Animal welfare, etológia és tartástechnológia

Animal welfare, ethology and housing systems

Volume 9 Issue 3

Különszám/Special Issue

Gödöllő

2013

THE EFFECT OF AN *IN VITRO* EXPOSURE TO OCTYLPHENOL ON BOVINE SPERMATOZOA

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Abstract

Octylphenol (OP) is an endocrine disruptor, that can affect male reproductive system and induce reproductive abnormalities, such as a structural alterations of testes, epididymis and prostate, a lower of spermatozoa count and motility. The aim of the present study was to investigate the dose- and time-dependent effect of octylphenol (1, 10, 100 and 200 µg/mL) dissolved in 1% ethanol on the spermatozoa motility during several time periods (0 h, 2 h, 4 h and 6 h) and superoxide formation after 6 h of *in vitro* cultivation. The spermatozoa motility was determined by CASA (Computer Assisted Semen Analyzer) system using the Sperm Vision^{TM} program. The following parameters were evaluated: percentage of motile spermatozoa (motility > 5 µm/s) and percentage of progressive motile spermatozoa (motility > 20 µm/s). The nitroblue-tetrazolium (NBT) test was used to assess the intracellular superoxide production.

The results from CASA system showed the decreased spermatozoa motility in all experimental groups with the addition of OP during all time periods. Significant differences (P<0.001 and P<0.05) between the control groups and the experimental groups were recorded. The NBT test revealed that at the dose 1 µg/mL occurred the slightly decrease of superoxide production compared to the control group. Conversely, the higher doses 10, 100 and 200 µg/mL of OP increased intracellular superoxide production in the bovine spermatozoa. Significant differences (P<0.05 and P<0.001) were observed between the groups C and D containing the highest doses of OP in comparison to the control group.

In conclusion, the results from our *in vitro* experiments confirm that the high doses of octylphenol have the negative effect on bovine spermatozoa motility and can generate the increased intracellular superoxide production causing oxidative stress.

Keywords: endocrine disruption, octylphenol, spermatozoa motility, CASA system, NBT test

Introduction and Literature survey

Alkylphenols (APs) and their metabolites are lipophilic substances exerting apparent estrogenic action in *in vitro* and *in vivo* testing system. With the widespread industrial use of alkylphenols, these are disseminated in the environment with sewage sludge. Alkylphenols can accumulate in vivo, at least in fish (*Shiraishi et al*, 1989). Domestic animals and humas are likely to exposed via the food chain.

Octylphenol (OP) is a ubiquitous chemical in the environment. Octylphenol is an alkylphenol that is an important intermediate in the production of a number of commercial materials. The major use of OP is for the production of alkylphenol ethoxylates (APEs), a class of nonionic surfactants with a wide range of applications (*White et al*, 1994; *Pocar et al*, 2003). APEs are commonly found in industrial processing and in household and institutional cleaning products (*Ying et al*, 2002; *Qian et al*, 2006).

Exposure to OP is matter of concern because it is both estrogenic and toxic to cells (*Blake et al*, 2004; *Calafat et al*, 2008).

4-*tert*-octylphenol can affect invertebrates, amphibians and fish, possibly by endocrine disrupters through an estrogen mediated mode of action, with some evidence of thyroidal activity in amphibians (*ECETOC*, 2009, *Evans et al*, 2011).

4-tert-octylphenol accumulation was observed in liver, muscle and plasma up to 12 h whereas in testis 18 h post administration (*Madsen et al*, 2006).

OP is more persistent than its parent compounds and can mimic naturally produced estrogen by interacting with estrogen receptors (*Blake and Ashiru*, 1997).

OP is the most potent estrogenic alkylphenol i*n vitro* and displaying approximately 1/1000 the extragenicity of the potent estrogen 17β-estradiol (E_2) (*White et al*, 1994).

In male, OP can act as a „xenoestrogen“ to disrupt testicular development and fertility (*Hossaini et al*, 2001). OP can reduce the size and weight of the rat testis (*Kim et al*, 2004; *Bian et al*, 2006; *Chen et al*, 2006), epididymis, and prostate (*Chen et al*, 2006), can decrease sperm count of rats (*Bian et al*, 2006; *Herath et al*, 2004) and rams (S*weeney et al*, 2007), can lower daily sperm production (*Vom Saal and Hughes*, 2005; *Bian et al*, 2006) and spermatozoa motility of rats (*Bian et al*, 2006; *Gregory et al*, 2009).

