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Rhodium and iridium salts inhibit proliferation and induce DNA damage in rat fibroblasts *in vitro*

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ABSTRACT

Environmental concentration of the platinum group elements is increased in the last years due to their use in automobile catalytic converters. Limited data are available on the effects of such elements at a cellular level and on their toxicity, especially for rhodium and iridium which have been more recently introduced in use.

The toxic effects of rhodium and iridium salts were analyzed on a normal diploid rat fibroblast cell line *in vitro*. Both salts halted cell growth in a dose- and time-dependent fashion by inhibiting cell cycle progression, inducing apoptosis and modulating the expression of cell cycle regulatory proteins. In fact, they both caused an accumulation of cells in the G2/M phase of the cell cycle and affected the expression levels of pRb, cyclins D1 and E, $p21^{Waf1}$ and $p27^{Kip1}$. DNA strand breaks, as assessed by comet test, and an increase in the intracellular levels of reactive oxygen species also occurred in exposed cell cultures.

These findings suggest a potential toxicity of both iridium and rhodium salts and emphasize the need for further studies to understand their effects at a cellular level to enable a better assessment of their toxic effects and to identify ways for their modulation and/or prevention.

1. Introduction

The platinum group elements (PGEs) comprise rare metals such as platinum (Pt), palladium (Pd), rhodium (Rh), ruthenium, iridium (Ir) and osmium. These chemically very similar metals have exceptional catalytic qualities, are very resistant to chemical corrosion over a wide temperature range and have high melting point, high mechanical strength and good ductility. These physicochemical properties allow to use the PGEs in various sectors of industry such as chemicals, petrochemicals, electrical and electronics, glass production, jewellery production, medical sector and in dentistry (Pyrzyñska, 1998; Ravindra et al., 2004; Brook, 2006; Resano et al., 2007). Moreover, presently Pt, Pd and Rh are extensively employed, in various combinations, in the manufacture of three-way catalyst (TWC) for the abatement of emissions from petrol/rich-burn engines (Gagnon et al., 2006). TWCs simultaneously convert over 90% of hydrocarbons, carbon monoxide and nitrous oxides from exhaust emissions into less harmful carbon dioxide, nitrogen and water vapor (Onovwiona and Ugursal, 2006; Twigg, 2007).

The wide use of Rh and the recent application of Ir in the motor industry is leading to increasing concentrations of these metals in the environment, as a result of abrasion and surface deterioration of the catalysts (Barefoot, 1997; Artelt et al., 1999). Consequently, the deposition in different environmental compartments could result in a higher exposure by inhalation not only for occupational categories exposed to vehicle traffic but also for the general population (Botrè et al., 2007). Recently, our group has conducted several studies to monitor Rh and Ir levels in the atmosphere of Rome demonstrating significant airborne levels of both metals and increased levels of urinary Rh, but not Ir, in subjects occupationally exposed to PGEs emitted from automotive catalysers (Iavicoli et al., 2007; 2008a,b).

The constant growth of the environmental dispersion of PGEs and the consequent increase of their concentrations in road dust, airborne particulate, soil and groundwater tables is raising concerns about the environmental impact and toxicity of these elements. However, the adverse effects of PGEs on human health are still in dispute and incompletely elucidated (Sheard, 1955; Bergman et al., 1995; Merget et al., 2010; Bedello et al., 1987; De la Cuadra and Grau-Massanés, 1991; De la Fuente et al., 2003; Murdoch et al., 1986; Murdoch and Pepys, 1987; Santucci et al., 2000; Cristaudo et al., 2005).

Few *in vivo* and *in vitro* studies have been published that address bioavailability, mode of penetration into live organisms and

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toxicity of Ir and Rh (Landolt et al., 1972; Zimmermann et al., 2002). Recently, we investigated the effects of Ir (III) chloride hydrate on the immune system of female Wistar rats and found that Ir exposure produced an immunological imbalance altering the levels of Th1 and Th2 cytokines (Iavicoli et al., 2010).

Limited literature is also available regarding *in vitro* studies to evaluate the toxic effects of Rh, while no studies concerning the *in vitro* effects of Ir are present. Bünger et al. (1996) assessed the cytotoxic and mutagenic effects of eight water-soluble complexes of Pt, Pd and Rh and suggested that Rh complexes are less toxic than Pt and Pd compounds. Evaluation of the genotoxic properties of Rh ($RhCl_3$) compounds in the human lymphocyte micronucleus (MN) assay displayed a statistically significant increase in MN frequency above the dose of 100 μM (Migliore et al., 2002). It was also shown that $RhCl_3$ is able to cause oxidative DNA damage inducing a statistically significant increase in oxidized bases at all tested concentrations (10–1000 μM). Finally, a significant decrease of DNA migration was observed at doses of 100 and 250 μM. The effects of inorganic PGEs salts were also analyzed on human bronchial epithelial cells reporting a slight toxicity for Rh chloride associated with no increase in the relative reactive oxygen species (ROS) levels for concentrations up to 0.09 mol/L (Schmid et al., 2007).

