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New investigations into the genotoxicity of cobalt compounds and their impact on overall assessment of genotoxic risk

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ABSTRACT

The genotoxicity of cobalt metal and cobalt compounds has been widely studied. Several publications show induction of chromosomal aberrations, micronuclei or DNA damage in mammalian cells *in vitro* in the absence of S9. Mixed results were seen in gene mutation studies in bacteria and mammalian cells *in vitro*, and in chromosomal aberration or micronucleus assays *in vivo*. To resolve these inconsistencies, new studies were performed with soluble and poorly soluble cobalt compounds according to OECD-recommended protocols. Induction of chromosomal damage was confirmed *in vitro*, but data suggest this may be due to oxidative stress. No biologically significant mutagenic responses were obtained in bacteria, $Tk^{+/-}$ or *Hprt* mutation tests. Negative results were also obtained for chromosomal aberrations (in bone marrow and spermatogonia) and micronuclei at maximum tolerated doses *in vivo*. Poorly soluble cobalt compounds do not appear to be genotoxic. Soluble compounds do induce some DNA and chromosomal damage *in vitro*, probably due to reactive oxygen. The absence of chromosome damage in robust GLP studies *in vivo* suggests that effective protective processes are sufficient to prevent oxidative DNA damage in whole mammals. Overall, there is no evidence of genetic toxicity with relevance for humans of cobalt substances and cobalt metal.

Abbreviations: 2-AA, 2-aminoanthracene; 2NF, 2-nitrofluorene; 4-NOPD, nitro-*o*-phenylenediamine; 6 TG, 6-thioguanine; 8-OH-dG, 8-hydroxy-2-deoxyguanosine; 9AA, 9-aminoacridine; AAF, artificial alveolar fluid; B[a]P, benzo(a)pyrene; CA, chromosomal aberrations; CE, cloning efficiency; $CoSO_4$, cobalt sulphate heptahydrate; CoOct, cobalt octoate (more correctly known as cobalt bis(2-ethylhexanoate)); CPA, cyclophosphamide; DCF, dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco's minimal essential medium; DMH, 1,2-dimethylhydrazine monohydrate; EDX, energy dispersive X-ray spectroscopy; EMS, ethyl methanesulfonate; GEF, global evaluation factor; GLP, Good Laboratory Practice; *gpt*, glutamic-pyruvate transaminase gene; HBSS, Hank's balanced salts solution; hOGG1, human 8-hydroxyguanine DNA-glycosylase 1; *Hprt*, hypoxanthine-guanine phosphoribosyl transferase gene; ICP-MS, inductively coupled plasma mass spectrometry; IWGT, International Workshops on Genotoxicity Testing; LMF, log mutant frequency; MC, methyl cellulose; MF, mutant frequency; MI, mitotic index; MMC, mitomycin C; MMS, methyl methanesulfonate; MN, micronucleus or micronuclei; MTD, maximum tolerated dose; NA, nuclear anomalies; NADP, nicotinamide adenine dinucleotide phosphate; NCE, normochromatic erythrocytes; NQO, 4-nitroquinoline-1-oxide; NTP, National Toxicology Program; OECD, Organisation for Economic Co-operation and Development; PCE, polychromatic erythrocytes; PE, plating efficiency; PHA, phytohaemagglutinin; PSD, particle size distribution; ROS, reactive oxygen species; RPMI 5, RPMI 10 and RPMI 20, RPMI medium containing 5%, 10% and 20% heat-inactivated horse serum, respectively; RS, relative survival; RTG, relative total growth; S9, 9000 *g* supernatant from livers of rats induced either with Aroclor-1254 or phenobarbital and β-naphthoflavone; SD, standard deviation; SEM, scanning electron microscopy; SHE, Syrian hamster embryo; SIN-1, 3-morpholinosydnonimine hydrochloride; TI, tail intensity; TFT, trifluorothymidine; *Tk*, thymidine kinase gene.

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1. Introduction

Cobalt metal has many important uses in industry. The most important use of cobalt metal is as an alloying element in superalloys, magnetic and hard-metal alloys, such as stellite and cemented carbides, cobalt-containing high-strength steels, electrodeposited alloys and alloys with special properties. Cobalt salts and oxides are used as pigments in the glass and ceramics industries, as catalysts in the oil and chemical industries, as paint and printing ink driers and as trace metal additives for agricultural and medical uses. Cobalt, in the form of cobalamin, is also an essential trace element in human nutrition, as cobalt is found as the active centre in cobalamin coenzymes. Given the widespread use of and exposure to cobalt and cobalt compounds, its safety for humans is of prime importance. The potential health hazards resulting from exposure to cobalt compounds have recently been reviewed (Paustenbach et al., 2013), but mainly focussed on the chemistry, pharmacokinetics and systemic toxicity of cobalt. In this paper we review the existing published genotoxicity data for cobalt substances and present diverse new experimental data from studies with a variety of different cobalt substances that should be considered in the overall assessment of cobalt and its genotoxic potential.

2. Review of published data

The genotoxic potential of soluble cobalt compounds and cobalt metal itself has been widely studied and reported.