Octylphenol can generate reactive oxygen species (ROS) which are cytotoxic agents that lead to significant oxidative damage by attacking biomolecules such as membrane lipids and DNA in cells (*Kabuto et al*, 2003). When ROS are generated in living systems, a wide variety of antioxidants have a role to reduce the effects of oxidative stress. Antioxidants neutralize ROS by donating one of their own electrons, ending the electron-"stealing" reaction. They act as scavengers, helping to prevent cell and tissue damage that could lead to cellular damage and disease (*Halliwell*, 1996). But, when the excessive production of ROS for any reason, the oxidative damage occurs in the cells. Many environmental contaminants reported to disrupt the prooxidant/antioxidant balance of cells by inducing oxidative stress (*Ho et al*, 1998). One of these contaminants is OP that caused tissue injury in the liver, kidneys, brain, and other organs by leading to formation of ROS (*Bindhumol et al*, 2003; *Kabuto et al*, 2004; *Chitra et al*, 2003). Octylphenol can also cause oxidative stres and damage rat testes (*Aydogan et al*, 2010).

OP can cause apoptosis of testicular germ cells (*Zhou* e*t al*, 2001; *Kim et al*, 2004) and Sertoli cells (*Qian et al*, 2006).

The objective of this *in vitro* study was to investigate the effect of various concentrations of octylphenol dissolved in 1% ethanol during several time periods (0 h, 2 h, 4 h and 6 h) on the motility and the intracellular superoxide production in the bovine spermatozoa.

Material and methods

Semen samples

Bovine semen samples were obtained from 10 adult breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). The samples had to accomplish the basic criteria given for the corresponding breed. After collecting the samples were stored in the laboratory at room temperature (22-25°C). Each sample was diluted in physiological saline solution (PS) (sodium chloride 0.9% w/v, Bieffe Medital, Italia), using a dilution ratio of 1:40, depending on the original spermatozoa concentration.

***In vitro* culture**

Spermatozoa were incubated with various concentrations of octylphenol (4-OP; Sigma-Aldrich, St. Louis, USA) dissolved in 1% ethanol (Sigma-Aldrich, Bratislava, Slovakia) (group A – 1; B – 10; C – 100; D – 200 µg/mL of OP). The control spermatozoa group was cultured with physiological saline solution. The positive control group was cultured with 1% ethanol. Spermatozoa were cultivated in the laboratory at room temperature (22-25°C). The control group (medium without OP) was compared to the experimental groups (exposed to different concentrations of OP).

Computer-assisted semen analysis (CASA)

The motility analysis was carried out using a CASA (Computer Assisted Semen Analyzer) system – SpermVision™ program (MiniTűb, Tiefenbach, Germany) with the Olympus BX 51 microscope (Olympus, Tokyo, Japan) at cultivation times 0 h, 2 h, 4 h and 6 h. Each sample was placed into the Makler Counting Chamber (deph 10 µm, Sefi-Medical Instruments, Haifa, Izrael) and the following parameters were evaluated: percentage of motile spermatozoa (motility > 5 µm/s; MOT) and percentage of progressive motile spermatozoa (motility > 20 µm/s; PROG). This study was performed

in ten replicates at each concentration (n = 10). 1000-1500 spermatozoa cells were analyzed in each sample.

Nitroblue-tetrazolium (NBT) test

The intracellular formation of superoxide radical was observed by the nitroblue-tetrazolium (NBT) test (*Esfandiari et al*, 2003). This assay is conducted by counting the cells containing blue NBT formazan deposits, which are formed by reduction of the membrane permeable, water-soluble, yellow-colored, nitroblue tetrazolium chloride (2,2´-bis(4-Nitrophenyl)-5,5´-diphenyl-3,3´-(3,3´-dimethoxy-4,4´-diphenylene)ditetrazolium chloride; Sigma, St. Louis, USA) and superoxide radical. Formazan can be measured spectrophotometrically at a measuring wavelenght of 570 nm as reference by a microplate ELISA reader (Multiskan FC, ThermoFisher Scientific, Finland). The data were expressed in percentage of control (i.e. optical density of formazan from cells not exposed to octylphenol). Results from the analysis were collected during four repeated experiments at each concentration (*Tvrdá et al*, 2013).

