

In Vitro Assessment of the Impact of Nickel on the Viability and Steroidogenesis in the Human Adrenocortical Carcinoma (NCI-H295R) Cell Line

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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 (NiCl₂) 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 NiCl₂ and compared with control group (culture medium without NiCl₂). 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 NiCl₂ 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 NiCl₂ (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 NiCl₂. The cell viability remained relatively unaltered up to 125 µM (P>0.05) and slightly decreased from 250 µM of NiCl₂ (P<0.05). Our results indicate endocrine disruptive effect of NiCl₂ on the release of progesterone and testosterone in the NCI-H295R cell line.

Although no detrimental effect of NiCl₂ (≤62.50 µM) could be found on 17β-estradiol production, its toxicity may reflect at other points of the steroidogenic pathway.

Key words

Nickel chloride • Sexual steroid hormones • Cell viability • NCI-H295R cell line • Endocrine disruption

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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/m³ of air; 150 ng of Ni/m³ 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 (Ni²⁺) have potential toxic effects on the reproductive system (Das and Dasgupta 2000). Animal studies referred to the negative effects of Ni²⁺ 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 (NiCl₂) on the viability and steroidogenesis of

the NCI-H295R cell line. Specifically, we examined the dose-dependent changes of NiCl₂ 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 (-196 °C). The cells for the experiments were cultured for a minimum of five additional passages using new NCI-H295R batches from frozen stocks prior to initiation of the exposure studies. The cells were grown in 75 cm² plastic cell culture flasks (TPP Techno Plastic Products AG, Switzerland) in an incubator under standard conditions (37 °C and 5.0 % CO₂ atmosphere). Subsequently, the cells were grown in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient mixture (DMEM/F12; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1.2 g/l NaHCO₃ (Sigma-Aldrich, St. Louis, MO, USA), 5.0 ml/l of ITS+Premix (BD Bioscience, San Jose, CA, USA) and 12.5 ml/l of BD Nu-Serum (BD Bioscience, San Jose, CA, USA). The medium was changed 2-3 times per week and cells were detached from flasks for sub-culturing using sterile 0.25 % trypsin-EDTA (Sigma-Aldrich, St. Louis, MO, USA). After trypsinization, cells were plated at the appropriate density to obtain 90-100 % confluency. Cell density was determined using a hemocytometer (Fig. 1) and adjusted with culture medium to a final concentration of 300000 cells/ml. The cell suspensions were plated (with final volume of 1.0 ml/well) into sterile plastic 24-well plates (TPP, Grainer, Germany) for estimation of sexual steroid hormones (50-60 % confluency of cells). For cytotoxicity evaluation, the cells (100 µl/well) were seeded into 96-well plates (MTP, Grainer, Germany). The seeded plates were incubated at 37 °C and 5.0 % CO₂ atmosphere for 24 h to allow the cells to attach to the wells (Knazicka *et al.* 2013).

