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Reactive oxygen species and oxidative DNA damage mediate the cytotoxicity of tungsten–nickel–cobalt alloys in vitro

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Abstract

Tungsten alloys (WA) have been introduced in an attempt to find safer alternatives to depleted uranium and lead munitions. However, it is known that at least one alloy, 91% tungsten–6% nickel–3% cobalt [WNC-91–6–3], causes rhabdomyosarcomas when fragments are implanted in rat muscle. This raises concerns that shrapnel, if not surgically removable, may result in similar tumours in humans. There is therefore a clear need to develop rapid and robust in vitro methods to characterise the toxicity of different WAs in order to identify those that are most likely to be harmful to human health and to guide development of new materials in the future. In the current study we have developed a rapid visual method to detect toxicity mediated by individual WA particles in cultured L6-C11 rat muscle cells. Using a variety of techniques (histology, comet assay, caspase-3 activity, microarrays) we show that, in agreement with the in vivo rat carcinogenicity studies, WNC-91–6–3 was the most toxic of the alloys tested. On dissolution, it produces large amounts of reactive oxygen species, causes significant amounts of DNA damage, inhibits caspase-3, triggers a severe hypoxic response and kills the cells in the immediate vicinity of the alloy particles within 24 h. By combining these in vitro data we offer a mechanistic explanation of the effect of this alloy in vivo and show that in vitro tests are a viable alternative for assessing new alloys in the future.

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Introduction

Whilst it undoubtedly makes effective weapons, there is widespread public concern that depleted uranium may have unintended detrimental consequences for both human health and the environment. Tungsten alloys (WA) have been introduced in an attempt to find safer alternative materials both for the military and in civilian applications where it has been used by wildfowlers as a “less toxic” replacement for lead shot in shotguns. One of the drawbacks of using tungsten is that its high melting point (3422 °C) makes it difficult to form the required shapes not only for weapons but also other applications. This is solved by mixing tungsten powder with small quantities of nickel and other metals including cobalt, copper and iron. The mixture is heated until the other metals melt to form an alloy that dissolves some of the tungsten and binds the remainder into a solid mass that can be machined as required. Although usually referred to as an alloy, the final product is not homogeneous and consists of the remains of the particles of tungsten powder embedded in a binding matrix that is a true alloy made up of the dissolved tungsten and the other metals (Gero et al., 2001). One of the advantages of WA-based munitions is that the properties of the alloy component (e.g. hardness and ability to fragment) can be tailored to specific applications by altering both the composition and the manufacturing process (Gero et al., 2001).

Powdered nickel and cobalt have a well established history of causing rhabdomyosarcoma when injected into the thigh muscles of rats; other metals including copper, iron and tungsten do not have this effect (Heath and Webb, 1967). When pellets of a specific WA (91% tungsten–6% nickel–3% cobalt [WNC-91–6–3]) were implanted into the thigh muscles of F344 rats, it was found that all of the animals developed aggressive rhabdomyosarcomas that rapidly metastasised to the lung resulting in 100% lethality (Kalnich et al., 2005). Similar results were obtained with nickel pellets but not tantalum, tungsten or another formulation WNF-97–2–1 (where F is iron) (Kalnich et al., 2005; Roszell et al., 2008). These in vivo animal data have raised concerns that embedded fragments of WA which are not amenable to surgical excision, might, over time, lead to formation of tumours in military and civilian casualties.

Since the long-term in vivo testing required to determine potential carcinogenicity of different munitions alloys is both expensive and time-consuming, there is an urgent requirement to develop rapid and robust in vitro methods to characterise the toxicities of different WAs.
This will allow the identification of alloys most likely to be harmless to human health prior to more rigorous testing (either detailed mechanistic in vitro studies or long-term in vivo rodent carcinogenicity studies), and allow rational risk decisions to be made about whether to pursue development of a particular alloy or to look for replacements. Furthermore, an understanding of the mechanism of WA-mediated toxicity would facilitate assessment of whether the carcinogenicity of WAs is likely to be a species-specific effect and may also be helpful in guiding therapeutic interventions to minimise the harmful effects of embedded WA fragments in shrapnel wounds.

In this study we have developed a simple visual cytotoxicity assay utilising a cultured rat muscle cell line (L6-C11) where individual alloy particles can be tested and assessed for their relative ability to cause cell death. We provide evidence that as they dissolve, WA particles generate reactive oxygen species (ROS) and that in the case of WNC-91–6–3 this is sufficient to cause significant DNA damage. Using a combination of these results and those from caspase 3 assays and microarrays, we offer a mechanistic explanation as to why WNC-91–6–3 was the most toxic of the alloys tested both in vitro and in vivo.