Statistical analysis

Statistical analysis was carried out using the GraphPad Prism 3.02 program (GraphPad Software Incorporated, San Diego, California, USA). Descriptive statistical characteristics (mean, minimum, maximum, standard deviation and coefficient of variation) were evaluated. One-way analysis of variance (ANOVA) with 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 and discussion

Octylphenol is an environmental contaminant, that can induce male reproductive abnormalities, such as a reduction of the size and weight of testis, epididymis and prostate, lower daily sperm production and count or a decrease spermatozoa motility.

Evaluation of the percentage of spermatozoa motility showed slightly decreased values in all experimental groups with addition of octylphenol dissolved in 1% ethanol compared to the control group during time 0 h of *in vitro* cultivation. Medium with 1% ethanol (positive control group) didn´t significantly ($P>0.05$) influenced the spermatozoa motility during all time periods. In this time, the lowest spermatozoa motility and significant differences were recorded in the groups B, C and D ($P<0.001$) with the doses of OP (10 µg/mL; 100 µg/mL; 200 µg/mL) in comparison with the control group (84.02 ± 6.95%; 83.91 ± 9.96% and 82.00 ± 10.10% versus 93.62 ± 3.39%). The results are shown in the Table 1 and Figure 2.

The decreased spermatozoa motility was also found in all doses of OP in comparison with the control group after 2 h of cultivation and significant differences were observed between all experimental groups ($P<0.001$) compared to the control group. The lowest spermatozoa motility was detected at the dose 200 µg/mL of OP (69.12 ± 9.65%).

All doses of OP decreased the average spermatozoa motility also after 4 h, but significant differences ($P<0.001$) were found only between the group D and the control group.

After 6 h of *in vitro* cultivation a decrease of motility in all experimental groups in comparison to the control group was found. A significant decrease of spermatozoa motility ($P<0.05$ and $P<0.001$) was found in the groups B, C and D.

Table 1: Bovine spermatozoa motility (MOT; %) exposed to OP dissolved in 1% ethanol in various time periods

Groups	Control	Positive control	1 A	10 B	100 C	200 D
	µg/mL of OP					
Time 0						
x	93.62	92.00	92.36	84.02***	83.91***	82.00***
minimum	83.33	85.75	83.33	69.76	61.90	60.00
maximum	100.0	97.72	100.0	96.00	96.15	97.43
S.D.	3.39	3.25	3.38	6.95	9.96	10.10
CV (%)	3.62	3.58	3.66	8.27	11.87	12.32
Time 2						
x	87.05	83.36	80.60***	75.36***	72.94***	69.12***
minimum	70.76	69.23	57.89	56.66	46.15	42.85
maximum	98.27	94.91	93.22	94.85	91.46	89.18
S.D.	6.39	7.38	7.64	10.83	12.44	9.65
CV (%)	7.33	8.85	9.47	14.37	17.06	13.95
Time 4						
x	78.84	76.74	76.84	74.89	70.24	63.60***
minimum	67.39	50.00	54.16	60.00	41.17	43.24
maximum	93.75	95.74	88.88	92.98	89.65	89.74
S.D.	5.63	12.67	6.84	8.63	12.81	10.85
CV (%)	7.14	16.51	8.90	11.52	17.73	17.06
Time 6						
x	74.87	71.18	72.90	68.22*	65.59***	59.68***
minimum	64.86	56.00	55.55	51.42	39.17	34.48
maximum	88.23	87.05	88.46	87.09	88.88	78.46
S.D.	5.48	10.48	8.30	10.89	13.04	12.30
CV (%)	7.32	14.73	11.38	15.97	19.88	10.61

Legend: x – mean, SD – standard deviation, CV (%) – coefficient of variation
***=*P*<0.001; **=*P*<0.01; *=*P*<0.05