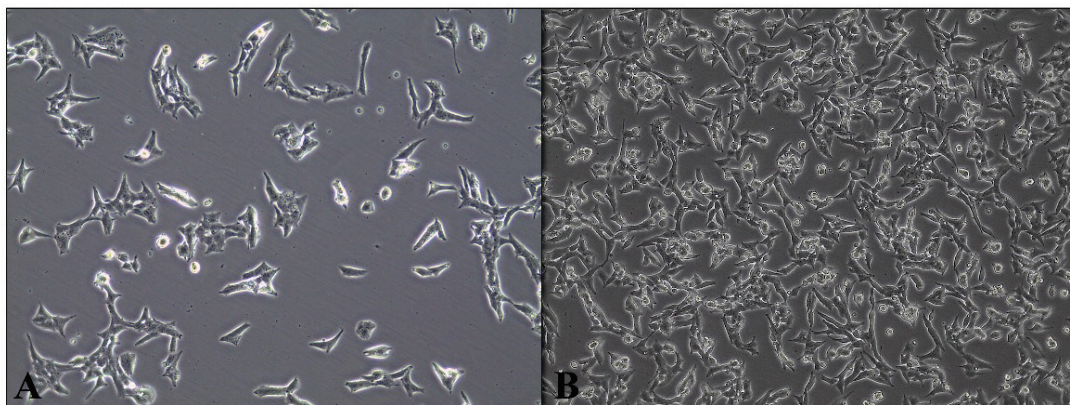


Fig. 1. Monolayer of human adrenocortical carcinoma (NCI-H295R) cell line. (A) low density; (B) high density (magnification 100x).

In vitro exposure

After 24 h attachment period, the cell culture medium was removed from the plates and replaced with a new medium supplemented with 3.90; 7.80; 15.60; 31.20; 62.50; 125; 250 and 500 μM nickel chloride (NiCl_2 ; $\geq 98\%$; Sigma-Aldrich, St. Louis, MO, USA), respectively. Cell cultures were set in 24 and 96-well plates (MTP, Grainer, Germany). 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-dimethylthiazol-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 (37 °C, 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 -80 °C until sexual steroid hormones measurements. Enzyme linked immunosorbent assay (ELISA) was used for the quantification of progesterone, testosterone and 17 β -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 5 α -dihydrotestosterone was 16.0 %. The

sensitivity of progesterone assay was 0.05 ng/ml, and the intra- and inter-assay coefficients of variation were $\leq 4.0\%$ and $\leq 9.3\%$, respectively. The intra- and inter-assay coefficients of variation for the 17 β -estradiol assay were $\leq 9.0\%$ and $\leq 10.0\%$, 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 \pm 4.00 ng/ml). Lower levels of progesterone were detected in groups with higher doses ($\geq 125\ \mu\text{M}$) of NiCl_2 ($P < 0.01$) as shown in Table 1. In the control group, progesterone production (100 %) was 21.05 \pm 4.40 ng/ml. The percentage changes of progesterone release after NiCl_2 exposure are presented in Fig. 3.

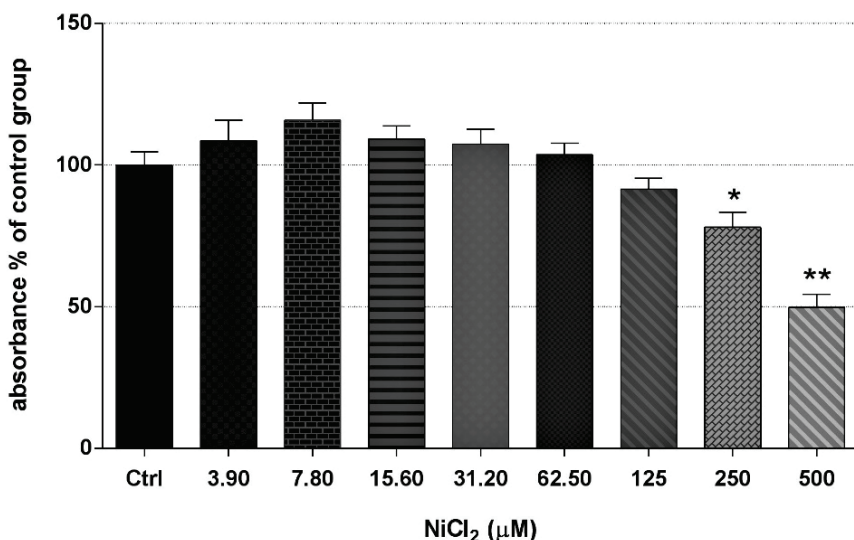


Fig. 2. The viability of NCI-H295R cell line in culture after 48 h of NiCl₂ exposure. The cytotoxicity was assessed using the MTT assay following NiCl₂ exposure. Each point represents the arithmetic mean (\pm S.D.) absorbance in % of (untreated) controls (Ctrl) determined in three independent experiments. The number of replicate wells was 22-32 at each point. A decline in absorbance reflects a decline in cell viability. The statistical difference between the values of Ctrl and treated cells was indicated by asterisks *** (P<0.001); ** (P<0.01) and * (P<0.05) (One-way ANOVA with Dunnett’s multiple comparison test).

