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SUB-CHRONIC ORAL EXPOSURE TO IRIDIUM (III) CHLORIDE HYDRATE IN FEMALE WISTAR RATS: DISTRIBUTION AND EXCRETION OF THE METAL

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□ Iridium tissue distribution and excretion in female Wistar rats following oral exposure to iridium (III) chloride hydrate in drinking water (from 1 to 1000 ng/ml) in a sub-chronic oral study were determined. Samples of urine, feces, blood and organs (kidneys, liver, lung, spleen and brain) were collected at the end of exposure. The most prominent fractions of iridium were retained in kidney and spleen; smaller amounts were found in lungs, liver and brain. Iridium brain levels were lower than those observed in other tissues but this finding can support the hypothesis of iridium capability to cross the blood brain barrier. The iridium kidney levels rose significantly with the administered dose. At the highest dose, important amounts of the metal were found in serum, urine and feces. Iridium was predominantly excreted *via* feces with a significant linear correlation with the ingested dose, which is likely due to low intestinal absorption of the metal. However, at the higher doses iridium was also eliminated through urine. These findings may be useful to help in the understanding of the adverse health effects, particularly on the immune system, of iridium dispersed in the environment as well as in identifying appropriate biological indices of iridium exposure.

Keywords: Iridium; Sub-chronic exposure; Rats; Distribution; Excretion.

INTRODUCTION

Catalytic converters were first fitted to light duty vehicles in the USA and Japan in 1975, in response to new emission standards, such as the US Clean Air Act Amendement (Beneman *et al.* 2005). Many other countries with large vehicle markets have followed since then, including South Korea (1987), Mexico (1989), the member states of the European Union (1993), Brazil (1994) and China (2000) (Kendall 2004). These devices simultaneously convert more than 90% of carbon monoxide (CO) and nitrous oxides (NO_x) and 80% of unburned hydrocarbons (HCs) from exhaust emissions into less harmful carbon dioxide, nitrogen and water

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vapour (Onowwiona and Ugursal 2006; Twigg 2007). The catalytic converter initially employed a combination of platinum (Pt), palladium (Pd) and rhodium (Rh), with Pt and Pd being used to oxidize CO and HCs and Rh facilitating the reduction of NO_x. Today, there is a wide range of possible combinations and concentrations of Pt, Pd and Rh required by car manufacturers that are used to achieve different catalyst performance features. Moreover, recently, iridium (Ir) has been incorporated into catalytic converter technology (the so called "De" catalyst) based on its capacity to drastically reduce NO_x emission in the exhausts of lean burning engines (Ravindra *et al.* 2004).

Therefore, it is reasonable to assume in the next years a possible increase of Ir concentrations in the environment and consequently a higher exposure both for the general population and subjects occupationally exposed to vehicle traffic (Botrè *et al.* 2007). In fact, the rapidly changing redox conditions, high temperature, mechanical friction, abrasion and surface deterioration of the catalysts lead to Pt group elements (PGEs) emission into the various compartments of the environment at ng/km rates (Barefoot 1997; Artelt *et al.* 1999; Moldovan *et al.* 1999; Ek *et al.* 2004).

As in the case of the other PGEs, the recent application of Ir in the motor industry raised concerns about its increasing environmental levels and the potential adverse health effects. However, the toxicological literature concerning Ir effects on human health is very limited. Indeed, it was reported a case of contact dermatitis probably caused by exposure to a Pt and Ir alloy (Sheard 1955) and a case of contact urticarioid response with anaphylactic reactions in a worker of an electrochemical factory exposed to Ir chloride (Bergman *et al.* 1995). There are also some studies that, evaluating the effects of PGEs in workers of Pt refinery and catalyst manufacturing and recycling factory, revealed positive prick test reactions to Ir (Santucci *et al.* 2000; Cristaudo *et al.* 2005), while Marcusson *et al.* (1998) found positive reactions to Ir trichloride in 1% of 205 subjects with implanted dental alloys. These findings suggested the ability of this metal to cause immune sensitization and toxic responses in humans. In this regard, we have recently demonstrated that exposure of Wistar rats to different doses of Ir (III) chloride hydrate was able to induce an immunological imbalance, with Th1 cytokines displaying a marked dose-dependent decrease, whereas the Th2 cytokine IL-4 showed the opposite response (Iavicoli *et al.* 2010).

