# Original Article

# The Impact of 4-Nonylphenol on the Viability and Hormone Production of Mouse Leydig Cells

(mice / Leydig cells / 4-nonylphenol / viability / testosterone / cyclic adenosine monophosphate / androstenedione)

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**Abstract. Exogenous substances altering the function of the endocrine system and exhibiting adverse health effects on the organism are defined as endocrine disruptors. Nonylphenol is one of the most abundant alkylphenol ethoxylate derivatives, being detected in food products. Diverse studies have classified nonylphenol as hazardous to the health, especially to male reproduction. This *in vitro* study aimed to examine the effects of 4-nonylphenol on androstenedione and testosterone production as well as on the viability of Leydig cells of NMRI mice. The cells were cultured for 44 h with addition of 0.04; 0.2; 1.0; 2.5 and 5.0 µg/ml of 4-nonylphenol and compared to the control. Quantification of testosterone and androstenedione directly from aliquots of the medium was performed by enzyme-linked immunosorbent assay. Cell viability was measured by the metabolic activity assay for mitochondrial functional activity. Androstenedione production significantly ($P < 0.001$) increased with 1.0; 2.5 and 5.0 µg/ml 4-nonylphenol. Although cAMP-stimulated testosterone production was not significantly affected by 4-nonylphenol, a tendency to attenuate the level of testosterone in the Leydig cells treated with 2.5 and 5.0 µg/ml 4-nonylphenol was observed. The viability of mouse Leydig cells was slightly increased at the lowest doses of 4-nonylphenol (0.04 and 0.2 µg/ml). We also observed an increase at higher concentrations of the substance (1.0; 2.5 and 5.0 µg/ml), but this increase was not significant. Further investigations are required to establish the biological significance and possible reproductive implications.**

## Introduction

In the last decade, research has focused on the potentially hazardous effects of a wide range of chemicals present in the human or wildlife environment. Exogenous substances having the potential to alter the endocrine integrity with subsequent adverse health effects on the organism are called endocrine disruptors (EDs). Pesticides, pollutants, heavy metals and other chemicals have the potential to jeopardize proper endocrine functions in exposed organisms (Sanderson, 2006). ED may lead to inhibition of critical cellular processes controlling steroidogenesis, as well as malformations in the male reproductive tract of both humans and animals. EDs interfere with the synthesis, metabolism or action of hormones crucial in reproductive processes. Moreover, it is likely that EDs are structural analogues of steroids, having similar effects as true hormones, high levels of which may have disproportionate consequences (Svechnikov et al., 2010). Prominent EDs are nonylphenol polyethoxylates (NPEs), which are highly cost-effective surfactants with exceptional performance, and are widely used in institutional, commercial and industrial applications. NPEs are frequently applied in pesticides, detergents, insecticides and other synthetic products (Langford and Lester, 2002). The primary final biodegradation product of NPEs is nonylphenol (NP). Due to its wide usage, a large amount of NP was discharged into the ecosystem, especially into water. Several studies have reported that NP may exhibit adverse effects on the male reproductive system, translated into reduced weight of

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Abbreviations: 4-NP – 4-nonylphenol, cAMP – cyclic adenosine monophosphate, ED – endocrine disruptor, ELISA – enzyme-linked immunosorbent assay, FBS – foetal bovine serum, HSD – hydroxysteroid dehydrogenase, MEM – minimum essential medium, MTT – metabolic activity assay, NP – nonylphenol, NPE – nonylphenol polyethoxylate, SEM – standard error of the mean.

reproductive organs, decreased spermatozoa or testosterone production, as well as testicular abnormalities. NP represents a public health concern; however, data regarding its impact on human or wildlife are very limited and its exact mechanism of action is not clear yet (Lee et al., 1999; Berryman et al., 2004).