Table 1. Effect of 48 h NiCl₂ exposure on the release of progesterone (ng/ml) by human adrenocortical carcinoma (NCI-H295R) cell line.

Groups	Control	3.90	7.80	15.60	31.20	62.50	125	250	500
	Ctrl	H	G	F	E	D	C	B	A
	NiCl ₂ (µM)								
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
\pm 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

X – arithmetic mean, \pm 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.

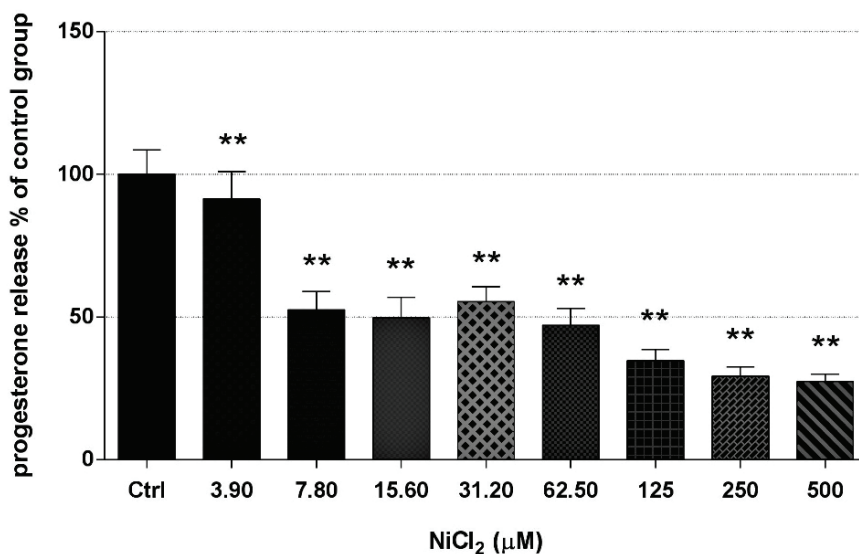


Fig. 3. Progesterone release (%) by NCI-H295R cell line in culture after 48 h of NiCl₂ exposure. Each point represents the arithmetic mean (\pm S.D.) progesterone % of (untreated) controls (Ctrl) determined of three repeated experiments. The number of replicate wells was 4-6 at each point per experiment. The statistical difference between the values of Ctrl and treated cells was indicated by asterisks *** (P<0.001); ** (P<0.01) and * (P<0.05) (One-way ANOVA with Dunnett’s multiple comparison test).

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 $125 \mu\text{M}$ of NiCl_2 ($1.22 \pm 0.74 \text{ ng/ml}$) in comparison with control group ($10.75 \pm 3.45 \text{ ng/ml}$). The percentage changes of testosterone release after NiCl_2 exposure are presented in Fig. 4.

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 $62.50 \mu\text{M}$) 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 ($\geq 125 \mu\text{M}$) of NiCl_2 , which released similar levels of 17β -estradiol (Table 3).

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

Table 2. Effect of 48 h NiCl_2 exposure on the release of testosterone (ng/ml) by human adrenocortical carcinoma (NCI-H295R) cell line.

Groups	Control	3.90	7.80	15.60	31.20	62.50	125	250	500
	Ctrl	H	G	F	E	D	C	B	A
	NiCl ₂ (μM)								
<i>X</i>	10.75	4.42**	3.18**	1.98**	4.96**	1.46**	1.22**	2.18**	1.86**
<i>minimum</i>	6.54	2.12	1.84	0.48	3.02	0.27	0.25	0.88	0.57
<i>maximum</i>	16.44	7.28	5.87	3.54	7.12	2.71	2.33	3.19	3.21
\pm 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

X – arithmetic mean, \pm 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.

