Metal-specific differences in levels of DNA damage caused by synovial fluid recovered at revision arthroplasty

A. P. Davies, A. Sood, A. C. Lewis, R. Newson, I. D. Learmonth, C. P. Case

From Southmead Hospital, Bristol, England

Previous research has shown an increase in chromosomal aberrations in patients with worn implants. The type of aberration depended on the type of metal alloy in the prosthesis. We have investigated the metal-specific difference in the level of DNA damage (DNA strand breaks and alkali labile sites) induced by culturing human fibroblasts in synovial fluid retrieved at revision arthroplasty.

All six samples from revision cobalt-chromium metal-on-metal and four of six samples from cobalt-chromium metal-on-polyethylene prostheses caused DNA damage. By contrast, none of six samples from revision stainless-steel metal-on-polyethylene prostheses caused significant damage. Samples of cobalt-chromium alloy left to corrode in phosphate-buffered saline also caused DNA damage and this depended on a synergistic effect between the cobalt and chromium ions.

Our results further emphasise that epidemiological studies of orthopaedic implants should take account of the type of metal alloy used.

The long-term biological effects of joint replacement surgery are not known. Because metal implants contain potentially mutagenic metals such as chromium, cobalt, nickel, several epidemiological studies have investigated whether there is a risk of malignancy after joint replacement surgery. A review performed by the International Agency for Research on Cancer (IARC) in 2000 summarised the findings of 14 epidemiological cohort studies in six countries, after total knee or total hip replacement. One study included only patients with metal-on-metal implants, five included only those with polyethylene-on-metal implants, while the remaining studies had patients with mixed or unspecified types of implant. One study showed a small increase while the remainder showed a decrease in the overall incidence of cancer. Four of these studies suggested that there was an excess risk for specific types of cancer, including Hodgkin’s disease, non-Hodgkin’s lymphoma, leukaemia and renal cancer. The others, however, were inconsistent.

Most of these epidemiological studies did not have information on possible confounding variables, such as immunosuppressive therapy, rheumatoid arthritis and the use of analgesics. The follow-up in most of the studies may have been too short to investigate cancer occurring many years after exposure; in some studies with longer follow-up the numbers of long-time survivors were small. The IARC placed orthopaedic implants of complex composition under the group-3 classification (not classifiable as to their carcinogenicity to man). More recently, a very large long-term epidemiological study from Sweden has identified that patients with the longest follow-up periods of 15 years or more after total hip replacement are at increased risk of multiple myeloma compared with the general population.

The mutagenicity and carcinogenicity of any given biomaterial is determined by the characteristics of both the composition of the material and its presentation within the human body. The characteristics include rates of release of leachable material into the local and distant biological environment, the nature of that local environment, corrosion or degradation of a biomaterial resulting in the potential formation of compounds with different mutagenic properties and the surface properties of the material, all of which in turn influence the speed and effects of corrosion. There are very few data available for the assessment of these effects and the research methods used are often not validated.

Metal prostheses implanted into the human body are subject to wear and corrosion which result in the release of a wide range of degradation products. Selective removal, or
chemical modification, of individual components of a prosthesis can alter the composition of the remaining surface of the material and of the degradation particles. In the case of metal alloys commonly used in orthopaedic prostheses, the release of one type of metal ion can be strongly influenced by the electrochemical properties of other metals within the alloy. There are few studies which provide adequate data of these effects, but there is the potential for the release of chemical species of known mutagenicity or carcinogeticity.

In view of these uncertainties, we have performed a study of the mutagenicity of the synovial fluid from within the pseudocapsule of worn implants. This fluid was chosen because it is the material to which the patient is exposed. We have followed up a previous study, which showed that patients with worn implants had an increased level of chromosomal aberrations in peripheral blood lymphocytes. The type of aberration seen depended on the type of alloy in the prosthesis. Patients with worn titanium prostheses showed an increase in aneuploidy with no increase of chromosomal translocations. Patients with worn cobalt-chromium implants had increases in both aneuploidy and of chromosomal translocations, the latter implying the presence of chromosomal breakage. Since metal ions such as chromium and cobalt are known to cause damage at the DNA level, with DNA adduct formation and strand breaks, we have now investigated whether there is any evidence for metal-specific damage at the DNA level as well as at the chromosomal level. To do this we have used the single-strand gel electrophoresis or ‘comet’ assay which measures the sum of DNA strand breaks, DNA cross-links and alkali labile sites. The comet assay has been widely validated in studies of exposure to mutagens.

