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In vitro assessment of the impact of nickel on the viability and steroidogenesis in the human adrenocortical carcinoma (NCI-H295R) cell line

Norbert Lukac[1], Zsolt Forgacs[2], Hana Duranova[3*], Tomas Jambor[1], Jirina Zemanova[1], Peter Massanyi[1], Barbara Tombarkiewicz[4], Shubhadeep Roychoudhury[5] and Zuzana Knazicka[6]

[1]Department of Animal Physiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovak Republic.

[2]Independent Researcher, Baross u. 135/AVI/3, H-1089, Budapest, Hungary.

[3]AgroBioTech Research Centre, Slovak University of Agriculture in Nitra, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovak Republic.

[4]Department of Zoology and Animal Welfare, Faculty of Animal Sciences, University of Agriculture in Krakow, Al. Mickiewicza 24/28, 31-059 Krakow, Poland.

[5]Department of Life Science and Bioinformatics, Assam University, 788 011 Silchar, India.

[6]Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovak Republic.

*Corresponding author

E-mail addresses: hana.duranova@uniag.sk

RNDr. Hana Duranova, Ph.D.

AgroBioTech Research Centre

Slovak University of Agriculture in Nitra

Tr. Andreja Hlinku 2, 949 76 Nitra

Slovak Republic

Tel. : +421 37 641 4929

Short title: Nickel in Relation to Sexual Steroid Hormones

Summary

Nickel is a ubiquitous environmental pollutant, which has various effects on reproductive endocrinology. In this study, human adrenocortical carcinoma (NCI-H295R) cell line was used as an *in vitro* biological model to study the effect of nickel chloride ($NiCl_2$) on the viability and steroidogenesis. The cells were exposed to different concentrations (3.90; 7.80; 15.60; 31.20; 62.50; 125; 250 and 500 μM) of $NiCl_2$ and compared with control group (culture medium without $NiCl_2$). The cell viability was measured by the metabolic activity assay. Production of sexual steroid hormones was quantified by enzyme linked immunosorbent assay. Following 48 h culture of the cells in the presence of $NiCl_2$ a dose-dependent depletion of progesterone release was observed even at the lower concentrations. In fact, lower levels of progesterone were detected in groups with higher doses (≥ 125 μM) of $NiCl_2$ ($P < 0.01$), which also elicited cytotoxic action. A more prominent decrease in testosterone production ($P < 0.01$) was also noted in comparison to that of progesterone. On the other hand, the release of 17β-estradiol was substantially increased at low concentrations (3.90 to 62.50 μM) of $NiCl_2$. The cell viability remained relatively unaltered up to 125 μM ($P > 0.05$) and slightly decreased from 250 μM of $NiCl_2$ ($P < 0.05$). Our results indicate endocrine disruptive effect of $NiCl_2$ on the release of progesterone and testosterone in the NCI-H295R cell line. Although no detrimental effect of $NiCl_2$ (≤ 62.50 μM) could be found on 17β-estradiol production, its toxicity may reflect at other points of the steroidogenic pathway.

Keywords: nickel chloride, sexual steroid hormones, cell viability, NCI-H295R cell line, endocrine disruption

Introduction

Several environmental contaminants are recognized as endocrine disruptors (EDs), which may adversely affect the reproductive functions of humans, as well as wildlife species (Kabir *et al.* 2015, Vitku *et al*. 2015, Yang *et al.* 2015, Roychoudhury *et* al. 2016, Kolatorova *et al*. 2017, Jambor *et al.* 2018, Jambor *et al.* 2019). This heterogeneous group of exogenous substances has the ability to alter functions of the endocrine system with a subsequent negative impact on the cellular behaviour and health in an intact organism. Endocrine disruptors may be found in a variety of products, such as pesticides, household items, cosmetics or plastic packaging. It is likely that some EDs are structural analogues of steroids, having similar effects as true hormones, high levels of which may have disproportionate consequences (Sanderson 2006, Svechnikov *et al.* 2010). They can strongly affect reproductive and endocrine functions in several ways (Andersen *et al.* 2002), either by directly affecting the hormone production through interaction with the appropriate enzymes, or through interfering with their transport to target organs to alter natural hormone metabolism or even to inactivate the function of steroidogenesis regulatory proteins (e.g., Steroidogenic Acute Regulatory – StAR) (Sanderson and van den Berg 2003).

Nickel (Ni) is a widely distributed metal that is industrially applied in various mineral forms (Lu *et al.* 2005). Dusts from volcanic emissions, the weathering of rocks and soils, biological cycles and solubilisation of Ni compounds from soils (Sunderman 2004) represent the main natural sources of atmospheric (7.0-12.0 ng of Ni/m^3 of air;

150 ng of Ni/m^3 of air near point sources) and aqueous (3-10 μg of Ni/l of water in surface water and groundwater) Ni (ATSDR 2005). Anthropogenic sources of Ni pollution include mining, smelting and refining activities, burning of fossil fuels, sewage incineration and plastic production (Yu 2005). The major source of exposure to Ni for the general population is the food chain (Pandey and Srivastava 2000, Llamas and Sanz 2008). It usually enters the body via food and water consumption, although inhalation exposure in occupational settings is the primary route for Ni-induced toxicity (Ankel-Fuchs and Thauer 1988). Based on the average levels of Ni consumption through water (4.0-8.6 μg/day) and food (69.0-162.0 μg/day), the daily per oral intake of the metal was estimated to be 0.001-0.0024 mg/kg/day for an average adult human being weighing 70 kg (ATSDR 2005). Another source of non-occupational exposure to Ni is tobacco smoking, and each cigarette is estimated to contain 1.1-3.1 μg of Ni (Cempel and Nikel 2006).

