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In vitro and *in vivo* biological responses to a novel radiopacifying agent for bone cement

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Iodixanol (IDX) and iohexol (IHX) have been investigated as possible radiopacification agents for polymethylmethacrylate (PMMA) bone cement, to replace the currently used barium sulphate and zirconia. IDX and IHX are both water-soluble iodine-based contrast media and for the last 20 years have been used extensively in clinical diagnostic procedures such as contrast media enhanced computed tomography, angiography and urography. One of the major reasons to remove the current radiopacifying agents is their well-documented cytotoxicity and their potential to increase bone resorption.

Using *in vitro* bone resorption assays, the effect of PMMA particles plus IDX or IHX to induce osteoclast formation and lacunar resorption on dentine slices has been investigated. These responses have been compared with the *in vitro* response to PMMA particles containing the conventional radiopacifying agents, that is, barium sulphate and zirconia. In parallel, the *in vivo* reaction, in terms of new bone formation, to particles of these materials has been tested using a bone harvest chamber in rabbit tibiae.

In vitro cell culture showed that PMMA containing IHX resulted in significantly less bone resorption than PMMA containing the conventional opacifiers. *In vivo* testing, however, showed no significant differences between the amounts of new bone formed around cement samples containing the two iodine-based opacifying agents in particulate form, although both led to fewer inflammatory cells than particles of PMMA containing zirconia. Our results suggest that a non-ionic radiopacifier could be considered as an alternative to the conventional radiopacifying agents used in biomaterials in orthopaedic surgery.

Keywords: bone cement; radiopacifing agent; particles; biocompatibility; bone resorption

1. INTRODUCTION

Total joint replacement is the most successful method of treating end-stage arthritis. It significantly improves the quality of life and the functional capability of patients suffering from arthritic disease. Currently an estimated 1 million total joint arthroplasties are performed each year worldwide. In Sweden, more than 22% are performed in patients less than 65 years old (Malchau *et al.* 2002), and in the UK over 42% are in this younger age group (Department of Health Research and Development Directorate, Biomaterials and Implants Advisory Group 1995; Royal College of Surgeons 2000). Despite the attractions of non-cemented fixation, the vast majority of joint arthroplasties still involve some polymethylmethacrylate (PMMA)-based cement for fixation. In Sweden, over 96% of hip arthroplasties (Malchau *et al.* 2002) and over 98% of knee arthroplasties (Swedish Knee Register 2003) use bone cement for the fixation of at least one component. Similarly, a recent UK audit report has indicated that bone cement is used in over 90% of total joint replacements (Royal College of Surgeons 2000). The use of cemented fixation is particularly high in both Sweden and the UK, but is also popular elsewhere, particularly for more elderly patients.

Bone cement consists of a polymer powder, usually PMMA or a PMMA co-polymer, which is polymerized in the operating theatre with a liquid monomer. Contrast agents, notably barium sulphate ($BaSO_4$) and zirconium dioxide (ZrO_2), are commonly added to the bone cement in order to confer radiopacity and thus aid in the radiographic assessment of the implants. The addition of radiopacifying agents to PMMA is, however, not achieved without altering the properties of the bone cement. Although changes in the mechanical strength of the bone cement may be insignificant (Chan & Ahmed 1991; Ginebra *et al.* 2002), radiopacifying agents are harder than metallic femoral heads and, if they enter the joint space, may cause third body

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wear, damaging both the articulating surfaces. Damage to the metal component is more serious as plastic deformation of the surface leads to the production of ridges which consequently scratch, and continue to scratch, the opposing, usually polyethylene surface, leading to the accelerated production of wear debris (Issac *et al.* 1987; Caravia *et al.* 1990).