Gonads are complex organs containing various cell types that differ morphologically and functionally. In most species, the testes are comprised of seminiferous tubules and the interstitial compartment. Both contain endocrine secretory elements such as Sertoli and Leydig cells (Stoklosowa, 1982). The Leydig cells are of about 4–20 μm in size and have a spherical shape. It has been shown that the postnatal development of Leydig cells in rodents follows a specific pattern: mesenchymal Leydig cells will proliferate into progenitor Leydig cells, followed by newly formed adult Leydig cells. Immature adult Leydig cells will eventually develop into mature adult Leydig cells. The adult type of Leydig cells originates postnatally within the mouse testis by day 56. Their formation is the result of active proliferation and differentiation of undifferentiated stem cells. Circulatory hormones as well as locally produced growth factors are shown to have important effects on the differentiation and maturation of the adult Leydig cell population, and it appears that different factors regulate each stage of the Leydig cell lineage. These cells are often used as a model for steroidogenic studies (Mendis-Handagama and Ariyaratne, 2001).

Treatment with high concentrations of NP may cause a significant decrease in the levels of testosterone and other steroid hormones. Testosterone is the most important hormone in the male reproductive system, as it is responsible for the maintenance of spermatogenesis and secondary sexual characteristics in the male (Payne and Sha, 1991; Laurenza et al., 2002). Leydig cells are known to be the primary producers of steroid testosterone in response to gonadotropic stimulation. Biosynthesis of this hormone requires specific sequential actions converting cholesterol into various steroid classes. Cytochrome P450 catalyses the cleavage of cholesterol to pregnenolone. 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β- hydroxysteroid dehydrogenase (17β-HSD) also catalyse other intermediate stages of steroidogenesis. NP may directly or indirectly affect steroidogenic enzymes or cyclic adenosine monophosphate (cAMP) required for the biosynthesis of testosterone (Payne and Sha, 1991; Thoreux-Manlay et al., 1995). Methods needed to isolate interstitial cells (Fig. 1) are significantly laborious, as the cells are particularly sensitive to mechanical intrusion and long laboratory procedures may seriously damage them.

The present *in vitro* study was conducted to evaluate the possible effect of 4-nonylphenol (4-NP) on the male reproductive system of NMRI mice, with a special emphasis on interstitial (Leydig) cells.

## Material and Methods

### *Animals*

NMRI mice (Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dobrá Voda, Slovak Republic), 30–40 g of weight and 8–9 weeks of age, were kept in a room and maintained in a 12 h light:dark cycle and 20–25 °C. The animals were kept in plastic cages