Although Ni is considered to be an essential micronutrient (Eisler 1998), it has a number of effects in the cell (Das 2009). It plays an important role in DNA, RNA and protein structure and/or function (Pandey and Srivastava 2000). Nickel also serves as a cofactor or a structural component of several metalloenzymes (Przybyla *et al.* 1992). Deficiency is rare due to a low level of requirement, and relatively high availability in the diet, but experiments have shown that at cellular levels Ni deprivation may result in changes in the membrane properties and other structures (Das and Dasgupta 1997, Das 2009). On the contrary, high quantity of Ni is injurious for animal and human health (Pandey *et al.* 1999, Pandey and Srivastava 2000). More recently, several reports have showed that Ni is able to induce toxicological, physiological and histopathological alterations in a number of animal species (Pane *et al.* 2003, Bersenyi *et al.* 2004, Brix *et al.* 2004, Gupta *et al.* 2006, Krockova *et al.* 2011, Lukac *et al.* 2011). Soluble Ni compounds are likely to be human carcinogens (Costa 1991, Costa *et al.* 2005), and

toxic and/or carcinogenic effects of such Ni compounds may be associated with Ni-mediated oxidative damage to DNA, proteins and inhibition of cellular antioxidant defences (Rodriguez *et al.* 1996). There is sufficient evidence that Ni ions (Ni^{2+}) have potential toxic effects on the reproductive system (Das and Dasgupta 2000). Animal studies referred to the negative effects of Ni^{2+} on the structure and function of testis, seminal vesicles, prostate gland (Pandey *et al.* 1999, Forgacs *et al.* 2001, Massanyi *et al.* 2003, Massanyi *et al.* 2007, Zemanova *et al.* 2007), and spermatozoa concentration as well as motility (Das and Dasgupta 2000, Lukac *et al.* 2011). Nickel salts are also capable of inducing morphological changes such as, degeneration of testicular germinal epithelium (Benson *et al.* 1988, Pandey *et al.* 1999), testicular sarcomas as well as functional disorders including inhibition of spermatogenesis (Mathur *et al.* 1977, Yokio *et al.* 2003) and steroidogenesis (Das and Dasgupta 2002, Krockova *et al.* 2011). Such negative effects may ultimately lead to sterility (Massanyi *et al.* 2007).

Steroidogenesis can be tested using a number of cell lines or primary culture with gonadal tissue, but the most widely used assay utilizes a human adrenocortical carcinoma (NCI-H295R) cell line. Such *in vitro* steroidogenesis screening assays are used to examine the impact of endocrine active chemicals/substances (EACs) capable of altering steroid biosynthesis (Ding *et al.* 2007, Fialkova *et al.* 2018). Progesterone, testosterone and estradiol are the main steroid hormones that play essential roles during the regulation of reproduction in vertebrates and are also involved in numerous other processes related to development and growth (Hecker and Giesy 2008). The present study investigated the effects of nickel chloride ($NiCl_2$) on the viability and steroidogenesis of the NCI-H295R cell line. Specifically, we examined the dose-dependent changes of $NiCl_2$ as a potential endocrine disruptor in relation to the release of progesterone, testosterone and 17β-estradiol by NCI-H295R cell line *in vitro*. The NCI-H295R cell line was derived from H295 cells which were established from a

primary hormonally active adrenocortical carcinoma (Gazdar *et al.* 1990, Rainey *et al.* 2004). This cell line has physiological characteristics of zonally undifferentiated human fetal adrenal cells (Staels *et al.* 1993, Harvey and Everett 2003), and represent an unique *in vitro* model system having the ability to produce all of the steroid hormones found in the adult adrenal cortex and the gonads, allowing testing the effects on both corticosteroid synthesis together with the production of sexual steroid hormones (Gazdar *et al.* 1990). Another advantage of the H295R cell bioassay is that it can be used to evaluate the enzymatic activities of steroidogenic genes (Hilscherova *et al.* 2004). In fact, the NCI-H295R Steroidogenesis Assay has been included in the Tier1 Screening Battery of the United States Environmental Protection Agency's (EPA) Endocrine Disruptor Screening Program (EDSP). The test guideline of the H295R Steroidogenesis Assay (TG 456) has been further validated by the Organization for Economic Cooperation and Development (OECD 2011).

Materials and Methods

Cell culture

The NCI-H295R cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in a Good Laboratory Practice (GLP) certified laboratory (National Institute of Chemical Safety, Budapest, Hungary; OGYI/31762-9/2010) according to previously established and specifically validated protocols (Hilscherova *et al.* 2004, Zhang *et al.* 2005, Hecker *et al.* 2006, Hecker and Giesy 2008, OECD 2011).

After initiation of the NCI-H295R culture from the original ATCC batch, cells were cultured for five passages and these cells were split and frozen down in liquid nitrogen (-196ºC). The cells for the experiments were cultured for a minimum of five additional