**Methods**

**Materials and reagents.** All chemicals and cell culture products were bought, at the highest grade available, from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK); unless otherwise stated: DePex mounting medium from BDH; EnzChek Caspase-3 assay kit #2 and Superscript II reverse transcriptase from Invitrogen Ltd. (Paisley, UK); heat-inactivated foetal bovine serum from PAA Ltd. (Yeovil, Somerset, UK); frozen stocks of L6-C11 rat skeletal muscle myoblasts from ECACC (now part of HPACC); *E. coli* formamidopyrimidine-DNA glycosylase (FPG) from Trevigen Inc. (Gaithersburg, Maryland, USA); RNeasy minikit and QIAquick PCR purification kit from Qiagen (Crawley, West Sussex, UK); DNA-free kit from Ambion (Austin, Texas, USA); Cy3- and Cy5-labelled deoxyctydine triphosphate from GE Healthcare (Little Chalfont, Buckinghamshire, UK); Giemsa’s stain solution from VWR International Ltd. (Poole, Dorset, UK); Jenner Stain from Raymond A. Lamb Ltd. (Eastbourne, Sussex, UK); particles of cobalt, iron, nickel and tungsten from Alfa Aesar (Heysham, Lancashire, UK). Tungsten heavy metal alloys and tantalum chips were a generous gift from Rheinmetall Waffe Munition GmBH. The chips were cleaned and sterilised by ultrasonicating three times in acetone and dried and packaged aseptically under argon by Intrinsiq Materials Ltd. (Farnborough, Hampshire, UK).

**Cell culture.** Cells were resuscitated, cultured and passaged as directed by ECACC (using high-glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with heat-inactivated foetal bovine serum (10% v/v), l-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (0.1 mg/ml)) to give a stock of aliquots at passage 4 that were stored in liquid nitrogen. These cells were then resuscitated as required to ensure that all experiments used cells aged between passages 6 and 7 to minimise variation due to changes in morphology.

**Histology.** Cells were grown in 75 cm² culture flasks until almost confluent. They were then trypsinised, diluted 1:10 and seeded onto 22 mm diameter coverslips placed in 6-well culture plates. After achieving confluence, culture was continued for four days to allow the formation of myotubes. Prior to dosing, the medium on the cells was removed by aspiration and replaced with 1.5 ml fresh media. Small quantities of particles were placed in sterile micro-centrifuge tubes with 0.5 ml medium and temporarily suspended by repeated vigorous aspiration using a 1 ml Finnpipette. The suspended particles were then dripped onto the medium immediately over the coverslips from a height of about 2 cm; the resulting splashes were found to be the most effective way to distribute the particles across the coverslip. After 24 h incubation at 37 °C and 5% CO₂, the medium was removed, the cells rinsed first with phosphate-buffered saline then with methanol, and then fixed in 1 ml fresh methanol for 5 min. During this time the coverslips were loosened and a small piece of plastic slide under each slip to prevent it adhering to the culture plate.

After fixing, the cells were stained with Jenner-Giemsa stain. Briefly, After the methanol was removed, the cells were allowed to dry for 5 min and then 1 ml Jenner stain (diluted 1 part with 2 parts 4 mM sodium phosphate buffer, pH 5.6) was added to each well. After 5 min, the cells were washed with repeated changes of deionised water until the water remained almost colourless. As much water as possible was removed by pipette and 1 ml Giemsa stain (diluted 1 part with 19 parts 4 mM sodium phosphate buffer, pH 5.6) was added to each well and the cells allowed to stain for 10 min. The cells were then washed with deionised water as previously. When the coverslips were completely dry they were stuck, cell-side down, to 39 × 76 mm glass slides using sufficient DePex to form a thick ring around the outside of the coverslip. The excess adhesive was necessary as the metal particles lifted the coverslip enough to allow air to be sucked underneath if the edge was not completely sealed. Digital photographs were taken of the cells at 40× magnification using an Olympus Camedia C-5050 Zoom camera via C 3040 and U-TVIX-2 adaptors.

