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Effects of Cobalt on DNA Double Strand Break Repair-Deficient Cells

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Abstract

Epidemiology studies of human workers exposed to particulate cobalt have found increased incidence of lung cancer. A hallmark of lung cancer, chromosome instability (CIN) manifests as either numerical or structural abnormalities, which are thought to result from unrepaired or miss-repaired DNA double strand breaks. Cells have two major protective mechanisms to repair double strand breaks, homologous recombination (HR) and non-homologous end joining (NHEJ). Previous studies have shown that HR protects cells against metal induced CIN while NHEJ does not. We investigated whether this outcome occurs for cobalt. To test this possibility, we assessed the cytotoxicity and genotoxicity of cobalt in Chinese hamster cell lines that are deficient in HR and NHEJ repair. We found cobalt to be cytotoxic to all Chinese hamster cells, and compared to the wild type and complemented, in which the essential proteins have been reintroduced into the cellular genome, more complex genotoxicity was observed in the repair-deficient cells. Interestingly this complex damage manifested as a greater number of chromatid exchanges. When treated with 2 ug/cm^2 cobalt oxide the HR-deficient cells had 90 chromatid lesions and 20 chromatid exchanges per 100 metaphases analyzed compared to the wildtype cell line which had 25 and 0, respectively. When treated at the same concentration the NHEJ-deficient cells had 73 chromatid lesions and 19 exchanges as compared to the wildtype cell line which had 30 and 2, respectively. This is the first study to investigate the roles of HR and NHEJ in cobalt induced chromosome aberrations. Altogether these data suggest that HR and NHEJ are required to protect against cobalt induced CIN.

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Introduction

Historic and Anthropogenic Uses of Cobalt

Cobalt is a naturally occurring element that can be found in the air, water or soil as a result of either natural or anthropogenic activities (CDC, 2004). The 27th element on the periodic table, cobalt can be found in multiple valance states in a variety of compounds (CDI, 2014). Cobalt received its name from the German "kobalt" (CDI, 2013). German silver and nickel miners would attribute smelting problems to the "kobolds" (goblins) of which cobalt ore was later found to be the cause of the problems (CDI, 2013). Pure cobalt metal was not isolated until 1735 by the Swedish scientist G. Brandt (CDI, 2013). Naturally occurring cobalt is often found in salts, exhibits ferromagnetic properties and can be found in naturally occurring magnetic ores (CDI, 2013).

Anthropogenic activities are known to disperse cobalt into the surrounding environment from trash incineration, fuel combustion, radiation wastes, mining and smelting activities (CDC, 2004; CDI, 2013). It is believed that in instances such as with trash incinerators, cobalt particles are cast into the atmosphere where they bind with other particles and are potentially more lethal when inhaled (CDC, 2004). The health concern with inhalation of particulate depends on the size of the particle, the key factor dictating the depth of impaction in the respiratory tract. Larger particles tend to get trapped at the larger bronchial sites, while smaller particles are able to travel to the smaller branches where electrostatic precipitation and sedimentation effects particle deposition (CDC, 2004). Miners, smelters, industrial workers and individuals who

receive joint replacements are at an increased risk of cobalt exposure, relative to the general population (CDI, 2013; Lasfargues et al., 1994).

One of the earliest dated uses of cobalt is as a coloring agent primarily in ceramics, pottery and jewelry (CDI, 2013). Classically production of cobalt coloring agents involved grinding, heating and chemically activating various cobalt oxides and salts at high temperatures (CDI, 2013). Cobalt salts have also been used as drying agents in oil base paints, varnishes and inks (CDI, 2013). The cobalt salts accelerate and catalyze the oxidation and polymerization of paints, increasing flexibility and stability (CDI, 2013).

Cobalts Uses in Magnets and Super-alloys

Magnetic ores containing cobalt, nickel or iron can be found naturally around the world as relatively weakly magnetic (CDI, 2013). It was not until 1917 that the use of cobalt in hardened steel magnets began to increase (CDI, 2013). With the arrival of the industrial era magnets with stronger fields, greater malleability, and a resistance to high temperature driven loss of magnetism were in high demand. Magnet producers discovered that when cobalt was added to hardened magnets the Curie temperature, the temperature at which metals lose their magnetism (CDI, 2013), 1,121°C for cobalt, was significantly greater than any other material in use at the time (CDI, 2013). This and other cobalt derived advantages in magnets are what allowed development of more advanced technologies (CDI, 2013). The use of hardened steel magnets began to decline in the 1930's mostly due to the invention of Alnico magnets (aluminum, nickel, iron and cobalt) (CDI, 2013). Important factors for all cobalt containing magnets are the

significantly greater Curie temperature, permanence of magnetism and resistance to corrosion (CDI, 2013).

The super-alloy and cemented carbide industries have been relying on incorporating a greater number of cobalt-based alloys since the 1930's (CDI, 2013). It was the demand for reliable high temperature alloys for turbines that brought cobalt to its current place in the super-alloy family (CDI, 2013). The increased use of cobalt based alloys has been a result of three key characteristics which cobalt has over nickel in super-alloys; (1) cobalt based alloys have a higher melting point, (2) cobalt-containing alloys have greater heat corrosion resistance, and (3) cobalt alloys are more resistant to thermal fatigue and are therefore better suited for welding (CDI, 2013).

Petrochemical Uses of Cobalt

The petrochemical and plastic industries use approximately 5-thousand tons of cobalt a year, as reported in 2005 (CDI, 2013). Petroleum refineries use cobalt oxide for the desulphurization and hydro-treating of oil and gas (CDI, 2013). These processes remove organic sulphur, a health hazard, from crude oil, converting it to hydrogen sulfide (CDI, 2013). The chemical industry uses cobalt primarily in the production of precursor compounds to plastics and resins, terephthalic acid (TPA) and di-methylterephthalate (DMT) (CDI, 2013). The reactions essential to create both TPA and DMT from praxylene require cobalt compounds (cobalt acetate or bromide) in different ratios (CDI, 2013). Interestingly cobalt compounds are also used to catalyze the reactions essential to create polyethylene terephthalate (PET) bottles (CDI, 2013). The use of cobalt has also made an appearance in the adhesive industry (CDI, 2013).

By adding cobalt to the adhesives that hold the rubber of tires to their steel rims manufacturers found significantly stronger bonds, and a lower separation rate at the site of rubber-steel contact (CDI, 2013).

Cobalts Use in Cemented Carbide Industry

The cemented carbide industry involves the use of hard, resilient compounds to cut through various materials (CDI, 2013). Prior to the addition of cobalt, many steel based cutting tools were only able to cut for short periods of time and required frequent replacement (CDI, 2013). By adding cobalt to steel and carbide blades and drills the cemented carbide industry has been able to increase the hardness and resilience of their cutting tools significantly (CDI, 2013). Importantly the procedure by which cobalt is added to carbides requires sintering of cobalt oxide powders, which could be inhaled by those working with the powder (CDI, 2013). Also despite the increased toughness and resistance to wear of cobalt containing products eventually wear down. As high use drills and cutting tools the particles of cobalt could disperse into the air exposing those workers using or in close proximity to the equipment (CDI, 2013).

Additional Industrial Uses of Cobalt

Several industries, including power production, food packaging, and medical safety have used the cobalt radio-isotopes $cobalt^{60}$ and $cobalt^{58}$ since the mid 1900's (CDI, 2013; Diehl, 2002; Nansai et al, 2014; Pauli et al, 1986; Tauxe et al, 2001; Wilburn et al, 2012). The use of irradiation to kill bacteria on food was first investigated in the 1920's at the suggestion of the USDA (CDI, 2013; Diehl, 2002; Nansai et al, 2014; Pauli et al, 1986; Tauxe et al, 2001; Wilburn et al, 2012). It was not

until World War II that advancements in the available technology allowed for the practical application of food irradiation (CDI, 2013; Diehl, 2002; Nansai et al, 2014; Pauli et al, 1986; Tauxe et al, 2001; Wilburn et al, 2012). Despite the technology available it was determined that too little was known about the potential effects of food irradiation (CDI, 2013; Diehl, 2002; Nansai et al, 2014; Pauli et al, 1986; Tauxe et al, 2001; Wilburn et al, 2012). In 1970 the International Project in the Field of food Irradiation (IFIP) was created to evaluate the safety of irradiating food (CDI, 2013; Diehl, 2002; Nansai et al, 2014; Pauli et al, 1986; Tauxe et al, 2001; Wilburn et al, 2012). The IFIP's 1980 findings concluded that food irradiated by 10 kGy was not a toxilogical hazard (CDI, 2013; Diehl, 2002; Nansai et al, 2014; Pauli et al, 1986; Tauxe et al, 2001; Wilburn et al, 2012). Radio-isotopes of cobalt^{60} have also been used for medical sterilization of surgical equipment (CDI, 2013; Diehl, 2002; Nansai et al, 2014; Pauli et al, 1986; Tauxe et al, 2001; Wilburn et al, 2012).

Due to the castability, high load reliability and erosion resistance of cobalt based super-alloys they have been incorporated into a number of industries (CDI, 2013). It has been because of their resilience that cobalt super-alloys were once frequently used in cutting tools and have transitioned to being used in hip and knee replacement joints (CDI, 2013). Recent production of cobalt containing 'high-speed steels' and other alloys have created a significant increase in the global demand and consumption of cobalt since 2006 (CDI, 2013).

Due to the increased demand for cobalt and cobalt containing products a greater number of the population is at risk for exposure and development of related diseases (CDI, 2013). Cobalt can get into the environment as a result of natural or anthropogenic

events. Natural events which have been found to deposit cobalt particles into the air include forest fires and volcanic eruptions (CDC, 2004). The cobalt found in the air after these natural events often joins with other particulate matter and settles in the soil where the mobility is limited depending on solubility (CDC, 2004). Cobalt ions have been observed in various water systems as a result of erosion and exposure of cobalt containing salts to weathering (CDC, 2004). Once in the environment not all cobalt species are bio-available to all species (CDC, 2004; CDI, 2013). Some animals, specifically ruminants, are able to utilize Co (II) directly, while many other animals such as humans are only able to make use of cobalt from sources such as vitamin B12 (CDC, 2004). Some plants are able to take up cobalt from the surrounding soil, and only certain microorganisms are able to directly use cobalt (CDC, 2004).

Importance of Researching Cobalt's Potential Health Effects

Studies linking cobalt exposure to CIN and cancer have been inconclusive as a result of multiple confounding factors. Though, exposure to cobalt has been directly linked to the development of several lung diseases including asthma and lung cancer (CDI, 2013; Lasfargues et al., 1994). Epidemiology studies of workers exposed to cobalt encounter the confounding factors of co-exposures to other metals and smoking (Lasfargues et al., 1994; Wild et al., 2000). While cobalt co-exposure is important to investigate, understanding the independent interactions and mechanisms of cobalt alone are essential to establish potential carcinogenicity. Investigations into the incidence of lung cancer among workers of cobalt smelters and refineries have found enough of a link between cobalt exposure and lung cancer for the IARC to classify cobalt as a potential human carcinogen (CDC, 2004; IARC, 2006). More epidemiology studies are

needed to understand the relationship of cobalt exposure and carcinogenesis. Interestingly patients who receive cobalt containing hip replacements were similarly observed to have greater numbers of single strand breaks in blood samples (Davies et al., 2005; Witzleb et al., 2006).

Previous studies investigating cobalt carcinogenesis in rats found several cobalt species able to induce tumorigenesis after inhalation and intramuscular injection (Gilman, 1962). It was also observed that rats who developed tumors at the site of injection experience metastasis to the lungs (Gilman, 1962). It has also been observed in rats that inhalation of cobalt induces tumorigenesis, as well as other respiratory diseases (Gilman, 1962; Lasfargues et al., 1995). Interestingly it has been observed that co-exposure of cobalt with tungsten and titanium significantly increases the development of respiratory diseases and tumors in rats (Gilman, 1962; Lasfargues et al., 1995). Studies investigating cobalt exposure in other species have also observed increased genotoxicity as in male zebrafish (Reinardy et al., 2013). As cobalt ore is often found with other metals ore and cobalt is found in various alloys, exposure to cobalt is a serious health concern.

