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Published in:
Journal of Orthopaedic Research

DOI:
10.1002/jor.21526

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Download date: 23-09-2023
The Influence of Co–Cr and UHMWPE Particles on Infection Persistence: An In Vivo Study in Mice

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Received 18 May 2011; accepted 29 July 2011
Published online 22 August 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jor.21526

ABSTRACT: Wear of metal-on-metal (cobalt–chromium, Co–Cr particles) and metal-on-polyethylene (ultra-high-molecular-weight polyethylene, UHMWPE particles) bearing surfaces in hip prosthesis is a major problem in orthopedics. This study aimed to compare the influence of Co–Cr and UHMWPE particles on the persistence of infection. Bioluminescent Staphylococcus aureus Xen36 were injected in air pouches prepared in subcutaneous tissue of immuno-competent BALB/c mice (control), as a model for the joint space, in the absence or presence of Co–Cr or UHMWPE particles. Bioluminescence was monitored longitudinally up to 21 days, corrected for absorption and reflection by the particles and expressed relative to the bioluminescence found in the presence of staphylococci only. After termination, air pouch fluid and air pouch membrane were cultured and histologically analyzed. Bioluminescence was initially lower in mice exposed to UHMWPE particles with staphylococci than in mice injected with staphylococci only, possibly because UHMWPE particles initially stimulated a higher macrophage presence in murine air pouch membranes. For mice exposed to Co–Cr particles with staphylococci, bioluminescence was observed to be higher in two out of six animals compared to the presence of staphylococci alone. In the majority of mice, infection risk in the absence or presence of Co–Cr and UHMWPE particles appeared similar, assuming that the longevity of an elevated bioluminescence is indicative of a higher infection risk. However, the presence of Co–Cr particles yielded a higher bioluminescence in two out of six mice, possibly because the macrophage degradative function was hampered by the presence of Co–Cr particles. © 2011 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 30:341–347, 2012

Keywords: Co–Cr; wear particles; infection; in vivo; bioluminescence

Metal-on-metal (MOM) bearings have been favored compared to conventional metal-on-polyethylene (MOP) bearings, because of their low wear in hip arthroplasties for young and active patients. However, with up to three million gait cycles, active MOM patients still accumulate nearly 1 mg of nanosized cobalt–chromium (Co–Cr) wear particles per year. Patients with MOP bearings gather approximately 20- to 100-fold more ultra-high-molecular-weight polyethylene (UHMWPE) particles after the same number of cycles.

Conflicting results have been reported concerning the influence of Co–Cr wear particles on bacterial growth and associated infection persistences of total hip arthroplasties with MOM bearings. Co–Cr debris has been demonstrated to promote bacterial growth in vitro, but on the other hand high concentrations of Co and Cr ions appeared to possess bacteriostatic properties as well. Recently, Co ions were reported to inhibit neutrophil proton pumps and Co–Cr particles induced toxic effects when phagocytosed, frustrating the degradative function of macrophages. These processes hamper the immune system and might predispose MOM patients to infection at the implant site.

In vitro it is impossible to determine the net effect of wear particles in the presence of bacteria on infection persistences. Clinically, investigating the influence of wear particles on the development of an infection requires large patient groups as infection only occurs in 0.5–3% of patients. Moreover, it is not feasible to collect pseudosynovial fluid for infection analysis from such a large patient group, as this invasive procedure bears the persistence of introducing an infection.

An in vivo murine air pouch model has been developed in order to mimic the joint lining around failed prostheses for comparing the biocompatibility of particulate debris. Using this model, the presence of Co–Cr, UHMWPE, Ti–6Al–4V, and polymethylmethacrylate (PMMA) particles in such pouches were found to increase the air pouch membrane thickness, and the number of macrophages in the pouches as well as the IL-1 response. Forty-eight hours after the introduction of particles into such pouches, Ti–6Al–4V showed the most pronounced increase in membrane thickness and IL-1 response, while UHMWPE particles were responsible for the highest increase in number of macrophages. In addition, both shape and texture of the particles were found to influence the severity of inflammatory response, with rough debris surface texture exerting the most adverse tissue response. The effects of the combined presence of bacteria and particulate debris in an air pouch model of the joint lining around a prosthesis has never been performed.