157 passages using new NCI-H295R batches from frozen stocks prior to initiation of the
158 exposure studies. The cells were grown in 75 cm^2 plastic cell culture flasks (TPP
159 Techno Plastic Products AG, Switzerland) in an incubator under standard conditions
160 (37°C and 5.0% CO_2 atmosphere). Subsequently, the cells were grown in a 1:1 mixture
161 of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient mixture
162 (DMEM/F12; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1.2 g/l $NaHCO_3$
163 (Sigma-Aldrich, St. Louis, MO, USA), 5.0 ml/l of ITS+Premix (BD Bioscience, San
164 Jose, CA, USA) and 12.5 ml/l of BD Nu-Serum (BD Bioscience, San Jose, CA, USA).
165 The medium was changed 2-3 times per week and cells were detached from flasks for
166 sub-culturing using sterile 0.25% trypsin-EDTA (Sigma-Aldrich, St. Louis, MO, USA).
167 After trypsinization, cells were plated at the appropriate density to obtain 90-100%
168 confluency. Cell density was determined using a hemocytometer (Fig. 1) and adjusted
169 with culture medium to a final concentration of 300 000 cells/ml. The cell suspensions
170 were plated (with final volume of 1.0 ml/well) into sterile plastic 24-well plates (TPP,
171 Grainer, Germany) for estimation of sexual steroid hormones (50-60% confluency of
172 cells). For cytotoxicity evaluation, the cells (100 µl/well) were seeded into 96-well
173 plates (MTP, Grainer, Germany). The seeded plates were incubated at 37ºC and 5.0%
174 CO_2 atmosphere for 24 h to allow the cells to attach to the wells (Knazicka *et al.* 2013).
175
176 *In vitro exposure*
177 After 24 h attachment period, the cell culture medium was removed from the plates and
178 replaced with a new medium supplemented with 3.90; 7.80; 15.60; 31.20; 62.50; 125;
179 250 and 500 µM nickel chloride ($NiCl_2$; ≥ 98%; Sigma-Aldrich, St. Louis, MO, USA),
180 respectively. Cell cultures were set in 24 and 96-well plates (MTP, Grainer, Germany).
181 Following treatment, the cells were maintained for 48 h. The experimental groups A - H

(exposed to different concentrations of $NiCl_2$) with control group (Ctrl) (culture medium without $NiCl_2$) were compared.

Cell viability

The viability of the cells exposed to $NiCl_2$ was evaluated by the metabolic activity (MTT) assay (Mosmann 1983). This colorimetric assay measures the conversion of a yellow tetrazolium salt [3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide i.e. MTT), to blue formazan particles by mitochondrial succinate dehydrogenase enzyme of intact mitochondria of living cells. Formazan was measured spectrophotometrically. Following the termination of $NiCl_2$ exposure, the cells were stained with MTT (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 0.2 mg/ml. After 2 h incubation (37ºC, and 5.0% CO_2 atmosphere), the cells and the formazan crystals were dissolved in 150 µl of acidified (0.08 M HCl) isopropanol (CentralChem, Bratislava, Slovak Republic). The absorbance was determined at a measuring wavelength of 570 nm against 620 nm as reference by a microplate reader (Anthos MultiRead 400, Austria). The data were expressed in percentage of the control group (i.e., absorbance of formazan from cells not exposed to $NiCl_2$).

Hormonal analysis

At the end of 48 h $NiCl_2$ exposure, the aliquots of the culture medium were removed from the 24-well cell culture plates and after centrifugation the supernatant was collected and frozen at -80ºC until sexual steroid hormones measurements. Enzyme linked immunosorbent assay (ELISA) was used for the quantification of progesterone, testosterone and 17β-estradiol (Dialab GmbH, Wiener Neudorf, Austria) directly from the aliquots of the medium. According to the manufacturer's data, the sensitivity of testosterone assay was 0.075 ng/ml, and the intra- and inter-assay coefficients of

variation were 4.6% and 7.5%, respectively. Cross-reactivity with 5α-dihydrotestosterone was 16.0%. The sensitivity of progesterone assay was 0.05 ng/ml, and the intra- and inter-assay coefficients of variation were ≤ 4.0% and ≤ 9.3%, respectively. The intra- and inter-assay coefficients of variation for the 17β-estradiol assay were ≤ 9.0% and ≤ 10.0%, and the sensitivity was 8.68 pg/ml. The absorbance was determined at a wavelength 450 nm using a microplate reader (Anthos MultiRead 400, Austria) and the data were evaluated by WinRead 2.30 computer software. Values were expressed in percentage of the untreated control (control groups served as 100%). Forscolin, prochloraz and aminoglutethimide (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.1% DMSO were used as positive controls.

Statistical analysis

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

Results

Cell viability

The cell viability remained relatively unaltered up to 125 μM ($P > 0.05$) and slightly decreased from 250 μM of $NiCl_2$ ($P < 0.05$). The cytotoxic effect of $NiCl_2$ (< 50%) was

very distinct ($P < 0.01$) in the group with the highest concentration (500 μM) of $NiCl_2$ (Fig. 2).

Release of progesterone by human adrenocortical carcinoma (NCI-H295R) cell line

Following 48 h culture of NCI-H295R cell line in the presence of $NiCl_2$, a dose-dependent depletion ($P < 0.01$) of progesterone release was observed in all the experimental groups, even at the lowest concentration (3.90 μM) of $NiCl_2$ used in the study (19.56 ± 4.00 ng/ml). Lower levels of progesterone were detected in groups with higher doses (≥ 125 μM) of $NiCl_2$ ($P < 0.01$) as shown in Table 1. In the control group, progesterone production (100%) was 21.05 ± 4.40 ng/ml. The percentage changes of progesterone release after $NiCl_2$ exposure are presented in Fig. 3.

Table 1. Effect of 48 h $NiCl_2$ exposure on the release of progesterone (ng/ml) by human adrenocortical carcinoma (NCI-H295R) cell line. *Abbreviations:* x – arithmetic mean, ±S.D. – standard deviation, CV (%) – coefficient of variation. The level of significance was set at *** ($P < 0.001$), ** ($P < 0.01$) and * ($P < 0.05$). Ctrl – control group.