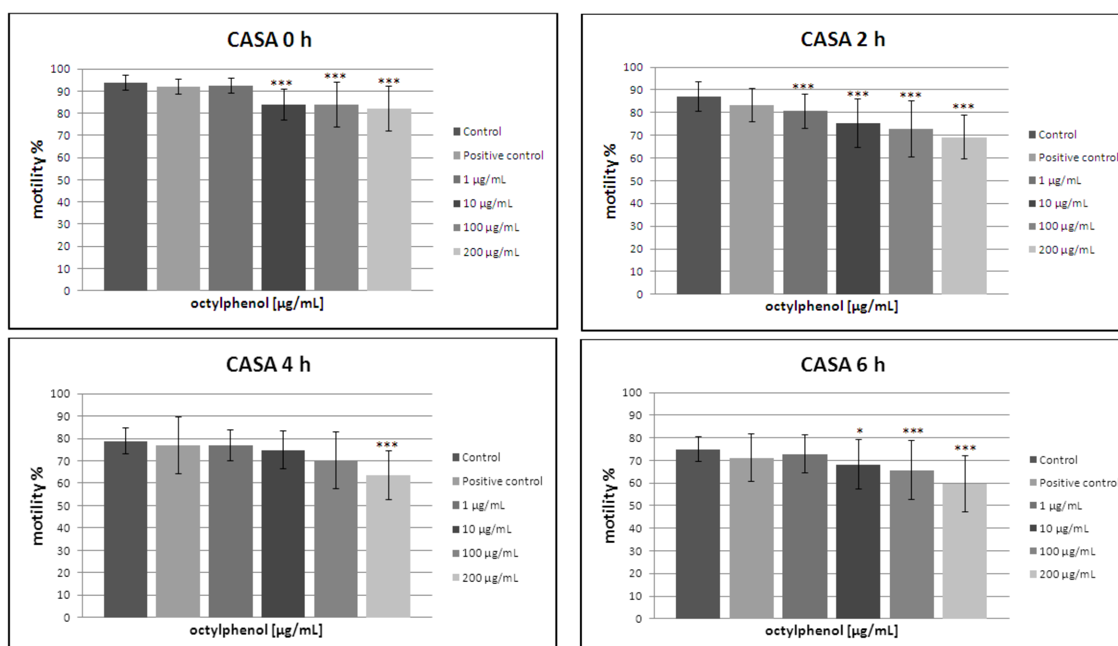

*Figure 1: Effect of various concentrations of octylphenol dissolved in 1% ethanol on bovine spermatozoa motility (%) at 0, 2, 4 and 6 h of in vitro cultivation. The level of significance was set at ***P<0.001; **P<0.01; *P<0.05.*

An identical decrease of spermatozoa motility was detected also for the percentage of progressive motile spermatozoa in all time periods. The results are shown in the Table 2 and Figure 2.

Table 2: Bovine progressive spermatozoa motility (PROG; %) exposed to OP dissolved in 1% ethanol in various time periods

Groups	**Control**	**Positive control**	**1 A**	**10 B**	**100 C**	**200 D**
	µg/mL of OP					
Time 0						
x	92.42	89.61	90.98	81.55***	80.68***	79.86***
minimum	81.31	76.56	75.86	63.82	50.00	59.45
maximum	98.88	95.65	96.96	94.00	93.10	93.54
S.D.	3.57	4.97	4.36	8.22	11.78	9.77
CV (%)	3.86	5.56	4.80	10.08	14.60	12.24
Time 2						
x	85.45	81.04	79.03***	73.54***	71.54***	67.75***
minimum	70.46	64.70	57.33	50.00	46.05	42.25
maximum	96.66	91.13	91.66	91.83	89.83	89.18
S.D.	6.28	6.95	7.78	13.53	11.98	10.04
CV (%)	7.36	8.57	9.85	18.40	16.74	14.82
Time 4						
x	76.14	74.72	75.36	71.22	68.77	61.41***
minimum	59.09	50.00	50.20	59.00	41.00	40.54
maximum	91.30	92.30	88.58	88.61	88.23	87.50
S.D.	6.14	12.49	7.67	7.27	12.24	10.84
CV (%)	8.06	16.72	10.18	10.22	17.30	17.66
Time 6						
x	71.36	70.47	69.35	65.24***	63.61***	58.03***
minimum	59.09	44.61	54.54	44.44	34.14	34.408
maximum	84.55	85.00	83.33	80.00	85.00	77.71
S.D.	6.42	10.17	8.07	11.08	14.76	12.21
CV (%)	9.82	14.02	10.18	13.95	14.42	9.51

Legend: x – mean, SD – standard deviation, CV (%) – coefficient of variation
***=P<0.001; **=P<0.01; *=P<0.05