In the current manuscript, we aim to compare the influence of Co–Cr and UHMPE particles on the persistence of infection in an animal model.

The authors declare no potential conflict of interest.

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an in vivo bio-optical imaging system was used to longitudinally monitor the growth of bioluminescent staphylococci in air pouches prepared in the subcutaneous tissue of mice in the absence and presence of Co–Cr or UHMWPE particles. After sacrifice, microbiological and histological analyses of surrounding tissue were performed.

METHODS

Wear Particles

Commercially available pure UHMWPE particles (Ceridust 3615, 7 μm mean diameter) were generously provided by the manufacturer (Clariant, Coventry, RI). Co–Cr particles were derived from ISO 5832-4 cast Co–Cr alloy Micro-Melt® dust (Carpenter Powder Products, Wyomissing, PA), with courtesy of Biomet (Warsaw, IN). In order to obtain Co–Cr particles with a size range similar to UHMWPE particles, we used a pre-separator attached to an inhaler 2000 adapter (Sympatec GmbH, Clausthal-Zellerfeld, Germany) to remove larger particles. Subsequently, the smaller particles were trapped in an aerosol and liquid impinger in ethanol. Both Co–Cr and UHMWPE particles were washed in 70% ethanol solution to remove possible bound endotoxin from environmental bacteria and ethanol was subsequently evaporated under vacuum. The absence of endotoxins was confirmed using the Limulus assay (Endosafe, Charlestown, SC). Prior to inoculation, particles were sonicated for 30 min to prevent aggregation. Final particle size distributions were measured with a Sympatec HELOS compact KA laser diffraction apparatus (Sympatec GmbH), using a ROODS dry powder disperser at 3.0 bar. A lens of 200 mm was used and calculations were based on the Fraunhofer diffraction theory. Particle morphology was visualized with a JEOL JSM6301 scanning microscope (JEOL, USA Inc., Peabody, MA) at 3 kV, after sputter-coating the particles with a 5 nm thick, conductive film of Pd/Au.

Bioluminescent Staphylococcus aureus Xen36

Staphylococcus aureus Xen36 (commercially obtained from Xenogen, Alameda, CA) was derived from S. aureus ATCC 49525. The parental strain was a clinical isolate from a bacteremic patient, made bioluminescent by stably integrating a native plasmid. This modification enables the strain to produce luciferase and its substrate, resulting in a photon-emitting state when in presence of ATP, the reduced biomolecule flavin mononucleotide, NADPH, and oxygen. Staphylococcus aureus Xen36 was cultured from cryopreservative beads (Protect Technical Surface Consultants Ltd, Lancashire, UK) onto tryptone soya broth (TSB; Oxoid, Basingstoke, UK) agar plates supplemented with 200 μg/ml kanamycin at 37°C in ambient air. One colony was used to inoculate 10 ml TSB supplemented with kanamycin for 24 h at 37°C. Main cultures were grown overnight by inoculating 10 ml TSB supplemented with kanamycin with 0.5 ml preculture. Subsequently, main cultures were centrifuged and rinsed three times with sterile phosphate-buffered saline (PBS) and resuspended in PBS. The inoculum was diluted to 9 × 10^7 cells/ml by counting in a Bürker-Türk counting chamber. Aliquots of the inoculum were incubated on agar plates for 48 h at 37°C to yield the concentration of CFUs in the inoculum, which was found to be 5 × 10⁹ CFU/ml.