Groups	**Control** **Ctrl** **$NiCl_2$ (μM)**	**3.90** **H**	**7.80** **G**	**15.60** **F**	**31.20** **E**	**62.50** **D**	**125** **C**	**250** **B**	**500** **A**
X	21.05	19.56**	11.07**	10.59**	11.70**	9.93**	7.33**	6.18**	5.79**
minimum	15.24	15.64	6.24	6.84	8.27	6.88	5.27	4.25	3.55
maximum	28.25	24.12	14.25	15.26	14.85	14.49	10.58	8.18	7.58
±S.D.	4.40	4.00	3.26	3.06	2.65	3.00	1.99	1.62	1.35
CV (%)	20.91	20.46	29.48	28.96	22.96	30.24	27.15	26.13	23.41
%	100.00	92.92	52.59	47.51	55.58	47.19	34.83	29.37	27.48

Release of testosterone by human adrenocortical carcinoma (NCI-H295R) cell line

Testosterone production decreased significantly ($P < 0.01$) at all the concentrations of $NiCl_2$ used in the study (Table 2). Furthermore, this decline was more prominent in comparison to that of progesterone. The lowest release of testosterone was ($P < 0.01$) noted at 125 μM of $NiCl_2$ (1.22 ± 0.74 ng/ml) in comparison with control group (10.75 ± 3.45 ng/ml). The percentage changes of testosterone release after $NiCl_2$ exposure are presented in Fig. 4.

Table 2. Effect of 48 h $NiCl_2$ exposure on the release of testosterone (ng/ml) by human adrenocortical carcinoma (NCI-H295R) cell line. *Abbreviations:* x – arithmetic mean, ±S.D. – standard deviation, CV (%) – coefficient of variation. The level of significance was set at *** ($P < 0.001$), ** ($P < 0.01$) and * ($P < 0.05$). Ctrl – control group.

Groups	**Control** **Ctrl**	**3.90** **H**	**7.80** **G**	**15.60** **F**	**31.20** **E**	**62.50** **D**	**125** **C**	**250** **B**	**500** **A**
	$NiCl_2$ (μM)								
X	10.75	4.42**	3.18**	1.98**	4.96**	1.46**	1.22**	2.18**	1.86**
minimum	6.54	2.12	1.84	0.48	3.02	0.27	0.25	0.88	0.57
maximum	16.44	7.28	5.87	3.54	7.12	2.71	2.33	3.19	3.21
±S.D.	3.45	2.02	1.47	1.18	1.71	0.90	0.74	0.89	1.07
CV (%)	32.12	45.66	46.33	59.52	34.45	61.85	60.43	41.05	57.51
%	100.00	41.10	29.55	18.41	46.14	13.53	11.30	20.23	17.26

Release of 17β-estradiol by human adrenocortical carcinoma (NCI-H295R) cell line

The 17β-estradiol production was substantially increased at low concentrations (3.90 to 62.50 μM) of $NiCl_2$. However, the increment was not statistically significant ($P > 0.05$) in comparison with control group (Fig. 5). The lowest release of 17β-estradiol by NCI-

H295R cell line was recorded in groups with high concentrations (≥ 125 μM) of $NiCl_2$, which released similar levels of 17β-estradiol (Table 3).

Table 3. Effect of 48 h $NiCl_2$ exposure on the release of 17β-estradiol (pg/ml) by human adrenocortical carcinoma (NCI-H295R) cell line. *Abbreviations:* x – arithmetic mean, ±S.D. – standard deviation, CV (%) – coefficient of variation. The level of significance was set at *** ($P < 0.001$), ** ($P < 0.01$) and * ($P < 0.05$). Ctrl – control group.

Groups	**Control** **Ctrl**	**3.90** **H**	**7.80** **G**	**15.60** **F**	**31.20** **E**	**62.50** **D**	**125** **C**	**250** **B**	**500** **A**
	$NiCl_2$ (μM)								
X	1.10	1.69	2.08	1.66	1.42	1.34	0.89	0.86	0.91
Minimum	0.74	0.94	1.02	0.80	0.84	0.94	0.64	0.76	0.51
Maximum	1.68	2.88	2.57	2.39	2.78	2.51	1.30	1.05	1.06
±S.D.	0.29	0.67	0.57	0.68	0.71	0.60	0.20	0.09	0.18
CV (%)	26.32	39.86	27.57	41.23	49.90	45.05	21.87	10.49	20.35
%	100.00	154.70	167.00	151.70	129.60	122.40	81.73	78.37	83.21

Discussion

Hormonal effects are believed to play an important role in the reproductive toxicology of Ni at both the neuroendocrine and gonadal levels in the hypothalamic-pituitary-gonadal (HPG) axis (Forgacs *et al.* 2012). The effects of Ni on steroidogenesis have been described recently; however, the results vary depending on the experimental model, duration of exposure as well as the doses used. The present study on the impact of $NiCl_2$ on the NCI-H295R cell line suggests a direct action of $NiCl_2$ on the steroid-

