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The potential impact of 4-octylphenol on the basal and stimulated testosterone formation by isolated mice Leydig cells

Účinok 4-octylphenolu na bazálnu a stimulovanú produkciu testosterónu myších Leydigových buniek

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Abstract

Octylphenol is biodegradation product of alkylphenolethoxylates frequently used in detergents, paints and other industrial applications. This compound is classified as an endocrine disruptor. Recent studies have hypothesized that occupational exposure to octylphenol poses adverse effects on reproductive system of humans and wildlife species. Enzymes involved in the steroid biosynthesis pathway are really sensitive targets for the action of various endocrine-disrupting chemicals. Aim of *in vitro* study was determined the effect of 4-octylphenol on basal and human chorionic gonadotropin stimulated testosterone formation of ICR mice Leydig cells. On the other hand, was classified potential impact of mentioned endocrine disruptor on Leydig cell viability after 44 h of cultivation. Cell suspension was cultured with addition of 0.04; 0.2; 1.0; 2.5 and 5.0 $\mu g*mL^{-1}$ of 4-octylphenol and compared to the control. Hormone quantification from the medium was performed by enzyme-linked immunosorbent assay. Viability of cell suspension was determined by the metabolic activity assay. Unstimulated testosterone production significantly ($P<0.001$) increased with 2.5 and 5.0 $\mu g*mL^{-1}$ 4-octylphenol. Cell viability was also significantly ($P<0.001$; $P<0.05$) stimulated by 4-octylphenol. Although human chorionic gonadotropin stimulated testosterone secretion was significantly ($P<0.05$) affected by the lowest concentration (0.04 $\mu g*mL^{-1}$) in the cell viability was recorded significantly ($P<0.001$; $P<0.05$) higher mitochondrial activity (1.0; 2.5 and 5.0 $\mu g*mL^{-1}$). Considerably more detailed and systematic research in this area is required for a better understanding of potential risk to humans or animals.

Keywords: Leydig cells, mice, octylphenol, testosterone

Abstrakt

Octylphenol je biodegradačný produkt alkylphenol-ethoxylátov, ktorý je široko využívaný v detergentoch, farbivách a ďalších industriálnych produktoch. Zároveň je klasifikovaný ako endokrinný disruptor. Nedávne štúdie poukazujú na fakt, že pôsobenie octylphenolu vyvoláva negatívne účinky v reprodukčnom systéme ľudí a zvierat. Enzýmy, ktoré sú zapojené do procesu biosyntézy steroidov sú veľmi citlivé na pôsobenie endokrinných disruptorov. Táto *in vitro* štúdia bola zameraná na sledovanie účinku 4-octylphenolu na bazálnu a hCG stimulovanú produkciu testosterónu u ICR myších Leydigových buniek. Rovnako bol klasifikovaný účinok spomínaného endokrinného disruptora na viabilitu týchto buniek po 44 hodinovej kultivácií. Bunková suspenzia bola kultivovaná s prídavkom 0,04; 0,2; 1,0; 2,5 a 5.0 $\mu g*mL^{-1}$ 4-octylphenolu a porovnávaná s kontrolou. Kvantifikácia množstva testosterónu bola vykonávaná priamo z média pomocou imunoanalýzy. Pre stanovenie viability Leydigových buniek bol využitý MTT test. Bazálna produkcia testosterónu bola signifitantne ($P<0.001$) zvýšená v koncentráciách 2,5 a 5,0 $\mu g*mL^{-1}$ 4-octylphenolu. Pri sledovaní životaschopnosti bol zaznamenaný signifikantný ($P<0.001$; $P<0.05$) nárast v koncentráciách 1,0 ; 2,5 a 5,0 $\mu g*mL^{-1}$ 4-octylphenolu v porovnaní s kontrolou. Stimulovaná produkcia testosterónu pomocou hCG spolu s pôsobením 4-octylphenolu spôsobila siginifikantý nárast produkcie v najnižšej koncentrácií testovanej látky. V prípade bunkovej viability bola zaznamenaná signifikantne($P<0.001$; $P<0.05$) vyššia mitochondriálna aktivita exponovaných buniek (1,0; 2.5 a 5.0 $\mu g*mL^{-1}$). Ďalší systematický výskum v oblasti pôsobenia endokrinných disruptorov považujeme za veľmi potrebný pre lepšie pochopenie potenciálnych rizík pre ľudí a zvieratá.