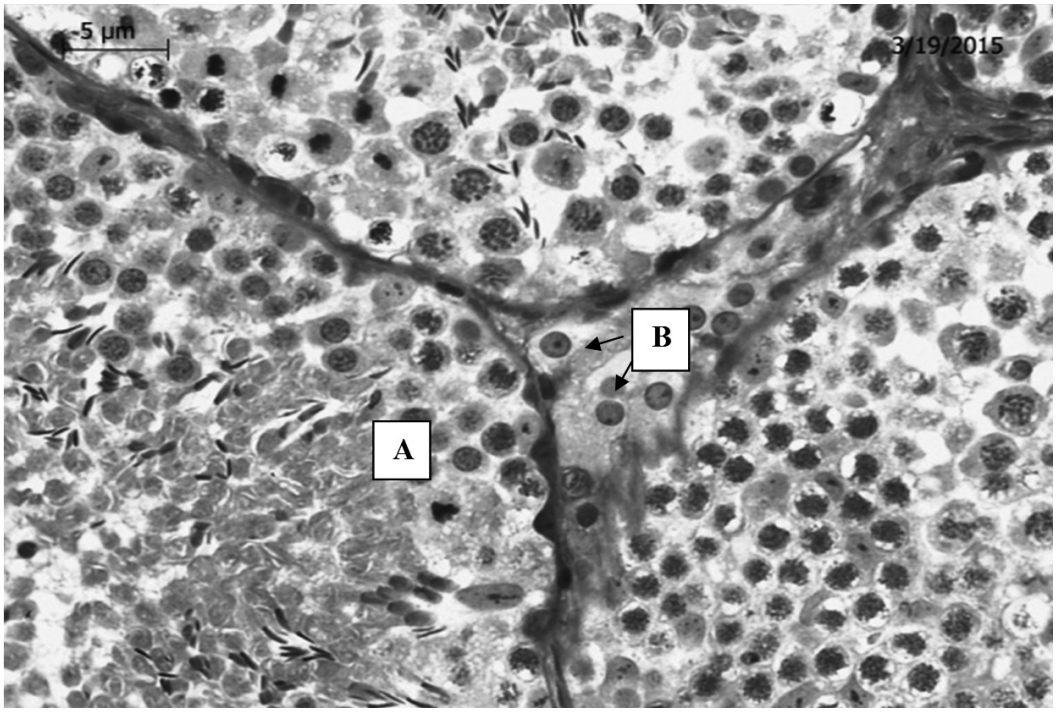


*Fig. 1.* Interstitial cells were isolated from NMRI mouse testis.
**A** – seminiferous tubule, **B** – interstitial Leydig cells, magnification 400×

and allowed free access to standard laboratory pellets and tap water.

### *Isolation of interstitial cells from the mouse testis*

Interstitial (Leydig) cells can be isolated without enzyme treatment by mechanical dissociation. We followed the protocol established by Stoklosowa (1982) with a slight modification. Mice were sacrificed following the procedure approved by Act No. 377/2012, the testes were immediately removed and transferred to a sterile Petri dish containing a small amount of minimum essential medium (MEM, Live Technologies, Bratislava, Slovak Republic). The testes were carefully decapsulated using tweezers, and placed on a nylon sieve over a beaker. All subsequent steps were performed under sterile conditions. Interstitial cells were rinsed out with a vigorous stream of MEM without serum to a beaker placed on ice. The cell suspension was subsequently collected and centrifuged. The cell suspension was centrifuged (300 × *g*, 4 °C, 10 min) interstitial cells were washed twice and resuspended. Subsequently, the cell culture was adjusted with culture medium (MEM) supplemented with 10% foetal bovine serum (FBS; Biochrom AG, Berlin, Germany), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich, Bratislava, Slovak Republic) to a final concentration of $10^6$ cells/ml. Leydig cells were cultured in sterile 24-well culture plates (Nunclon, Denmark) adjusted to a final volume of 500 µl/well. After seeding the cell suspension, different concentrations (0.04; 0.2; 1.0; 2.5 and 5.0 µg/ml) of 4-NPl (Fluka, Buchs, Switzerland) were administered to the culture. Cells destined for the determination of testosterone production were cultured in the presence of cyclic AMP solution (db-cAMP, Sigma-Aldrich, Bratislava, Slovak Republic). All interstitial cells were incubated for 44 h at 34 °C under a humidified atmosphere of 95% air and 5% $CO_2$. After respective cell treatments, the media were removed and frozen at -20 °C for subsequent hormone assays. The resulting cell suspension was used for cell viability assessment.

### *Quantification of androstenedione*

Determination of androstenedione directly from aliquots of the culture media was performed by enzyme-linked immunosorbent assay (ELISA). The ELISA kits were purchased from Dialab (Androstenedione Cat. #K00197, Wiener Neudorf, Austria). Absorbance was measured at 450 nm.

### *Quantification of cAMP-stimulated testosterone secretion*

The testosterone level in the incubation medium was determined by ELISA. The ELISA kits were purchased from Dialab (Testosterone Cat. #K00234). Absorbance was measured at 450 nm.