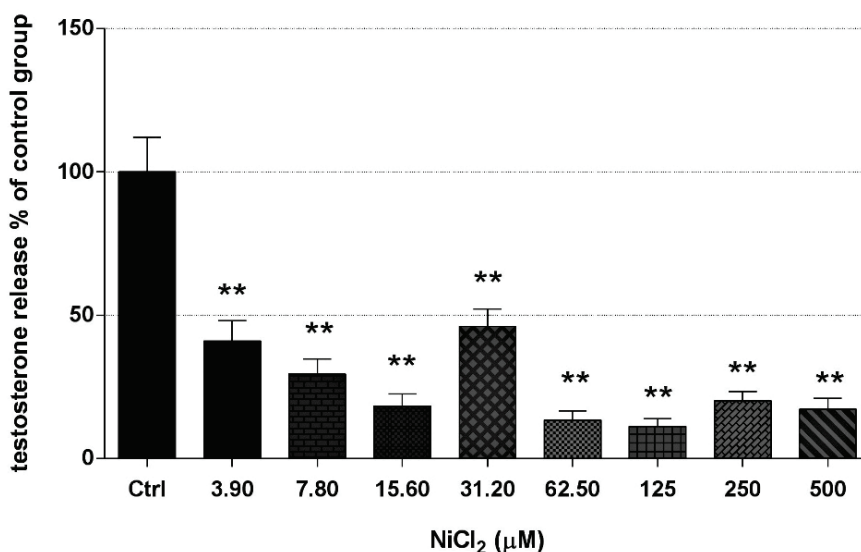


Fig. 4. Testosterone release (%) by NCI-H295R cell line in culture after 48 h of NiCl_2 exposure. Each point represents the arithmetic mean (\pm S.D.) testosterone % of (untreated) controls (Ctrl) determined of three repeated experiments. The number of replicate wells was 6-10 at each point per experiment. The statistical difference between the values of Ctrl and treated cells was indicated by asterisks *** ($P < 0.001$); ** ($P < 0.01$) and * ($P < 0.05$) (One-way ANOVA with Dunnett's multiple comparison test).

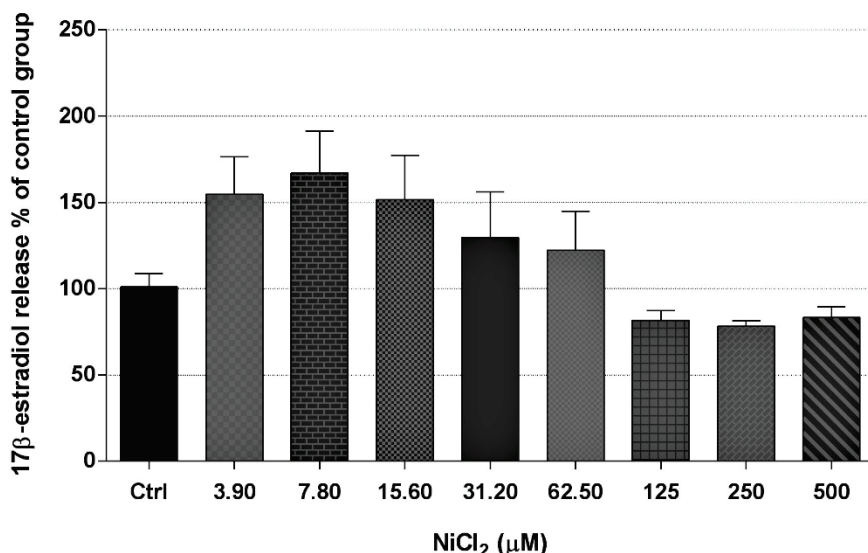


Fig. 5. 17β-estradiol release (%) by NCI-H295R cell line in culture after 48 h of nickel chloride (NiCl₂) exposure. Each point represents the arithmetic mean (± S.D.) 17β-estradiol % of (untreated) controls (Ctrl) determined of three repeated experiments. The number of replicate wells was 6-12 at each point per experiment. No statistical difference between the values of Ctrl and treated cells was recorded (P>0.05) (One-way ANOVA with Dunnett's multiple comparison test).