Risks and Assessments of Cobalt Exposure

Recent cell culture studies have found several cobalt species to be cytotoxic and genotoxic to human cells (Green et al., 2013; Smith et al., 2014). Both human and animal studies have investigated the potential genotoxicity and carcinogenicity of several cobalt species ability to induce chromosomal aberrations, CIN and cell transformation (NTP, 1998; Lasfargues et al., 1994; Smith et al., 2014). Previous cell

culture studies have established that particulate cobalt oxide is cytotoxic as well as genotoxic, causing single and double strand breaks, and sister chromatid exchanges (Da Boeck et al., 2003; Beyersmann and Hartwig, 2008; Smith et al., 2014). Cobalt was also observed to cause single strand breaks in blood cells taken from samples of French industrial workers exposed to particulate cobalt (Lasfargues et al., 1994). Earlier studies also established that cellular internalization of cobalt is required to induce genotoxicity (Smith et al., 2014), but they did not investigate the major double strand break repair pathways.

Another population with a high risk of cobalt exposure is individuals with metal joint replacements. Interestingly a younger population, 45 to 55 years old versus the typical age of 65+ years old, is receiving hip replacements (Papageorgiou et al., 2007; Onega, 2006; Schaffer et al., 1999; Shettlemore and Bundy, 2001). The concern for the younger hip replacement population is the greater duration of exposure to potential carcinogenic compounds such as cobalt and nickel (Papageorgiou et al., 2007). Artificial hip and knee replacements contain various combinations of alloys some of which contain cobalt (Davies et al., 2005; Haddad et al., 2011; Lhotka et al., 2003). Despite being more resistant to fracture, dislocation and corrosion than other arthroplasty options the metal on metal (MoM) hip replacements do wear down (Davies et al., 2005). MoM arthroplasties wear down and emit particles into the surrounding tissue (Jakobsen et al., 2007). These alloy particles, which contain cobalt, are then corroded by the immune system response and surrounding tissues are exposed to the metal ions (Germain et al., 2003). An important characteristic of particles is their constant presence until completely dissolved (Shettlemore and Bundy 2001). This

increases the duration of exposure of surrounding tissue to cobalt and the other metals contained in the particles emitted from the hip replacements (Shettlemore and Bundy, 2001). It is important to continue to study the population of individuals with MoM hip replacements to better understand the effect of cobalt exposure and potential carcinogenesis.

Mechanisms of Cobalt Induced DNA Damage

Previous studies have investigated the ability of cobalt to induce oxidative stress, interfere with base and nucleotide excision repair, interfere with zinc finger repair proteins and enhance topoisomerase II (Asmuß et al., 2000; Baldwin et al., 2004; Beyersmann and Hartwig, 2008; Hengstler et al., 2003; Lee et al., 2012; Liu et al., 2012; Valko et al., 2006; Whiteside et al., 2010; Witkiewicz-Kucharzyk and Bal, 2006). Cobalt, like nickel and other toxic metals has been previously shown to inhibit DNA repair by interfering with the zinc finger repair proteins (Witkiewicz-Kucharzyk and Bal, 2006). Specifically cobalt has been shown to inhibit the DNA damage recognition protein xeroderma pigmentosum group A (XPA) (Asmuß et al., 2000). The XPA protein is essential for nucleotide excision repair in human cells (Asmuß et al., 2000) and contains a DNA binding zinc finger motif (Asmuß et al., 2000). Cobalt is believed to displace the zinc finger in the XPA DNA binding motif, thus inhibiting its function (Asmuß et al., 2000). It has also been observed that cobalt induces a hypoxic environment for the cell which increases the oxidative stress put on the cell (Lee et al., 2012). Oxidative stress has been shown to induce an array of chromosomal aberrations (Lee et al., 2012), and can potentially induce carcinogenesis (Lee et al., 2012). Interestingly some species of cobalt have also been observed to act similarly to

topoisomerase II poisons (Baldwin et al., 2004), acting by enhancing the activity of topoisomerase II while inhibiting other repair proteins (Baldwin et al., 2004; Hengstler et al., 2003; Witkiewicz-Kucharzyk and Bal, 2006). Topoisomerase II is a protein which temporarily cleaves double stranded DNA ahead of replication machinery to alleviate super-coil stress caused by the unwinding of DNA (Baldwin et al., 2004). By enhancing the activity of topoisomerase II cobalt induces more double strand breaks to occur and by potentially inhibiting other repair proteins allows those DSBs to persist (Baldwin et al., 2004; Morgan et al., 1998).

Introduction to Carcinogenesis and Molecular Mechanisms Potentially Linked to Cobalt

Carcinogenesis is the process of transition from a normal cell to a cancerous cell (Klassen, 2013; Lee et al., 2012; Morgan et al., 1998). Cancer development occurs in three stages; (1) initiation, (2) promotion, and (3) progression (Klassen, 2013; Lee et al., 2012; Morgan et al., 1998). Initiation involves the development of a heritable change that is passed on to the daughter cells (Klassen, 2013; Lee et al., 2012; Morgan et al., 1998). The initiating cell is not itself a cancer cell and may grow normally. It is future daughter cells that have the potential to develop into cancerous cells (Klassen, 2013; Lee et al., 2012; Morgan et al., 1998). After the initiation stage promotion of the altered cell(s) occurs in which selective clonal expansion produces a pre-tumorous growth (Klassen, 2013; Lee et al., 2012; Morgan et al., 1998). Clonal expansion involves selection of cells that are best suited to resist or evade the immune response and pass on heritable changes gained from the initiation stage (Klassen, 2013; Lee et al., 2012; Morgan et al., 1998). The final stage in carcinogenesis, progression, involves

the transition of a benign lesion into a malignant growth (Klassen, 2013; Lee et al., 2012; Morgan et al., 1998). At this point a greater number of chromosomal aberrations may arise as a result of rapid DNA synthesis (Klassen, 2013; Lee et al., 2012; Morgan et al., 1998).

Chromosome instability (CIN), an early event in carcinogenesis and tumor development (Morgan et al., 1998) is characterized by either chromosome structural or numerical aberrations as a result of miss-repair (Morgan et al., 1998; Moynahan et al., 2010). Double strand breaks (DSB) are one of the most lethal chromosomal lesions that can result from exposure to particulate metals (Xie et al., 2007). DSBs are so severe that a single break persisting can result in cell death, while repair may result in CIN (Sonoda et al., 2006). Damage from DSBs can also lead to more complex aberrations such as chromatid exchanges when cells fail to properly repair (Morgan et al., 1998). Chromatid exchanges occur when the damaged chromosome attempts to use HR and finds a seemingly homologous region (Jackson et al., 2001; Kasparek and Humphrey 2011; Moynahan et al., 2010), this could result in a chromosomal translocation (Morgan et al., 1998). Under normal circumstances cells are able to repair DSBs with either of two repair pathways, homologous recombination (HR) or non-homologous end-joining (NHEJ) (figure 1A and B) (Moynahan et al., 2010). Previous studies have observed significant incidence of CIN as a result of increases in DSBs and chromatid exchanges with exposure to particulate hexavalent chromium (Stackpole et al., 2007). Other related studies investigating the carcinogenesis of chromium have also observed a significant increase in the incidence of cellular transformation (Xie et al., 2007). Recent studies have established that, like chromium, cobalt is able to induce DSBs

(Smith et al., 2014) which have the potential, if miss-repaired, to result in an increase in CIN and potentially carcinogenesis (Jackson et al., 2001; Morgan et al., 1998).

The mechanism(s) by which particulate cobalt induces carcinogenesis are not yet established. However, rodent and human studies have demonstrated that cobalt is genotoxic and results in chromosome aberrations, single and double strand breaks, and sister chromatid exchanges (Beyersmann and Hartwig 2008; Lasfargues et al., 1992; Smith et al., 2014). Despite previous findings the major repair pathway essential to protect against cobalt induced CIN has yet to be established. Investigations into established carcinogens, such as hexavalent chromium, have reported significant increases in CIN as a result of a deficient HR not NHEJ repair pathway (Camyre et al, 2007; Stackpole et al., 2007; Wise et al., 2002).

DNA Double Strand Break Repair Mechanisms Homologous Recombination and Non-homologous End Joining

Homologous recombination (HR), an error-free repair (Moynahan et al., 2010; Wyman et al., 2004), requires an intact sister chromatid, an exact copy of the region of damaged DNA, to complete repair of the DNA double-strand breaks (DSB) (Gildemeister et al., 2009). Due to the requirement of the sister chromatid for successful repair HR is only a viable option for the cell during S/G_2 (Moynahan et al., 2010; Wyman et al., 2004). HR consists of three stages: presynapsis (single strand stabilization), synapsis (sister chromatid strand invasion), and postsynapsis (Holiday junction formation and resolution) (Gildemeister et al., 2009; Liu et al., 2004). During presynapsis RPA proteins bind to the single strand of the damaged chromosome.

Replacement of RPA by RAD51 is mediated and stabilized by RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) (French et al., 2002; Lundin et al., 2003). After RAD51 has successfully replaced RPA, the RAD51 strand invades the homologous region of the sister chromatid, which leads to the formation and stabilization of the Holiday junction (Gildemeister et al., 2009). The Holiday junction requires all of the previously mentioned RAD51 paralogs involved in order to be maintained and prevent collapse (Gildemeister et al., 2009; Liu et al., 2004). After the RAD51 loaded invading strand has completed replication of the homologous region it is thought that a complex containing XRCC3 and RAD51C resolve the Holiday junction (Gildemeister et al., 2009; Liu et al., 2004).

Recent studies have further investigated the role of RAD51C in HR when CIN occurs and found that RAD51 is essential for HR (Lundin et al., 2003; Tarsounas et al., 2004). It has been shown that RAD51C transports its paralog RAD51 into the nucleus to localize at the site of the DNA DSB (Gildemeister et al., 2009). It is proposed that the absence of RAD51 at the site of DNA DSB results in the cell abandoning HR in place of non-homologous end-joining (NHEJ) (Sonoda et al., 2006; Wyman et al., 2004).

Non-homologous end-joining (NHEJ) is the primary alternative to HR and is error-prone (Moynahan et al., 2010). While HR is only accessible during S/G_2, NHEJ is available throughout the cell cycle (Moynahan et al., 2010). NHEJ involves DNA-dependent protein kinase (DNA-PK) and several ligase proteins (Lees-Miller et al., 2003; van Gent et al., 2007). In order for DNA-PK to localize to the site of the DSB the Ku80/Ku70 complex must first localize to the site of damage (Lees-Miller et al., 2003).

DNA-PK and its catalytic subunit bind to the Ku80/Ku70 complex and auto-phosphorylates in order to transducer the NHEJ signal (Jackson et al., 2002; Lees-Miller et al., 2003). The Ku80 gene is an essential sensory component required for NHEJ (Jackson et al., 2002). The process by which NHEJ repairs DNA DSBs involves removal of several base pairs around the initial break site (van Gent et al., 2007). After the ends of the damaged DNA have been processed the two 'smooth' ends are ligated together resulting in a loss of genetic material (van Gent et al., 2007). Reliance on NHEJ in instances of particulate carcinogen exposure has been linked to CIN and carcinogenesis (Kasparek and Humphrey 2011; Morgan et al., 1998).

It is unknown whether HR or NHEJ are required to protect the cell against cobalt induced CIN. Therefore, the purpose of this study is to establish whether HR or NHEJ protects against particulate cobalt induced CIN. In order to determine the importance of HR and NHEJ in protecting against cobalt induced CIN we have investigated the significance of RAD51C- and Ku80-deficiencies, respectively.