producing cells and subsequent changes in hormonal release. Nickel significantly decreased the release of progesterone and testosterone in the entire range of concentrations of $NiCl_2$ used in the study whereas the cell viability remained relatively unaltered up to 125 μM ($P > 0.05$) and slightly decreased from 250 μM of $NiCl_2$ ($P < 0.05$). The cytotoxic effect of $NiCl_2$ (< 50%) was evident ($P < 0.01$) in the group with the highest concentration (500 μM) of $NiCl_2$. These results clearly confirm reports of Forgacs *et al.* (2011) and Ocztos *et al.* (2011), who observed similar effects of Ni^{2+}, Hg^{2+} and Cd^{2+} on the release of progesterone and testosterone by NCI-H295R cell line. Using primary gonadal culture, these authors also confirmed that Ni^{2+} is able to disturb the sexual steroid production far below its cytotoxic concentration. Similar effects of other metals (cadmium, mercury, copper) have also been reported by our group from studies in the NCI-H295R cell line (Knazicka *et al.* 2013, 2015, Bilcikova *et al.* 2020). Earlier, Krockova and Massanyi (2010) reported a dose-dependent decrease in progesterone production by the Leydig cells at the highest concentration of 1000 μmol/l of $NiCl_2$. Revesz *et al.* (2004) previously exposed human ovarian granulosa cells (obtained from women undergoing *in vitro* fertilization) to 15.60 to 1000 μM of Ni^{2+} for 48 h in order to determine the site of action of Ni^{2+}. The granulosa cells were stimulated to produce progesterone by using maximally stimulating amounts of human chorionic gonadotropin (0.10 IU/ml hCG) or dibutyryl cyclic adenosine monophosphate (1.00 mM db-cAMP). Dose-dependent depression in both hCG and db-cAMP stimulated progesterone production was seen at 15.60 μM or higher concentration of Ni^{2+} which is not cytotoxic to human ovarian granulosa cells. The viability of cells remained unaffected up to 31.25 μM of Ni^{2+} and decreased significantly at 62.50 μM of Ni^{2+}. Their data further indicated that the effect of Ni^{2+} on the progesterone production is not due to cytotoxicity, and the cellular site(s) of inhibitory action appears to be subsequent to the membrane receptor and production of db-cAMP. The inhibition of progesterone

secretion by granulosa cells (Roychoudhury *et al*. 2014a, 2015) or rat ovarian fragments (Roychoudhury *et al*. 2014b) were also induced by other metals. The effect of Ni^{2+} may be associated with its interactions with other essential divalent metal cations, blocking functional groups, displacing essential metal ions or modifying active conformation of biomolecules (Coogan *et al.* 1989). Ni^{2+} is known to inhibit calcium (Ca^{2+}) channels. On the other hand, Ca^{2+} plays an important role in the regulation of progesterone production as shown in the rat granulosa cells (Tsang and Carnegie 1983). In addition, Ni has been demonstrated to alter the metabolic activity of microsomal monoxygenases, some of which are essential for steroid metabolism (Mattison *et al.* 1983). Thus, above mentioned findings could also participate in Ni-triggered alterations of progesterone release by NCI-H295R cell line.

Our presented data showed that testosterone seemed to be more vulnerable than progesterone and 17β-estradiol to $NiCl_2$ exposure suggesting multiple sites of action of this metal in steroidogenesis. Disorders of the testosterone synthesis could result in a reduced activity of the key enzymes involved in the biosynthesis of testosterone. Das and Dasgupta (2002) reported that nickel sulphate ($NiSO_4$) affects steroidogenic enzymes (3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase) causing alterations in the testosterone formation in adult rat testes. In another study, Krockova *et al.* (2011) investigated the effects of $NiCl_2$ on the testosterone secretion, cell viability and apoptosis in mouse Leydig cells *in vitro*. They demonstrated that $NiCl_2$ decreased the testosterone production at a low dose (15.67 μmol/l) and subsequently confirmed Ni-induced structural and functional alterations in the Leydig cells. Testosterone production by mouse primary Leydig cells culture following an *in vitro* Ni^{2+} exposure (62.50 to 1000 μM) was also evaluated by Forgacs *et al.* (1998). Dose-dependent depression in hCG-stimulated testosterone production was found at ≥ 125 μM or higher dose of Ni^{2+}, while basal testosterone production remained

unaffected. They further showed the effect to be dose-dependent, and is not due to cytotoxicity. Previously, Laskey and Phelps (1991) examined the effect of Ni^{2+} and other metal cations (Co^{2+}, Cu^{2+}, Hg^{2+}, Cd^{2+} and Zn^{2+}) on *in vitro* Leydig cell testosterone production. The results showed no change in Leydig cell viability with any metal cation treatment during the 3 h incubation. Dose-response depression in both hCG- and db-cAMP-stimulated testosterone production was noted with Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+} and Zn^{2+} treatment. Surprisingly, Cd^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+} caused a depletion in hCG- and db-cAMP-stimulated testosterone production, also caused significant increases in 20α-hydroxycholesterol- and pregnenolone-stimulated testosterone production over untreated and similarly stimulated cultures. This indicates that these cations may act at multiple sites within the Leydig cells. Sun *et al.* (2003) studied the mechanisms of changes in the genital system caused by nickel sulfate ($NiSO_4$) in male rats. They observed that the contents of testicular Ni were increased; however, the blood serum contents of testosterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were reduced. It was assumed that the Ni-induced genital system injury in male rats may be related to the decrease in the content of these hormones.

The present study noted that the 17β-estradiol production was increased (although non-significantly) at low concentrations (3.90 to 62.50 μM) of $NiCl_2$ ($P > 0.05$). In agreement with our results, no significant changes were observed in serum estradiol levels in rats intraperitoneally injected with $NiCl_2$ (4 mg/kg body weight) (Hfaiedh *et al.* 2007). In the treated rats, demonstrably increased activity of testicular aromatase was also reported. Taking into account these considerations we presume that the considerably decreased levels of testosterone together with non-significant alterations in release of 17β-estradiol in the present study could be associated with higher aromatase

activity leading to stable estrogen levels as an adaptive response of NCI-H295R cell line to Ni exposure.

As a metalloestrogen, Ni activates estrogen receptor-α (ERα) (Darbre 2006, Forgacs *et al.* 2012). Martin *et al.* (2003) examined the ability of metal ions to activate ERα in the human breast cancer cell line (MCF-7). Similar to estradiol, treatment of cells with Cu, Co, Ni, Pb, Hg, Sn, Cr or V stimulated cell proliferation. The metals also decreased the concentration of ERα protein and mRNA, and induced expression of the estrogen-regulated genes, progesterone receptor and pS2. The ability of such metals to alter gene expression was blocked by an anti-estrogen, suggesting that their activity is probably mediated by ERα. The estrogenic potency of Ni was comparable to that of estradiol.