### *Cytotoxicity evaluation*

Viability of the Leydig cells exposed to 4-NP was evaluated by the metabolic activity (MTT) assay. After removing the medium for steroid measurements, Leydig cells were stained with a tetrazolium salt ((3-4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide – MTT) and the plates were inserted into a $CO_2$ incubator. After 3 h of incubation (34 ºC, humidified atmosphere of 95% air and 5% $CO_2$), the reaction was stopped with 1 ml/well of isopropanol. The optical density was determined at a measuring wavelength of 570 nm against 620 nm by an ELISA reader (Multiscan FC, ThermoFisher Scientific, Vantaa, Finland).

### *Statistics*

The data were statistically analysed using GraphPad Prism 3.02 (GraphPad Software Incorporated, San Diego, CA). Descriptive statistical characteristics (minimum, maximum, standard error, mean, etc.) were evaluated at first. One way analysis of variance (ANOVA) with Dunnett's post test was 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-NP on androstenedione production*

Interstitial (Leydig) cells isolated from the testis of adult laboratory NMRI mice were cultured with five different concentrations of 4-NP for 44 h. This substance had dose-dependent effects on androstenedione production. As seen in Fig. 2, the androstenedione production was significantly ($P < 0.001$) increased in experimental groups supplemented with 1.0–5.0 µg/ml of 4-NP. All

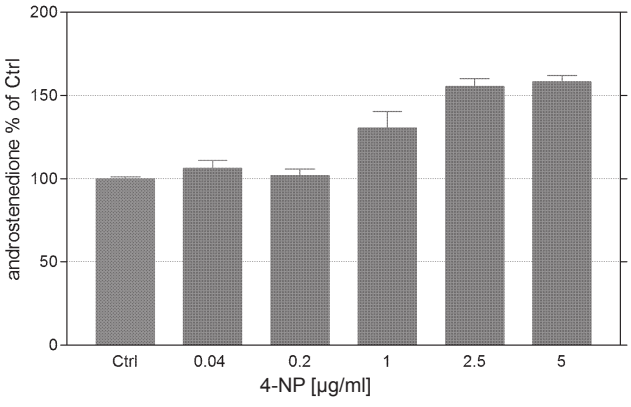


*Fig. 2.* The effects of 4-NP on androstenedione production after 44 h of *in vitro* cultivation
Each bar represents the mean (± SEM) androstenedione percentage of the control (untreated) and treated groups. The level of significance was set at *** ($P < 0.001$), ** ($P < 0.01$), * ($P < 0.05$). Ctrl – control group

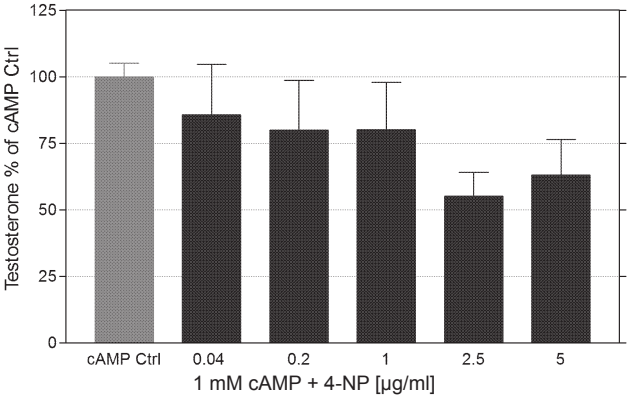


*Fig. 3.* The effects of 4-NP on cAMP-stimulated testosterone production after 44 h of *in vitro* culture
Each bar represents the mean (± SEM) testosterone percentage of the control (untreated) and treated groups. The level of significance was set at *** ($P < 0.001$) ** ($P < 0.01$, * ($P < 0.05$). Ctrl – control group

