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REGULAR ARTICLE

THE MERCURY AS ENDOCRINE DISRUPTOR ON THE ADRENOCARCINOMA CELL LINE H295R

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ABSTRACT

Mercury (Hg) is one of the oldest heavy metals, which has various effects on the endocrine system. Target of this *in vitro* study was to determine the effects of mercuric chloride ($HgCl_2$) on the steroidogenesis in adrenocarcinoma cells isolated from the cell line H295R. We examined the dose-dependent changes of $HgCl_2$ on the production of testosterone (T). Release of steroid hormone by adrenocarcinoma cells was determined after 48 h $HgCl_2$ exposure (1.0; 5.0; 25; 50; 100 $\mu mol.dm^{-3}$) using an ELISA assay. Decreased hormone production was detected in all experimental groups with the addition of $HgCl_2$. In regards to the release of T, significant differences ($P<0.01$) between the control group and all experimental groups was recorded. The lowest amount of T was found after administration at doses >50 $\mu mol.dm^{-3}$ of $HgCl_2$. Obtained data indicate, that Hg has toxic effect on the testosterone production and its toxicity can reflect also in the others pathways of the cells.

Keywords: mercury chloride, testosterone, cell line H295R

INTRODUCTION

Mercury (Hg) is one of the oldest toxicants known **(Zhu *et al.,* 2000)**, which is considerable risk factors of environment and food chain **(Tazisong and Senwo, 2009)**. Mercury occurs as elemental Hg as well as in inorganic and organic compounds, although all having different toxicological properties. This heavy metal is circulated naturally in the biosphere. In addition Hg is released into the environment each year by human activities, such as combustion of fossil fuels and other industrial releases **(Berlin *et al.,* 2007)**. However, recent studies indicate that anthropogenic sources have the greatest contribution in the environment **(Tan *et al.,* 2009)**. The usual ways, how humans get exposed to Hg, is through of food, dermal absorption as well as by inhalation of vapour **(Zhu *et al.,* 2000)**.

Mercuric chloride ($HgCl_2$) is a highly reactive compound, which can harm cells by a variety of mechanisms including direct interaction with sulfhydryl (SH^-) groups of proteins and enzymes **(Einollahi *et al.,* 2006)**. Mercuric ion (Hg^{2+}) is able to form many stable complexes with biologically important molecules, such as SH^- groups. The affinity of Hg for sulfur and sulfhydryl groups is a major factor underlying the biochemical properties of Hg and mercury compounds **(Berlin *et al.,* 2007)**. SH^- groups constitute an important component in proteins. Mercury binding to these groups can produce a change in the proteins structure and alter binding conditions in prosthetic groups in enzymes **(Freitas *et al.,* 1996)** and block receptor binding **(Albrecht and Matyja, 1996)** K^+ or Ca^{2+} ion flows in the cell membranes **(Aschner *et al.,* 1996)**. This can affect cell membrane potentials and intracellular and intercellular signals. Mercury is a potent neurotoxic agent and neurotoxicant **(Pugach and Clarkson, 2009)**, which accumulates in female follicular fluid **(Al-Saleh *et al.,* 2008)**, the liver and kidney **(Lukáč *et al.,* 2006; Massányi *et al.,* 2007)** and brown hares **(Kolesárová *et al.,* 2008)** and muscle of pigs **(López-Alonso *et al.,* 2007)**. Its presence in agricultural systems is of concern due to its potential toxicity **(Tazisong and Senwo, 2009)**. Exposition to a high concentration of metallic mercury causes an increase in reproductive problems **(Schuurs, 1998)**, which can be strongly reflected in the process of steroidogenesis. The endocrine disruptive effects of Hg have recently become one of the major public concerns. There is sufficient evidence from animal studies supporting the disruptive effects of mercurial on the functions of the thyroid, adrenal, ovary, and testis, although several factors make it difficult to extrapolate the animal data to the human situation **(Zhu *et al.,* 2000)**.