Materials and Methods

Chemicals and reagents

Dulbecco's minimal essential medium and Ham's F-12 (DMEM/F-12) purchased from Mediatech Inc. (Herndon, VA). Sodium pyruvate, penicillin/streptomycin, L-glutamine, phosphate buffered saline (PBS), and Gurr's buffer were purchased from Invitrogen Corporation (Grand Island, NY). Crystal violet, ethanol, and methanol were purchased from J.T. Baker (Phillipsburg, NJ). Cobalt (II) oxide (CoO), and demecolchicine were purchased from Sigma/Aldrich. Giesma stain was purchased from Biomedical Specialties Inc. (Santa Monica, CA). Ethanol was purchased from Pharmco-AAPER (Brookville, CT). Tissue culture dishes, flasks and plastic ware were purchased from Corning Inc. (Acton, MA).

Cell Culture

The cell lines used in this study are listed in Table 1. To study homologous recombination we focused on the RAD51C gene and used the Chinese hamster lung (CHL) fibroblast cell lines: V79, irs3 and irs3#6. The irs3 cell lines, derived from the wildtype V79 cells, are deficient in RAD51C, the irs3#6 cell line is complemented with RAD51C cDNA (Stackpole et. Al, 2007). Complementation in this instance is referring to the reintroduction of the genetic material which had originally been excluded from a cell line, in this thesis these were the RAD51C and Ku80.

To study non-homologous end-joining, we focused on the Ku80 gene and used the Chinese hamster ovary (CHO) cell lines: CHO-K1, xrs6, and 2E. The xrs6 cell

lines, derived from the wildtype CHO-K1 cells, are deficient in Ku80 and used with their complemented cell line 2E (Camyre et al., 2007).

All cells were maintained in media (DMEM/F-12 supplemented with 15% CCS, 100 u/ml penicillin/100 ug/ml streptomycin, 2 mM glutaMAX, and 0.1 mM sodium pyruvate) and incubated in 5% carbon dioxide (CO_2) at 37°C as sub-confluent monolayers. Media was replaced with fresh warm media every second day. Cells were sub-cultured every two to three days using 0.25% trypsin/1mM EDTA solution as previously described (Stackpole et. al, 2007).

Clonogenic Survival Assay / Cytotoxicity Assay

To asses altered plating efficiency a clonogenic assay was utilized which compared treated populations decreased plating efficiency relative to an untreated control as previously described (Wise et al 2002). Cells were seeded in individual wells then reseeded after treatment in 60 mm culture dishes at colony forming densities (200 cells/ml), and allowed to grow for 5-7days and then the number of colonies was counted.. Cells which survived were those which attached and grew on the surface of the dish used. Those cells that are damaged or dead fail to attach to the dish or proliferate. All experiments were conducted at least three times, with four dishes per treatment per experiment.

Chromosomal Aberration Assay / Clastogenicity Assay

Cobalt induced clastogenicity was evaluated using a chromosome aberration assay which quantifies chromosome damage in treated and untreated cell populations as previously described (Wise et al 2002). After the cells were exposed for the duration of

their treatment times, cells were arrested in metaphase using demecolchicine, fixed, dropped on slides and stained with crystal blue dye. One hundred metaphases per data point were evaluated in each experiment and each experiment was repeated at least three times. Aberrations scored due to frequency were chromatid lesions, isochromatid lesions and chromatid exchanges.

Cobalt Preparation

Cobalt oxide (CAS# 1307-96-6, $\geq$ 99.99% trace element) was used as a representative particulate cobalt compound. Suspended CoO particles were prepared as previously described (Smith et al., 2014). The particles were kept in suspension using a vortex mixer and diluted in appropriate suspensions for specific treatment concentrations. Treatment concentrations were directly dispensed into cultures from these suspensions.

Intracellular and Extracellular Cobalt Ion Measurements

To determine cobalt ion levels inductively coupled plasma optical emission spectroscopy (ICP-OES) was used as previously described (Holmes et. al, 2005). Cells were treated with varying concentrations of cobalt oxide for 0 h or 24 h exposure durations at the same density of cell used in the cytotoxicity and chromosome damage assays. After treatment cells were harvested and placed in hypotonic solution followed by 2% SDS to degrade the cellular membranes. The solution was sheered through an 18 gauge needle and filtered through a 0.2 um filter. Samples were then diluted in 2% nitric acid and passed through a 0.2 um filter. To account for potentially any present particles the 0 h cobalt ion levels were subtracted from the 24 h ion levels.

Statistics

The Students t-test was used to calculate the p-values of the raw data in order to determine statistical significant of the difference in means. Estimates of differences are based on Students t-test distribution.

Results

Cobalt Ion Uptake

Intracellular cobalt ion levels increase with dose and are not significantly affected by the absence of either the RAD51C or Ku80 genes

Intracellular cobalt ion levels were measured for all cell lines. The RAD51C-wildtype (V79) and RAD51C-complemented (irs3#6) cell lines exhibited similar intracellular cobalt ion levels, which were lower than the RAD51C-deficient (irs3) levels treated with the same concentrations (figure 2A). For example, at 2 ug/cm^2 cobalt oxide the wild type (V79) and RAD51C-complemented (irs3#6) cells had intracellular cobalt ion levels of 1000 and 938 uM, compared to the RAD51C-deficient (irs3) which had 1546 uM (figure 2A).

The Ku80-wildtype (CHO-K1) and Ku80-deficient (xrs6) had similar intracellular cobalt ion levels, while the Ku80-complemented (2E) cell line showed higher intracellular cobalt ion levels (figure 2B). For example when treated with 1 ug/cm^2 cobalt oxide the wild type (CHO-K1) and Ku80-deficient (xrs6) have intracellular cobalt ion levels of 379 and 536 uM, respectively, while the Ku80-complemented (2E) had intracellular cobalt ion levels of 728 uM (figure 2B).

Clonogenic survival

Deficiency in RAD51C did not significantly affect the clonogenic survival of Chinese hamster cells exposed to cobalt oxide

RAD51C is involved in the transport of RAD51 to the RPA loaded single strand as well as the resolve and presynapsis steps of HR (Moynahan et al., 2010; Tasounas et al., 2004). We found RAD51C deficiency did not significantly affect the cells cytotoxic sensitivity to cobalt, compared to their respective wild type cell line (figure 3). For example at 1 ug/cm^2 cobalt oxide, both the RAD51C-wildtype (V79) and RAD51C-deficient (irs3) cell lines each exhibited approximately 80% relative survival (figure 3). While the RAD51C complemented (irs3#6) cells showed a significantly greater sensitivity to cobalt (figure 3).

Ku80 deficient Chinese hamster cells were not clonogenically sensitive when exposed to cobalt oxide

Ku80 is involved in the sensing of double strand breaks and providing a support structure for DNA-PKcs at the double strand break during NHEJ (Lees-Miller et al., 2003).The Ku80 wild type (CHO-K1), Ku80 deficient (xrs6) and Ku80 complemented (2E) all had ~70% relative survival at 1 ug/cm^2 of cobalt oxide (figure 4). Correcting for intracellular cobalt levels shows the same conclusion; deficiencies in Ku80 do not significantly affect the cytotoxic effect of cobalt oxide exposure (figure 5B).

Clastogenicity

RAD51C deficient Chinese hamster cells were significantly more sensitive to the genotoxic effects of cobalt oxide

RAD51C deficient cells (irs3) were significantly more sensitive to the genotoxic effects of cobalt oxide. At low concentrations loss of RAD51C increased the percent of metaphases with damage (figure 6A). For example, when exposed to 1 ug/cm^2 cobalt

oxide the wild type (V79) cells had 12% of metaphases with damage while the RAD51C-deficient cells had 30% and the RAD51C-complemented cells exhibited 8.7% of metaphases with damage (figure 6A).

The total amount of chromosome damage in 100 metaphases was less in the wildtype (V79) than in the RAD51C-deficient (irs3), and the RAD51C-complemented (irs3#6) cells responded similarly to the wildtype cell line (figure 6B). For example, when treated with 1 ug/cm^2 cobalt oxide the wildtype (V79) exhibited 15 aberrations compared to 75 and 10 in the RAD51C-deficient (irs3) and complemented (irs3#6) cell lines, respectively (figure 3B). Correcting for intracellular cobalt ion levels did not significantly change the effect seen between the wildtype (V79), RAD51C-deficient (irs3) and complemented (irs3#6) cell lines (figure 10B).

We also considered the spectrum of damage in each cell line. Figures 7 A, B and C show the spectrum of damage with the most common aberrations in the cell lines exposed to cobalt oxide being chromatid lesions, isochromatid lesions and chromatid exchanges. The wildtype (V79) and RAD51C-complemented (irs3#6) cells had less damage than the RAD51C-deficient (irs3) cells which exhibited significantly greater amounts of damage in each of the three categories of aberrations previously mentioned (figure 7). For example at 1 ug/cm^2 cobalt oxide the RAD51C-wildtype (V79) exhibited 9 chromatid lesions (figure 7A), 4 isochromatid lesions (figure 6B) and 0 chromatid exchanges (figure 7C) compared to the RAD51C-deficient (irs3) and RAD51C-complemented (irs3#6) cell lines, which exhibited 65, 2, 7 and 9, 1 and 1 aberrations, respectively (figure 7 A, B and C).

NHEJ

Chinese hamster cells deficient in Ku80 were sensitive to the genotoxic effects of cobalt oxide

We observe the Ku80-deficient (xrs6) cell line to have a greater percent of metaphases with damage than the wildtype (CHO-K1) and complemented (2E) cell lines respectively (figure 8A). For example, at 1 ug/cm^2 cobalt oxide the wildtype (CHO-K1) exhibited 10 percent of metaphases with damage compared to the Ku80-deficient (xrs6) and Ku80-complemented (2E) cell lines which exhibited 45 and 19 percent, respectively (figure 8A). Correcting for intracellular cobalt ion levels did not significantly alter the effect seen in the Ku80-deficient cells (figure 9C). For example at an intracellular cobalt ion concentration of 1000 uM the wildtype (CHO-K1) exhibited 21 percent of metaphases with damage while the Ku80-deficient (xrs6) and Ku80-complemented (2E) cells had 27 and 18 percent of metaphases with damage, respectively (figure 10C).

The Ku80-deficient (xrs6) cell line had a greater number of aberrations, which include chromatid lesions, isochromatid lesions and chromatid exchanges, than the Ku80-wildtype (CHO-K1) and complemented (2E) cell lines, respectively (figure 8B). For example, when treated with 1 ug/cm^2 cobalt oxide the wildtype (CHO-K1) cell line exhibited 15 aberrations while the Ku80-deficient (xrs6) and Ku80-complemented (2E) exhibited 50, 25 aberrations, respectively (figure 8B). At the highest dose administered, 4 ug/cm^2, the Ku80-deficient (xrs6) cell line had no metaphases (figure 8B). Correcting for intracellular cobalt ion levels did not significantly alter the observed difference

between the wildtype (CHO-K1), Ku80-deficient (xrs6) or complemented (2E) cell lines (figure 10D).

The spectrum of damage for the Ku80-deficient cell lines also included chromatid lesions, isochromatid lesions and chromatid exchanges. The Ku80-deficient (xrs6) cell line had increased number of aberrations in the spectrum of damage compared to the wildtype (CHO-K1) and complemented (2E) cell lines (figure 9 A, B and C). For example at 1 ug/cm^2 cobalt oxide the wildtype (CHO-K1) had 12 chromatid lesions compared to the Ku80-deficient (xrs6) and complemented (2E) cell lines 38 and 18, respectively (figure 7A). Ku80-deficiency (xrs6) did not increase the number of isochromatid lesions (figure 8B). The number of chromatid exchanges was greater in Ku80-deficient (xrs6) cells compared to the Ku80-wildtype (CHO-K1) and complemented (2E) cell lines, respectively (figure 9C). For example at 1 ug/cm^2 cobalt oxide wildtype (CHO-K1) cells had 1 chromatid exchange compared to Ku80-deficient (xrs6) and Ku80-complemented which had 9 and 2 exchanges, respectively (figure 9C).