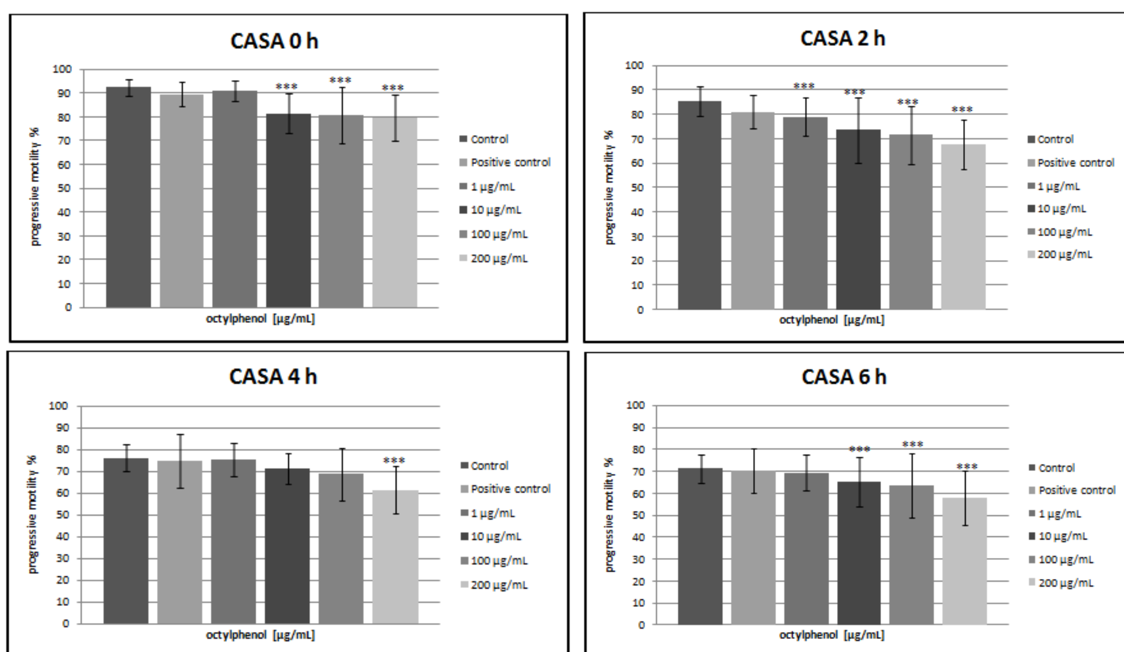

Figure 2: Effect of various concentrations of octylphenol dissolved in 1% ethanol on bovine progressive spermatozoa motility (%) at 0, 2, 4 and 6 h of in vitro cultivation. The level of significance was set at $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$.

Many *in vivo* and *in vitro* studies suggest that octylphenol induces abnormities in male reproductive system. The data obtained from our study indicate, that octylphenol has also a negative *in vitro* inhibitory effect on spermatozoa motility during short-term cultivation. Our results showed a decreased spermatozoa motility in all concentrations of OP (1, 10, 100 and 200 µg/mL) dissolved during all time periods. The similar results reached a study by *Bian et al.* (2006), that demonstrated the toxic effect of various concentrations of 4-tert-octylphenol (50, 150 and 450 mg/kg/d) on testicular functions of rats and found out, that the size and weight of the testis, epididymis and prostate were reduced in all the three dosages. The dose of 150 mg/kg/d of OP significantly lowered sperm motility, raising the dose to 450 mg/kg/d also significantly decreased testicular sperm count and daily sperm production.

Gregory et al. (2009) evaluated the effects of chronic exposure to OP on male reproduction and recorded a significant decrease in body weight in the 125 mg/kg group after 60 day of treatment. Total percent sperm motility was significantly lower in rats exposed to the intermediate dose (50 mg/kg of body weight).

Sweeney et al. (2007) determined if maternal exposure to octylphenol pre- and/or postnatally influenced FSH concentrations and semen quantity and quality in postpubertal rams. They found out, that the exposure to OP from birth to weaning increased the number of morphologically abnormal sperm cells in the ejaculates of these rams.

*Herath et al. (*2004) also confirmed that OP can reduce sperm counts resulting from lowered plasma testosterone in male rats just after puberty.

Vom Saal et al. (1998) found that mice exposed to OP experienced significantly lower daily sperm production.

Blake and Boockfor (1997) demonstrated that chronic administration of 4-tert-octylphenol (OP) to adult male rats resulted in lower levels of LH, FSH, prolactin and testosterone, and proposed that OP inhibited the secretion of reproductive hormones by mimicking estrogen. The same authors also established that chronic administration of 80 mg/kg OP caused shrinkage of the testis. Male accessory sex organs and spermatogenesis were disrupted, spermatozoa deformities were observed (*Boockfor and Blake*, 1996).