Various cell lines and cells in primary culture have been used for the investigation of effects of xenobiotics on steroidogenesis. In the present study was used the human cell line

H295R as a model system for detection of toxic effect of $HgCl_2$ on the production of sex steroid hormones *in vitro*. This cell line was derived from NCl-H295 cells, which were established from a primary hormonally active adrenocortical carcinoma. The cells represent unique *in vitro* model system in that they have the ability to produce all of the steroid hormones found in the adult adrenal cortex and the gonads, allowing testing for effects on both corticosteroid synthesis and the production of sex steroid hormones **(Gazdar *et al.*, 1990)**. As well as permitting the measurement of hormone production, another advantage of the H295R cell bioassay is that it can be used to evaluate the enzymatic activities of steroidogenic genes **(Zhang *et al.*, 2005)**.

The objective of our study was to determine the effects of various concentrations of mercuric chloride ($HgCl_2$) on the steroidogenesis in adrenocarcinoma cell line H295R. We examined the dose-dependent changes of $HgCl_2$ as endocrine disruptor on the steroid hormone production – testosterone (T).

MATERIAL AND METHODS

H295R cell bioassay

The H295R human adrenocarcinoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and stored (liquid nitrogen -196 ºC) in a certified laboratory (National Institute of Chemical Safety, OGYI/31762-9/2010; GLP – Good Laboratory Practice, Budapest, Hungary) to execution of analysis. The protocols for culturing and exposure of H295R cells have been previously established and validated **(Hilscherová *et al.*, 2004; Zhang *et al.*, 2005; Hecker *et al.*, 2006)**. The H295R cells were grown in an incubator at 37 ºC with a 5% CO_2 atmosphere. The cells were cultured with supplemented medium containing Dulbecco´s Modified Eagle´s Medium with Ham´s F-12 Nutrient mixture (DMEM/F12) (Sigma D-2906; Sigma, St. Louis, MO, USA) (1:1) supplemented with 1.2 $g.l^{-1}$ Na_2CO_3, 5.0 $ml.l^{-1}$ of ITS+ Premix (BD Biosciences; 354352, San Jose, CA, USA) and 12.5 $ml.l^{-1}$ of BD Nu-Serum (BD Biosciences; 355100, San Jose, CA, USA) according to the protocol **Hilscherová *et al.* (2004)**. The adrenocarcinoma cells were incubated in plate wells at 37 ºC and 5% CO_2 in humidified air until a 70-75% confluent monolayer was formed. Cells density was determined using hemacytometer to a final concentration of approximately 10^6 cells per ml. Following incubation for 48 h, the aliquots of

the culture medium were centrifuged (at 2000 rpm, for 10 min, 4 °C) and the supernatant was collected and frozen at -20 ºC until hormone determination.

In vitro exposure

The adrenocarcinoma cells were exposed to concentrations (1.0, 5.0, 25, 50, 100 µmol.dm^{-3}) of mercuric chloride ($HgCl_2$, Sigma-Aldrich, St. Louis, USA). The cells were analyzed in 96-well plates (MTP, Grainer, Germany) after 48 h $HgCl_2$ exposure. We compared the control group (medium without mercury) with the experimental groups (exposed to different concentrations of mercury).

Hormone measurement

Quantification of steroid hormone – testosterone (T) was performed after Hg exposure using enzyme linked immunosorbent assays (ELISA, Dialab GmbH – testosterone, Austria). The absorbance was determined at a wavelength 450 nm on the microplate ELISA reader (Anthos MultiRead 400, Austria).

Statistical analysis

Obtained data were statistically analyzed by the PC program GraphPad Prism 3.02 (GraphPad Software Incorporated, San Diego, California, USA). Descriptive statistical characteristics (mean, minimum, maximum, standard deviation and coefficient of variation) were evaluated. One-way analysis of variance (ANOVA) and the Dunnett´s multiple comparison test were used for statistical evaluations. The level of significance was set at *** (P<0.001); ** (P<0.01); * (P<0.05).

RESULTS AND DISCUSSION

Sex hormones and other reproductive indices are often used as biomarkers of effects on the reproductive system **(Drevnick and Sandheinrich, 2003)**. Reports concerning the effects of mercuric chloride are sufficient presented at cellular and molecular levels **(Tan *et al.*, 2009)**, but research in the area of hormonal system is insufficient. Therefore, the general objective of this study was to provide other information its impact on steroidogenesis.