Discussion

Global expansion of cobalt mining operations has been on the rise as demand for cobalt containing products has increased as a result exposure to cobalt is also expected to increase. Due to inconclusive epidemiology studies the IARC has classified cobalt as a potential human carcinogen (IARC, 2006), when in fact animal and cell culture studies have found cobalt to be carcinogenic (Lasfargues et al., 1995; Smith et al., 2014). Previous cell culture studies have established that cobalt induces double strand breaks, one of the most lethal lesions to chromosomes, and chromatid exchanges, which could potentially lead to chromosome translocations (Kasparek et al., 2011). It has also been previously determined that HR is required to protect against DSB induced CIN (Moynahan et al., 2010; Stackpole et al., 2007). The understanding of the repair mechanisms required to protect against cobalt induced DSBs and CIN are not yet established. Therefore it was the purpose of this study to establish whether HR and or NHEJ protect against cobalt induced CIN.

Both RAD51C and Ku80 genes are involved in the repair of double strand breaks (Jackson et al., 2001; Sonoda et al., 2011). RAD51C is involved in the transport of RAD51 to the RPA loaded single strand as well as the resolve and presynapsis steps of HR (Moynahan et al., 2010; Tasounas et al., 2004). While Ku80 is involved in the sensing of double strand breaks and providing a support structure for DNA-PKcs at the double strand break (Lees-Miller et al., 2003). Our data suggest that cobalt induced damage likely occurs during replication, as indicated by the large number of chromatid exchanges. The increase in the number of double strand breaks and chromatid exchanges indicate cobalt's ability to amplify the incidence of CIN, an established

hallmark of carcinogenesis (Kasparek and Humphrey, 2011; Weinstock et al., 2006). The literature shows that failure to correctly repair and resolve breaks in DNA can result in more complex aberrations, which can in turn develop into translocations and in turn CIN (Agarwal et al., 2006; Kasparek and Humphrey, 2011; Weinstock et al., 2006).

Interestingly some species of cobalt have also been observed to act as topoisomerase II poisons (Baldwin et al., 2004) by enhancing the activity of topoisomerase II while inhibiting repair proteins (Baldwin et al., 2004; Hengstler et al., 2003; Witkiewicz-Kucharzyk and Bal, 2006). Topoisomerase II is a protein which temporarily cleaves double stranded DNA ahead of replication machinery to alleviate super-coil stress caused by the unwinding of DNA (Baldwin et al., 2004). By enhancing the activity of topoisomerase II cobalt induces more double strand breaks and by inhibiting other repair proteins allows DSBs to persist (Baldwin et al., 2004; Morgan et al., 1998). These studies have established potential mechanisms by which cobalt is able to induce and promote chromosome damage but fail to elucidate the DSB repair pathway required to protect against CIN.

These studies provide likely mechanisms behind cobalt induced chromosomal aberrations which explain the damage we observed in this study. This is the first study which has investigated the role of HR and NHEJ in protecting against cobalt induced chromosome damage. By using the RAD51C- and Ku80-deficient cell lines we have established that HR and NHEJ protect against cobalt induced chromosomal aberrations. Deficiency in either RAD51C or Ku80 saw significant increases in aberrations compared to their respective wildtype and complemented cell lines, though the

RAD51C deficient cell line saw the greatest overall increase in chromatid exchanges and DSBs. These are the first data to investigate genes involved in HR and NHEJ repair in cobalt induced chromosome aberrations. These data suggest that HR is essential in protecting the majority of cells from cobalt induced DNA DSBs and CIN. Previous studies investigating the role of HR in protecting against chromium induced CIN have observed similar increases in DSBs and chromatid exchanges in HR deficient cell lines (Stackpole et al., 2007). Interestingly earlier studies have established that NHEJ is not required to protect against chromium induced CIN (Camyre et al., 2007). This is the first study to demonstrate that homologous recombination more so than non-homologous end-joining, is required to protect the cell against cobalt induced chromosome damage.

We observed that both RAD51C- and Ku80-deficient cells were not significantly more sensitive than the wildtype or complemented cell line to the cytotoxic effects of particulate cobalt. Our data suggests that while cobalt is cytotoxic to the cell lines used, deficiencies in RAD51C and Ku80 were not significant factors in the ability of cells to attach and propagate in our assays. These cytotoxicity data are consistent with previous findings in which cobalt oxide was found to induce similar levels of increased cytotoxicity with resulting cobalt exposure (Smith et al., 2014). Interestingly previous studies found that cobalt inhibited apoptosis at greater intracellular ion concentrations when compared to nickel (Green et al., 2013). This suggests that cobalt, as we and others have shown, is cytotoxic, Green et al (2013) have shown that cells exposed to cobalt hyper-load with the ion and temporarily inhibit cell

death mechanisms, and is also able to inhibit cell death even in repair deficient cell lines, RAD51C and Ku80 respectively (Green et al., 2013; Smith et al., 2014).

Interestingly the RAD51C-complemented (irs3#6) cell line exhibited a greater sensitivity to cobalt cytotoxity than either the wildtype (V79) or RAD51C-deficient (irs3) cell lines. The RAD51C-comeplemented (irs3#6) cells were transfected with human cDNA of the RAD51C gene (French et al., 2002). The RAD51C-complemented (irs3#6) cell line was more sensitive to mytomycin C and x-ray than the wildtype cell line (French et al., 2002). The increased sensitivity observed in the RAD51C-complemented (irs3#6) cells could be due to the fact that hamster and human RAD51C proteins are only 77% identical (French et al., 2002). The disparity in protein homology between the species could account for the varied sensitivity to different toxicants.

We also observed that the RAD51C and Ku80-deficient cells were significantly more sensitive to the genotoxic effects of cobalt as compared to their respective wildtype and complemented cell lines. Interestingly previous studies have found similar increases in sensitivity to genotoxicity in RAD51C and Ku80 deficient cells when exposed to particulate hexavalent chromium (Camyre et al., 2007; Stackpole et al., 2007). Our data are also consistent with previous studies that investigated the genotoxicity of cobalt to cultures of human lung cells (Smith et al., 2014). These data suggest that RAD51C and Ku80 deficiencies increase the sensitivity to cobalt induced damage which manifested primarily as double strand breaks and chromatid exchanges. Interestingly it was the RAD51C deficient cells which showed the greatest number and diversity of damage, with a significant increase in chromatid exchanges compared to all

other cell lines used. Taken together these data suggest that HR is more protective of cells against cobalt induced chromosome aberrations.

Interestingly previous studies have observed increased incidences of translocations and CIN as a result of miss-repaired double strand breaks and chromatid exchanges (Agarwal et al., 2006; Morgan et al., 1998; Weinstock et al., 2006). It is well established that in situations with a deficient DNA repair double strand breaks and chromatid exchanges can result in translocations (Agarwal et al., 2006; Weinstock et al., 2006). These translocations are categorized as structural chromosomal instability (CIN) a hallmark of carcinogenesis (Kasparek and Humphrey, 2011; Morgan et al., 1998). The data presented in the present study have shown that when HR is deficient cobalt oxide increases the incidence of chromatid exchanges. These data suggest that cobalt induces CIN and in the absence of HR is carcinogenic.

An interesting difference was observed in the sensitivity to genotoxic effect of cobalt between the RAD51C- (irs3) and Ku80-deficient (xrs6) cell lines when treated with cobalt oxide. Interestingly at 4 ug/cm2 cobalt oxide the Ku80-deficient (xrs6) cell line was more sensitive as these cells had too few metaphases as a result of possible increased cell death and inhibited metaphases, present to assess the damage, while the RAD51C-deficient (irs3) still had sufficient metaphases to analyze. Also of interest was the difference in the amount of aberrations between the RAD51C- and Ku80-deficient cell lines, with the RAD51C-deficient (irs3) cells exhibiting a significantly greater amount of chromosome aberrations. It could be that the HR deficient cells were able to rely on NHEJ to prevent even severely damaged cells from undergoing apoptosis, while the NHEJ deficient cells which were severely damaged underwent apoptosis. When a

normal cell experiences chromosome damage cells rely on HR to repair the damage, the preferred repair pathway, when HR is not available the cell will resort to NHEJ but if NHEJ fails the cell enters apoptosis (Camyre et al., 2007; Green et al., 2013; Sonoda et al., 2006). These data suggest interesting conclusions: (1) a damage threshold exists for cobalt induced apoptosis in the absence of NHEJ resulting in fewer damaged cells surviving with less total damage incurred, (2) HR-deficiency increases the reliance on NHEJ to repair damaged chromosomes, and (3) repair of severely damage cells by NHEJ results in a greater number of cells avoiding apoptosis resulting in a greater incidence of CIN.

In summary we have shown for the first time the relationship that HR and NHEJ genes, RAD51C and Ku80, play in protecting against cobalt induced CIN. We demonstrated that HR is more necessary to protect cells against cobalt induced chromosome damage and CIN. Thus, implicating HR as the primary repair pathway to resolve cobalt induced DSBs, chromatid exchanges and in protecting against cobalt induced CIN.

Future directions

There are many potential directions for future studies directed at cobalt and double-strand break repair mechanisms associated with cobalt induced cyto- and genotoxicity. These studies could involve investigations into the apoptotic and necrotic cell death pathways in repair deficient cells exposed to cobalt. Fluorescent microscopy could be used to identify protein localization after cobalt exposure. Pathways to be analyzed would include essential and known pathways and proteins affected by cobalt such DNA replication scission protein topoisomerase II, zinc finger proteins, essential to DNA repair and other DNA repair related proteins. Future clonogenic studies meant to investigate inherited, epigenetic, effects of cobalt exposure and co-exposure, with such metals as chromium and nickel, could be conducted to better expand the understanding of those affected mechanisms. Some such epigenetic pathways to investigate would be the mitotic spindle assembly pathway and its related proteins, as well as apoptosis and caspase proteins and cobalts impact on their function and expression. As with all studies the potential number of candidates to investigate far surpasses the abilities of a single individual and more time and research are required to understand the mechanisms of cobalt in inducing and perpetuating carcinogenesis.

Tables and Figures

Table 1.

Cell phenotype	RAD51C (HR)	Ku80 (NHEJ)
Wildtype	V79	CHO-K1
Gene deficient	irs3	xrs6
Gene restored	irs3#6	2E

Table 1: Chinese hamster cells used to study homologous and non-homologous end-joining repair.