Materials and Methods

We obtained 24 samples of synovial fluid from 24 patients at the time of primary or revision arthroplasty surgery. Great care was taken in the collection of these samples to avoid contamination with other metals. The joint was aspirated before it was opened using a plastic cannula after withdrawal of the trochar. All samples were retrieved in the same way to minimise confounding variables. All patients had been treated for osteoarthritis and patients with inflammatory arthropathies were excluded. They were aged between 45 and 80 years.

Implants in group 1 consisted of six contemporary types of cobalt-chromium alloy metal-on-metal hip replacement prosthesis at revision. These were all resurfacing designs and included three Wright Conserve+ (Wright Medical Technologies, Arlington, Tennessee), two Corin McMinn (Corin, Cirencester, UK) and one Birmingham Hip Resurfacing (Midland Metal Technologies, Birmingham, UK) prosthesis. Three were revised at two years, two at five years and one at six years after implantation. All were cemented on the femoral side and uncemented on the acetabular side. All had failed clinically because of aseptic loosening of the acetabular component. None showed any evidence of impingement between the components. Implants in group 2 consisted of types of cobalt-chromium-on-polyethylene knee replacement prosthesis at revision. There were five total knee replacements, namely, two PFC (DePuy, Warsaw, Indiana), two Kinemax and one Kineumatic (Stryker-Howmedica, Mahwah, New Jersey), and one St Georg Sled unicompartmental prosthesis (Waldemar Link GMBG, Hamburg, Germany). These were revised at six, six, seven, eight, eight and nine years after implantation, respectively. All had failed because of aseptic loosening of one or more components. There was no metal-on-metal contact in any of these prostheses. All components had been introduced with cement.

Implants in group 3 consisted of types of stainless-steel metal-on-polyethylene total hip replacement prosthesis at revision. There were three Charnley (DePuy, Warsaw Indiana), one Howse (DePuy, Leeds, UK), one CPS (Plus Endoprothetik AG, Rotkreuz, Switzerland) and one Müller (Protek AG, Bern, Switzerland). These had failed at two, ten, 12, 21, 22 and 26 years, respectively after implantation. The revision at two years was for instability, while the remainder were for aseptic loosening. All the components had been introduced with cement.

In group 4 there were six control samples of synovial fluid from patients with osteoarthritis obtained at the time of primary total joint replacement. None had had any form of metal implant in situ.

Primary human fibroblasts (20 x 10^6) were harvested from culture flasks and placed in each of 12 wells of an experimental culture plate (Orange Scientific, Braine-l’Alleud, Belgium). The cells were suspended in 2 ml of complete medium and 250 µm of test fluid were added. The test fluid comprised synovial fluid or a negative control of 250 µm of Hank’s balanced salt solution. The culture plates were sealed and incubated at 37°C for 48 hours. Each well was then incubated with 0.2 ml of 0.05% trypsin for four minutes. The freed cells were washed by repeat pipetting of 2 ml of complete medium, placed in sterile centrifuge tubes and refrigerated immediately. Each tube was centrifuged at 2500 G for five minutes at 4°C. The pellet was resuspended in 200 µl of HBSS and cell viability counts were performed. The contents of the tubes were centrifuged again at 2500 G for five minutes at 4°C. The pellet was resuspended in 180 µl of 0.8% low-melting-point Agarose and 90 µl of the resuspended cells were pipetted onto each of two slides. The cover slips were re-applied and the slides were refrigerated for 30 minutes. A final layer of 100 µl of 0.8% low-melting-point agarose gel was applied before immersion overnight in lysis solution in total darkness at 4°C. The slides were washed in electrophoresis buffer and electrophoresis was carried out at 40 volts and 300 (SD 2) mA for 30 minutes. The slides were then washed with distilled water, stained with 50 µl of etidium bromide (20 µg/ml) and cover slips applied. They were stored at 4°C in total darkness and scored within 72 hours. Scoring was performed using a commercially-available image-analysis sys-
tem (Perceptive Instruments Ltd, Suffolk, UK). Fifty cells were scored for each slide and the Olive tail moment recorded. The Olive-tail moment data were pooled for the two slides from each well and compared with the duplicate experimental well run at the same time. The HBSS control data were compared between experiments to ensure consistency of results.