Moreover, the cytotoxic effect of $NiCl_2$ (< 50%) was evident ($P < 0.01$) in the group with the highest concentration (500 μM/ml) of $NiCl_2$ used in the study. The cell viability remained relatively unaltered up to 125 μM ($P > 0.05$) and slightly decreased from 250 μM of $NiCl_2$ ($P < 0.05$). Ng and Liu (1990) noted that Ni (1.0; 10.0 and 100 μM of $NiCl_2.6H_2O$) and other metals tested (including $PbCl_2$, $ZnCl_2$, $AlCl_3$, $CrCl_3$, $FeCl_2$ and LiCl) had no deleterious effect on viability and hormone-induced steroidogenesis of Leydig cells and the cells in the adrenal gland.

Conclusion

The results of the present study indicate the endocrine disruptive effect of $NiCl_2$ on the release of sexual steroid hormones (progesterone and testosterone) in the human adrenocortical carcinoma (NCl-H295R) cell line even at low (minimum) concentrations. Testosterone release seemed more vulnerable whereas no detrimental effect of $NiCl_2$ could be seen at concentrations ≤ 62.50 μM of $NiCl_2$ on 17β-estradiol production thereby suggesting multiple sites of action of this metal in the steroidogenic pathway.

Further research may clarify the precise molecular mechanism of action of $NiCl_2$ on the sexual steroid production and their metabolites whose production is conditioned by the steroidogenic enzymes.

Conflict of interest

There is no conflict of interest.

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644 **Figure 1.** Monolayer of human adrenocortical carcinoma (NCI-H295R) cell line.
645 *Abbreviations:* A – low density; B – high density (magnification 100x).
646
647 **Figure 2.** The viability of NCI-H295R cell line in culture after 48 h of $NiCl_2$ exposure.
648 *Abbreviations:* The cytotoxicity was assessed using the MTT assay following $NiCl_2$
649 exposure. Each point represents the arithmetic mean (±S.D.) absorbance in % of
650 (untreated) controls (Ctrl) determined in three independent experiments. The number of
651 replicate wells was 22-32 at each point. A decline in absorbance reflects a decline in cell
652 viability. The statistical difference between the values of Ctrl and treated cells was
653 indicated by asterisks *** ($P < 0.001$); ** ($P < 0.01$) and * ($P < 0.05$) (One-way
654 ANOVA with Dunnett's multiple comparison test).
655
656 **Figure 3.** Progesterone release (%) by NCI-H295R cell line in culture after 48 h of
657 $NiCl_2$ exposure. *Abbreviations:* Each point represents the arithmetic mean (±S.D.)
658 progesterone % of (untreated) controls (Ctrl) determined of three repeated experiments.
659 The number of replicate wells was 4-6 at each point per experiment. The statistical
660 difference between the values of Ctrl and treated cells was indicated by asterisks ***
661 ($P < 0.001$); ** ($P < 0.01$) and * ($P < 0.05$) (One-way ANOVA with Dunnett's multiple
662 comparison test).
663
664 **Figure 4.** Testosterone release (%) by NCI-H295R cell line in culture after 48 h of
665 $NiCl_2$ exposure. *Abbreviations:* Each point represents the arithmetic mean (±S.D.)
666 testosterone % of (untreated) controls (Ctrl) determined of three repeated experiments.
667 The number of replicate wells was 6-10 at each point per experiment. The statistical
668 difference between the values of Ctrl and treated cells was indicated by asterisks *** (P

669 < 0.001); ** (P < 0.01) and * (P < 0.05) (One-way ANOVA with Dunnett's multiple
670 comparison test).
671
672 **Figure 5.** 17β-estradiol release (%) by NCI-H295R cell line in culture after 48 h of
673 nickel chloride ($NiCl_2$) exposure. *Abbreviations:* Each point represents the arithmetic
674 mean (±S.D.) 17β-estradiol % of (untreated) controls (Ctrl) determined of three repeated
675 experiments. The number of replicate wells was 6-12 at each point per experiment. No
676 statistical difference between the values of Ctrl and treated cells was not recorded (P >
677 0.05) (One-way ANOVA with Dunnett's multiple comparison test).
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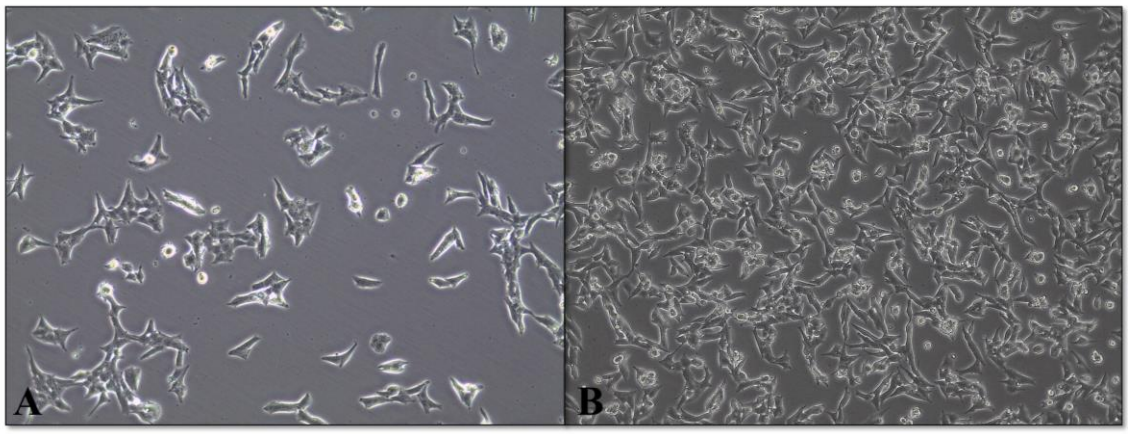