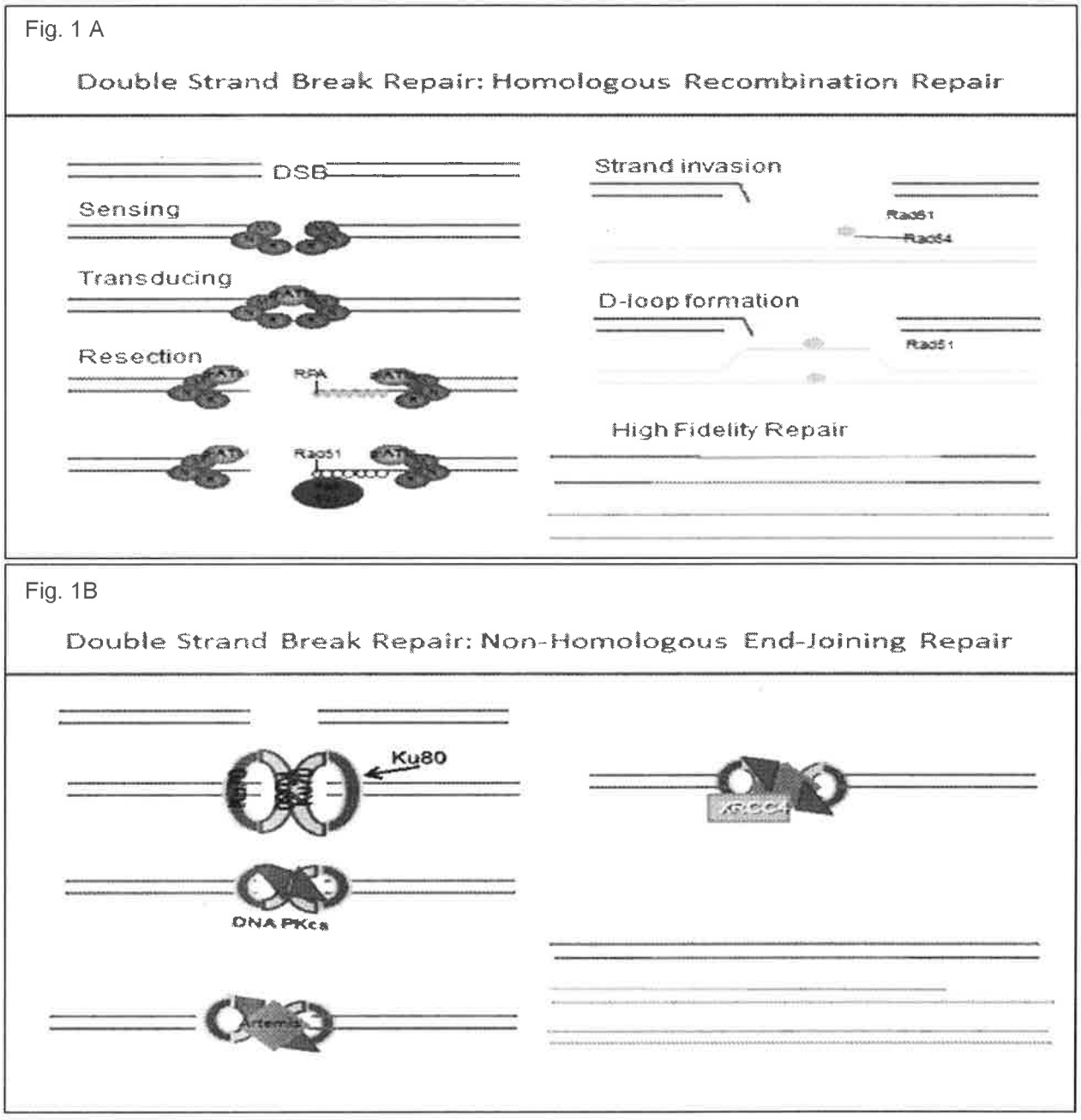


Figure 1. Homologous recombinant (A) and non-homologous end-joining (B) major repair pathways for DNA double strand break repair. (A) Homologous recombination requires the Rad51C protein to maintain the Holiday junction, D-loop structure of the strand invasion repair pathway. (B) Non homologous end-joining requires functional Ku80 proteins to provide a structural platform for DNAPKcs and additional repair proteins.

Fig. 2A

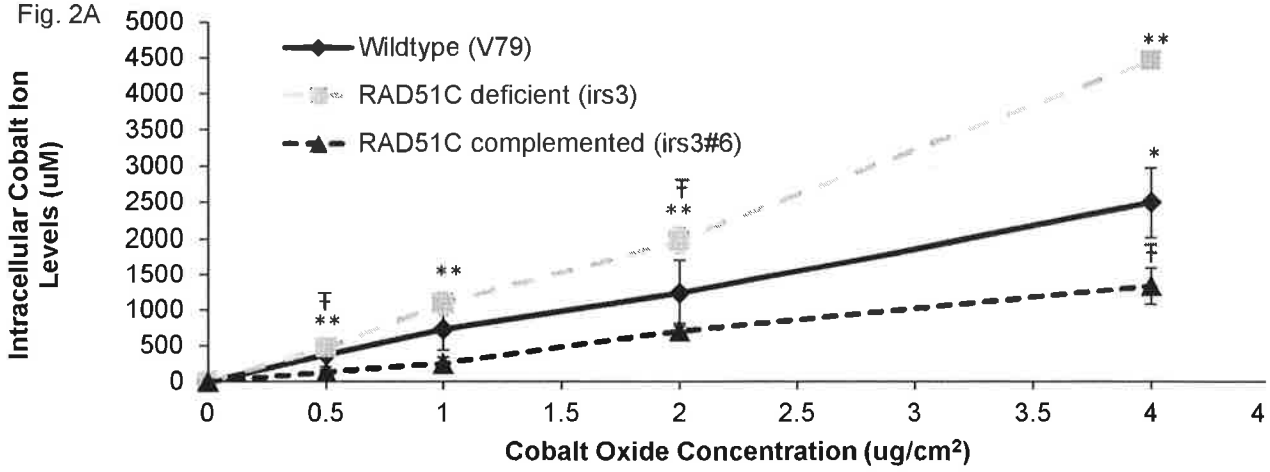


Fig. 2B

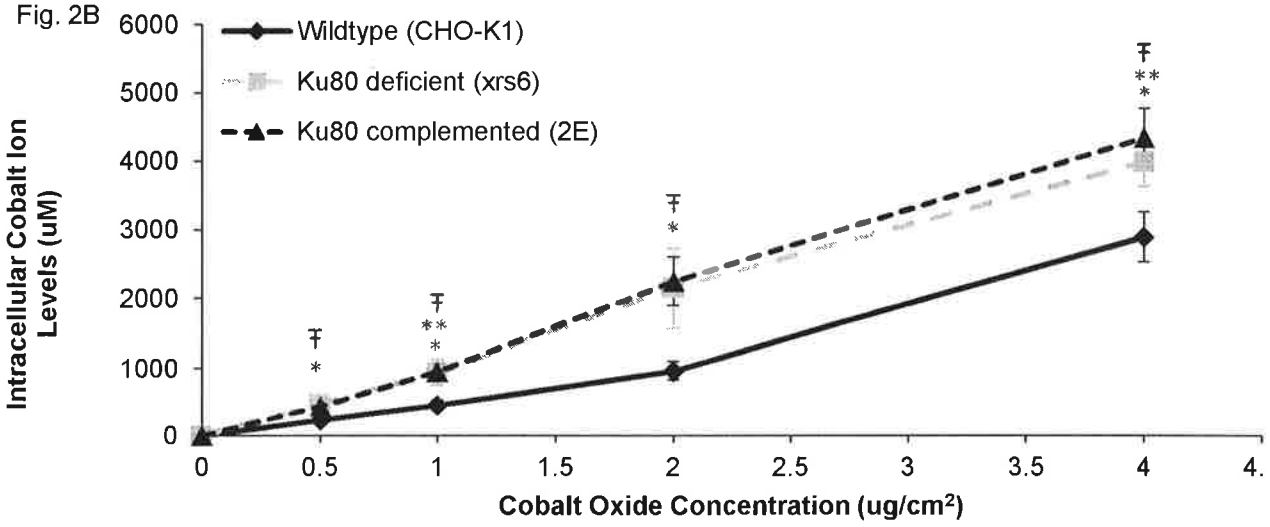


Figure 2. Intracellular cobalt ion concentration in wild-type, DNA-repair deficient and hcDNA-complemented Chinese hamster cell lines. This figure shows that exposure to particulate cobalt for 24 h increases the intracellular cobalt ion levels in a concentration dependent manner. (A) This figure shows that intracellular cobalt ion levels in RAD51C-deficient cells is greater than in the wildtype and complemented cell lines. (B) This figure shows that intracellular cobalt ion levels are higher in the hcDNA-complemented cell line than in the wildtype or Ku80-deficient cell lines. Data represent an average of three independent experiments ± standard error of the mean (P-values less than or equal to 0.05 are denoted as "*, **, or Ŧ" for the wildtype, deficient and complemented cell lines respectively).

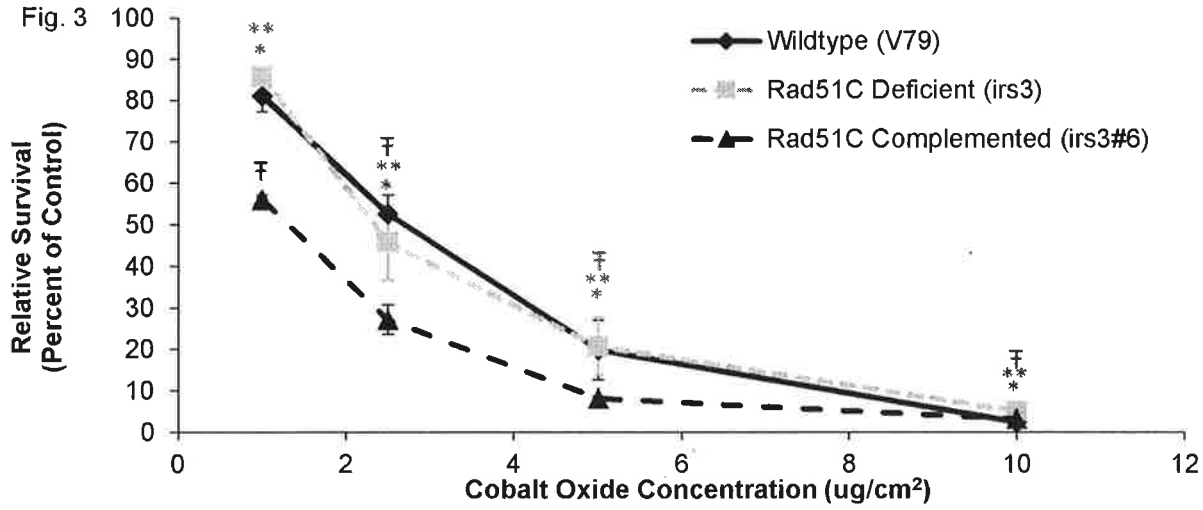


Figure 3. Particulate cobalt oxide cytotoxicity in wild-type, HR-repair deficient and hcDNA-complemented Chinese hamster cell lines. Controls for each cell line were from untreated cultures seeded and cultured for the same duration of time as exposed cultures. This figure shows that exposure to particulate cobalt for 24 h induces a concentration dependent decrease in relative survival in all cell lines tested. These data show that RAD51C-deficiency does not increase the sensitivity to particulate cobalt-induced cytotoxicity; Data represent an average of three independent experiments ± standard error of the mean (P-values less than or equal to 0.05 are denoted as "*, **, or Ŧ" for the wildtype, deficient and complemented cell lines respectively).

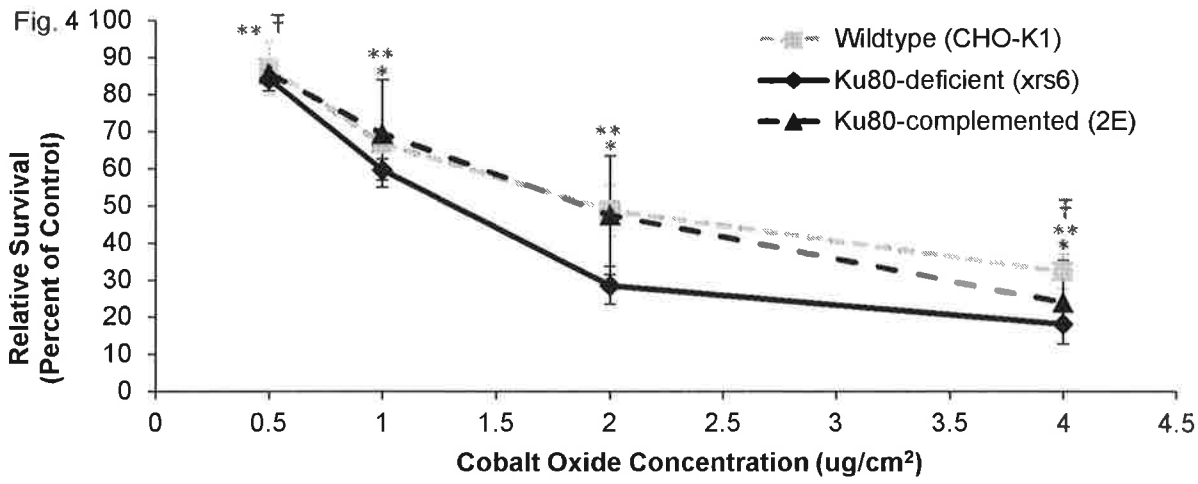


Figure 4. Particulate cobalt oxide cytotoxicity in wild-type, NHEJ-repair deficient and hcDNA-complemented Chinese hamster cell lines. Controls for each cell line were from untreated cultures seeded and cultured for the same duration of time as exposed cultures. This figure shows that exposure to particulate cobalt for 24 h induces a concentration dependent decrease in relative survival in all cell lines tested. These data show that Ku80-deficiency does not increase the sensitivity to particulate cobalt-induced cytotoxicity; Data represent an average of three independent experiments ± standard error of the mean (P-values less than or equal to 0.05 are denoted as "*, **, or Ŧ" for the wildtype, deficient and complemented cell lines respectively).