Electrothermal atomic absorption spectroscopy (Perkin Elmer ZL 4100, Wellesley, Massachusetts) was used to analyse the metal content of the samples of synovial fluid used in the comet assay experiments. Synovial fluid (200 µl) was placed into a polytetrafluoroethylene block with 18 x 3 ml wells and evaporated to dryness: 200 µl of 60% nitric acid (Merck Ultrapur, Merck, Whitehouse Station, New Jersey) and 50 µl of 30% hydrochloric acid (Merck Ultrapur) were added to each well. These were sealed and heated to 120˚C for two hours. The digested samples were evaporated to dryness. After cooling to room temperature, 200 µl of a solution containing nitric acid and 5000 ppm magnesium nitrate were accurately pipetted into each well to redissolve the digested samples of synovial fluid. The samples injected automatically into the atomic absorption spectroscope were 20 µl and the furnace programme used is given in Table I.

The detection limit for these experiments was 0.5 parts per billion (ppb) for cobalt and chromium. Each sample analysed was compared with two sets of reference standard solution both before and after the measurement had been made. Each experiment thus involved five measurements to derive a reproducible result for the metal content of the synovial fluid.

In order to test the hypothesis that the observed differences in levels of DNA damage caused by cobalt-chromium and stainless-steel prostheses were related to the concentrations of cobalt and chromium within the synovial fluids, we performed additional experiments using a series of artificial metal solutions. A model of corrosion of P21 cobalt-chromium alloy (Sulzer Medica, Winterthur, Switzerland) was generated by incubating alloy pellets in buffered salt solution at 37°C. P21 cobalt-chromium alloy comprises 26% to 30% chromium, 5% to 7% molybdenum, 1% nickel, 1% manganese, 1% silicon and small amounts of iron and carbon: the balance is cobalt. Graphite furnace atomic absorption spectroscopy measurements of the cobalt and chromium content of the alloy solution were then used to create solutions of cobalt and chromium salts containing these same metal ion concentrations either alone or in combination. The artificial fluids were made by dissolving salts of Cr(VI), Cr(III) and Co(II) in buffered salt solution. Potassium dichromate (CR VI), Chromium chloride (Cr III) and cobalt chloride (Co II) were used for this purpose. These artificial solutions were used to treat fibroblasts in an identical way to the experiments with synovial fluid described above.

**Statistical analysis.** This was carried out using the Stata statistical package (StataCorp, College Station, Texas). The outcome variable was the comet-tail moment, measured on each cell in each sample in each group. Each of these samples contained at least 150 cells. We fitted a log-linear regression model with Huber variances for each group of samples to calculate confidence intervals (CI) for the arithmetic mean comet-tail moment for each sample, and also calculated CIs for the ratio of arithmetic mean comet-tail moments between each treated sample and the control sample in the same group. CIs and p values were therefore calculated for six treated/control arithmetic mean ratios in each of five groups, giving a total of 30. This number of ratios measured is fairly large, and we might expect 5% of them to be significantly different from one at the 5% level by chance alone. We therefore used the Simes procedure, controlling the false discovery rate at 0.05<sup>17</sup> to define a subset of arithmetic mean ratios which might still be viewed as significantly different from one, even allowing for multiple testing.<sup>18</sup>

**Results**

All six samples of synovial fluid from the cobalt-chromium metal-on-metal implants (Fig. 1a) and four of the six samples from cobalt-chromium metal-on-polyethylene implants (Fig. 1b) caused statistically significant increases in the level of DNA damage compared with control values in human fibroblasts *in vitro*. None of the samples of synovial fluid from stainless-steel metal-on-polyethylene prostheses (Fig. 1c) caused increased levels of DNA damage. All six control synovial fluid samples from osteoarthritic joints (Fig. 1d) caused a very low level, but statistically significant, increased DNA damage.