For animal experiments, a 5% weight/volume concentration was chosen for Co–Cr particles (25 mg/ml) as suspended in syringes with 1 ml of the staphylococcal suspension. Particles were gently added in the syringes after removing the aspirator. Next the aspirator was replaced, the syringe was hold upside down to remove redundant air from the syringe, and the bacterial inoculum was aspirated. The concentration of UHMWPE particles was adjusted to match the number of Co–Cr particles using the particle size distributions listed in Table 1. Since the UHMWPE particles were smaller than the Co–Cr particles, this resulted in a lower concentration of 5 mg/ml. Although the bioluminescence yield per bacterium should not a priori be influenced by its environment, the presence of light reflecting or absorbing surfaces like Co–Cr or UHMWPE particles may influence the bioluminescence captured in bio-optical imaging. Therefore, syringes filled with only S. aureus Xen36 and with Co–Cr or UHMWPE particles added were imaged with the bio-optical imaging system, as described below. The ratio between the bioluminescence yields in the absence and presence of Co–Cr or UHMWPE particles was used to correct the in vivo bioluminescence for possible effects of absorption and reflection by the particles, assuming that the influence of differences in geometry between the syringes and the air pouches can be neglected.

Murine Air Pouch Infection Model

Seven weeks old male BALB/c OlaHsd immune competent mice (Harlan Netherlands BV, Horst, Netherlands) with specified pathogen free conditions were quarantined in our animal facility for 2 weeks prior to experiments. All animals were housed per groups of three in individual ventilated cages with Cellu-dri Soft R bedding (Shepherd Specialty papers, Kalamazoo, Michigan, US). The mice were weighed daily and evaluated on the basis of activity, illness, and alternative behavior. All mice weighed 20 g or over at the start of the experiment (average 22.2 ± 1.0 g) and had ad libitum access to food and water throughout the experiment.

Air pouches were prepared according to a protocol developed in our laboratory.6 Briefly, an area of 2 cm² dorsal skin was shaved and cleaned with alcohol to provide the pouch site. Sterile air (1 ml) was injected subcutaneously at a single site with a 25-gauge needle syringe on alternate days for 5 days to establish a definitive pouch, which filled spontaneously with serum. Buprenorphine (0.03 mg/kg) was administered subcutaneously 30 min prior to injections. During pouch preparation, anesthesia was induced with a 3.5% Isoflurane/O₂ (Zeneca, Zoetermeer, Netherlands) gas mixture and maintained at 1.5%.

Six days after air pouch creation, pouches were injected with 1 ml of a bioluminescent staphylococcal suspension...
(six mice) or a combination of bioluminescent bacteria with Co–Cr or UHMWPE particles (also six mice per group). In addition, three smaller control groups (three mice per group) were injected with Co–Cr or UHMWPE particles or only PBS in the absence of any bacteria. Subsequently, bacterial presence was monitored for a period of 21 days by bioluminescence imaging, as described below. At the end of the experimental period, animals were euthanized under anesthesia by cervical dislocation. Experiments were performed in accordance to federal legislation regarding the protection of animals and were approved by the Animal Experiments Committee at the University of Groningen.

Bioluminescence Imaging

Bioluminescence was imaged with a highly sensitive, cooled CCD camera (IVIS 100 Imaging System, Caliper Life Sciences, Hopkinton, MA). Bioluminescence images were obtained on days 5, 6, and then every 3 days up to 21 days with 5 min exposure time using a 15-cm field of view, binning of 4, 1/f stop (i.e., aperture is wide open for maximal light collection) and open filters. Pseudo-color images were obtained and overlaid on a gray scale photograph of the mice. Mice were kept under anesthesia during imaging. Bioluminescence was quantified as total bioluminescent flux (photons/s) within a circular region of interest (2.5 cm²) by using living image software (Xenogen). In case of imaging of the syringes containing bioluminescent bacteria in the absence and presence of particles, the area of the syringe was taken as the region of interest.

In order to account for effects of the presence of particles on staphylococcal bioluminescent fluxes measured, all measured fluxes were corrected for absorption and reflection using the in vitro bioluminescence ratios between syringes filled with bacteria in the absence and presence of particles. Subsequently, data of the groups of mice with bioluminescent bacteria in the absence and presence of particles were subsequently normalized by dividing the corrected bioluminescence flux of individual mice on each day by the average corrected bioluminescence flux observed in the presence of S. aureus Xen36 only.