Kľúčové slová: Leydigove bunky, myši, octylphenol, testosterón

Introduction

Recent studies have hypothesized that environmental exposure to endocrine-disrupting-chemicals has adverse effects on the reproductive system of humans and various wildlife species. The current definition of endocrine disruptors is an exogenous substance of mixture that alters functions of the endocrine system and consequently causes negative effects in an intact organism or its progeny or subpopulations (McPhaul et al., 1993). Endocrine disruptors play a critical role in modulating the steroidogenic function of the male gonads. Disruption of androgen biosynthesis and action of endocrine disrupting chemicals may inhibit critical cellular processes. In recent years, the link between endocrine disruptorswas evaluated and the increased incidence of male reproductive dysfunctions including decline in semen quality, a rise in the incidence of cryptorchidism and increase in the incidence of testicular cancer (Toppari et al., 1996). The group of known endocrine disruptors is extremely heterogeneous. One of the common environmental contaminant is 4-tert-octylphenol. It is environmentally persistent degradation product of alkylphenol-ethoxylates. It is a class of nonionic surfactants widely used in pesticides, detergents, paints, plastics and industrial applications. The highest concentrations of

alkylphenols appear to be found in water ways receiving industrial or municipal waste water (Naylor et al., 1992). Octylphenol is listed as a typical endocrine disruptors with estrogenic action. Octylphenol is accumulates in the adipose tissue on the basis of hydrophobic character of this substance. Half life of octylphenol in the environment is estimated as 5 days and in testes is accumulated even18 hours post administration (Madsen et al., 2006). Testes are complex organs containing different cell types that differ morphologically and functionally. Endocrine secretory elements such as Sertoli and Leydig cells are main part of these organs. Leydig cells are sensitive and essential part of reproductive system. They play critical role in the development and function of the testis (Stoklosowa, 1982). The main function of Leydig cells is the production and secretion of androgens. Testicular level of these hormones depends on the capacity of Leydig cells to produce steroids and on the number of the cells present in the interstitium (Teerds and Rijntjes, 2007). It has been shown that the postnatal development of Leydig cells in rodents follows a specific pattern: mesenchymal cells will proliferate into progenitor cells, followed by newly formed adult cells. These cells are appropriate as a model system for steroidogenic studies (Mendis-Handagama and Ariyaratne, 2001).Possible routes by which octylphenol can affect reproductive parts: (i.) an alteration of the hypothalamic -pituitary function, (ii.)4-tert-octylphenol has been reported to mimic the effects of estrogen – like activity in both *in vitro* and *in vivo* model system. It was demonstrated the ability to bind and activate the estrogen receptor , (iii.) the direct disruption of cells in testes, including the germ cells, Sertoli cells and Leydig cells (Nimrod and Benson, 1996). It is not clear so far through which pathway octylphenol affects reproductive parameters. In experimental studies on animal models diverse effects of octylphenol have been obtained depending on the choice of animal species, routes of administration and doses. In adult rats, chronic exposure to octylphenol was reported decrease testis size and sperm numbers. Lower concentrations of luteinizing and follicle-stimulating hormones were demonstrated after octylphenol exposure (Boockfor and Blake, 1997). Reports on the effects of lower doses of octylphenol administration on male reproductive system are contradictory (Bian et al., 2006; Kim et al., 2007). In cultured Leydig cells, octylphenol inhibited human chorionic gonadotropin (hCG) stimulated testosterone formation. On the other hand, in the recent study, it was evaluated that exposure of cultured Leydig cells from young adult rats to octylphenol alone for 4 h increased testosterone levels approximately two-fold above control levels (Murono et al., 2001). Little is known about the degree of exposure of human and other mammals to octylphenol. Estimated daily intake is up to 300 $pg.kg^{-1}$ body weight (Lee et al., 2002). Collectively, these studies suggest that octyplhenol induces abnormities in male reproductive system. Using an *in vitro* mice Leydig cell model, was examined the potential impact of 4-octylphenol on basal and hCG– stimulated testosterone production and cell viability.