**Caspase assay.** The method employed was based on that devised by Dusinska and Collins (1996). Following the appropriate treatment, cells were washed in cold phosphate-buffered saline (PBS) and gently scraped into fresh PBS (1 ml). Cells were centrifuged (200 × g, 5 min) and pellets re-suspended in PBS (150 μl). The suspension was divided into two parts with 135 μl re-pelleted, the PBS removed by aspiration and the dry pellet stored at −75 °C for use in the caspase assays. The remaining 15 μl aliquot of cell suspension was placed into a sterile tube containing 0.5% low melting point agarose (300 μl) and this cell suspension was transferred to two parallel glass microscope slides (150 μl per slide, BDH, UK), pre-coated with 0.5% normal melting point agarose. Glass coverslips (BDH, UK) were added and slides were placed on an aluminium block over ice for 10 min. Coverslips were removed and slides and were incubated for 1 h at 4 °C in lysis buffer (2.5 M NaCl, 0.1 M Na₂EDTA, 10 mM Tris base, 1% sodium N-lauryl sarcosinate, 10% DMSO and 1% Triton X-100).

Following lysis, slides were washed three times in cold FPG enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM Na₂EDTA, 0.2 mg ml⁻¹ BSA). FPG (1 unit) in FPG enzyme solution was subsequently added to the appropriate slides and glass coverslips added. In addition, a slide treated with buffer alone (50 μl) was prepared as a negative control. Slides were placed in a moist box, covered and incubated at 37 °C for 1 h. Slides were then transferred to a horizontal electrophoresis tank (Fisher, Loughborough, Leices- ter, UK) containing ice cold electrophoresis buffer (300 mM NaOH and 1 mM Na₂EDTA, pH 13.0) and DNA was allowed to unwind for 20 min. DNA was subjected to electrophoresis (32 V, 0.83 V cm⁻¹, 20 min) [both of these steps were carried out at room temperature] and slides neutralised by incubating (3 × 5 min) with 3 ml of neutralisation buffer (0.4 M Tris, adjusted to pH 7.5). Slides were subsequently stained with 50 μl of 10× Sybr Gold (Invitrogen).

The slides were examined at 320× magnification (32×/0.4 dry objective) using a fluorescence microscope (Zeiss Axiosvert 10, Germany), fitted with a 515–560 nm excitation filter and a barrier filter of 590 nm. A USB digital camera (Merlin, Allied Vision Technologies) captured the images, which were analysed using a personal computer-based image analysis system (Comet assay IV, Perceptive Instruments). Images of one hundred randomly selected nuclei were analysed per slide. Measurement of median percent tail DNA was chosen to assess the extent of DNA damage as it exhibits far less inter-run variability than other comet parameters due to its independence from electrophoresis voltage and run time (Olive and Durand, 2005). Median values of three separate experiments were analysed using a post-hoc Student’s t-test.
Caspase-3 assay. Caspase-3 activity was measured using an EnzChek Caspase-3 assay kit #2 in accordance to the manufacturer’s instructions with the slight modification that the reactions were incubated at 37 °C for 60 min. Cell pellets (prepared as above) were thawed and assayed in duplicate, and pellets from three control flasks and a standard curve (0–25 μM rhodamine 110) were included in each assay. Fluorescence was measured using an FL600FA fluorescence plate reader (Bio-Tek Instruments Inc., Winooski, Vermont, USA) using excitation and emission filters of 485 ± 20 and 530 ± 25 nm respectively.

ROS assay using 2′-7′-dichlorofluorescin diacetate. A stock solution of 2′-7′-dichlorofluorescin diacetate (3 mM) was prepared in ethanol and stored in 1 ml aliquots at −75 °C. Tissue culture medium (DMEM, supplemented as above, 50 ml) was placed in 75 cm² vented culture flasks with 167 μl 2′-7′-dichlorofluorescin diacetate stock solution (final concentration 10 μM). Residual esterase activity in the foetal bovine serum was sufficient to cleave the acetate groups from the probe allowing oxidation of the fluorescein group. Metal particles were added and dispersed as evenly as possible by agitating the flasks, which were incubated at 37 °C and 5% CO₂. Samples (100 μl) were withdrawn at 1, 2, 4, 6, 8, and 24 hour intervals and placed on ice. These were diluted to 2 ml with deionised water and the fluorescence was measured using a Perkin Elmer LS 50B luminescence spectrophotometer at excitation and emission wavelengths of 502 nm and 520 nm respectively.