Conventional radiopacifying agents used in bone cements have been shown to evoke a significant pathological response *in vivo* and *in vitro*. $BaSO_4$ injected intradermally into experimental animals is known to cause a foreign body inflammatory reaction (Adams 1976); barium has also been shown to intensify the release of inflammatory mediators in response to PMMA particles (Lazarus *et al.* 1994). Very few reports are available with regards to ZrO_2, although the development of epithelioid granulomas after the injection of elemental zirconium has been reported (Adams 1976). It has been shown by various groups that polymerized PMMA particles containing barium or zirconium can induce a greater osteolytic response, both *in vitro* (Sabokbar *et al.* 1997, 1998) and *in vivo* (Wimhurst *et al.* 2001), than PMMA particles with no added radiopacifying agents. As there is an increase in the number of joint replacements (notably with the use of PMMA bone cement), it is imperative to find alternative radio-contrast media in order to reduce the failure of prosthetic implants or aseptic loosening caused by the biological responses evoked by PMMA particles containing $BaSO_4$ or ZrO_2. Some groups have attempted to use iodine as an opacifier, in the form of methacrylates containing iodine that are used to replace the monomer phase while retaining the conventional PMMA powder phase (Davy *et al.* 1997; Artola *et al.* 2004; van Hooy-Corstjens *et al.* 2004). These cements are prepared in the same way as the conventional MMA/PMMA cements.

A different approach has been used in this paper. Iodixanol (IDX) and iohexol (IHX) are non-ionic monomers and dimers, respectively, containing iodine, and were originally developed as water-soluble contrast media to be used in radiographic investigations such as angiography (Almén 1995). Since then, they have been used in millions of investigations annually worldwide with minimal side effects (Davenport *et al.* 1999; Nakamura 2003). Both these contrast media have now been produced as fine ground powder, which is mixed in with the powder phase of bone cement prior to cement polymerization. As radiopacifiers, they have been shown not to reduce the mechanical properties of the resulting cement compared with cements manufactured with conventional radiopacifiers (Kjellson *et al.* 2001). Additionally, IDX and IHX provide suitable levels of radiopacification for clinical applications (Kjellson *et al.* 2004). Prior to their clinical application, the effect they have when combined with bone cement on both osteoclasts and osteoblasts needs to be investigated. The biologically based clinical requirements for novel opacifiers are that they produce less effect on osteoclasts and do not reduce osteoblast activity compared with PMMA bone cement containing the conventional barium and zirconium. The aim of the current study, is therefore, to evaluate the *in vitro* and *in vivo* effects of PMMA bone cement containing IDX and IHX, and compare their cellular response to PMMA bone cement containing conventional radiopacifiers.

In vitro cell co-culture with PMMA particles has been used to assess the level of resorption produced on dentine slices by murine osteoclasts. Osteoclasts are multinucleated bone-resorbing cells formed from phagocytes (Fujikawa *et al.* 1996). Reproducible models have been produced using both human and mouse precursor peripheral blood monocytes (Sabokbar *et al.* 1998; Neale *et al.* 2000) and used to assess the response to a range of different biomaterials. It is now known that for the pre-osteoclastic monocytes to differentiate into active bone resorbing osteoclasts the cooperation of osteoblasts with monocytes is essential (Lacey *et al.* 1998). Osteoblasts are known to express a novel membrane-bound factor, RANKL. The interaction of RANKL with RANK, a receptor specific to osteoclast precursor cells, and macrophage colony stimulating factor (M-CSF), is essential (Lacey *et al.* 1998). The transformation of pre-osteoclasts into osteoclasts can be followed during *in vitro* cell culture by the expression of various factors. Early on, pre-osteoclasts are able to express tartrate resistant acid phosphatase (TRAP). Once mature, osteoclasts are able to resorb bone or any similar substrate on which they are being cultured. In the current investigation, we aim to study the ability of PMMA particles containing IDX or IHX to induce osteoclast formation in monocytes/osteoblast co-culture bone resorption assay systems. The extent of their osteoclastic reaction will be assessed in terms of TRAP expression on glass coverslips and lacunar resorption on dentine slices.