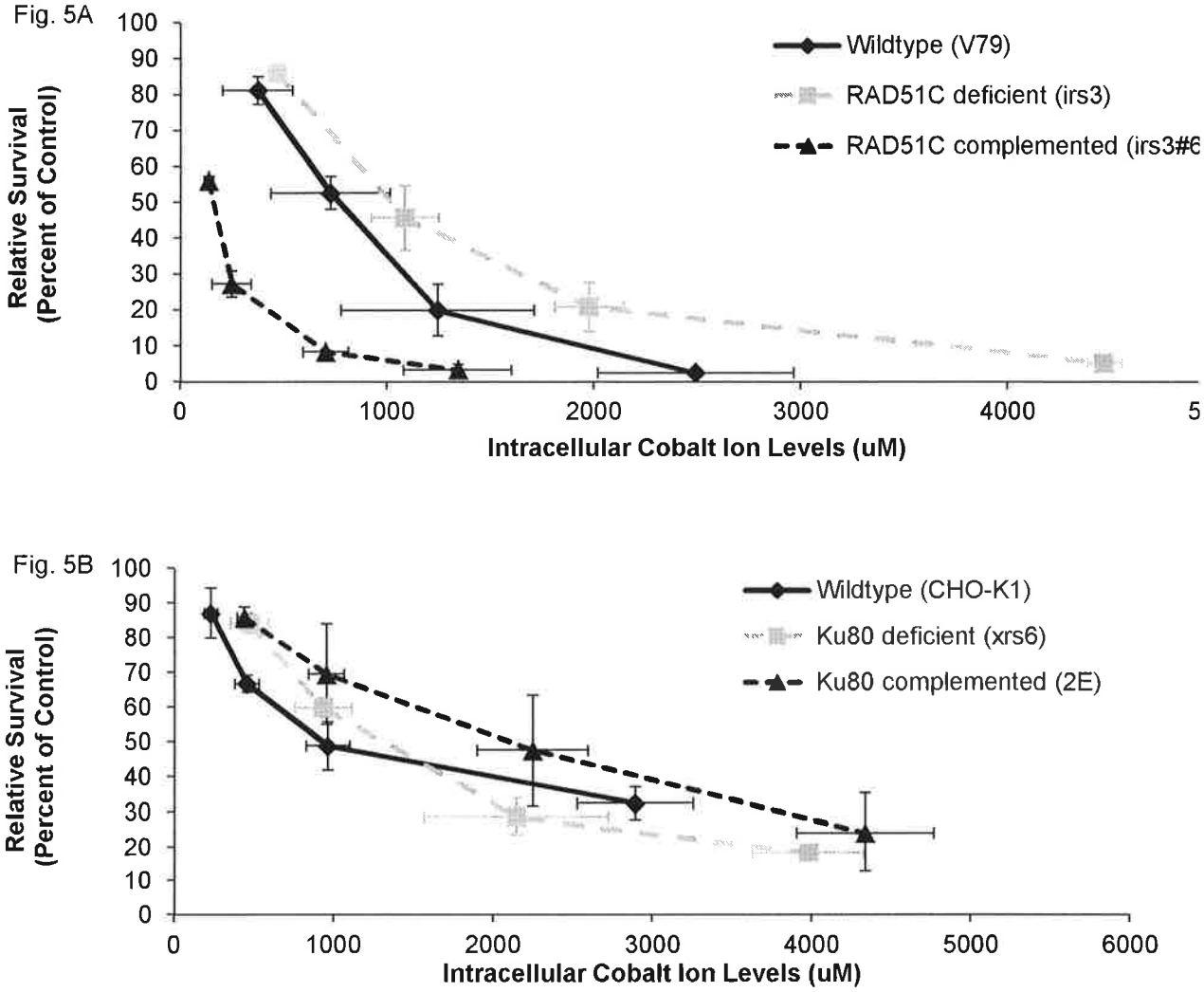


Figure 5. Particulate cobalt oxide induces similar cytotoxicity in wild-type, DNA-repair deficient and hcDNA-complemented Chinese hamster cell lines. Controls for each cell line were from untreated cultures seeded and cultured for the same duration of time as exposed cultures. (A) This figure shows that at similar intracellular cobalt ions levels RAD51C-deficient cells have a similar cytotoxic response to the wildtype and complemented cell lines. (B) These data show that at similar intracellular cobalt ion levels Ku-80 deficient cells experience lower relative survival when compared to the wildtype and complemented cell lines. Data represent an average of three independent experiments $\pm$ standard error of the mean.

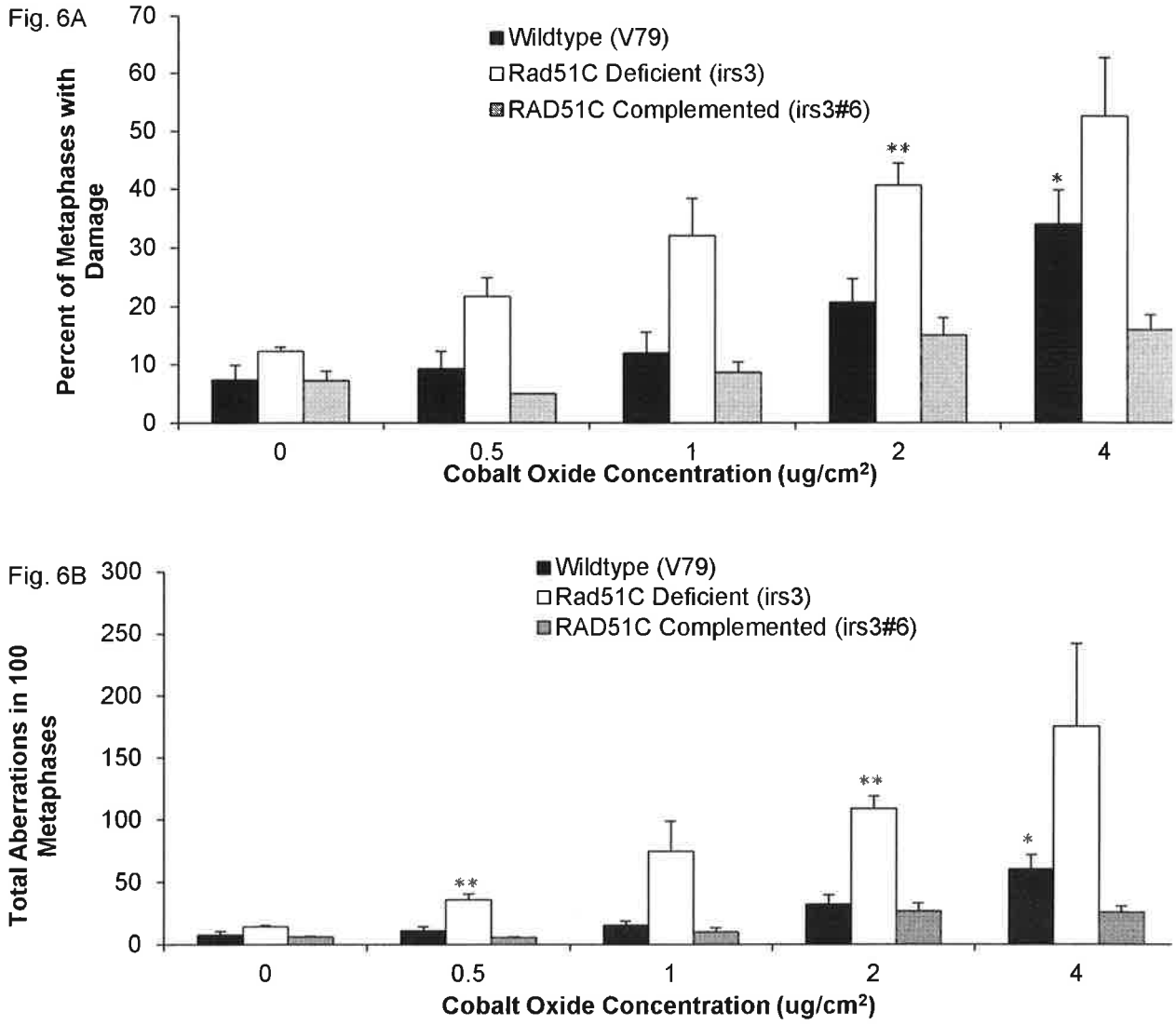


Figure 6. Particulate cobalt oxide induces concentration dependent genotoxicity in wild-type, HR-repair deficient and hcDNA-complemented Chinese hamster cell lines. This figure shows that 24 h exposure to particulate cobalt induces a concentration dependent increase in percent of metaphases with chromosome damage and total aberrations in 100 metaphases. (A) These data show that RAD51C-deficient cells experience a significantly greater percent of metaphases with damage, and (B) also that RAD51C-deficient cells have greater total aberrations in 100 metaphases compared to the wildtype and complemented cell line. Data represent an average of three independent experiments ± standard error of the mean (P-values less than or equal to 0.05 are denoted as "*, **, or Ŧ" for the wildtype, deficient and complemented cell lines respectively).