Microarrays. Cells were grown and treated (six flasks were dosed with a metal plus a total of twenty-four undosed control flasks) in 75 cm² flasks, washed in phosphate-buffered saline, scrape harvested in 500 μl RNA later and then stored at −75 °C. After the samples had been collected they were randomised before further processing. Briefly, total RNA was extracted from the cells using an RNeasy minikit and any residual DNA was degraded using DNA-free™. Fluorescent-labelled cDNA was synthesised from the RNA using Superscript II reverse transcriptase and Cy3- or Cy5-labelled deoxyctydine triphosphate as appropriate. The labelled cDNA was purified using a QIAquick PCR purification kit, the amount of product was calculated spectrophotometrically and then stored at −20 °C until required. Microarray slides were prepared from a set of *Rattus norvegicus* (rat) ARoS V3.0 oligonucleotide chips (Eurosphere MWG Operon, Alabama, USA) by the Functional Genomics Facility (School of Biosciences, University of Birmingham, UK).

Hybridization was performed at random such that half the samples in each test and control group were applied as Cy3-labelled cDNA and the other half as Cy5-labelled. Labelled cDNA (20 pmol) was taken from each sample and used to prepare two pooled mixtures (one Cy3-labelled, the other Cy5-labelled). Labelled cDNA (with 15 pmol dye incorporated) was mixed with an equal amount of the corresponding pool and hybridised to the slides, which were then incubated at 42 °C for 16 h in a slide booster (Advalytix, Munich, Germany) set to sonicate at maximum power with a pulse:pause ratio of 3:7. Solutions of 20× SSC buffer (trisodium citrate (0.3 M) with sodium chloride (3 M)) and 10% w/v sodium dodecyl sulphate (SDS) were diluted and mixed appropriately and then filtered (0.22 μm pore size) to prepare washing solutions.

The hybridised slides were stringently washed in an “AdraWash” (Advalytix, Munich, Germany) (wash programme: 1 × 5 min pulse with 2× SSC/0.1% w/v SDS at 42 °C; all subsequent steps at room temperature, 1 × 5 min pulse with 0.1× SSC/0.1% w/v SDS; 3 × 7 min pulse with 0.1× SSC; 2 × 3 min with deionised water; 1 × 5 min with ethanol) and dried by centrifugation at 2000 rpm. The slides were checked for smears and, if necessary, manually re-washed in water, ethanol and then re-centrifuged. The dried slides were scanned using an Axon 4000B dual wavelength laser scanner, which detects emissions from Cy3 at 572 nm and Cy5 at 635 nm; the data were analysed using GeneSpring version 7.3.1 (Agilent Technologies, Santa Clara, California, USA).

Before the data were analysed, they were normalised and a lowess curve was used to correct for differences in intensity between the two fluorescent dyes. After curve fitting, the data were filtered to remove extraneous data points; the filters excluded data where the control signal was less than 38 in at least 3 of 5 conditions, the raw expression data were less than 10 in at least 3 of 5 conditions, and that data for a particular gene were detected in fewer than 24 of 48 samples. Statistical tests were carried out within Genespring. The probability that a particular gene ontology (GO) term was enriched amongst responsive genes compared with non-responsive genes was calculated using a hypergeometric distribution model as implemented by Genespring, employing a P-value cut-off of 1 × 10⁻⁴.

**Results**

**Cytotoxicity**

To investigate WA-mediated cytotoxicity, WA particles were added to monolayers of rat L6-C11 cells that had been maintained at confluence for four days to allow the formation of myotubes. No cell death was observed in the presence of either the tantalum control particles (Figs. 1 A–B) or WNC-97–2–1 particles (Figs. 1 C–D) for up to 48 h. In contrast, in the presence of either WNC-97–2–1 (Figs. 1 E–F) or WNC-91–6–3 (Figs. 1 G–H) there was clear evidence of cytotoxicity to surrounding cells after 24 h of incubation as shown by the almost complete absence of viable cells and the presence of large numbers of chromatin droplets in the area immediately surrounding the particles. Interestingly, the effect was reversible in the case of the WNC-97–2–1 particles with cells re-populating the area surrounding the particle after approximately 72 h. In the case of WNC-91–6–3 the cell-free area continued to expand for several days and re-population did not begin until 2–3 weeks after dosing, by which time it is estimated that the binder matrix will have completely dissolved from chips of these dimensions.