A rabbit model utilizing a bone harvest chamber has been used to assess the biocompatibility of the materials and degree of bone regrowth around the materials. This model has been used to assess the effects of biomaterials as solids and particles, including bone cement, polyethylene, titanium alloy, cobalt-chrome and hydroxyapatite particles on bone formation (Goodman *et al.* 1993, 1996, 1999; Goodman 1994; Wang *et al.* 1994).

2. MATERIALS AND METHODS

2.1. Material preparation

The particles were produced from five different samples based on Palacos bone cement (Schering-Plough AB, Stockholm, Sweden), prepared as instructed by the manufacturer. These samples were:

(i) PMMA with no radio-contrast agent (Palacos-based PMMA; as negative control);
(ii) PMMA with 15% (w/w) ZrO_2, which is reported (Buchhorn & Willert 2001) to be particles of 8 μm mean diameter which agglomerate to produce up to 50 μm diameter clusters (Palacos R; as positive control);
(iii) PMMA with 10% (w/w) $BaSO_4$, which is reported (Buchhorn & Willert 2001) to be particles of 2 μm mean diameter which agglomerate to produce up

to 100 μm diameter clusters (as positive control; from Stryker-Howmedica);
(iv) PMMA with 10% (w/w) IDX mean particle size <20 μm (from Nycomed Amersham, Norway); and
(v) PMMA with 10% (w/w) IHX mean particle size <20 μm (from Nycomed Amersham, Norway).

The cement was ground down using an ultracentrifuge mill with liquid nitrogen to embrittle the cement. Size distribution was determined by scanning electron microscopy and by laser light particle size analysis (Malvern Instruments, MS20, UK). These results demonstrated that there was a limited range of particle sizes and that 99% were less than 10 μm, with the majority (>80%) of the particles between 1 and 5 μm. To ensure that the particles were sterile and endotoxin-free they were washed three times in absolute alcohol and rinsed in MilliQ water. The absence of endotoxins in all samples was checked using E-Toxate limulus assay (Sigma-Aldrich Chemicals, UK).

2.2. In vitro *studies*

For cell culture, alpha minimal essential medium (MEM; Gibco, UK) was supplemented with 100 IU ml^{-1} penicillin, 10 μg ml^{-1} streptomycin and 10 mmol L-glutamine (Gibco, UK), and 10% foetal calf serum (FCS; Gibco, UK). Recombinant mouse soluble RANKL and M-CSF were purchased from Peprotech (London, UK) and R&D Systems Europe (Abingdon, UK), respectively. All incubations were carried out at 37 °C in 5% CO_2.

2.2.1. Preparation of monocyte cultures. The whole blood of mice was collected and diluted in culture media, and peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation technique. The PBMCs were used either to investigate the cytotoxic effect of the particles or to determine the *in vitro* effect of PMMA particles on the extent of osteoclast formation and subsequent lacunar resorption.

2.2.2. Cytotoxicity tests. For the cytotoxic assays, 10 μg of particles per millilitre of MEM/FBS were exposed to 1.5×10^5 PBMCs for 24–48 h, and then centrifuged to remove the particles and cell debris. Once prepared, the solutions were stored at −20 °C until ready for testing. Cell damage was measured by the release of the cytoplasmic enzyme lactate dehydrogenase (LDH), using a commercially available Cytotox 96 kit (Catalogue no. G1780, Promega, Madison, WI).

2.2.3. Preparation of plates for bone resorption assay. Isolated PBMCs were seeded at 1.5×10^5 cells $well^{-1}$ onto 4 mm diameter dentine slices or 6 mm diameter glass coverslips and placed in the 7 mm diameter wells of a 96-well tissue culture plate. The cells were allowed to settle on their substrates for 2 h and then removed, washed and placed on their substrate in 24-well culture plates. The MEM/FBS was further supplemented by soluble RANKL (30 ng ml^{-1}) and M-CSF (25 ng ml^{-1}). To exaggerate the cellular responses and hence facilitate the comparison between the samples, cultures were also supplemented with 10^{-8} M dexamethasone (Sigma-Aldrich Chemicals). Particle samples were re-suspended in MEM and sonicated for 10 min prior to addition to 24 well plates (at 10 μg ml^{-1} $well^{-1}$). Monocyte cultures ± particles were maintained for 7 or 14 days with the supplemented media replaced every 3–4 days.