Each of the samples of synovial fluid from cobalt-chromium-alloy metal-on-metal prostheses showed elevated levels of cobalt and chromium. The concentration of chromium varied from 0.95 µM to 6.88 µM in this group. The concentration of cobalt varied from 0.92 µM to 2.64 µM. The group of samples of synovial fluid from cobalt-chromium metal-on-polyethylene prostheses showed much lower metal concentrations, but these were detectable in all six samples. The concentrations of chromium varied between 0.07 µM and 2.06 µM and those of cobalt between 0.01 µM and 0.62 µM. The group of samples of synovial fluid from stainless-steel metal-on-polyethylene prostheses showed measurable concentrations of chromium but in all

---

**Table I. Details of the furnace programme**

<table>
<thead>
<tr>
<th>Temperature (˚)</th>
<th>Ramp rate (s)</th>
<th>Hold time (s)</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>2</td>
<td>1</td>
<td>Drying</td>
</tr>
<tr>
<td>140</td>
<td>60</td>
<td>10</td>
<td>Carbonising</td>
</tr>
<tr>
<td>700</td>
<td>8</td>
<td>5</td>
<td>Ashing</td>
</tr>
<tr>
<td>1500</td>
<td>3</td>
<td>20</td>
<td>Atomising</td>
</tr>
<tr>
<td>2350 (Cr)</td>
<td>0</td>
<td>5</td>
<td>Atomising</td>
</tr>
<tr>
<td>2400 (Co)</td>
<td>0</td>
<td>5</td>
<td>Atomising</td>
</tr>
<tr>
<td>2450</td>
<td>0</td>
<td>3</td>
<td>Clean out</td>
</tr>
</tbody>
</table>

* Cr, Chromium; Co, Cobalt
cases the concentration of cobalt was very low. In four cases it was below the detection limit of the experiment. Concentrations of chromium varied between 0.07 μM and 2.76 μM. The two measurable concentrations were each 0.05 μM. The group of samples from osteoarthritic joints showed very low but measurable concentrations of chromium of between 0.05 μM and 0.13 μM. In every case, the concentration of cobalt was below the detection limit of the experiment.

The solution of corroded P21 cobalt-chromium alloy was found to contain 0.27 μM of chromium and 0.84 μM of cobalt. It produced a statistically significant increase in the 

![Graphs showing DNA damage results](image-url)
level of DNA damage compared with control values (Fig. 2). Artificial salt solutions containing CO(II) alone at 0.84 μM induced a lower level of DNA damage than that produced by the P21 solution. Similarly, spiked salt solutions containing either CR(III) or CR(VI) alone at 0.27 μM induced lower levels of DNA damage than the P21 alloy solution. Spiked salt solutions containing combinations of both chromium at 0.27 μM and cobalt at 0.84 μM demonstrated higher levels of damage of DNA which were comparable with those seen with the P21 alloy solution. CR(VI) with Co(II) caused the highest levels of damage while CR(III) with Co(II) caused slightly lower levels compared with the P21 alloy solution.

Discussion

Our study has shown that synovial fluids from failed cobalt-chromium-alloy prostheses, either metal-on-metal or metal-on-polyethylene, cause DNA damage to human fibroblasts in tissue culture. By contrast, synovial fluids from failed stainless-steel metal-on-polyethylene prostheses did not cause damage. Synovial fluid from osteoarthritic joints caused low levels of DNA damage, implying that some component of the arthritic process such as cytokines or other inflammatory factors is capable of causing such low damage in the absence of a prosthesis. The fact that samples of synovial fluid from failed stainless-steel metal-on-polyethylene prostheses did not demonstrate elevated levels of DNA damage indicates that failure of the implant is not responsible for this damage and that the effect of the original arthritic process is no longer active once the joint has been replaced. By contrast, failed cobalt-chromium prostheses demonstrated much larger increases in DNA damage implying that the important factor in causing this is some difference between the constituents of the alloys cobalt chromium and stainless steel.