**WA particles result in the formation of ROS**

To investigate whether ROS were produced as WA particles dissolved in tissue culture medium, WA and tantalum control particles with a total surface area of 207 mm² (estimated statistically from the size distribution provided by the manufacturer) were incubated in complete medium (50 ml) in the presence of the redox sensitive dye dichlorofluorescin diacetate. There was no evidence of ROS formation in the presence of the control tantalum particles (Fig. 2A). All the other particles investigated produced detectable levels of ROS at a rate that was approximately linear over the time period investigated (0–24 h). The propensity of the different alloys to form ROS when WA particles were produced was WNC-91–6–3 > WNC-97–2–1 > WNC-97–2–1, which correlates with their relative cytotoxicity. When pure metals were investigated, only tungsten and cobalt generated detectable levels of ROS (Fig. 2B) although those produced by the former were only barely above background levels.

**WA-mediated oxidative DNA damage**

Having demonstrated that WA particles produce ROS when incubated in the presence of complete media, we investigated whether there were detectable biological consequences of this for cells in culture. Because WNC-91–6–3 is a known rat carcinogen (Kalinchik et al., 2005) we chose to investigate whether oxidative damage to genomic DNA could be detected. Following treatment of cells with either tantalum control, WNC-97–2–1 or WNC-97–2–1 particles for 24 h there was no statistically significant increase in levels of strand breaks compared to control cells either in the absence or presence of PFG. In contrast, when cells were incubated with WNC-
91–6–3, there was a statistically significant 19-fold increase in frank DNA strand breaks ($p < 0.001$, t-test with Bessel’s correction) compared to the untreated controls (Fig. 3). This clearly demonstrates that WNC-91–6–3, but not the other alloys investigated, causes genetic damage, including oxidative DNA damage, to cells in culture.

**Exposure to WA inhibits caspase-3 activity**

Caspase-3 is a key enzyme in apoptosis and preliminary experiments showed that it was inhibited by nickel and cobalt ions (data not shown). We found that all three WAs caused a significant inhibition of caspase-3 activity with a slight, non-significant, reduction in the presence of tantalum (Fig. 4).

**Microarray analysis**

The microarrays consisted of 26,985 probes, designed to screen the entire rat transcriptome. However, because muscle cells are highly specialised, many genes were not expressed at a detectable level and after filtering we found that only 11,906 of the probes produced
reliable data. Genes were identified as differentially expressed between treatment groups by analysis of variance (ANOVA) with a multiple testing correction for a false discovery rate (FDR) of \( \leq 0.05 \) (Benjamini and Hochberg, 1995). The expression of these genes relative to untreated controls is shown in Supplementary Table 1. An initial principal components analysis (PCA) was performed on these genes, summarising the data from all genes in this list for each individual sample. This clearly separated the treatment groups (Fig. 5) and showed that the two cobalt-containing alloys had very different expression patterns from the other materials. Interestingly, it was clear from the PCA that the tantalum “inert-metal control” particles were also producing an effect and that therefore this material cannot be regarded as completely inert. However, since tantalum has been used clinically for implants and other purposes for over 70 years without significant problems (Black, 1994), these effects are unlikely to be of medical relevance.

The data from each metal-treated group were then compared to controls. The numbers of genes assessed as differentially regulated versus controls at FDR \( \leq 0.05 \) were 324 for WNC-91–6–3; 638 for WNC-97–2–1; 1026 for WNF-97–2–1; and 151 for tantalum. As shown in Fig. 5, the treatment groups displayed differing variabilities that impacted on the number of significantly changing genes detected by these tests. WNF-97–2–1 samples, for example, showed low variability and therefore a high number of apparently statistically significant gene expression changes. Therefore, to display an overview of the expression responses, we applied a simple 1.5-fold change as the detection threshold, and the numbers of genes showing increased/decreased expression are shown in Fig. 6. The 1.5-fold change cut-off
was chosen due to the nature of the exposure, in that cells closest to the metal particles were likely to show a severe response, those further away a lesser response. Therefore employing the usual 2-fold change cut-off may be inappropriate in these non-homogenous samples. Although there was considerable overlap between the different groups of up-regulated and down-regulated genes there was little overlap between the up-regulated genes of one group and the down-regulated genes of the other groups. Statistical analyses carried out at the GO term representation level for the 1.5-fold responding genes showed that they largely fell into five broad classes as summarised in Table 1.