Analysis of Tissue Samples

Air pouch fluid was collected during anesthesia by flushing an air pouch with 1 ml sterile PBS, immediately before euthanizing the mice. Directly after euthanasia, air pouch membrane samples were collected from the dorsum of the air pouch for histological and culturing purposes. The pouch membrane was lifted in the center with sterile forceps and cut with sterile surgical scissors after which it was divided in two equally sized cranial and caudal tissue samples used for histology and culturing.

For histological analysis, tissue samples were taken from all animals. Part of a tissue sample was fixed in 10% buffered formalin, dehydrated, and embedded in paraffin blocks with particular care to preserve the original shape of the pouch tissue, while the other part was used for culturing. Sections were cut along the pouch middle line, mounted, and stained with hematoxylin and eosin stain. Cover slips were fitted to the slides using glass bonding adhesive.

After scanning with a NanoZoomer 2.0-HT system (Hamamatsu photonics, Hamamatsu, Japan), three separate sections per specimen were evaluated with NDP view software (Olympus, Center valley, PA) in a blinded fashion by three individuals. Tissue reaction was scored by measuring the thickness of the pouch membrane and enumeration of the number of macrophages per mm² of analyzed air pouch membrane.

For culturing, tissue samples were sonicated on ice with a Vibra Cell 375 (Sonics and Materials, Danbury, CT) and subsequently rubbed with sterile forceps on TSB agar plates. Both the sonication fluid from tissue samples and the air pouch fluid were serially diluted and spread on TSB agar plates. All plates were left to incubate for 24, 48, and 72 h at 37 °C and CFUs were counted.

Statistics

Normalized bioluminescence fluxes were compared at each observation day during the 21 days follow-up period by ANOVA followed by a Mann–Whitney U-test. In addition, air pouch membrane thickness and macrophage counts were analyzed by ANOVA followed by a Mann–Whitney U-test. p Values of <0.05 were considered significant.

RESULTS

Laser diffraction revealed volume size-distributions to be within the micrometer size range (Table 1). Differences between Co–Cr and UHMWPE particles were minor and confined to particle sizes above 10 μm. Scanning electron microscope (SEM) images demonstrated that Co–Cr particles are more spherically shaped, with UHMWPE particles having a more irregular flake-shape (Fig. 1).

The bioluminescence captured in the bio-optical imaging system was influenced by the presence of Co–Cr and UHMWPE particles. Bioluminescence arising from 1 ml syringes filled with 5 × 10⁶ S. aureus Xen36 in the presence of Co–Cr particles was significantly weaker (0.8×, p < 0.05) than in the absence of Co–Cr particles, due to absorption of light by the particles. On the other hand, extensive reflection by UHMWPE particles significantly increased the bioluminescence measured (1.6×, p < 0.05). These ratios were employed to correct the bioluminescence arising from mice infected with S. aureus Xen36 in the presence of particles.

Figure 2 shows examples of bioluminescence images of an individual mouse from each of the three experimental groups, followed longitudinally. Weakest bioluminescence is seen for the mouse injected with S. aureus Xen36 and Co–Cr particles, while more prominent and lasting bioluminescence arises from the mouse injected with S. aureus Xen36 and UHMWPE particles. These images however, may not be taken as indicative for the course of infection, as they do not account for absorption and reflection of the bioluminescence signal.

Bioluminescence from all three groups of mice, as corrected for absorption and reflection were subsequently normalized by dividing the corrected bioluminescence fluxes by the average bioluminescence flux at each day in the group of mice injected with bacteria only. Figure 3 reveals a clear difference in the course of infection in animals having received Co–Cr particles...
and UHMWPE particles. In the Co–Cr group, normalized bioluminescence was below unity in four out of the six mice, but all returned to unity within 9–21 days. In the UHMWPE group, bioluminescence was initially less than unity, with only one animal on day 3 emitting a slightly higher bioluminescence, but after 9 days there was no difference anymore in bioluminescence arising from mice injected with \textit{S. aureus} \textit{Xen36} only or with staphylococci and UHMWPE particles.