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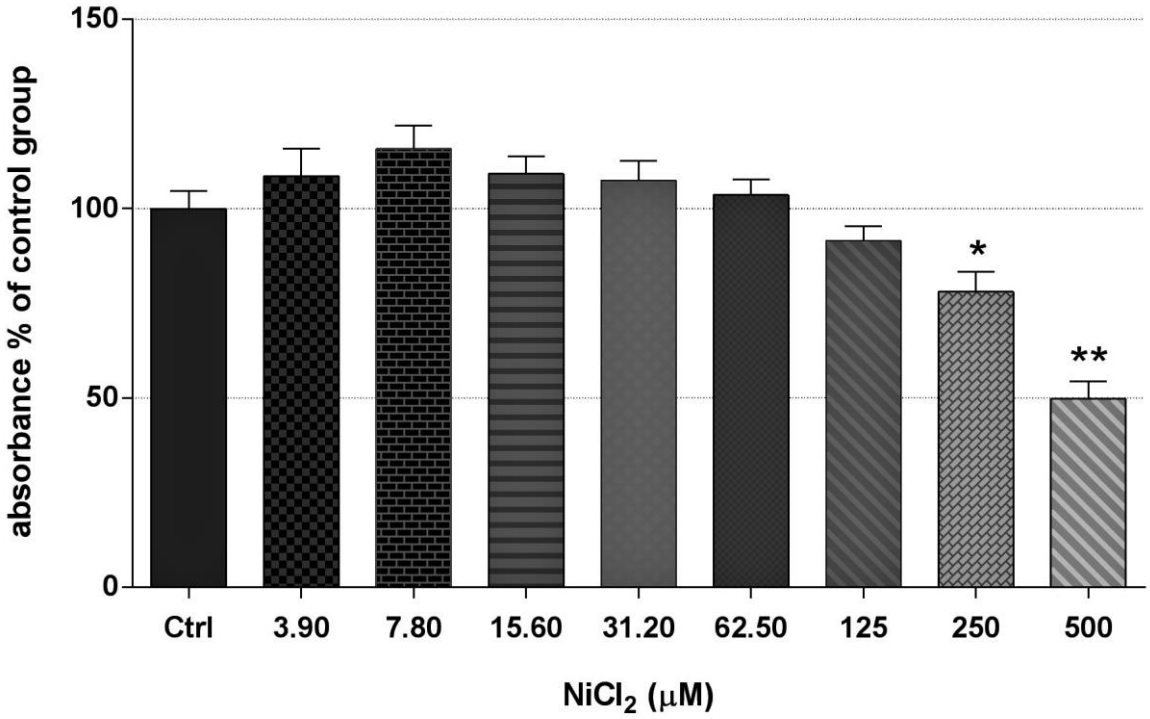

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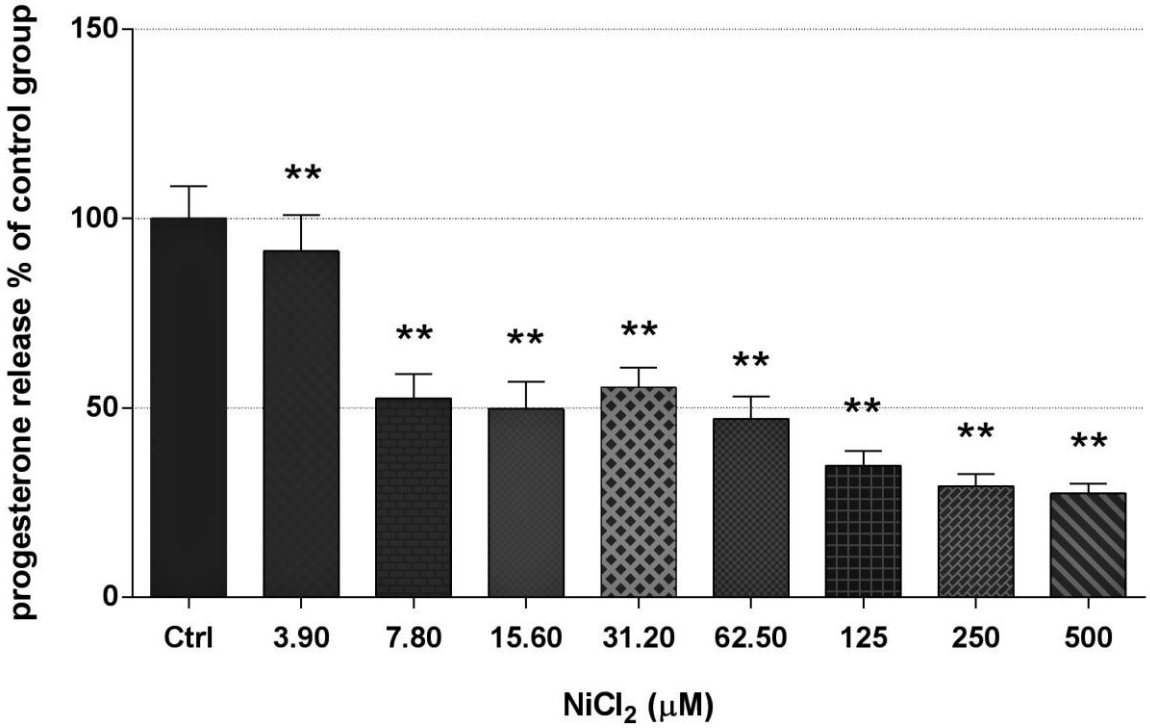

