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Evaluation of different methods to eliminate adherent endotoxin of polyethylene wear particles

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ABSTRACT

Wear particles have to be cleaned from any substances, which could eventually lead to confounding effects in studies concerning the *in vitro* or *in vivo* biological activity of wear debris. Several observations have demonstrated that lipopolysaccharide (LPS) as a component of the outer membrane of gramnegative bacteria can modulate the cell response to wear debris.

There are numerous methods described in the literature for the removal of LPS. But there is an exception for polyethylene particles, they cannot be treated with radiation and heating processes.

There remain four possible methods: Cleaning the particles with sodium hydroxide or with acetic acid, washing them with ethanol or via using a demanding ultracentrifugation procedure. Thus we decided to compare the different methods in consideration of the remaining LPS level and their effects on the particle numbers and morphology.

During the study several problems regarding these methods appeared: Either they could not remove the LPS below the demanded detection level, or the methods had influence on amount and morphology of the particles.

Due to these findings the authors developed a new method based on ultracentrifugation. With this method the LPS could be removed as required, amount and morphology of the particles were not affected.

1. Introduction

In arthroplasty, aseptic loosening is one of the major causes for revision surgery [1–3]. Wear debris plays an important role influencing the biological reaction against particles [1–3]. The complex mechanism of the particle induced biological response is not totally understood in detail [1]. Therefore, *in vivo* as well as *in vitro* are necessary to investigate the biological response of wear debris to map the signal pathways of aseptic loosening.

Several observations have demonstrated that lipopolysaccharide (LPS) can modulate the cell response to wear debris [4–8]. LPS, or bacterial endotoxin, is the major component of the outer membrane of gramnegative bacteria, which is a potent stimulus of the proinflammatory cascade in macrophages and results in the production and release of soluble inflammatory mediators [9–11]. LPS can be found on plastic, glass, and metal particle surfaces [5]. Such evidence indicates the importance of controlling for the potential confounding effects of LPS contamination in the models used to define the reactive nature of wear debris. Furthermore, to test the effects of wear particles from new prosthesis designs or current types of polyethylene, for example crosslinked or Vitamin E-doped polyethylene, it is essential to use material retrieved from simulator studies and not commercially available particles. Since numerous factors can influence the cellular response to wear particles including size, dose, volume, shape and composition, the particles used for *in vitro* as well as *in vivo* models should be as close to the clinical situation as possible [1–3].

Whereas several methods for the removal of endotoxin were described for metal or bone cement particles [7,8,12], there is a lack of data for removing endotoxin from polyethylene particles, one of the most used biomaterials in arthroplasty, that were generated in joint simulators [13,14].

In addition, some authors use industrial particles with uniform shape and size, which do not conform to particles from joint simulators or joint revision surgery [15]. Furthermore, the authors identified several studies with blank materials and methods section, simplifying the phrase “after removing of endotoxin” is used without specifying the used methods [3,16–18]. Detailed data concerning the elimination procedures and further particle analysis are missing. The efficiency of the different methods, especially regarding effects on size and morphology of the polyethylene wear debris was rarely point of view.

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Therefore, the purpose of the study was to compare common different methods for the removal of endotoxin from polyethylene particles as well as to develop a safe new method for endotoxin removal from polyethylene wear particles.

2. Material and methods

This study contains a multistep-procedure. First, polyethylene wear particles were generated in a knee simulator under non-sterile conditions. The particles were isolated from the simulator lubricant using hydrochloric acid. This particle-containing solution was fed to the various test procedures. Before and after testing each method, a particle analysis was performed using scanning electron microscopy to determine size and shape of the wear debris. Levels of endotoxin in the test lubricants were measured after the removal procedures.

2.1. Particle generation and isolation

Tibial inserts (ultra-high-weight-molecular-weight-polyethylene—UHMWPE, GUR 1020, Gamma sterilised) were used with the appropriate femoral and tibial component recommended from the particular manufacturer. The experimental knee designs (three inserts/material) were tested on a knee simulator (Stallforth/Ungethuem, Germany), involving three simultaneously running test stations over 5×10^6 cycles using an established method [19].

The simulator was run at a frequency of 1 Hz. The physiologic load (maximum 2,600 N), flexion/extension (0–60) motion and anterior/posterior translation waveforms were taken from the recommended ISO kinematics of the knee during normal gait. The lubricant used for testing was 25% (v/v) newborn calf serum (Heraeus Kulzer, Berlin, Germany) with 0.1% (m/v) sodium azide solution(Sigma-Aldrich, Munich, Germany) in sterile water (ISO 14243-1), fungicide antibiotics (Amphotericin B, VWR International GmbH, Darmstadt) and EDTA (3.95 g for 0.5 l serum). The lubricant was changed every 6 day [19].