Finally, some data concerning the toxicokinetics of Ir were provided by experimental studies conducted several years ago, mostly on rats (AECD 1951a, 1951b; Casarett *et al.* 1960). After inhalation of metallic-Ir aqueous aerosols, metal was found in bronchi, larger bronchioles and in parenchymal regions (Casarett *et al.* 1960). Ir was then eliminated by lungs, with a half time of 6 h, while metal particles accumulated in the

pulmonary parenchyma had a considerably slower clearance, with a half time of 22–24 days. Findings showed that the major route of excretion was *via* feces, for the 96% of the absorbed dose. The quantities of Ir redistributed to organs and excreted in the urine in the study of Casarett were much less than those observed by Hamilton (AECD 1951a, 1951b), probably because metal Ir is less soluble in water than Ir chloride and oxy-chloride. In more recent studies a size dependence of Ir particles distribution was observed; a predominant retention of ultrafine insoluble particles in the lung was shown 1 week after rat inhalation (Kreyling *et al.* 2002, 2009). The particles were then predominantly cleared *via* airways and larynx into the gastrointestinal tract and feces, but there was also a small traslocation from lungs to blood circulation and to secondary target organs (liver, spleen, heart, brain, and carcass). Also Semmler *et al.* (2004) found that the most Ir amount in rats was retained in the lungs 3 weeks after a 2 or 6 months inhalation of insoluble ultrafine Ir particles, while the clearance out of the body was solely *via* excretion. Moreover, the study showed that extrapulmonary particle uptake decreased with time in liver, spleen, heart and brain.

Recently a study of our group demonstrated that female Wistar rats exposed to Ir in the drinking water (from 1 to 1000 ng/ml) for 90 days displayed renal toxicity based on the elevated urinary retinol binding protein (RBP) and albumin (Iavicoli *et al.* 2011).

Apart from these few results, nothing is known about long-term Ir absorption, its clearance pathways out of the body and systemic uptake pathways toward secondary organs. In this context we carried out a 90-day oral administration study with female Wistar rats exposed to different Ir (III) chloride hydrate concentrations administered *ad libitum* in drinking water, to investigate the distribution in internal organs and the elimination routes after oral administration. This type of treatment may simulate the exposure humans receive in several environmental or occupational conditions.

MATERIALS AND METHODS

Animals' husbandry

The Experimental Animal Production Plant of the Università Cattolica del Sacro Cuore (Rome, Italy) supplied twenty-eight female Wistar rats used in this study. At the start of the experiment, all the animals were approximately three months old. The mean weight for the animals was 265 g with individual weights within ± 15% of the mean, and no significant changes in body weight were observed at the end of the experiments. Before being treated with Ir (III) chloride hydrate, the animals were acclimated for two weeks and examined in order to confirm ade-

quacy for the study. During the study the animals were individually housed in Macrolon® cages (Tecniplast S.p.A., Buguggiate, Italy).

Room temperature and relative humidity were monitored and kept in the range of 19.8-25.3 °C and 50-60%, respectively, while a 12-h day/night cycle was maintained. Animals were fed with the solid maintenance diet R (Altromin Rieper A. S.p.A., Vandoies, Italy). Diet was available to rats without restrictions, as well as pure water or drinking water spiked with iridium (III) chloride hydrate. Iridium was analyzed in the diet pellets but the metal was undetectable in diet samples.