Materials and methods

Animals

ICR mice (Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dobrá voda, Slovak Republic) 35-45 g of weight and 9-10 weeks of age were kept in plastic cages and allowed free access to standard laboratory pellets and tap water. The animals were housed in a temperature controlled room (23±1°C) with 12h : 12h light-dark cycle.

Isolation of mice interstitial cells

Mice (interstitial) Leydig cells can be isolated without enzyme treatment or by trypsinization. Interstitial cells were isolated by mechanical dissociation without enzyme treatment as previously described by Stoklosowa (1982) with slight modification. In brief, mice were sacrificed and the testes were immediately removed and put into sterile Petri dish containing a small amount of minimum essential medium (MEM, Live Technologies, Bratislava, Slovak Republic). Twenty-six testes were carefully decapsulated using tweezers and placed on a sieve (of about 300 μm opening size) over a beaker. All next steps were performed under sterile conditions. With the help of a 10 mL syringe and needle (Luer 22G), interstitial cells were rinsed out with a vigorous stream of MEM without serum to beaker placed on ice. The cell suspension was subsequently collected and centrifuged. After centrifugation (300 x g, for 10 min, 4°C) the cells were washed twice and resuspended. Subsequently the cell suspension was adjusted with culture medium (MEM) supplemented with 10% fetal bovine serum (FBS; BiochromAG, Berlin, Germany), 100 $IU*mL^{-1}$ penicillin and 100 $\mu g*mL^{-1}$ streptomycin (Sigma – Aldrich, Bratislava, Slovak Republic) to a final concentration of 10^6 cells per mL. The cells were seeded into sterile 24-well plates (TPP, Switzerland). Then were treated for 44h in MEM medium that contained different doses of 4-octylphenol (4-OP; Sigma- Aldrich, Bratislava, Slovak Republic), 0.04; 0.2; 1.0; 2.5 and 5.0 $\mu g*mL^{-1}$. Cells destined for the determination of testosterone production were cultured in the presence of hCG (human chorionic gonadotropin, Pharmacy Max, Nitra, Slovak Republic) or without any stimulation – basal. All incubations were carried out at 34°C under a humidified atmosphere of 95% air and 5% CO_2. After cell treatment the media were removed and frozen at -20°C until testosterone determination. The resulting cell suspension was used for cell viability assessment.

Quantification of basal testosterone production

After 44h relative treatment with 4-octylphenol, the medium was collected, immediately centrifuged at 300 x g, 4°C for 10 minutes. The supernatant was stored at -20°C. The concentrations of testosterone in the media were determined by ELISA kits (Testosterone Cat. # K00234, Dialab, Austria).The absorbance was measured at 450 nm by an ELISA reader (Multiscan FC, ThermoFisher Scientific, Vantaa, Finland).

Quantification of hCG-stimulated testosterone production

After exposure to different concentrations of 4-octylpehnol for 44h, medium was collected and centrifuged at 300 x g, 4°C for 10 minutes. The supernatant was transferred to eppendorf tubes and stored at -20°C until testosterone detection. The concentration of testosterone from the culture medium was evaluated using the ELISA kits (Testosterone Cat.#K00234, Dialab, Austria) according to the instructional manual. The absorbance was measured at 450 nm by an ELISA reader (Multiscan FC, ThermoFisher Scientific, Vantaa, Finland).