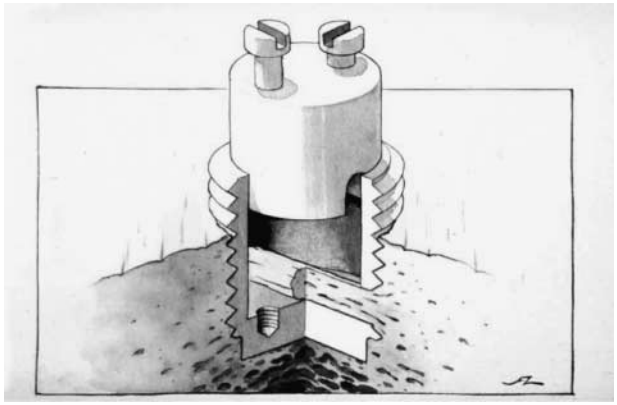

Figure 1. Schematic of the Bone harvest chamber used. (Reproduced with permission from Aspenberg *et al.* 1988.)

2.2.4. Detection of osteoclasts on coverslips and dentine slices. The cells on coverslips were cultured for 7 days and then were fixed in a citrate/acetone solution and stained for acid phosphatase, using naphthol AS-BI phosphate as a substrate. The product was reacted with fast garnet GBC salt in the presence of 1.0 M tartrate, and the multinucleated osteoclasts appeared red.

After 10 days in culture, dentine slices were treated with NH_4OH (1 N) for 30 min and cleaned by ultrasonication to remove adherent cells. The slices were then washed with distilled water and stained with 0.5% (w/v) toluidine blue for 3 min, before being air-dried. The surface of each dentine slice was examined for lacunar pit formation by light microscopy. The resorption was determined by calculating the percentage surface area of lacunar resorption on each dentine slice, using image analysis software (Photoshop 5.5, Adobe). Student's *t*-test was used to compare the different groups. For each treatment four dentine slices were used and each experiment was repeated at least three times in total.

2.3. In vivo *studies*

2.3.1. Experimental set-up. For the *in vivo* testing, the particles were sterilized with gamma irradiation. New Zealand White rabbits, over six months old, weight 3.3–4.3 kg and of mixed gender, were used for the study. Bone harvest chambers (figure 1) were implanted in both tibiae of each rabbit. Each chamber contains an inner core with a 1 mm diameter×5 mm groove that is coaxial with the holes in the outer cylinder, providing