The particles were separated from the lubricant using the acid digestion method [20]. 10 ml of each serum sample were added to 50 ml of hydrochloric acid (37% v/v; Merck, Darmstadt, Germany) and mixed with a magnetic stir bar at 60 °C for approximately one hour. 3 ml of this digestion solution were added to 150 ml of methanol (Merck, Darmstadt, Germany) and filtered through a 0.02 μm polycarbonate membrane (Anodisc 47, Whatman plc, Maidstone, Kent, United Kingdom) [21]. The filter membrane was then dried for at least 6 h and sputter-coated with gold.

The particles recovered on the filter membranes were imaged by scanning electron microscopy (SEM, Leica Stereoscan 420, Leica Microsystems, Wetzlar, Germany). The particles that were more than 1.0 μm of size were analyzed at a magnification of 2500 or 5000 diameters, the particles that were less than 1.0 μm at a magnification of 10,000 diameters. A minimum of 10 random, non-overlapping fields of view were analyzed at each magnification. Images of each field of view were captured, and the particles were measured using a digital image analysis program (Leica QWin, Image processing and analysis application, Leica Microsystems, Wetzlar, Germany).

The boundary of each particle was defined on the basis of a gray-scale level threshold.

The following parameters were recorded: mean diameter, perimeter, area, equivalent circle diameter (ECD), aspect ratio (AR) and the roundness (R) of the wear particles [22]. The particles analyzed before endotoxin removal were set as a comparison group.

2.2. Endotoxin removal

After determination of size and shape of the particles, several different methods were used for endotoxin removal. As a starting lubricant the particle containing lubricant from the knee simulator was used after the acid digestion procedure. Before the endotoxin removal steps, no further chemical solutions were added to this starting lubricant.

As described in the literature the authors compared two methods, one using acid and one using chemical base [6;23]. Furthermore, a method of washing the particles in ethanol [6] was tested. According to the ISO, the authors added a method using ultracentrifugation for the removal from polyethylene particles, which were retrieved from periprosthetic tissue [22]. Each of the described methods was repeated five times.

2.2.1. Chemical base method

For the chemical base method, the particle suspension from the knee simulator was mixed with distilled water [23]. Then 10 ml NaOH were added to 1 ml of the lubricant and stirred at 30 °C for 2 h. After a centrifugation at 3000 g for 20 min, the particles were washed with distilled water again. After this procedure, 5 ml of the final solution were pipetted into a vessel provided by Lonza (Verviers, Belgium).

2.2.2. Ethanol-washing method

According to Cho et al., the particle suspension was mixed with 70 vol% ethanol [6]. The ethanol was added to the particles, this solution were vortexed extensively for 24 h. The particles were then centrifuged, the supernatant discarded, and new 70 vol% ethanol was added. This process was repeated 3 times over the course of 3 days [6]. After this procedure, 5 ml of the final solution were pipetted into a vessel provided by Lonza (Verviers, Belgium).

2.2.3. Acetic acid method

First, the particles were washed with ethanol as described by the ethanol-washing method [6]. After that, the particle suspension was pipetted in acetic acid (1%). This suspension was put into a cooking water bath for 3 h. After this procedure, the suspension was washed with phosphate buffered saline (PBS) three times. Finally, 5 ml of the solution were pipetted into a vessel provided by Lonza (Verviers, Belgium).

2.2.4. Ultracentrifugation by ISO

In fact, this procedure as described by the ISO is developed for the removal of endotoxin from polyethylene wear particles, which were retrieved from periprosthetic tissue *in vivo*. Due to the enquiry of the literature performed by the authors, there is no specific procedure for endotoxin removal from particles generated *in vitro*.

After acid digestion of the particles [20], particle suspension was pipetted between two layers of saccharine-solutions into a ultracentrifugation tube (BECKMAN Centrifuge Tubes, Performance Validated for Beckman Coulter Inc. Rotors, Beckman Instruments, Inc., Spinco Division, Palo Alto, USA). The ultracentrifugation was set at 100,000 g for totally 3 h at 4 °C (Beckman Coulter, Beckman Optima LE 80 K, Krefeld, Germany). Then a white, particle containing layer was visible. This layer was pipetted between two layers 2-propanol (densities $\rho_1 = 0.96$ g/dl and $\rho_2 = 0.90$ g/dl, each 8.3 ml) into ultracentrifugation tubes. The second ultracentrifugation was set at 100,000 g for totally 3 h at 10 °C. Again a white layer was visible. The particle-solution was pipetted into endotoxin-free vessels, provided by Lonza (Verviers, Belgium).

As mentioned before five samples per method were tested. The endotoxin content of each sample was evaluated by a Limulus Amoebocyte Lysate (LAL) -Test (Lonza, Verviers, Belgium; acoording to the 'USP 28-NF 23, 2005, Bacterial Endotoxins Test' and 'Guidelines on Validation of the LAL test as an End-Product