Sub-chronic exposure

The twenty-eight female Wistar rats selected for the study were randomly divided into seven groups of four rats each. The preparation of Ir solutions was performed by high-purity deionized water obtained by a double demineralization system: a mixed bed Culligan cartridge (Culligan Italiana S.p.A., Cadriano di Granarolo Emilia, Italy) and a MilliQ A10 apparatus (Millipore, Bedford, MA, USA) connected in series. The water had a resistivity of 18.2 MΩ_cm compensated for temperature at 25 °C and total organic compounds of 2 ng/ml measured by photoxidation. The solutions used for Ir administration had the following concentration of iridium (III) chloride hydrate (Alfa Aesar GmbH & CO KG, Karlsruhe, Germany; as Ir) 0 (control group), 1, 10, 100, 250, 500, and 1000 ng/ml. The water solubility of the Ir salt used was sufficient to yield clear and homogeneous stock solution of 1 mg/L. The actual concentration of the stock solution was check by analyzing three replicates; results gave a maximum loss of the expected actual concentration <10%, fully satisfactory for the aim of the study. Water was given *ad libitum* to the animals of each group for the entire period of Ir administration, resulting in a daily ingestion of 19 ± 5 ml of Ir containing drinking water (averaged on a total of 90 days) for each rat. The animals were sacrificed at the 90th day, at the end of the exposure period, following the experimental protocol approved by the ethical committee of the Università Cattolica del Sacro Cuore.

Urine and feces collection

Urine and feces were collected during a 24-h period in individual metabolic cages at the 90th day. During the collection of the samples, no food was supplied as the experimental protocol scheduled only drinking water supplementation in the 24 h before sacrifice.

Blood collection and serum separation

On 90th day, rats were anesthetized with 0.5 mg of medetomine and 75 mg of ketamine *per* kg body weight. Blood was then collected from

each of the twenty-eight animals in a 1.5 ml vial (Eppendorf srl, Milan, Italy) by cardiac puncture. Serum was obtained after blood centrifugation at 3,500 rpm per 15 min and stored at -28 °C until analysis.

Organ collection

After the blood sampling, rats were euthanized by exsanguinations by cutting both the abdominal aorta and vena cava. The organs sampled for the study were kidneys, liver, lung, spleen and brain. These organs were selected in order to achieve comparable results with the few literature data available (AECD 1951a, 1951b; Casarett *et al.* 1960; Kreyling *et al.* 2002; Semmler *et al.* 2004; Kreyling *et al.* 2009).

Sample treatment

Samples of urine and serum were analyzed after 1:10 v/v dilution with 0.28 M HNO_3 solution (ultrapur grade, Carlo Erba, Milan, Italy). Organs (0.1-0.25 g) and feces (ca. 0.20 g) were dried and mineralized by a microwave-assisted acidic digestion in Teflon vessels, and the digests were diluted with high-purity deionized water to a volume of 20 ml prior to analytical measurements. The digestion procedure is deeply described elsewhere (Iavicoli *et al.* 2001a). Food pellets (ca. 0.25 g) used to feed the rats were freeze-dried (Lyolab 3000, Heto, Allerød, Denmark) for 72 h and, then, mineralized by a microwave-assisted acidic digestion according to the methods previously reported (Iavicoli *et al.* 2001a, 2001b). All concentration values in feces and organs hereafter are reported on dry weight.

Iridium determinations

Iridium was determined by using a sector-field inductively coupled plasma mass spectrometer (SF-ICP-MS) (Element-II, Thermo-Finnigan, Bremen, Germany) equipped with Meinhard glass type nebulizer, water-cooled spray chamber and nickel interface cones (Iavicoli *et al.* 2008). The most abundant isotope, i.e. ^{193}Ir, was selected for quantifications and the low resolution setting (m/Δm = 300) was used because no significant interference (i.e. ^{177}Hf^{16}O) affected the analytical mass. The addition calibration approach was adopted to quantify Ir in all the matrices, using spiked solutions of pooled samples. All calibrants were daily prepared from 1000 mg/L stock solutions of Ir (Spex Industries, Inc., Edison, NJ) after subsequent dilutions with high-purity deionized water. Indium was added at a concentration of 0.5 µg/l as internal standard in order to account for possible instrumental drifts. Recoveries of the method, performed by adding to control kidney (before digestion) a concentration of Ir of 100 ng/l and 200 ng/l were 102% and 100%, respectively. In serum and urine, the limit of detection (LoD) based on the 3σ criterion, was of 0.001 ng/l, while in tissues and feces was 0.002 ng/g.