Cytotoxicity evaluation

The viability of Leydig cells exposed to 4-octylhpenol *in vitro* was evaluated by the metabolic activity (MTT) assay (Mosmann et al., 1983).This colorimetric assay measures the conversion of a yellow water-soluble tetrazolium salt (3-(4,5 – dimetylthiazol – 2- yl)-2, 5 – diphenyl tetrazolium bromide) to purple formazan particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. The amount of formazan was measured spectrophotometrically. After removing the medium for steroid determination, Leydig cells were stained with a tetrazolium salt - (3-4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, St. Louis, USA) and the plates were inserted into a CO_2 incubator. After 3h of incubation (34°C, humidified atmosphere of 95% air and 5% CO_2) the reaction was stopped with 1 mL per well of isopropanol (2-propanol, p. a. CentralChem, Bratislava, Slovak Republic). The optical density was determined at a measuring wavelength of 570 – 620 nm by an ELISA reader (Multiscan FC, ThermoFisher Scientific, Vantaa, Finland). The data were expressed in percentage of control.

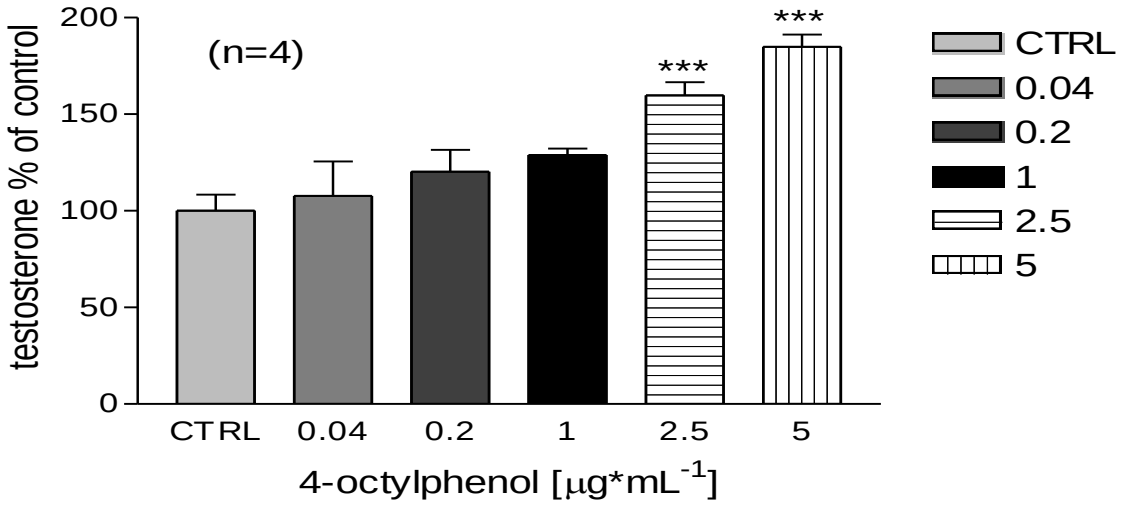
Statistical analysis

Obtained data were statistically analyzed using PC program GraphPad Prism 3.02 (GraphPad Software Incorporated, San Diego, California, USA). 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 ***($P<0.001$) ** ($P<0.01$) and * ($P<0.05$).

Results

The effect of 4-octylphenol on the basal testosterone secretion

Exposure of interstitial Leydig cells isolated from the testis of adult ICR mice were incubated with increasing concentrations of 4-octylphenol (0.04 – 5.0 $\mu g*mL^{-1}$) for 44h. As displayed in Figure 1, the testosterone formation was increased in all experimental doses of endocrine disruptor. The lowest doses (0.04; 0.2 and 1.0 $\mu g*mL^{-1}$) stimulate hormone production, but this increase was not significant. In response to 2.5 and 5.0 $\mu g*mL^{-1}$ of 4-octylpehnol, testosterone levels increased significantly ($P<0.001$), when compared to respective control (without 4-octyplhenol addition).