Genes controlling muscle structure and function were down-regulated in all treatment groups, although with WNF-97–97–21 the situation was somewhat complex as there were also some genes in this classification that were up-regulated. Very few genes in WNC–91–63 were down-regulated—the reasons for this are unclear but, from the results of the comet assays, it may be that there was such severe damage that the increased variability reduced the number of detectable changes. It was noticeable that genes associated with DNA damage and stress response were only significantly up-regulated in cells exposed to WNC–91–6–3. In addition, exposure to the two cobalt-based alloys caused a dramatic up-regulation of genes associated with glycolysis and other enzymes involved in carbohydrate metabolism and this effect was not seen with either tantalum or WNF–97–2–1.

Up-regulation of the genes for histone proteins was one of the most prominent changes caused by WNF–97–2–1 and lesser changes were also seen with tantalum and WNC–97–2–1. Whilst the other metals interacted with multiple histone-encoding genes, WNC–91–6–3 only caused the up-regulation of one of these (histone 2A). However, it did up-regulate genes for three histone-modifying enzymes: histone methylase, histone acetylase and histone deacetylase. Only histone deacetylase was also up-regulated by one of the other metals: WNF–97–2–1.

Discussion

Tungsten penetrator munitions are typically composed of tungsten, which has the high density necessary for a kinetic energy weapon, in a binder matrix of tungsten with nickel and a third component (X) that together provide strength and ductility. The binder matrix has a composition that is effectively constant for the initial Ni:X ratio; using a W:Ni:Co alloy where the Ni:Co ratio is 2:1, and the binder ratio is approximately 49:34:17 (Rheinmetall, 2007, private communication). In the study by Kalinich and co-workers it was found that, once the alloy pellets were embedded in the rat thigh muscles, the binder dissolves preferentially with steady state corrosion rates and loss of W, Ni and Co ions in urine in an almost identical ratio of approximately 3:2:1 (Kalinich et al., 2006).

In man, particles from nickel refining and water-soluble nickel salts have both been linked with cancers of the lung (Oller et al., 1997), possibly involving epigenetic effects. However, nickel-containing dental alloys are not associated with an increased cancer risk (Setcos et al., 2006). Although cobalt is known to be a carcinogen in its own right, in certain situations it appears to function as a co-
carcinogen. For example, inhalation of tungsten carbide (WC) or metallic cobalt separately is not associated with an increased risk of cancer. However, alloys consisting of WC and metallic cobalt not only cause tissue damage (lung fibrosis; ‘hard metal disease’) in exposed workers (Lison, 1996) but are also genotoxic in vitro and in vivo (Lison et al., 2001). Early work (Sunderman et al., 1990) found that the induction of sarcomas at the injection site from administration of nickel oxides and nickel/copper oxides depended on the bioavailability of the nickel (dissolution in body fluids) and the presence of contaminants such as cobalt.

Although often considered the gold standard, rodent in vivo carcinogenicity studies are expensive to undertake and there is a need to develop rapid in vitro tests to screen out new alloys that are most likely to be carcinogenic prior to further testing in vivo. Closely allied to this is the need to understand the mechanism of action of WA toxicity as this will facilitate a more informed risk assessment about the potential carcinogenicity of new materials to humans. We saw a clear dose–response with the cobalt-based alloys; WNC–91–6–3 was the most toxic to cells, causing an expanding zone of cell death around alloy particles that did not appear to repopulate until the binder matrix had dissolved. WNC–97–2–1 also demonstrated cytotoxicity but this was reversible and after 5–6 days cells had repopulated the region around the alloy particles. The other material tested (WNF–97–2–1) caused no visible toxicity. Our data therefore correlate with the observation that WNC–91–6–3 but not WNF–97–2–1 is carcinogenic in rats (Kalinich et al., 2006; Roszell et al., 2008). The ability of the different alloys to form ROS was also in the order: WNC–91–6–3 > WNC–97–2–1 > WNF–97–2–1, which correlates with their relative cytotoxicity. When pure metals were investigated, only cobalt generated ROS but levels were lower than expected based on the metric of surface area compared to WAs. This strongly suggests that cobalt is not simply the source of ROS in the alloy but that there is an interaction between different constituents of the alloy that greatly enhances the production of ROS. This supports the mechanism proposed by Lison et al. (1995) whereby interactions between cobalt, tungsten carbide and dissolved oxygen molecules generate ROS. With WA, this effect is likely to be heightened by the biphasic nature of the material as the presence of pure tungsten particles will cause a preferential galvanic corrosion of the binder matrix.