One difference between cobalt chromium and stainless steel is the lack of cobalt in the latter. For example, P21 cobalt chromium contains approximately 63% cobalt, 26% to 30% chromium, 5% to 7% molybdenum, 1% nickel, 1% manganese, 1% silicone and small amounts of iron and carbon. Stainless-steel 316 alloy contains 17% chromium, 12% nickel, 2.5% molybdenum, 2% manganese, 1% silicone and small amounts of sulphur and carbon. The balance (65%) is iron. Therefore a simple but untested hypothesis to account for the difference in the biological behaviour between the synovial fluids from cobalt-chromium-alloy and stainless-steel prostheses is that this may have been because of synergy of the effects of chromium and cobalt.

There are three reasons why this may be true. The first is suggested by the known biological effects of the respective ions. As previously mentioned the comet assay detects a combination of DNA strand breaks, cross-links and alkali labile sites. Both cobalt and chromium can cause this damage. CR(VI) induces DNA strand breaks, CR-DNA adducts, DNA cross-links and DNA-protein cross-links. Co(II) also causes DNA strand breakage. Although it is reported to be a 1000-fold less toxic than CR(VI), partly because of difficulties in entering the cell, this consideration may not apply to the release of CR(III) within the cell from particles after phagocytosis.

Co(II) also causes DNA strand breaks, but in addition it inhibits the incision and polymerisation step in DNA repair. Cobalt is therefore able, on theoretical grounds, to augment the damage caused by CR(VI) or CR(III). Secondly, other metals are known to have interactive effects which may be synergistic or antagonistic. Cobalt in particular has been shown to increase the mutagenicity of other metals in particulate form. Thirdly, in our experiments the amount of DNA damage caused by cobalt-chromium alloy after it had corroded in phosphate-buffered saline was only imitated by spiking native phosphate-buffered saline with the same concentration of both chromium (either VI or III) and cobalt ions together and not with either ion in isolation. It is therefore possible that the reason that there was more damage in synovial fluids from cobalt chromium compared with those from stainless-steel implants was because of a synergy between the chromium and cobalt in the alloy. The spiked salt solutions used in these experiments are highly simplified models of synovial fluid and will contain no inflammatory factors or proteins which could present or modify the metals that they contain. These fluids are simply solutions of free ionic cobalt and chromium, which may account for the lower levels of DNA damage seen in these experiments compared...
with experiments with samples of synovial fluids from failed cobalt-chromium-alloy prostheses.

It is extremely important to emphasise that the increased DNA damage in human fibroblasts in tissue culture after being exposed to synovial fluids from cobalt-chromium implants does not imply a greater risk of either local or systemic malignancy in vivo from cobalt-chromium prostheses. Although malignancy is characterised by the development of DNA damage and chromosomal aberrations it does not imply that an increase in DNA damage will lead to it. Molecular epidemiological studies suggest that DNA damage and chromosomal aberrations are very imprecise markers of future malignancy. Instead, these results should be used as a guide for future epidemiological studies. We have demonstrated that there is a difference in the biological activities of synovial fluids from around worn implants made of different metal alloys. It is therefore important that epidemiological studies should use this information and compare the long-term effects of stainless steel, cobalt-chromium and titanium alloy joint replacements rather than combining these data and comparing them with control values from the general population. A further advantage of this approach would be a partial elimination of confounding variables common to all patients with joint replacements and osteoarthritis, such as exposure to x-rays, drug therapy and genetic variables. This would allow the analysis to be restricted to the effects of the joint replacement alone. This may lead to a more informed opinion being made on the safest long-term joint replacement for young patients.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References