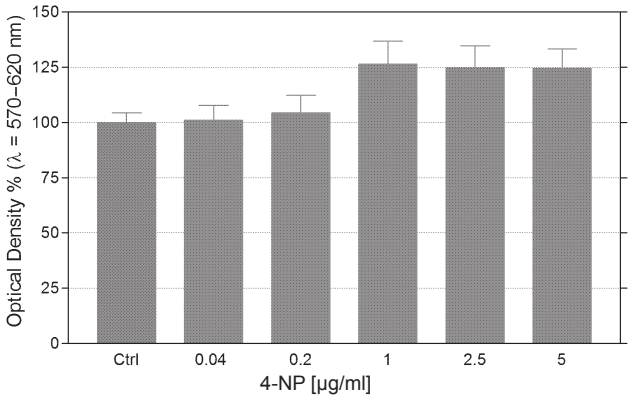


*Fig. 4.* The effect of 4-NP on cell viability after 44 h of *in vitro* cultivation
Each bar represents the mean (± SEM) viability percentage of the (untreated) control and treated groups. The level of significance was set at *** ($P < 0.001$), ** ($P < 0.01$), * ($P < 0.05$). Ctrl – control group

applied concentrations were compared with the control group (without 4-NP addition).

### *The effect of 4-NP on cyclic AMP-related testosterone secretion*

We found that 4-NP had a dose-dependent effect on the testosterone production in mouse Leydig cells. All five experimental groups (groups treated with 4-NP) responded to cAMP stimulation by decreasing the testosterone output. As seen in Fig. 3, testosterone production was decreased in the experimental groups supplemented with 0.04–5.0 µg/ml 4-NP in comparison with the control (without 4-NP treatment). However, this decrease was not significant.

### *The effects of 4-NP on cell viability*

The viability of mouse Leydig cells was detected by the MTT assay examining the mitochondrial functional activity. Figure 4 shows the impact of 4-NP on the Leydig cell viability after 44 h *in vitro* cultivation. Higher cell viability was recorded in the experimental groups supplemented with 1.0–5.0 µg/ml. However, this increase was not significant. The results suggest that lower doses of 4-NP do not damage the mitochondrial activity of mouse Leydig cells.

## Discussion

Various chemicals such as commercial products, factory contaminants, and EDs introduced into the environment have the potential to cause substantial damage to the whole organism. NP, an endocrine disruptor, has been reported to induce reproductive abnormalities and dysfunctions in males (Chapin et al., 1999; Soares et al., 2008). Based on these facts, our study focused on reproductive structures that could be affected by 4-NP. Leydig cells as one of the most interesting testicular cellular structures were incubated with different concentrations of 4-NP (0.04; 0.2; 1.0; 2.5 and 5.0 µg/ml) for 44 h. The results of our *in vitro* study indicate a dose-dependent increase in androstenedione production accompanied by an increase in cell viability. The changes in androstenedione production were significant ($P < 0.001$) with respect to 1.0; 2.5 and 5.0 µg/ml of 4-NP. A similar situation was observed in the case of cell viability. The results also indicate that treatment of interstitial cell suspension with experimental concentrations of 4-NP after 44 h caused a decrease in cAMP-stimulated testosterone production, compared with cells treated with cAMP alone (control). However, the differences were not significant.

In our *in vitro* study, androstenedione production was significantly increased at lower concentrations (1.0; 2.5 and 5.0 µg/ml) of 4-NP after 44 h cultivation. Several studies have expounded effects of NP on steroidogenesis of Leydig cells with an increase in production at low concentrations and a decrease at high concentrations (Murono et al., 1999; Wu et al., 2010). It has been documented that higher doses of 4-NP (5.0; 10.0 and 20.0 µmol/l) inhibit the level of testosterone in Leydig cells of experimental groups (Ying et al., 2012). A recent study has also indicated that lower concentrations of 4-NP lead to increased hormonal production in mouse Leydig cells. Testosterone production was significantly increased at the doses of 1.0; 2.5 and 5.0 µg/ml of 4-NP (Jambor et al., 2015). These findings are supported by the following *in vitro* study where the primary Leydig cells were exposed to NP for 48 h, including low concentrations (0.0011 mg/l to 0.022 mg/l) and high concentrations (0.11 mg/l to 5.5 mg/l). The increase of testosterone was observed at low concentrations of NP, while its reduction was detected at higher concentrations (Gong and Han, 2006). The same tendency was observed in our *in vitro* experiment. We may assume that applying higher concentrations of 4-NP in our experiment may have reduced androstenedione production