2.1. Studies with bacteria

In terms of mutagenic activity in bacteria, Zeiger et al. (1992) reported that **cobalt sulphate heptahydrate** was a weak mutagen in *Salmonella typhimurium* strain TA100 when using the pre-incubation method, and Pagano and Zeiger (1992) reported a stronger mutagenic response for **cobalt chloride** in TA97, again using the pre-incubation method with different aqueous solvents. **Cobalt chloride** was also reported to be mutagenic in the absence of S9 in strains TA98 and TA1537 using the plate incorporation method (Wong, 1988). However, the range of concentrations used was very toxic, no data from individual treatments were presented, and the control revertant numbers for strains TA1535 and TA1537 were unusually high. For these reasons these findings are less convincing. **Cobalt metal** itself was tested in *S. typhimurium* strains TA98 and TA100, and in *E. coli* WP2*uvrA*/pKM101 as part of the National Toxicology Program (NTP, 2013). No mutagenic responses were observed in *E. coli*, either in the absence or presence of S9 using the pre-incubation method. An equivocal response was observed in TA100 in the absence of S9, but mutagenic responses that were weak and not well correlated with dose level were observed in strain TA98 in the absence of S9. In a meeting abstract, Turoczi et al. (1987) reported very weak to weak mutagenic effects for **cobalt acetate** in 3 out of 5 *Salmonella* strains at a high dose (10 mg/plate), but no details were given.

2.2. Studies in mammalian cells in vitro

Mixed results have been reported for mutagenic activity of **cobalt chloride** in mammalian cells. Amacher and Paillet (1980) found no induction of *Tk* mutations in mouse lymphoma cells following 3-hrs treatment in the absence of metabolic activation. However, there was no indication what levels of toxicity (if any) these treatments induced, and the assay was not optimised to detect small colony mutants. By contrast, Hartwig et al. (1990, 1991) and Miyaki et al. (1979) reported that **cobalt chloride** induced *Hprt* mutations in V79 cells after longer (20 or 24 h) treatment in the absence of S9. Kitahara et al. (1996) reported that mutations were induced in Chinese hamster ovary cells, transfected with a bacterial *gpt* gene, by **cobalt chloride** and, to a lesser extent, by **cobalt sulfide**.

There are surprisingly few published studies on induction of chromosomal aberrations (CA) in mammalian cells with cobalt compounds. Olivero et al. (1995) found a weak, but insignificant induction of CA in human lymphocytes treated for 48 h in the absence of S9 with **cobalt chloride**, but not with **cobalt sulphate** or **nitrate**. Paton and Allison (1972) also reported no induction of CA by **cobalt nitrate** in two human cell lines, WI38 and MRC5, or in human lymphocytes when treated in the absence of metabolic activation for periods ranging from 2 to 24 h in the cell lines, and for 48 h in lymphocytes. However, the methods are far from standard and the negative result must be viewed with caution. Figgitt et al. (2010) used multicolour fluorescence *in situ* hybridisation (M-FISH) to show that divalent cobalt (**cobalt chloride hexahydrate**) was a weak inducer of aneuploidy, but a very weak inducer of chromosomal breaks in cultured human fibroblasts.

There are, in contrast, many more publications on induction of micronuclei (MN) in mammalian cells, most of which reported positive results for ultrafine cobalt metal and soluble cobalt salts. Daley et al. (2004) investigated MN induction in human lymphocytes by wear debris from hip or knee joints. The test material was therefore not a pure chemical (titanium, vanadium, aluminium, chromium, nickel and molybdenum were present) and therefore the reported increases in MN frequency cannot be exclusively attributed to cobalt. Van Goethem et al. (1997) reported significant induction of MN in human lymphocytes treated for short periods (15 min) with low concentrations of **ultrafine cobalt metal** in the absence of metabolic activation. These results were confirmed by De Boeck et al. (2003a) and Miller et al. (2001). As with the CA study described above Olivero et al. (1995) reported weak induction of MN in human lymphocytes treated with **cobalt chloride**, but not with **cobalt sulphate** or **nitrate**. However, the MN response with cobalt chloride was flat (a plateau) whilst toxicity (mitotic inhibition) increased with concentration, making it difficult to evaluate the results. On the other hand, Ponti et al. (2009) did not detect induction of MN in Balb/3T3 cells by concentrations of **cobalt chloride** up to 10 μM, while Gibson et al. (1997) reported dose-related induction of MN in Syrian hamster embryo (SHE) cells treated with **cobalt sulphate** for 24 h in the absence of metabolic activation.

There are several publications describing induction of direct DNA damage in mammalian cells, mainly using the comet assay as detection method. Caicedo et al. (2008), De Boeck et al. (1998), Hartwig et al. (1990, 1991) and Ponti et al. (2009) all reported induction of DNA damage by **cobalt chloride** in human Jurkat cells by the neutral comet assay, in isolated human lymphocytes using the alkaline comet assay, in HeLa cells by nucleoid sedimentation in sucrose density gradients, and in Balb/3T3 cells by the comet assay, respectively. De Boeck et al. (1998) and Van Goethem et al. (1997) also reported induction of direct DNA damage with low concentrations of **ultrafine cobalt metal** in human leukocytes and blood lymphocytes from three different donors using the alkaline version of the comet assay, although in a later paper, De Boeck et al. (2003a) were unable to reproduce the DNA damage responses. The differences did not appear to be due to particle size, cytotoxicity or oxidised bases.

A publication by Ponti et al. (2009) describes the induction of MN and DNA strand breaks (comet assay) in Balb/3T3 cells by cobalt nanoparticles. The induction of DNA damage appears to be due to oxidative stress caused by the nanoparticles according to Alarifi et al. (2013). However, nanosized cobalt is not commonly