Statistical Analysis

The statistically significant differences between the doses of exposure were analysed with the Kolmogorov-Smirnov test (p<0.05) that is more appropriate than the Mann-Whitney U-test when the sample size is small, as in this study. The correlation between doses of exposure and Ir concentrations in organs, feces, urine and serum was evaluated by a Spearman test. The relative values of p and ρ were given in the notes in each Table. It was also performed a statistical linear regression among tissues and doses.

RESULTS

Data obtained on Ir concentrations in tissues, in serum, urine and feces at the end of exposure period (90th day) are reported in Tables 1 and 2, respectively.

Statistically significant differences (p<0.01) between control group and rats exposed to 1 ng/ml, were observed for all tissues and fluids except for the feces. Likewise, statistically significant differences (p<0.01) between doses of 1 ng/ml and 10 ng/ml and between doses of 100 ng/ml

TABLE 1. Mean and standard deviation of Ir concentrations in tissues (ng/g, dry weight).

Dose (ng/ml)	Tissues				
	Liver*	Spleen*	Lung*	Kidney*	Brain*
0	0.015±0.02	0.007±0.002	0.015±0.02	0.03±0.02	0.005±0.001
1	0.13±0.05	0.41±0.07	0.17±0.04	0.95±0.17	0.02±0.01
10	0.86±0.13	4.06 ± 0.60	1.45±0.16	7.13±1.50	0.10±0.02
100	8.06±1.70	34.4 ± 8.30	12.8±2.30	66.2±27.6	2.80±4.30
250	33.3±2.60	114 ± 17.3	49.7±7.10	248±79.0	2.19±0.30
500	76.1±11.2	290 ± 53.0	118±20.0	507±182	4.31±1.40
1000	147±57.2	595 ± 222	258±101	1124±643	9.87±4.10

ρ: dose–response correlation, liver: 0.990; spleen:0.991; lung:0.990; kidney: 0.986; brain: 0.943; * p<0.01 for all matrices.

TABLE 2. Mean and standard deviation of Ir concentrations in serum and urine (ng/ml), and feces (ng/g, dry weight) in the different groups of exposure.

Dose (ng/ml)	Serum* (ng/ml)	Urine* (ng/ml)	Feces* (ng/g dry weight)
0	0.009±0.004	0.128±0.082	2.95±1.85
1	0.022±0.003	0.206±0.132	8.96±8.31
10	0.205±0.045	0.834±0.084	45.6±8.90
100	1.266±0.68	5.010±1.797	385±78.3
250	4.281±0.68	9.395±3.558	1412±380
500	13.00±6.30	26.26±7.325	1687±431
1000	32.10±10.2	51.64±12.97	4357±697

ρ: dose–response correlation serum: 0.988; urine: 0.981; feces: 0.973; * p<0.01 for all matrices.

and 250 ng/ml, were observed for all matrices except for brain. The difference between the doses 10 ng/ml and 100 ng/ml was statistically significant for all tissues. Instead, no difference between the doses 250 ng/ml and 500 ng/ml was found for kidney, brain and feces.

Moreover, the linear regression showed a goodness of fit in terms of a very high R^2 for urine and feces (0.92 and 0.94, respectively), while for the other organs it ranged from 0.75 (kidney) to 0.88 (spleen). The lowest R^2 (0.69) was observed for brain.

DISCUSSION AND CONCLUSIONS

The findings of this study demonstrated that the subchronic exposure determined in female rats an increase of Ir in all organs but also in serum, urine and feces.

Concerning the distribution, the greatest amount of Ir were found in kidney and spleen, but high levels of the metal were also observed in lung and liver. In particular, the high uptake in kidney could explain the renal tubule dysfunction that we observed in a previous study (Iavicoli *et al.* 2011) and it should be investigated to verify if the kidney is the critical organ. The elevated levels in spleen are very interesting and they could be correlated to the immunological alterations that were previously observed in the same model of animals subjected to the same experimental conditions (Iavicoli *et al.* 2010). In particular, it was observed that Ir exposure induced dose-dependent decrease ($p<0.01$) in T helper 1 (Th1) cytokines and increase ($p<0.01$) in a T helper 2 (Th2) cytokine. Such findings suggested that Ir altered T helper formation and the spectrum of cytokines produced, with skewing toward a Th2 ''bias''. Therefore, Ir would be able to facilitate Th2 development among precursor Th(0) cells while inhibiting development of a Th1 pattern. Literature data showed that at least one other metal, lead (Pb), exhibited a very similar behaviour to that shown by Ir (Sun *et al.* 2009). In fact, in mice, after intravenous injection of a lead nitrate solution, the highest Pb levels was observed in kidney, followed by liver and spleen. Moreover, Pb was also able to affect cytokines production in a similar manner to Ir.