Background bioluminescence flux was found to be below $1.2 \times 10^5$ (p/s) in control groups injected with PBS or particles only and coincided with the bioluminescence observed in all groups of mice after 21 days.

Histological analyses revealed no signs of inflammation in 26 out of the 27 mice in the different groups after sacrifice at day 21 (Fig. 4). Average air pouch membrane thickness after sacrifice was found to be $1.3 \pm 0.6$ mm with no significant differences across groups (Table 2). Similarly, mice injected with staphylococci in the absence or presence of particles, including control mice, had no significant difference in macrophage counts after sacrifice, with the exception of the animal in the Co–Cr and \textit{S. aureus} Xen36 group for which a high bioluminescent signal was measured until day 18. Micrographs of the pouch membrane of this mouse showed a high relative macrophage count of 12.4. In addition, there was a hematoma observed, which was not found in pouch membrane of other mice. Particulate debris was never detected in any of the air pouch membrane tissue samples, while extensive culturing did not reveal \textit{S. aureus} Xen36 bacteria in any of the pouch membrane samples.

**DISCUSSION**

Long-term clinical data on infection rates for specific bearing types are not yet available. Although there are in vivo studies available on the influence of bulk
material on the incidence of infection, this is the first in vivo study towards assessing the influence of wear particles on bacterial infection persistence. In this study, we used a novel method of in vivo imaging to longitudinally monitor the persistence of bioluminescent staphylococci in an artificial joint space. In the majority of mice, infection risk in the absence or presence of Co–Cr and UHMWPE particles appeared similar, assuming that the longevity of an elevated bioluminescence is indicative of a higher infection risk. However, the bioluminescence signal was initially lower in mice exposed to UHMWPE particles with staphylococci than in mice injected with staphylococci only, possibly because UHMWPE particles initially stimulated a higher macrophage presence in murine air pouch membranes, responsible for rapid eradication of bacteria. For mice exposed to Co–Cr particles with staphylococci, bioluminescence was observed to be higher in two out of six animals compared to the presence of staphylococci alone and accordingly a high number of macrophages was found in the pouch membrane of the mouse showing the most prolonged, elevated bioluminescence. Therefore, it can be concluded that whereas the presence of UHMWPE particles does not increase the persistence of infection, Co–Cr particles may have variable effects on infection persistence in different individuals. This study cannot provide a clue as to why two mice injected with Co–Cr particles and S. aureus showed elevated bioluminescence for prolonged periods of time as compared to the four other mice in this group, showing a response similar to the one of mice injected with UHMWPE particles and S. aureus.

Figure 3. Normalized bioluminescence flux as a function of time after correction for absorption and reflection due to the presence of Co–Cr or UHMWPE particles. The normalized bioluminescence flux denotes the flux arising from air pouches filled with particles and bacteria relative to the bioluminescence arising from pouches filled with S. aureus Xen36 only. Each symbol represents one mouse.