*** means significant difference (P<0.001)

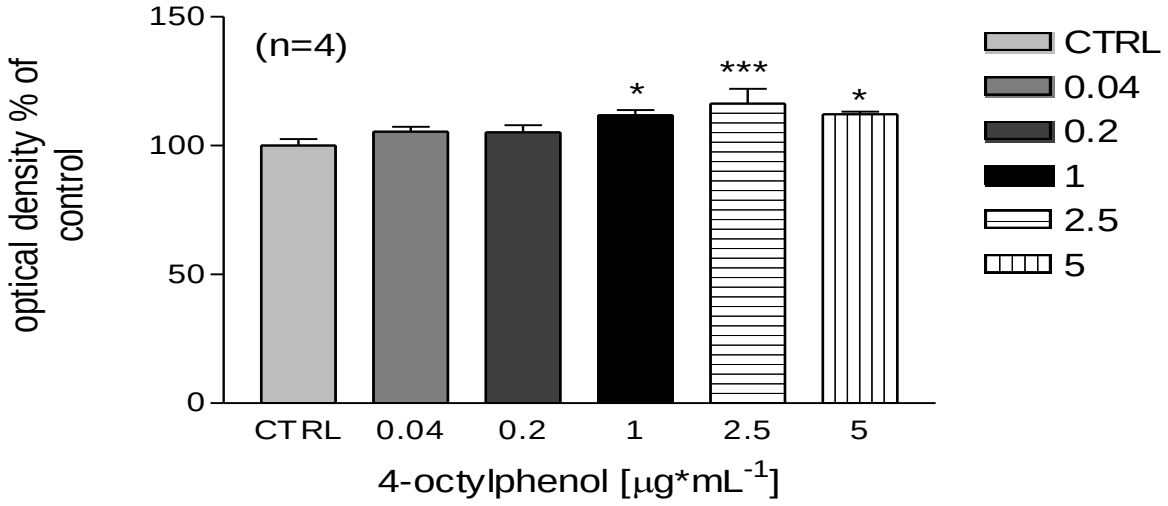
*** znamená signifikantný rozdiel (P<0.001)

Figure 1.The effect of 4-octylphenol on the basal testosterone production in mice Leydig cells after 44 hour of *in vitro* cultivation. CTRL – control group. Each bar represents the mean (±SEM) testosterone percentage of the control (untreated) and treated groups.

Obrázok 1.Vplyv 4-octylphenolu na bazálnu produkciu testosterónu myších Leydigových buniek po 44 h *in vitro* kultivácií. CTRL – kontrolná skupina. Jednotlivé stĺpce prezentujú priemernú (±SEM) produkciu testosterónu vyjadrenú v percentách a sú porovnávané s kontrolou.

The effect of 4-octylphenol on the basal cell viability *in vitro*

The viability of mice interstitial cells was detected by the MTT assay examining the mitochondrial functional activity. Figure 2 shows the impact of 4-octylphenol on the Leydig cells viability after 44h *in vitro* cultivation in the absence of hCG. In all experimental groups supplemented with 0.04-5.0 μg*mL^{-1} was recorded better viability as in control group (without 4-octylphenol treatment). In the last three concentrations (1.0; 2.5 and 5.0 μg*mL^{-1}) of test chemical was observed significant increase ($P<0.001$; $P<0.05$) of monitored parameter. Based on these facts, the concentrations of 4-octylphenol used in this study has no cytotoxicity effect after 44 hours treatment. The results also suggest that applied doses of 4-octylphenol do not damage the mitochondrial activity of mice Leydig cells.