Fig. 7A

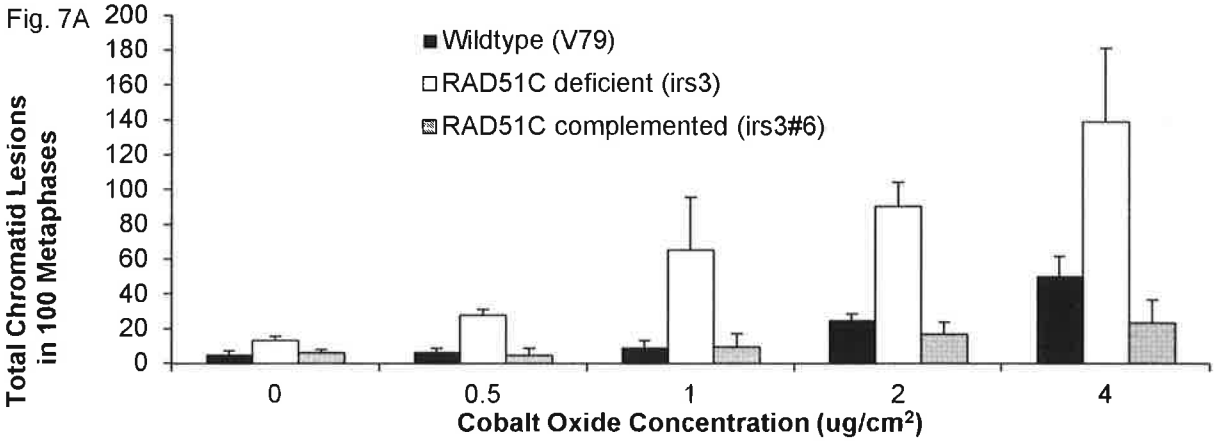


Fig. 7B

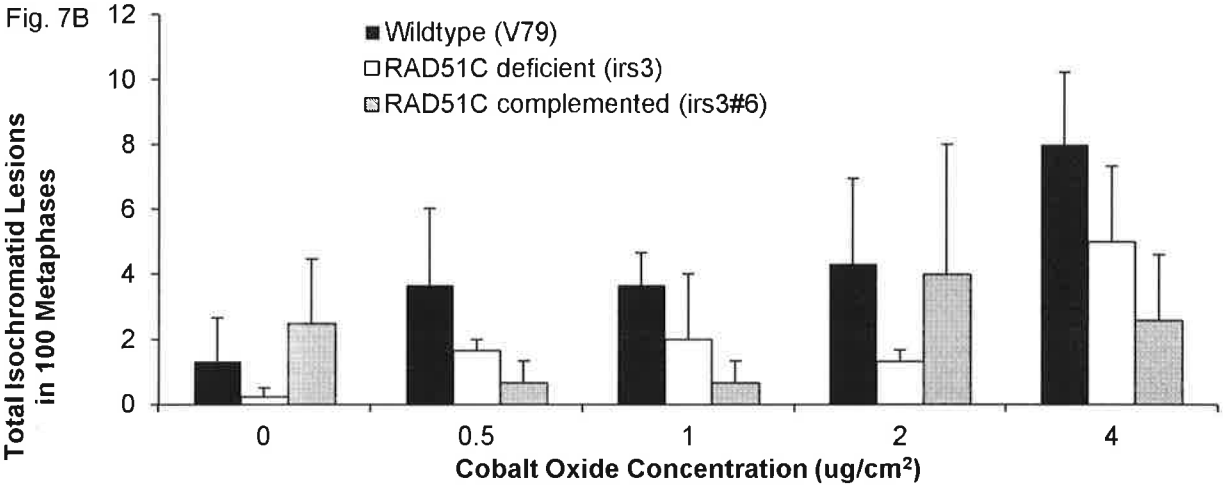


Fig. 7C

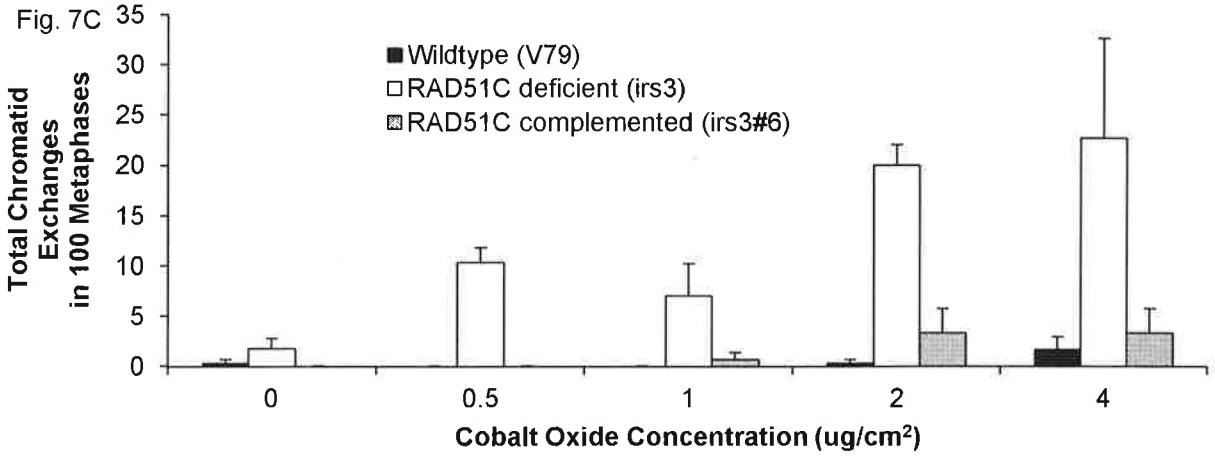
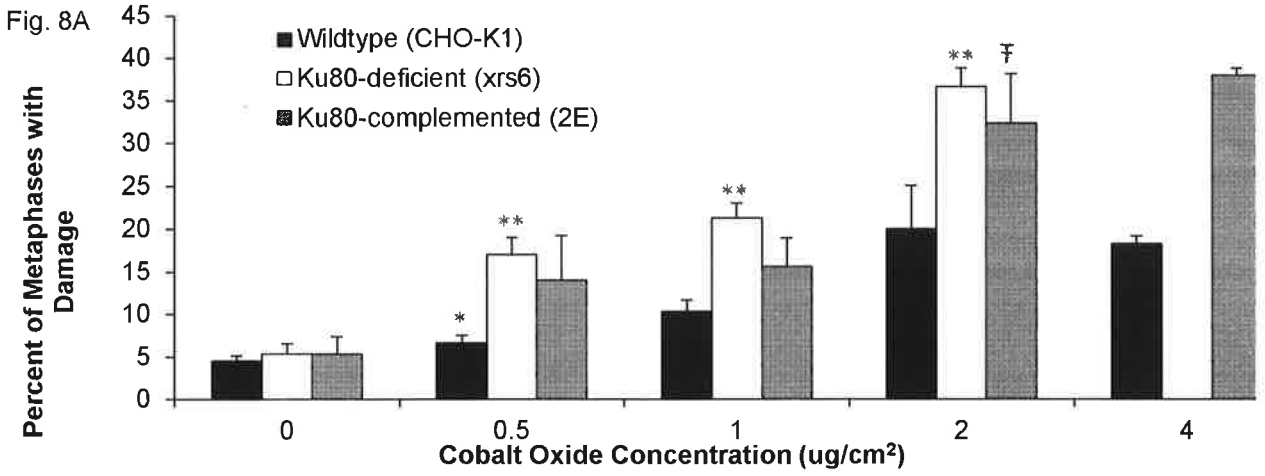


Figure 7. Spectrum of cobalt oxide induced chromosome damage in V79, irs3, irs3#6. Cobalt oxide induces a greater amount of, and more diverse, spectrum of damage in RAD51C-deficient cells after 24 h treatment. Data show total aberrations in 100 metaphases. Chromatid lesions were the most frequently occurring aberrations (A) and an increase in chromatid exchanges (C) was observed in the RAD51C-deficient cell line. Data represent an average of three independent experiments $\pm$ standard error of the mean.



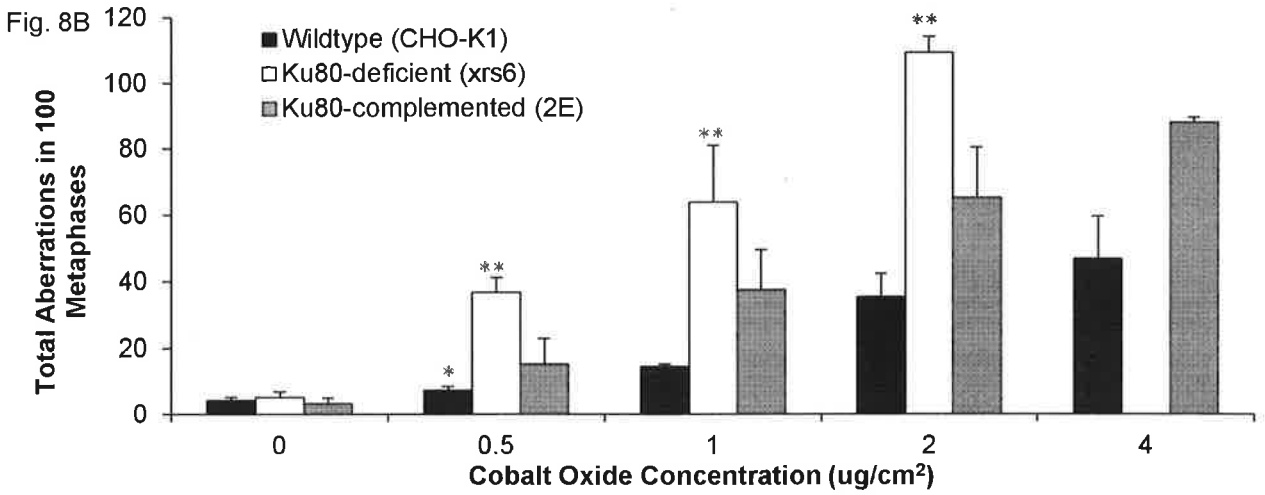
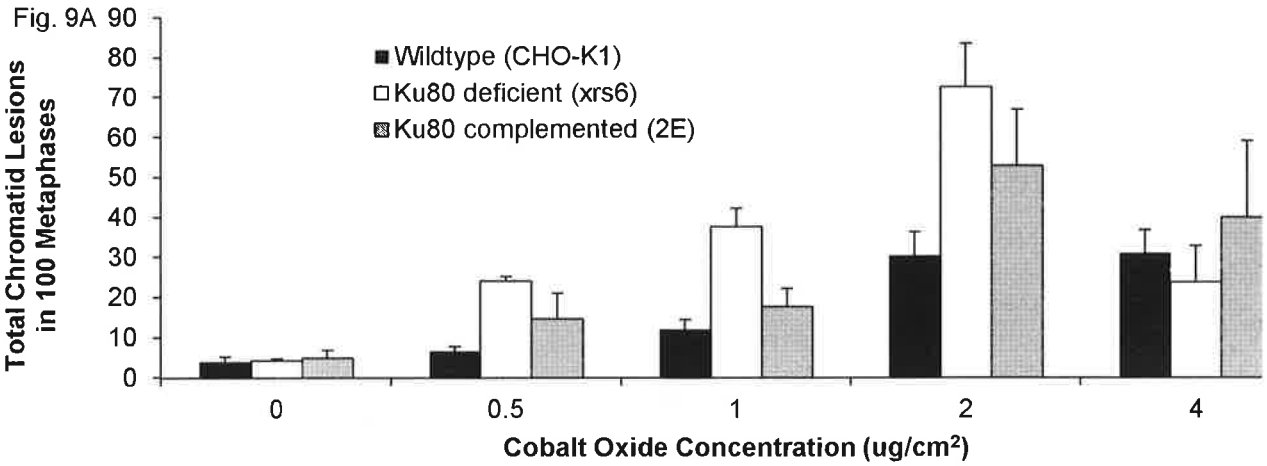
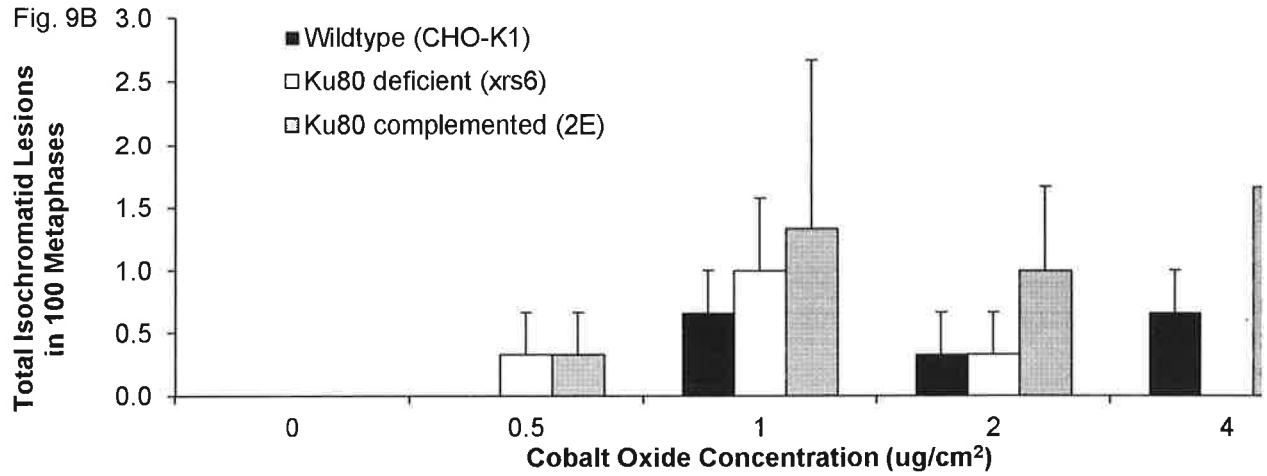


Figure 8. Particulate cobalt oxide induces concentration dependent genotoxicity in wild-type, NHEJ-repair deficient and hcDNA-complemented Chinese hamster cell lines. This figure shows that 24 h exposure to particulate cobalt induces a concentration dependent increase in percent of metaphases with chromosome damage and total aberrations in 100 metaphases. (A) These data show that Ku80-deficient cells experience significantly greater percent of metaphases with damage, as well as (B) significantly greater total aberrations, with no metaphases present at 4 ug/cm^2. Data represent an average of three independent experiments $\pm$ standard error of the mean (P-values less than or equal to 0.05 are denoted as "*, **, or Ŧ" for the wildtype, deficient and complemented cell lines respectively).