Both *in vivo* and *in vitro* experiments showed increased plasma IL-4 and IgE levels and a profound decrease of INF-γ and IL-2 production, indicating that Pb skews naïve T-cells toward Th2 (Heo *et al.* 1996, 1997; Iavicoli *et al.* 2004, 2006; Kasten-Jolly *et al.* 2010). Moreover, Heo *et al.* (1998) suggested that Pb inhibits Th1 development by increasing adenylate cyclase activity, and thereby increasing cyclic AMP levels. Considering the similarities in cytokines production induced by Ir and Pb, it could be interesting to verify if also Ir is able to use the same molecular mechanism of action and to study if this metal plays a role in promoting antibody production, including autoantibodies.

It is noteworthy that a slight increase of brain Ir levels with the increasing of the dose of exposure, was observed. This result could be very interesting because it suggests the ability of Ir to cross the blood-brain barrier and consequently it raises some concerns about a possible role of this metal as neuro-toxic substance. In this regard literature data are very limited. Göbbels *et al.* (2010), investigating the neurocompatibility of uncoated and coated (with Concanavalin A and poly-(D)-lysine) pure Ir and different Ir oxides in thoracic neurons from adult locusts and auditory brainstem neurons from embryonic chicken, found contrasting results. In fact, the evaluation of engraftment of cells, survival during the first three days in culture, and differentiation of neurons revealed that different Ir oxides are neurocompatible to neuronal cells of locusts and of limited neurocompatibility to chicken neurons from the auditory brainstem. In particular, chicken cells grown on uncoated substrates decreased in number to 50% and on coated substrates to 75% of starting cell density. A similar study conducted by Thanawala *et al.* (2007) on rat (Sprague-Dawley) cortical neurons, treated with amorphous and crystalline Ir oxide, showed that this metal is biocompatible and non-toxic for neural cells but it was also observed a poor cell attachment of neurons onto Ir oxides surfaces. Therefore, although Ir levels in soil (Müller and Heumann 2000; Fritsche and Meisel 2004), road dust (Müller and Heumann 2000; Djingova *et al.* 2003), plants or lichens (Djingova *et al.* 2003; Pino *et al.* 2010) and riverine or marine sediments (De Vos *et al.*, 2002; Lee *et al.*, 2003) are very low, in the order of ng/g or in the order of pg/m^3 as in the case of airborne Ir levels (Rauch *et al.* 2006; Iavicoli *et al.* 2008), special attention should be paid to the evaluation of possible neurotoxic effects in working and general population and, particularly, in children. Finally, the dose-dependent increase of Ir levels in serum showed that the metal was distributed to the blood compartment.

Concerning the excretion, the findings revealed that the body clearance of Ir took place mainly by elimination *via* feces and that this way of elimination was dose-related. This significant correlation with the ingested dose is likely due to low intestinal absorption of Ir. Also urinary Ir levels increased in a dose-dependent manner. Changes in urinary excretion among different groups of exposure could be due to altered conditions in the kidney of rats, as we previously observed in another study (Iavicoli *et al.* 2011). Therefore, considering that collection of urine is an easy, non-invasive and low-cost sampling method, these results suggested that urine could be an appropriate matrix to assess the environmental and/or occupational exposure to this metal. In this regard, our findings are particularly interesting although, to use the urinary levels of Ir as biomarkers of exposure, it is desirable that future studies assess the kinetics of urinary excretion of this metal.

In conclusion, the subchronic study here reported provides helpful data regarding the toxicokinetics of Ir and the target-organ toxicity of this metal. However, further studies are needed to assess the potential renal and neurological toxicity of Ir and to fully understand its effects on immune system.

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