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*** znamená signifikantný rozdiel (P<0.001)

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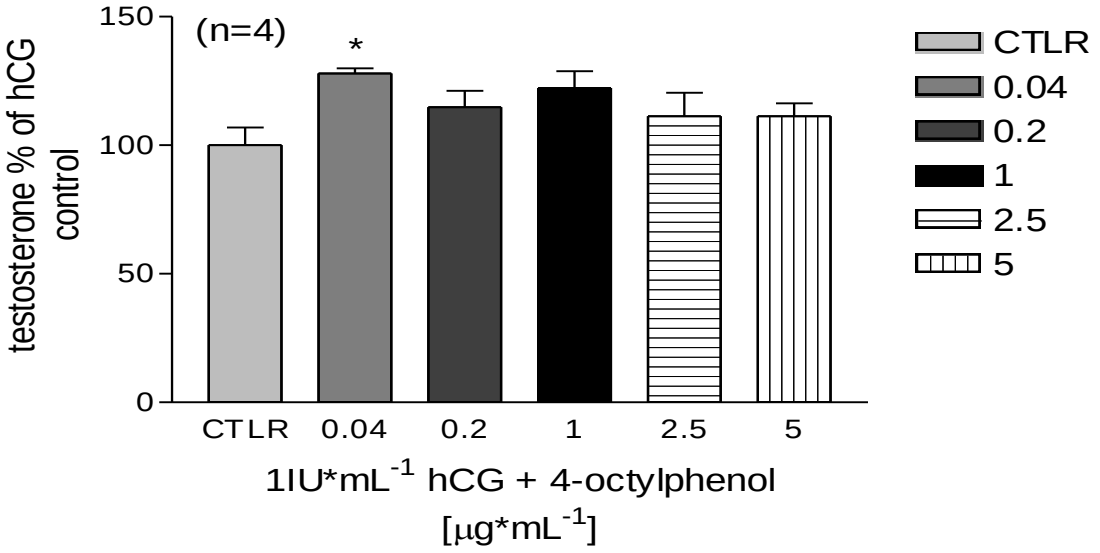
Figure 2.The effect of 4-octylphenol on cell viability after 44 hour of *in vitro* cultivation.Each bar represents the mean (±SEM) viability percentage of the control (untreated) and treated groups. CTRL- control group.

Obrázok 2. Vplyv 4-octylphenolu na životaschopnosť buniek po 44 h *in vitro* kultivácií. CTRL – kontrolná skupina. Jednotlivé stĺpce prezentujú priemernú (±SEM) životaschopnosť buniek vyjadrenú v percentách a sú porovnávané s kontrolou.

The effect of 4-octylphenol on the hCG-stimulated testosterone secretion

Interstitial Leydig cells were cultured with five different concentrations (0.04-5.0 μg*mL^{-1}) of 4-octylphenol in the presence of hCG during 44h. As seen in Figure 3, the lowest dose (0.04 μg*mL^{-1}) of endocrine substance significantly (P<0.05) increase testosterone secretion compared with control. Other experimental groups responded to 4-octylphenol and hCG stimulation by slight increase in testosterone output. However, this increase was not significant. All applied concentrations were compared with the control group (without 4-octylphenol treatment).



* means significant difference (P<0.05)

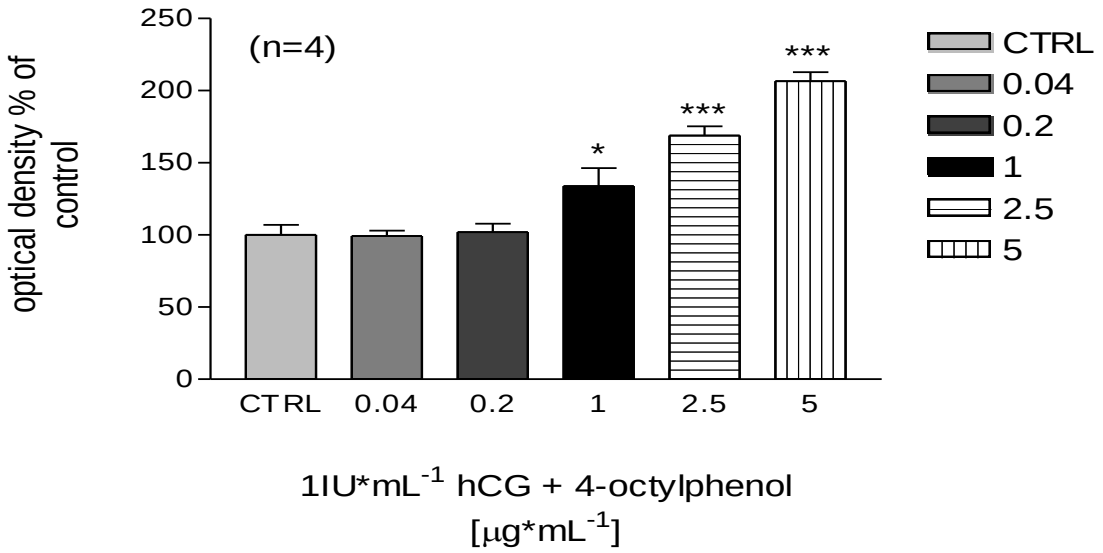
* znamená signifikantný rozdiel (P<0.05)