ROS are reactive molecules and are able to cause multiple forms of damage to cellular macromolecules including proteins, polyunsaturated lipids, RNA and DNA. Oxidative damage to genomic DNA is widely considered to be important in the process of carcinogenesis (Mates et al., 2008; Toyokuni, 2008). To test the hypothesis that WA-mediated ROS could cause oxidative DNA damage in cultured cells we utilised the FPG-modified comet assay, a rapid and sensitive test. Interestingly, of the particles tested only WNC–91–6–3 resulted in significant induction of strand breaks and oxidative DNA damage to cells. This is in agreement with its known carcinogenic properties in rats. In contrast, WNF–97–2–1, which is not a rodent carcinogen (Roszell et al., 2008), did not result in DNA damage either in the presence or absence of FPG. Therefore, although it is apparent that all the alloys generated some ROS in our cell-free test system, only WNC–91–6–3 generated ROS in sufficient quantities to overwhelm the antioxidant defences of cells (e.g. glutathione).

Further evidence to support the importance of ROS was provided by the microarrays which showed that genes associated with DNA damage and stress response were significantly up-regulated only by WNC–91–6–3. Likewise, genes associated with apoptosis and cell death, whose up-regulation would be a corollary of increased DNA damage, were up-regulated only by WNC–91–6–3. Although it is not possible to say for certain whether the changes in gene activity result in similar changes in either protein expression or enzyme activity, the fact that genes controlling more than one enzyme from the same pathway and that genes controlling enzymes in related pathways are being similarly affected makes it likely that this is the case. Although
ROS-instigated DNA damage alone is sufficient to raise concerns about the potential carcinogenicity of a substance, we have found evidence of other processes that allow us to speculate about the mechanisms underlying the potency with which WNC-91-6-3 can induce rhabdomyosarcoma.

Activation of glycolysis suggests that the cells are switching to an anaerobic metabolism that is associated with the hypoxic (low-oxygen) environment commonly found inside tumours. Cobalt has been used in tissue culture to mimic the effects of hypoxia (Du et al., 2008) and nickel is known to have a similar effect (Davidson et al., 2006). The cellular response to hypoxia is mediated by hypoxia inducible factor-1 (HIF-1) and the metals appear to act by inhibiting the activity of HIF-prolyl-hydroxylases (HIF-PHD). These enzymes negatively regulate the stability of HIF-1 and their inactivation allows the accumulation of HIF-1 to trigger a hypoxic response in the cell regardless of whether oxygen is absent or present. They require ferrous iron (Fe2+) for activity and this has led to the suggestion that the enzyme may be inactivated by a combination of two mechanisms: (a) direct substitution of iron by nickel or cobalt (Davidson et al., 2006; Vengellur and LaPres, 2004) and (b) the sequestration of cellular ascorbate (vitamin C) by nickel and cobalt; vitamin C is required to prevent the oxidation of iron to the ferric (Fe3+) form which also results in an inactive enzyme (Salnikow et al., 2004a). However, although nickel and cobalt have higher binding affinities for HIF-PHD than iron (Davidson et al., 2006), it would appear that the displacement of iron is unlikely to be energetically feasible in vivo (Kaczmarek et al., 2009) although it is still possible that newly synthesised proteins could incorporate nickel or cobalt resulting in loss of activity. Perhaps a more plausible interpretation of the protective effects seen by the co-administration of excess iron to cell cultures treated with nickel (Salnikow et al., 2004b; Kang et al. 2006) is that the iron is limiting the absorption of nickel by the cells (Tallkvist and Tjälve, 1997; Tallkvist et al., 2003; Salnikow et al., 2004b). In vivo, the iron protecting the cells will be made up partly from endogenous iron and partly from soluble iron dissolving from the alloy. Assuming that iron and nickel dissolve from the alloys in their atomic ratios (which, since their atomic weights are virtually identical, is almost the same as their proportions in the alloy by weight) an alloy with a higher proportion of nickel-iron (e.g. WNF-91-6-3) may release sufficient excess nickel to overwhelm the protective combination of endogenous plus alloy-derived iron and/or sequester cellular ascorbate sufficiently to inhibit HIF-PHD. Many of the pathways activated by HIF are associated with tumour formation and development, and what is seen here as essentially an anaerobic response could in effect be a form of neoplastic preconditioning.