Table 3. Effect of 48 h NiCl₂ exposure on the release of 17β-estradiol (pg/ml) by human adrenocortical carcinoma (NCI-H295R) cell line.

Groups	Control	3.90	7.80	15.60	31.20	62.50	125	250	500
	Ctrl	H	G	F	E	D	C	B	A
	NiCl ₂ (μM)								
<i>X</i>	1.10	1.69	2.08	1.66	1.42	1.34	0.89	0.86	0.91
<i>Minimum</i>	0.74	0.94	1.02	0.80	0.84	0.94	0.64	0.76	0.51
<i>Maximum</i>	1.68	2.88	2.57	2.39	2.78	2.51	1.30	1.05	1.06
<i>±S.D.</i>	0.29	0.67	0.57	0.68	0.71	0.60	0.20	0.09	0.18
<i>CV (%)</i>	26.32	39.86	27.57	41.23	49.90	45.05	21.87	10.49	20.35
<i>%</i>	100.00	154.70	167.00	151.70	129.60	122.40	81.73	78.37	83.21

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.

cell line suggests a direct action of NiCl₂ 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 NiCl₂ 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 NiCl₂ (P<0.05). The cytotoxic effect of NiCl₂ (<50 %) was evident (P<0.01) in the group with the highest concentration (500 μM) of NiCl₂. These results clearly confirm reports of Forgacs *et al.* (2011) and Ocztos *et al.* (2011), who observed similar effects of Ni²⁺, Hg²⁺ and Cd²⁺ on the release of progesterone and testosterone by NCI-H295R cell line. Using primary gonadal culture, these authors also confirmed that Ni²⁺ 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 NiCl₂. Revesz *et al.* (2004) previously exposed human ovarian granulosa cells (obtained from women undergoing *in vitro* fertilization) to 15.60 to 1000 μM of Ni²⁺ for 48 h in order to determine the site of action of Ni²⁺. 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 Ni²⁺ 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 monooxygenases, 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 17 β -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 (3 β -hydroxysteroid dehydrogenase and 17 β -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 $\mu\text{mol/l}$) 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 Forgács *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 20 α -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 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 17 β -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 17 β -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 (ER α) (Darbre 2006, Forgacs *et al.* 2012). Martin *et al.* (2003) examined the ability of metal ions to activate ER α 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 ER α 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 ER α . The estrogenic potency of Ni was comparable to that of estradiol.

Moreover, the cytotoxic effect of NiCl₂ (<50 %) was evident (P<0.01) in the group with the highest concentration (500 μ M/ml) of NiCl₂ used in the study. The cell viability remained relatively unaltered up to 125 μ M (P>0.05) and slightly decreased from 250 μ M of NiCl₂ (P<0.05). Ng and Liu (1990) noted that Ni (1.0; 10.0 and 100 μ M of NiCl₂.6H₂O) and other metals tested (including PbCl₂, ZnCl₂, AlCl₃, CrCl₃, FeCl₂ and LiCl) had no deleterious effect on viability and hormone-induced steroidogenesis of Leydig cells and the cells in the adrenal gland.

Conclusions

The results of the present study indicate the endocrine disruptive effect of NiCl₂ on the release of sexual steroid hormones (progesterone and testosterone)

in the human adrenocortical carcinoma (NCI-H295R) cell line even at low (minimum) concentrations. Testosterone release seemed more vulnerable whereas no detrimental effect of NiCl₂ could be seen at concentrations \leq 62.50 μ M of NiCl₂ on 17 β -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 NiCl₂ 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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