Figure 3.The effect of 4-octylphenol on the hCG-stimulated testosterone production in mice Leydig cells after 44 hour of *in vitro* cultivation.CTRL- control group. Each bar represents the mean (±SEM) testosterone percentage of the control (untreated) and treated groups.

Obrázok 3. Vplyv 4-octylphenolu na hCG-stimulovanú produkciu testosterónu myších Leydigových buniek po 44 h *in vitro* kultivácií. CTRL – kontrolná skupina. Jednotlivé stĺpce prezentujú priemernú (±SEM) produkciu testosterónu vyjadrenú v percentách a sú porovnávané s kontrolou.

The effect of 4-octylphenol on the hCG-stimulated cell viability *in vitro*

In order to rule out the cytotoxicity of 4-octylphenol on interstitial cells, MTT assay was performed in mice Leydig cells treated with various doses of 4-octylphenol in the presence of hCG (Figure 4). Cell viability was not reduced in any experimental groups following 44h incubation. Compared with the level of hCG group, the hCG plus 1.0 μg*mL^{-1} of 4-octylphenol group significantly (P<0.05) stimulated cell viability. Similar tendency was shown in the higher concentrations. In this case the applied doses demonstrate significant stimulation (P<0.001) of observed parameter. Mitochondrial activity of treated cells is not damaged in any concentration of test chemical.



*** means significant difference (P<0.001)

* means significant difference (P<0.05)

*** znamená signifikantný rozdiel (P<0.001)

* znamená signifikantný rozdiel (P<0.05)

Figure 4. The effect of 4-octylphenol on hCG-stimulated cell viability after 44 hour of *in vitro* cultivation. Each bar represents the mean (±SEM) viability percentage of the control (untreated) and treated groups. CTRL- control group.

Obrázok 4. Vplyv 4-octylphenolu na hCG-stimulovanú životaschopnosť buniek po 44 h *in vitro* kultivácií. CTRL – kontrolná skupina. Jednotlivé stĺpce prezentujú priemernú (±SEM) životaschopnosť buniek vyjadrenú v percentách a sú porovnávané s kontrolou.

Discussion

A lot of chemicals such as industrial products, commercial contaminants, and endocrine disruptors introduced into the environment have the potential to cause markedly cellular damage. They are able to interact with all parts of organism. One of the most sensitive part is reproductive system. Some *in vivo* and *in vitro* experiments about octylphenol are focused on their endocrine disrupting and potential adverse effects on developing reproductive system. The results of *in vitro* study indicate a dose-dependent increase in testosterone production accompanied by an increase in cell viability. The changes in unstimulated testosterone production were significant ($P<0.001$) with respect to 2.5 and 5.0 $\mu g*mL^{-1}$ of 4-octylphenol. The same tendency was observed in the case of cell viability.It has been documented that exposure to 1.0 to 500.0 nM octylphenol did not alter testosterone levels in exposed Leydig cells after 4h of cultivation. However, exposure to 2000.0 nM octylphenol significantly increased testosterone production. Similar tendency was observed after 24h treatment. Exposure to 1.0 to 100.0 nM octylphenol did not alter testosterone level, however exposure to 500.0 or 2000.0 nM octylphenol increased testosterone production (Murono et al.,1999).The results of study also demonstrate that treatment of interstitial cell suspension with experimental doses of test chemical after 44h