In this study the potential toxic effects of Ir and Rh salts were assessed using a series of *in vitro* assays for evaluation of basic biological aspects relating to biocompatibility. To determine the cytotoxic and bioactive effects of such compounds we measured viability, apoptotic and proliferation properties, fragmentation and oxidative damage of DNA in a culture system of normal fibroblasts exposed to both metals.

2. Materials and methods

All reagents were purchased from Sigma and were of the highest grade. All experiments were performed according to Good Laboratory Practice regulations and were repeated at least three times in triplicate. Data shown are mean ± standard deviation (SD). Salts of Iridium and Rhodium (Iridium III chloride hydrate and Rhodium III chloride hydrate, respectively) were provided by Alfa Aesar® (Karlsruhe, Germany) in sterile condition. Both salts were dissolved in sterile water and then overnight stirred on a magnetic stirrer to obtain a homogeneous solution. Both solutions were stocked for a few months at 4 °C.

2.1. Cell culture

The RAT-1 rat embryo fibroblasts (originally obtained from Dr. I.B. Weinstein, Columbia University, New York, NY, USA) were cultured in MEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.2. Cytotoxicity and cell proliferation assays

Cytotoxicity was evaluated using the MTT assay as an indicator of the metabolic competence of the cells (Sgambato et al., 2001). Briefly, 3×10^4 cells/well were seeded in 24-well culture plates, grown for a further 24 h and then incubated in medium containing increasing amount of each metals (from 0 to 3 mM). At the end of the incubation (48 h) the medium was removed and cultures were incubated with medium containing 1 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) for 2 h at 37 °C. The medium was then discarded and 250 μl acid–isopropanol (0.04 N HCl in isopropanol) was added to each well to stop the cleavage of the tetrazolium ring by dehydrogenase enzymes that convert MTT to an insoluble purple formazan in living cells. Plates were then kept in agitation at room temperature for about 15–20 min and the level of the colored formazan derivative was determined on a multiscan reader at a wavelength of 540 nm (reference wavelength 630 nm). Data are expressed as the percentage of surviving cells in treated cultures compared to control ones incubated with medium exposed to extracting conditions.

For cell proliferation assays, cells were plated at a density of 5×10^4 cells/well in 6-well culture plates. After 24 h, medium was changed and cultures were exposed to each of the metals. The number of cells per well was determined every day using a Coulter counter (Beckman Coulter, Fullerton, CA, USA) and medium was changed every 2 days.

2.3. Flow cytometry and apoptosis detection

For cell cycle analysis, exponentially growing cells were treated as previously described and were then collected, washed with phosphate-buffered saline (PBS), fixed in 5 ml of 70% ethanol and stored at 4 °C. For the analysis, cells were collected by centrifugation and the pellets were resuspended in 0.2 mg/ml of propidium iodide (PI) in Hank's balanced salt solution containing 0.6% NP-40 and RNase (1 mg/ml). The cell suspension was then filtered and analyzed for DNA content on a Coulter EPICS 753 flow cytometer, as previously described (Sgambato et al., 2001). The percentage of cells in different phases of the cell cycle and in the sub-G1 (subdiploid DNA content) peak were determined using a ModFit 5.2 computer program. The assays were repeated at least three times and gave similar results. The data reported are the results of a typical experiment. Apoptosis was detected using the Annexin-V-FLUOS Staining Kit (Roche Diagnostics Corporation, Indianapolis, Ind.), following the manufacturer's instructions. Briefly, cell pellets were resuspended in ice-cold binding buffer and annexin V-FITC and PI solution were added. After 15 min incubation in the dark, the cell suspension was filtered and analyzed by flow cytometry. The results shown are the mean of three replicate experiments. SD was <20% for all tested conditions.

2.4. Comet assay

Single cell gel electrophoresis or Comet assay was performed as previously reported (Sgambato et al., 2001). Briefly, after exposure to Ir or Rh salts, cells were collected, resuspended in low-melting agarose (1.0×10^4 cells/100 μl of 0.5% low-melting agarose in PBS) and immediately pipetted onto agarose-coated slides (1.5% in PBS containing 5 mM EDTA). Cells were then covered with a layer of agarose (0.5% in PBS) and allowed to solidify briefly. The slides were immersed in ice-cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Sarkosyl, 10% dimethyl sulfoxide and 1% Triton X-100 (pH 10.0)) for 60 min at 4 °C. They were then placed on an electrophoretic tray with an alkaline buffer (0.3 N NaOH, 1 mM EDTA) and allowed to equilibrate for 20 min at room temperature before the electrophoresis performed at 300 mA for 20 min in the same buffer. The slides were then washed, stained for 5 min with 2 mg/ml ethidium bromide (EB) and analyzed with a fluorescence microscope Eclipse E600 (Nikon Corporation, Tokyo, Japan). Images were acquired with a camera coupled with a computer and were analyzed using the software Image-Pro Plus 4.1 (Media Cybernetics, Silver Spring, MD), as previously reported (Sgambato et al., 2001 and 2010). In each experiment, at least 50 randomly selected cells were evaluated and results are expressed as mean ± SD of three independent experiments.