a lack of any demonstrated technique sensitivity (as defined by the minimum initial volume of particles that could be successfully extracted from a tissue sample), unsatisfactory results due to protein or bacterial contamination or the presence of extraneous impurities. Many of the methods also did not establish whether use of the isolation method caused any significant changes to particle measurements.

Although several recent publications have provided updated methods for isolating low-wearing materials from serum with proven sensitivity [27,28], such studies have not considered the isolation of particles from tissue, which can be useful when performed on periprosthetic tissue from around explants for diagnostic purposes. The method described by [28] to isolate Si_3N_4 particles from wear simulator serum using density gradient ultracentrifugation with novel sodium polytungstate gradients demonstrated no detectable particle loss at key stages of the procedure, and no detectable changes to particle size or morphology. The method is relatively quick to perform (the protocol requires approximately four days), is cost effective and does not require specialist equipment, making it an attractive technique.

The overall aim of this research study was to produce an improved method for the isolation of wear particles from tissue, using enzymatic digestion and sodium polytungstate density gradients, and test the method in a small animal model on a range of materials.

2. Materials and methods

2.1. Details of the particles employed in the study

Three clinically relevant materials were used in this study: silicon nitride (Si_3N_4), a ceramic material which has been used as an orthopaedic bearing material in cervical spacers and spinal fusion devices and which is currently being investigated as a potential joint replacement coating, cobalt chromium molybdenum alloy (CoCrMo) and titanium aluminium vanadium alloy (Ti-6Al-4V), both of which have been used extensively in various orthopaedic devices for several decades and are included here as intended substrate materials for the Si_3N_4 coating. The Si_3N_4 particles used were a commercially available nanopowder (<50 nm, Sigma-Aldrich). Wear debris of CoCrMo and Ti-6Al-4V were generated using a six-station multidirectional pin-on-plate wear simulator using sterile filtered water as a lubricant, as described previously [29]. Aliquots of each of the three particle types were inspected by scanning electron microscopy (SEM) prior to use to ensure that no contamination was present and that the particles were of a clinically relevant size range. Particles were thereafter sterilised by heat treatment at 180 °C for 4 h and sterile stock suspensions of 0.9 $mm^3 \cdot mL^{-1}$ of each particle type in phosphate buffered saline were produced. Prior to immediate use, the particle suspensions were vortexed and sonicated for 20 min three times to ensure a homogenous dispersion of particles.

2.2. Animal model

The animal test system used for this study consisted of male Wistar rats, 8–12 weeks old and with weights ranging from 297 to 342 g. Guidelines contained in EU Directive 2010/63/EU for animal experiments were followed [30], and the experiments were authorized by the ethical committee under the licence number 133/2014. The study involved a total of 18 rats, consisting of three material groups (Table 1). Tail marks and subcutaneous transponders were placed on the rats on arrival into the facility and the health of each rat was closely monitored; animals with any visible signs of illness were excluded from the study. The rats were acclimatised for at least eight days prior to the study. Stifle joints were chosen for treatment to allow more accuracy and repeatability compared to injection of the hip joints. Rats were injected in the right stifle joint using a glass Hamilton syringe (23 gauge) with 20 μL of a particle suspension (Si_3N_4, CoCrMo or Ti-6Al-4V), which equated to a dose of 0.018 mm^3 of particles per rat stifle joint. The left stifle joints were not injected and were instead used as untreated control joints. Thereafter rats were monitored daily over a seven day period, at which point animals were euthanised with a rising CO_2 concentration. Excess muscle tissue was removed from the stifle joints taking care not to open the joint compartment. The intact rat stifle joints (both treated and non-treated) were excised, formalin-fixed for a period of 48 h, and stored in 70% (v/v) ethanol.

Table 1
Particles used for injection into rat stifle joints.

Material group*	Details
Si_3N_4	Commercially available nanopowder (<50 nm, Sigma-Aldrich, UK)
CoCrMo (high carbon; >0.2%, [31])	Particles generated by pin-on-plate in house
Ti-6Al-4V	Particles generated by pin-on-plate in house

* Volume of particles injected was 0.018 mm^3 and particles were *in vivo* for a period of seven days for each material group (N = 6).

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