caused a slightly increase in hCG stimulated testosterone production. Significant differences was observed in the lowest concentration (0.04 μg*mL^{-1}) of 4-octylphenol. The rest of 4-octylpheno doses (0.2; 1.0; 2.5 and 5.0 μg*mL^{-1}) increase testosterone secretion, but not significantly. In case of cell viability was recorded significantly ($P<0.001$; $P<0.05$) higher mitochondrial activity compared with control group after 44 hours of treatment. Results of recent study evaluated the effect of octylphenol on hCG- stimulated testosterone production. After 24 hour of relative treatment was observed decrease in testosterone production in all applied doses of endocrine disruptor (1 – 2000nM) but differences were not significant (Murono et al., 2001). The conversion of cholesterol to testosterone involves four enzymatic steps: cytochrome P-450 cholesterol side – cleavage (P450scc), which converts cholesterol to pregnenolone; 3β-hydroxysteroid dehydrogenase-isomerase (3β-HSD), which converts pregnenolone to progesterone; 17α-hydroxylase/c17-20-lyase (P450c17), which converts progesterone to 17α-hydroxyprogesterone and androstenedione and 17β-hydroxysteroid dehydrogenase (17β-HSD), which converts androstenedione to testosterone. Murono´s et al. (2001) results suggest that *in vitro* octylphenol has the potential to inhibit one or more enzymatic steps involved in the conversion of cholesterol to testosterone. Although testosterone levels were not significantly reduced in Leydig cells following exposure to increasing concentrations of octylphenol (1.0 – 2000.0 nM) and hCG stimulation, the possibility that specific steroidogenic enzymes converting cholesterol to testosterone might be affected. The activity of enzymes involved in conversion of cholesterol to pregnenolone (P450scc), pregnenolone to progesterone (3β-HSD) and progesterone to androstenedione (P450c17) were decreased at the two highest concentrations (500.0 and 2000.0 nM) of octylphenol. In contrast, enzymatic activity of the conversion of androstenedione to testosterone (17β-HSD) over 4h after the initial 24h exposure to increasing octylphenol concentrations was unaffected. Previous study demonstrates that are differences in the pattern or sensitivity of testosterone response to direct octylphenol exposure of Leydig cells from animals of different ages (Picon and Gangneau, 1980). With respect to how the inhibiting effects of octylphenol on cholesterol conversion to testosterone are mediated, the possibility that octylphenol is able to generate reactive oxygen species and inhibit testosterone secretion was examined. Cytochrome P450scc and P450c17 are involved in converting cholesterol to testosterone in Leydig cells. They use molecular oxygen and electrons from NADPH for substrate hydroxylation. During normal steroidogenesis, reactive oxygen species are produced by electron leakage outside the electron transfer chains and these radicals can initiate lipid peroxidation to inactivate P450 enzymes (Hanokoglu et al., 1993; Hornsby, 1980).

Conclusions

Recent studies have hypothesized that environmental exposure to endocrine disruptors poses adverse effects on reproductive system of humans and wildlife species. Therefore the exposure to octylphenol and other substances in humans and animals must be seriously observed. The action of these substances on important structures of the reproductive system depends on the dose, cell type and many other factors. *In vitro* study was aimed to determine the potential effects of 4-octylphenol on the interstitial Leydig cells after 44h treatment. The results showed that some

experimental concentrations of endocrine disruptor may significantly stimulate hormone production and also cell viability. Previous experimental studies confirmed, that higher concentrations of 4-octylphenol are able to disrupt hormonal profile and mitochondrial activity in exposed cells. In this case, the range of applied doses is not too strong. These results together with the present findings therefore strongly suggest that applied concentrations of 4-octylphenol are not able to damage function and viability of interstitial cell suspension. Further investigations are definitely required to establish the biological significance and possible reproductive implications.

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