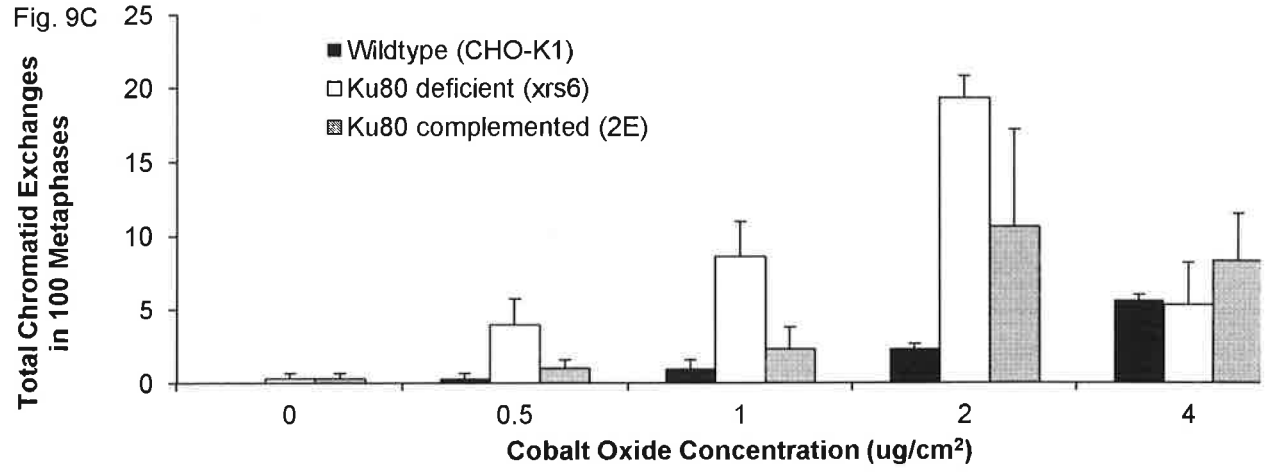


Figure 9. Spectrum of cobalt oxide induced chromosome damage in CHO-K1, xrs6 and 2E cells. Cobalt oxide induces a greater amount of and more diverse spectrum of damage in Ku80-deficient cells after 24

h treatment. Data show total aberrations in 100 metaphases. Chromatid lesions (A) were the most frequently occurring aberrations and an increase in chromatid exchanges (C) was observed in the Ku80-deficient cell line. Data represent an average of three independent experiments $\pm$ standard error of the mean.

Fig. 10A

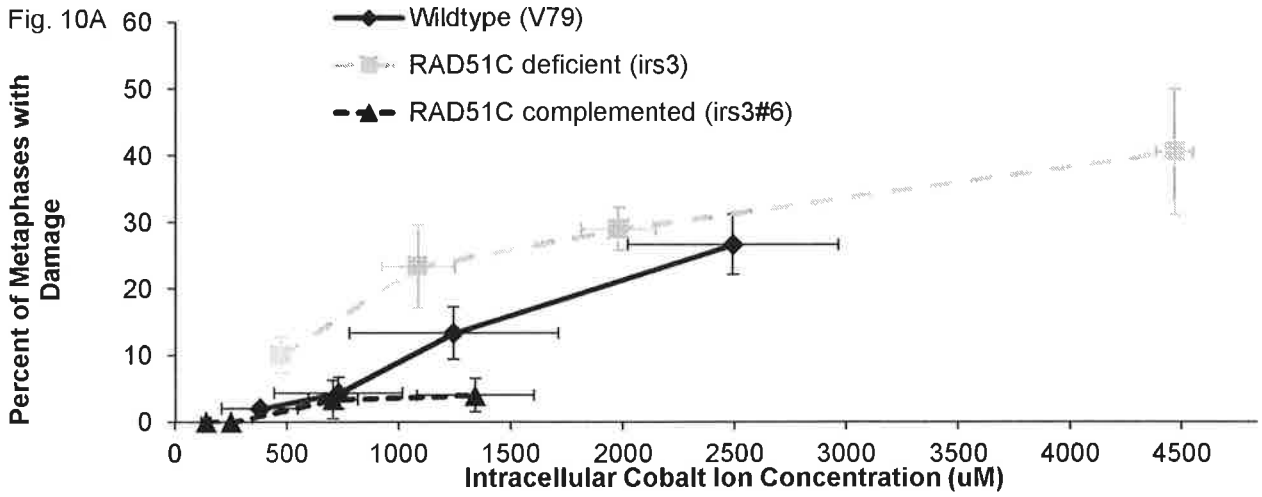


Fig. 10B

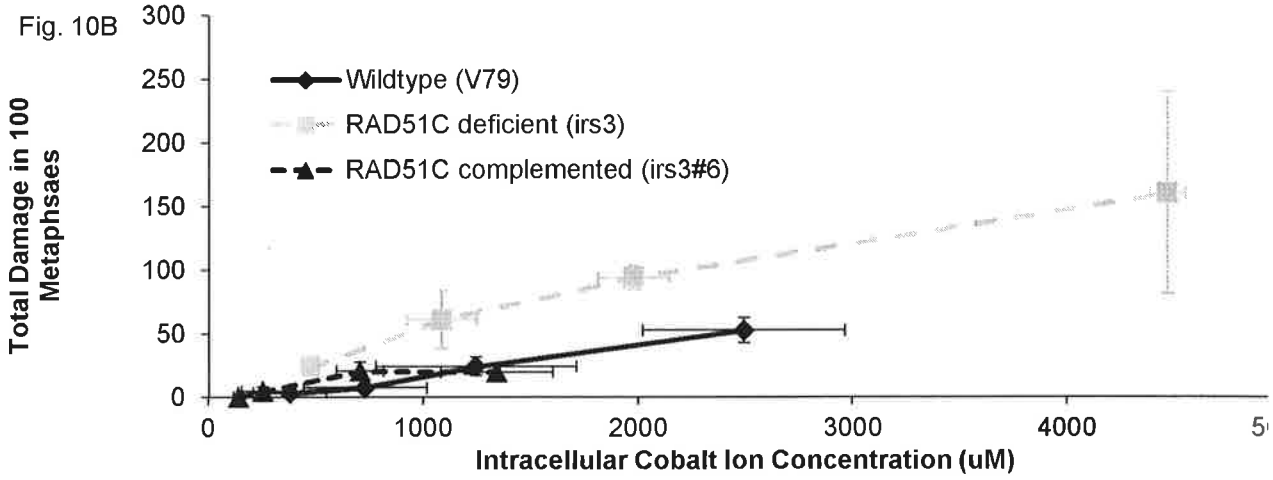
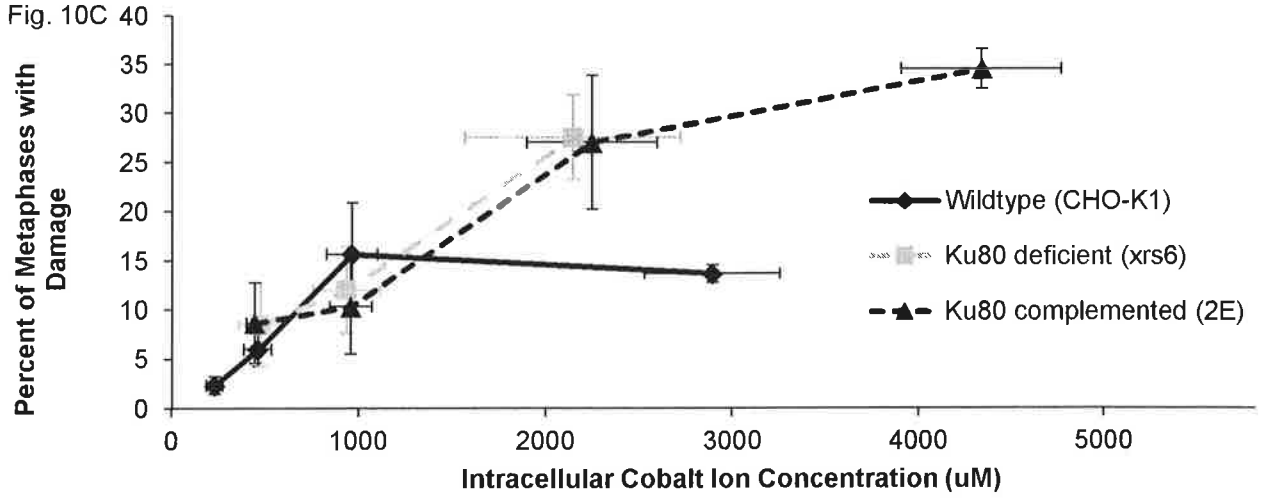


Fig. 10C



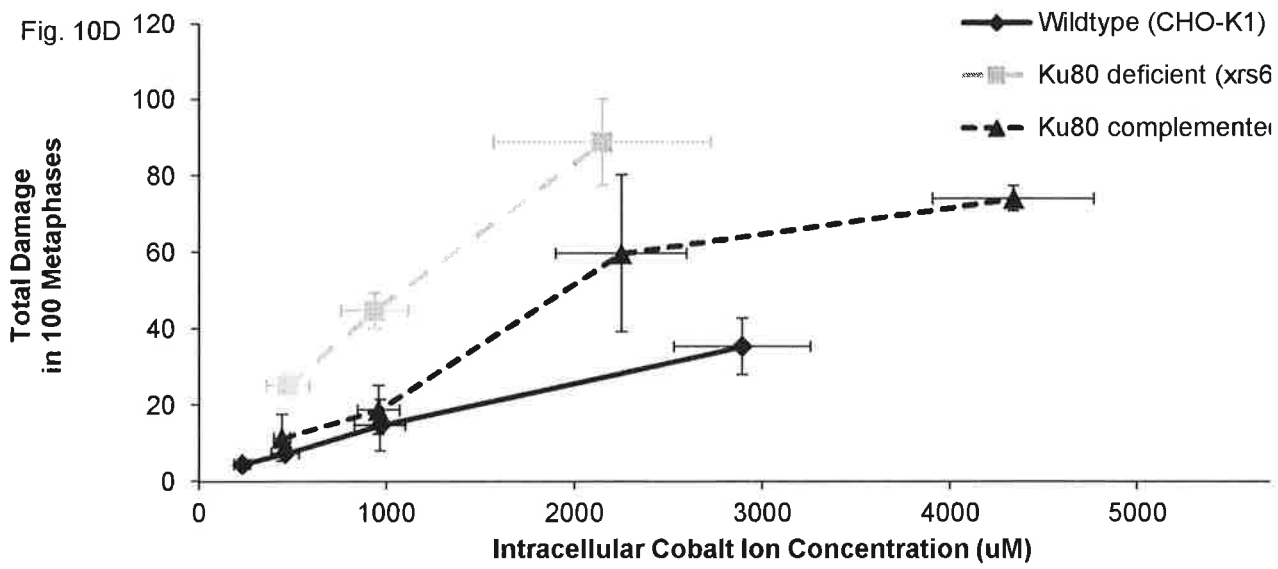


Figure 10. Exposure to cobalt oxide increases the percent of metaphases and total aberrations in wild-type, DNA DSB-repair deficient and hcDNA-complemented Chinese hamster cell lines. (A) At similar intracellular cobalt ion levels RAD51C-deficient cells experienced greater percent of metaphases with damage, as well as (B) total damage in 100 metaphases compared to their wildtype and complemented cell lines. (C) Ku80-deficient cells experienced greater percent of metaphases with damage as well as significantly greater (D) total aberrations in 100 metaphases at similar intracellular cobalt ion levels, when compared to their wildtype and complemented cell lines. Data represent an average of three independent experiments $\pm$ standard error of the mean.

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