Figure 4. Air pouch membranes after hematoxylin and eosin staining of three mice exposed to Co–Cr particles and S. aureus Xen36 (a), UHMWPE particles and S. aureus Xen36 (b) and S. aureus Xen36 only (c) showing no signs of infection or inflammation due to particles or bacteria. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jor]
Although an increased number of macrophages was reported 48 h after injection of Co–Cr or UHMWPE particles into murine air pouches,\textsuperscript{15,23} it is clear from the present study that macrophage numbers in the pouch membrane reduce to control levels within 21 days even during simultaneous exposure to S. aureus Xen36. If, for unknown reasons, a relatively fulminant infection occurs, higher numbers of macrophages persist for longer periods of time. This occurred in two mice of the Co–Cr group, which may not be a coincidence since it has been shown that macrophages in the presence of Co–Cr particles with staphylococci can be prone to toxic effects of Co ions,\textsuperscript{8–10} frustrating their degradative function. This implies a higher number of macrophages is needed to eradicate the bacteria present. This evidently does not necessarily occur in all animals. Generally, histological studies of tissues retrieved from revised prostheses have shown infiltration of surrounding tissues with Co–Cr or UHMWPE debris.\textsuperscript{21} In our study, particulate debris was not visible in any histological sample, nor was there any sign of inflammation after 21 days observed in five out of six animals injected with Co–Cr particles and S. aureus Xen36. This may imply transport of particles to other parts of the body, as described earlier in patients where metal wear debris has been found in lymph nodes, liver, and spleen,\textsuperscript{22} despite the fact only 10% of our particles were sized beneath 4 μm. Clinically derived particles from periprosthetic tissues size between 40 and 120 nm for Co–Cr and are around 660 nm for UHMWPE.\textsuperscript{23,24} However, more than 50% of the particles used were in the phagocytosable size range smaller than 10 μm\textsuperscript{26} and may be expected to provoke inflammatory reactions in vivo.\textsuperscript{27} Moreover, micro- and nanosized particles have been shown to provoke similar inflammatory reactions in vivo\textsuperscript{27} with Ceridust particles having been reported earlier to resemble UHMWPE wear debris derived from MOP implants.\textsuperscript{26,28}

One of the benefits of using bioluminescence is the relative ease with which bacterial presence can be monitored longitudinally in one and the same animal.\textsuperscript{29} This is the first study to combine the use of an in vivo air pouch model with bioluminescence measurements to evaluate bacterial persistence in the absence and presence of wear debris. The mouse's own joint space was not deemed suitable for bioluminescence evaluation as bony structures were expected to block bioluminescence significantly. All other current studies employing bioluminescence measurements to assess the longevity and severity of biomaterials-associated infections have used subcutaneous implantation of biomaterials. Kadurugamuwa et al.\textsuperscript{30} pre-inoculated a Teflon catheter and measured significant in vivo bioluminescence signals up to 20 days. Engelsman et al.\textsuperscript{31} pre-inoculated surgical meshes of different composition and found higher levels of bioluminescence up to 10 days that coincided with ex vivo counts of the number of colony forming units that could be retrieved from explanted meshes. The detection limit of bioluminescence measurements for bacterial presence is higher than of, for instance, plate counting, while bacterial persistence in tissues surrounding infected biomaterial has been demonstrated despite the absence of measurable bioluminescence.\textsuperscript{18} In fact, it has been suggested that bacteria on and around an implanted biomaterial not only find protection against antibiotics and the host immune system in their biofilm mode of growth, but also seek protection in surrounding tissue.\textsuperscript{32} It might well be a unique feature of this air pouch model that it does not allow bacteria to migrate and find shelter in surrounding tissue, possibly because the air pouch membrane is heavily vascularized although we admittedly cannot rule out bacterial presence below the detection limit of bioluminescence in tissue surrounding the air pouches.

In conclusion, the presence of staphylococci in combination with UHMWPE particles did not increase the persistence of infection, possibly as a result of initially higher macrophage levels. The combination of staphylococci with Co–Cr particles is known to frustrate the degradative function of macrophages and accordingly bioluminescence was higher and more prolonged in
two out of six mice, suggesting that Co–Cr particles may increase the persistence of infection. However, influences of the presence of particulate material in combination with staphylococci, had disappeared after 21 days, regardless of the type of particles.

ACKNOWLEDGMENTS
The authors acknowledge with gratitude the help of Floris Grasmeijer and Prof. Erik Frijlink in resizing the Co–Cr particles. They would also like to thank Imran Khan from Biomet and Philippe van den Broeck from Clariant for their help in providing us with Co–Cr and UHMWPE particles. In addition they would like to thank Jeroen Kuipers for his valuable technical support in the field of scanning electron microscopy and Greg Hugenholtz for his expertise in the tissue analyses. This work was supported by the University Medical Center Groningen.

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