It has long been established that histones protect DNA from iron-induced free radical damage (Enright et al., 1992) and both cobalt (Li et al., 2009) and nickel (Ke et al., 2008) are known to interact with histones. Hence it is not surprising that there should be changes in the expression of genes encoding histones. However, it is not clear why tantalum should also cause an up-regulation of these genes nor why WNF-97-2–1 should be the only metal to up-regulate histone 2B. Overall, the results from WNF-97-2–1, WNC-97-2–1 and, to a lesser extent, tantalum suggest that the chromatin structure has become more open; indeed genes controlling chromatins formation were also affected. The finding that WNC-91-6–3 also caused the up-regulation of the genes for histone methylase, histone acetylase and histone deacetylase is also of concern. These three enzymes, by modifying the histone proteins, regulate the activity of other genes; generally methylating a histone will down-regulate these genes. Such epigenetic changes are known to play an important role in the carcinogenic process and it is interesting that genes for all three enzymes are affected by the only alloy proven to cause rhabdomyosarcoma.

Caspase 3 is known to play an important role in regulation of both differentiation and apoptosis in muscle cells (Fernando et al., 2002; Murray et al., 2008) and has previously been shown to be inhibited by both ferric iron (Sliskovic and Mutus, 2006) and cobalt porphyrins (Blumenthal et al., 2005). The latter could be particularly relevant in vivo where the destruction of muscle tissue would result in the release of porphyrins. Our results show that ions from a relatively small amount of metal can significantly inhibit enzyme activity throughout a whole flask of cells and it would not be unreasonable to expect cells in the vicinity of a piece of shrapnel to be similarly affected in vivo.

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**Table 1**

<table>
<thead>
<tr>
<th>Functional area</th>
<th>Ta</th>
<th>WNF 97–2–1</th>
<th>WNF 97–2–1</th>
<th>WNC 91–6–3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle structure and function</td>
<td>↑</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↓</td>
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<tr>
<td>DNA organisation/regulation</td>
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<td>↑↑</td>
<td>↑↑</td>
<td>↓</td>
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<tr>
<td>Carbohydrate metabolism</td>
<td>–</td>
<td>–</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
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<tr>
<td>DNA damage and stress response</td>
<td>–</td>
<td>–</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
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<tr>
<td>Apoptosis and cell death</td>
<td>–</td>
<td>–</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
</tr>
</tbody>
</table>

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![Fig. 6](image-url) Two-dimensional representation of three-dimensional Venn diagrams showing distribution of genes that have been (A) up-regulated and (B) down-regulated by at least 1.5-fold following the 24-hour exposure of Li-C11 cells to metal particles when compared to undosed controls. A small dotted/dashed circle enclosed within a larger set indicates the overlap between the corresponding dotted/dashed set and the enclosing set.

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This mechanism, in combination with our results described here, would potentiate the carcinogenic properties of these materials. In conclusion, our data not only correlate with the carcinogenic properties of WA but also suggest a likely mechanism by which these materials cause cancer in rodents. The implanted material is a source of ROS; this causes local toxicity and oxidative DNA damage to cells surrounding the implanted material. Occasionally such cells will acquire mutations in key genes regulating cell proliferation and apoptosis. Our results suggest that this process is initiated by the mixture of nickel and cobalt in the binding alloy that gives synergistic effects, although the proportions must be critical since WNC-91 is much more toxic in vitro than WNC-97-2. Individually, the ions are known to have different mechanisms of action; cobalt generates free radicals whilst nickel binds to thiol groups. In addition, they inhibit key enzymes at the micromolar concentrations that could easily occur within the immediate vicinity of WA particles. Together, they appear to dysregulate gene expression whilst weakening the apoptotic response and thereby hindering the removal of abnormal cells. Linked with the over-supply of ROS, the damaged DNA is not removed and normal controls on cell growth are lost, leading to initiation of a tumour (Fig. 7). Our in vitro results are therefore coherent and entirely consistent with those found in vivo (Kalinich et al., 2005; Roszell et al., 2008). Although we have shown that relatively minor changes to the chemical composition of the alloys can produce major changes to their toxicological properties, the same is also true of their metallurgical properties. To find an acceptable balance between the two is not likely to be a simple task. However, by using in vitro assays that have been validated by reference to in vivo results, rapid profiling of new materials for likely toxicity and potential carcinogenicity may be carried out without the requirement for expensive in vivo studies.

Conflict of interest
The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at doi:10.1016/j.taap.2010.09.020.

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