after 44 h of cultivation. This can be partially explained by activation (lower doses) or inhibition (higher doses) of the key enzymes required for the androstenedione biosynthesis in mouse Leydig cells. It is known that steroidogenesis in Leydig cells is characterized by sequential actions converting cholesterol into different steroid classes during which 3β-HSD, CYP11A1 and StAR are responsible for the rate-limiting steps (Lavoie and King, 2009). Based on our results we may assume that the applied concentrations of 4-NP are not able to affect expression of genes involved in the androstenedione production.

Our study also demonstrated that different doses of 4-NP affected cAMP-stimulated testosterone production in mouse Leydig cells after 44 h cultivation. We found a decrease in testosterone production, but this decrease was not significant. On the other hand, Laurenzana et al. (2002) suggested the impact of NP on the activity of P450c17, indispensable in steroidogenesis, with a subsequent alteration in testosterone levels. Another study has shown that significant inhibition was observed in the testosterone release in cells treated with some NP concentrations (4.25 and 127.5 μM) in response to 8-Br-cAMP. Administration of 8-Br-cAMP alone stimulates the testosterone release. In contrast, the stimulatory effect was reduced in the presence of NP. We observed the same situation in our *in vitro* study. The mechanism by which NP modulates steroidogenesis has not been well defined, but a number of *in vitro* studies have shown that many compounds may directly or indirectly affect the enzymes required for the biosynthesis of testosterone in Leydig cells, including $P450_{scc}$, P450c17, 3β-HSD and 173β-HSD (Thoreux-Manlay et al., 1995). Wu et al. (2010) found that activities of some important enzymes in testosterone biosynthesis were inhibited by exposure to NP. These findings further implied that the combination of cAMP and 4-NP displayed diverse mechanisms of effect on inhibiting testosterone production. In our *in vitro* experiment we found that different doses of 4-NP affected the Leydig cell viability after 44 h of cultivation. Some concentrations (0.2; 1.0; 2.5 and 5.0 μg/ml) of 4-NP increased the cell viability, but not significantly.

Mitochondria could be damaged by different environmental compounds and chemicals including NP. Wu et al. (2010) treated rat Leydig cells with different concentrations (42.5; 127.5 and 425.0 μM) of 4-NP. The highest dose (425.0 μM) exhibited a cytotoxic effect. Another study presented significant alterations in the Leydig cell viability at concentrations higher than 10 μmol/l, suggesting that the response was partly caused by the toxicity of NP isomers (Ying et al., 2012). Han et al. (2004) also reported that NP exposure resulted in testicular degeneration and increased cell apoptosis in a dose-dependent manner. Based on our results we suppose that the applied concentrations of 4-NP were too low for initiating a response and changes in cell viability. With higher doses of the endocrine disruptor, decreased Leydig cell viability can be demonstrated in the same period of cultivation. We concur with Yao et al. (2007), who investigated the effects of NP on the mitochondrial membrane potential and confirmed that the mitochondrial permeability transition was an important step in the induction of cellular apoptosis.

### *Conclusion*

The interest to study the interactions between endocrine disruptors and the reproductive system has continued to increase over the last decade. The action of these substances on important structures of the reproductive system depends on the dose, cell type and other factors. Our *in vitro* study was aimed to determine the effects of 4-NP on the interstitial (Leydig) cells after 44 h treatment. We can conclude that some experimental concentrations may significantly increase androstenedione production; however, the testosterone production stimulated by cAMP was decreased in all applied concentration of this endocrine disruptor. In the case of cell viability, a non-significant increase was observed. Further investigations are definitely required to establish the biological significance and possible reproductive implications.

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