A study of *Kim et al.* (2004) looked at the effects of OP on the testicular development of prepubertal rats and showed that OP is able to reduce the size and function of the male reproductive organs due to increased apoptosis of testicular germ cells and the decreased biosynthesis of testosterone.

Raychoudhury et al. (1999) observed, that octylphenol is directly toxic to cultured rat spermatogenic cells and Sertoli cells and suggested that this toxic effect in Sertoli cells is exerted through Ca(2+)- independent apoptosis.

The NBT test revealed after 6 h of *in vitro* cultivation with observed endocrine disruptor that the doses 10, 100 and 200 µg/mL of OP increased intracellular superoxide production in the bovine spermatozoa (Figure 3). However, significant differences ($P<0.05$ and $P<0.001$) were found out only between groups C and D containing the highest doses of OP in comparison to the control group. At the dose 1 µg/mL we recorded slightly decrease of superoxide production compared to the control group.

Aydogan et al. (2010) demonstrated that OP at dose 25 mg/kg/day orally administered to rats three times a week for 45 days can generate reactive oxygen species that cause oxidative damage in testes of Wistar rats.

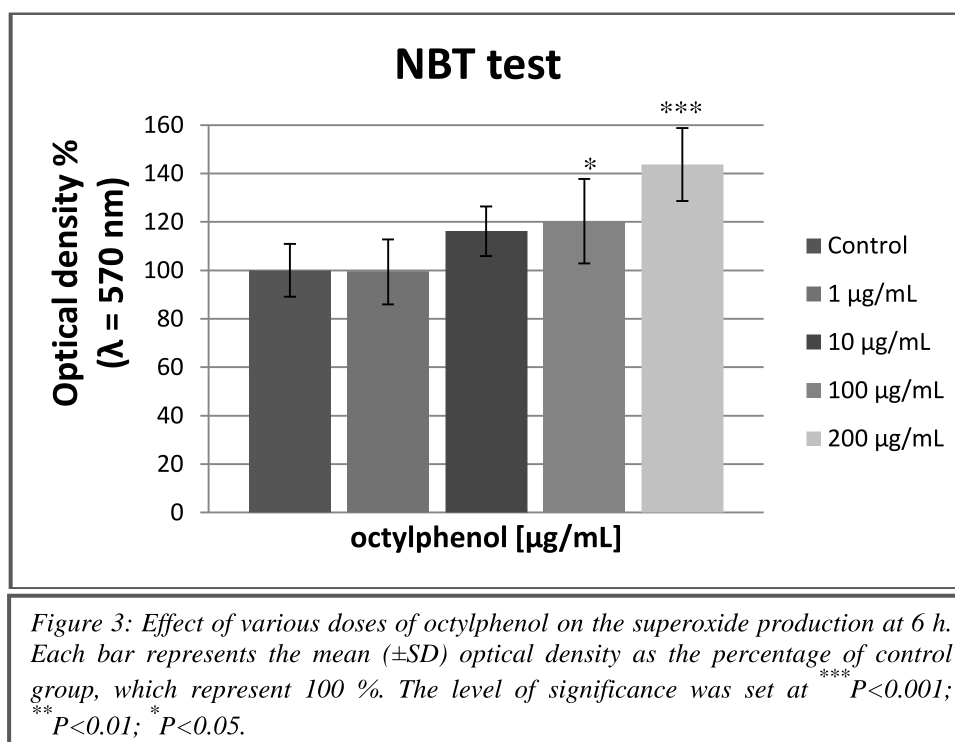

*Figure 3: Effect of various doses of octylphenol on the superoxide production at 6 h. Each bar represents the mean (±SD) optical density as the percentage of control group, which represent 100 %. The level of significance was set at ***P<0.001; **P<0.01; *P<0.05.*

Conclusion

The present data show that some endocrine disruptors may markedly affect the health and reproduction of animals or human. Octylphenol is one of these chemicals that can disrupt testicular development and ultimately reduce male fertility. The results obtained from our *in vitro* study confirm that the higher concentrations of octylphenol (100 and 200 µg/mL) have the toxic effect on spermatozoa motility during short-term cultivation. Octylphenol in these higher doses is also able to increase intracellular superoxide production causing oxidative stress.

Acknowledgments: This work was supported by the Scientific Agency of the Slovak Republic VEGA No. 1/0532/11.

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