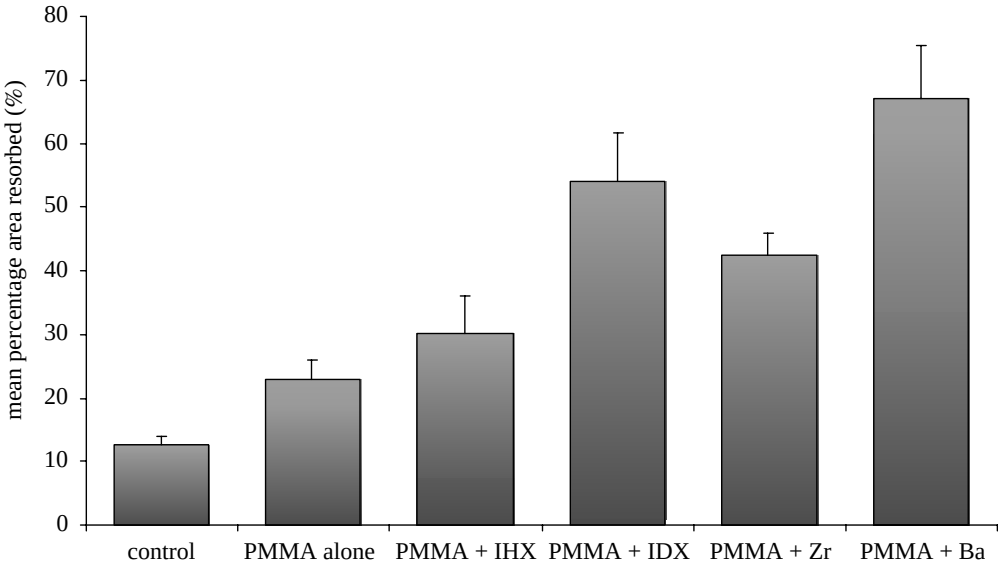

Figure 2. Amounts of dentine resorbed by peripheral blood mononuclear cells (PBMCs) co-cultured with no particles (control) or particles of bone cements containing different opacifiers.

a continuous canal through the chamber for tissue ingrowth. The chambers were implanted in the proximal tibiae and allowed to osseointegrate for six weeks.

Following osseointegration, 10 mg of particles of one of the four cement types (PMMA with barium sulphate was omitted from this section of the study) were mixed in a carrier of 0.4 ml hyaluronate gel (Healon, Kabi Pharmacia, Uppsala, Sweden). This dilution yielded an approximate concentration of 1×10^8 particles ml^{-1} (Goodman 1994). One cement type was added to the left tibia chamber of each rabbit and a different type added to the contralateral tibia chamber. After three weeks, tissue from both chambers was harvested and prepared for histology. The chambers were then cleaned and the test was repeated with the left–right assignment of the cement types reversed. These repeat tests allowed test reproducibility to be assessed. These series of tests were repeated for the other cement types in different combinations. Each material was implanted repeatedly four times in the left or right sides of six rabbits, giving a total of 20–22 implantations per material. In addition, 12 implantations of the carrier without cement particles were performed as controls.

2.3.2. Histological analysis. A total of 96 specimens were studied histologically. The specimens were decalcified, embedded in paraffin, cut into 6 μm sections and stained with haematoxylin and eosin. A foreign body reaction between ingrown tissue and cement particles was indicated by the presence of macrophages, giant cells, polymorphonuclear leucocytes and lymphocytes.

The amount of new bone formation was measured blindly using an imaging system (analySIS, Soft Imaging System, Munster, Germany). The percentage of bone formation was calculated as the area of trabecular bone divided by the total area of tissue. These values were statistically analysed by ANOVA.

3. RESULTS

3.1. In vitro *studies*

There was no significant change in LDH release by monocytes cultured in the presence of various PMMA particles as compared with cultures with no added particles; thus suggesting that the particle concentration used for the current study was not toxic to the monocyte cultures.

Osteoclast differentiation, evidenced by the expression of TRAP and lacunar resorption, was noted in all co-cultures (figure 2). Although the number of TRAP positive multinucleated cells was not significantly different in treated wells, significant differences in the mean percentage area lacunar resorption were observed between treatments. Relative to controls (i.e. those with no added particles and those containing pure Palacos bone cement with no additives), cultures containing cement particles with added $BaSO_4$ and 10% IDX showed a significant increase in osteoclast formation. Similarly, PMMA particles with added ZrO_2 resulted in a significant osteoclast formation as compared with cultures with no added particles, and those cultured in the presence of PMMA particles with no additive. However, the extent of osteoclast formation in response to PMMA + ZrO_2 was markedly less than that observed in cultures incubated with PMMA + $BaSO_4$.

In contrast, there were no significant differences between control cultures (PMMA alone) and cultures containing cement particles with 10% IHX. Moreover, cultures containing cement particles with added IHX showed considerably less osteoclast formation and bone resorption than cultures containing cement particles with added $BaSO_4$, ZrO_2 or 10% IDX. Additionally,