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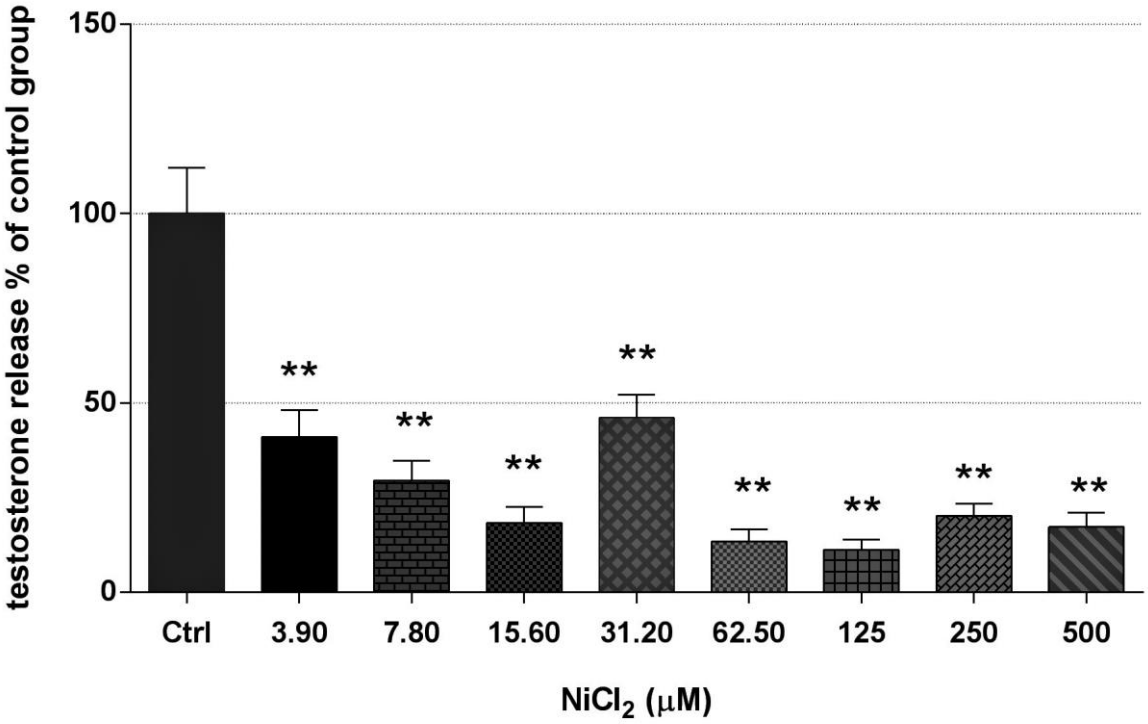

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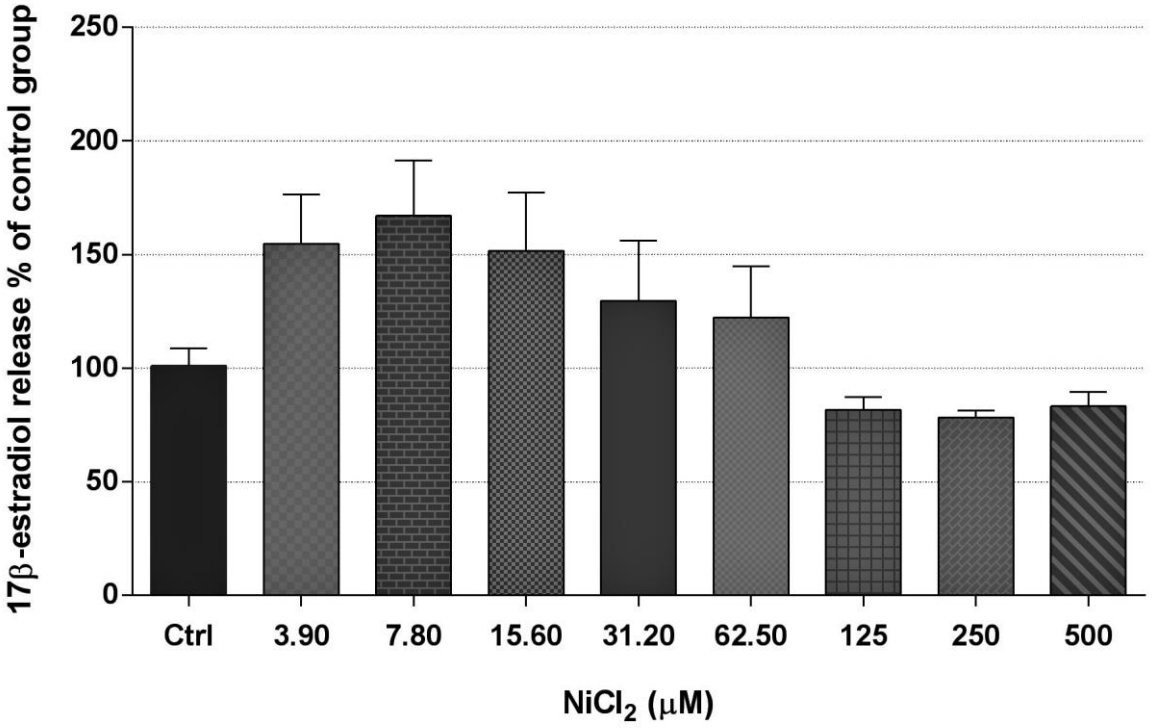

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