Specifically, we examined concentrations of testosterone (T) release by adrenocarcinoma cells in relation to $HgCl_2$ concentration. Data obtained from this *in vitro* study indicate that the hormonal release of steroid hormone by adrenocarcinoma cells is associated with the dose of Hg administration. In regards to the release of T into the media, significant differences ($P<0.01$) was recorded between the control group and experimental groups. The highest release of steroid hormone T by adrenocarcinoma cells was recorded in the control group (12.21 ± 4.90 ng.ml^{-1}). Decreased hormone production was significantly ($P<0.01$) detected in all experimental groups with the addition of $HgCl_2$. Results are shown in the Table 1. The lowest amount of T was found after administration at the doses >50 μmol.dm^{-3} of $HgCl_2$.

Table 1 Effect of $HgCl_2$ on testosterone release (ng.ml^{-1}) by adrenocarcinoma cells

Groups	**Control** **Ctrl**	**1.0** **E**	**5.0** **D**	**25** **C**	**50** **B**	**100** **A**
$HgCl_2$ (μmol.dm^{-3})						
Testosterone (ng.ml^{-1})						
x	12.21	4.85^{B}	5.51^{B}	1.06^{B}	0.36^{B}	0.88^{B}
minimum	6.69	2.59	2.84	0.89	0.10	0.05
maximum	18.5	8.29	8.24	1.38	0.78	2.05
S.D.	4.90	2.45	2.59	0.22	0.30	0.92
CV (%)	40.16	50.51	47.04	20.27	83.40	104.53

Legend: x – mean, S.D. – standard deviation, CV (%) – coefficient of variation

$^{A}P<0.001$; $^{B}P<0.01$; $^{C}P<0.05$

Hg ions has been found to be an endocrine system disrupting, function of the pituitary gland, thyroid gland, thymus gland, adrenal gland, enzyme production process and affecting many hormonal functions at very low levels of exposure **(Kumar *et al.*, 2006)**. Mercury has specific effects on the endocrine system, which may be linked to sex steroids as well as other hormones. Mercury can affect multiple points in the steroidogenesis pathway, inhibiting enzymes important for hormone synthesis **(Tan *et al.*, 2009)**. According to **Veltman and Maines (1986)** Hg is capable of directly inhibit enzymes within the steroidogenesis pathway, leading to decreases in hormone production **(McVey *et al.*, 2008)**. Our results also showed a direct toxic action of Hg on the steroid-producing cell and it caused changes of hormone concentration.

Vachhrajani and Chowdhury (1990) examined of testicular steroidogenesis after intraperitoneal administration of mercuric chloride (5; 10 μg.kg^{-1} $HgCl_2$) and methylmercury (50; 100 μg.kg^{-1} MeHg) for 90 days. Both ($HgCl_2$, MeHg) showed, that inhibited the activity

of 3β-hydroxysteroid dehydrogenase (3β-HSD) in the rat, leading to a significant decrease in serum testosterone levels and induced cellular disintegration of Leydig cells. **Nishiyama *et al.* (1985)** reported that the adrenocorticotropic hormone (ACTH) induced corticosteroid production in cultured rat adrenal cortical cells was not affected by Hg application at 1 μM. In contrast, when the concentration reached 100 μM, Hg exerted an adverse effect on the viability of isolated rat adrenal cortical cells. Thus, the concentration of Hg applied to the culture system seems to be critical. There was a reduction in ACTH-stimulated corticosterone production by adrenal decapsular cells as well as luteinizing hormone-stimulated testosterone production by Leydig cells **(Ng and Liu, 1990; Zhu *et al.*, 2000)**. Our presented data showed, that all concentrations of $HgCl_2$ (1.0-100 $\mu mol.dm^{-3}$) inhibited release testosterone by cell line H295R. The disorder of the synthesis of testosterone could result in a reduction in the activity of key enzymes implied in the biosynthesis of testosterone **(McVey *et al.*, 2008)**. Similar results were also found in our previous study with cadmium and copper on the human cell line H295R **(Kňažická *et al.*, 2011a, b)**.

CONCLUSION

Data obtained from this *in vitro* study indicate that the release steroid hormone - testosterone by adrenocarcinoma cells is associated with the dose of Hg administration. All chosen concentrations of $HgCl_2$ inhibited the testosterone release. The results suggest that low concentrations of testosterone can cause affect their metabolites, whose production is conditioned by steroid enzymes. Findings of the present study confirm the toxic effect of Hg and its toxicity can reflect also in the others pathways of the cells.

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