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REGULAR ARTICLE

THE IMPACT OF NONYLPHENOL (NP) ON THE SPERMATOZOA MOTILITY *IN VITRO*

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ABSTRACT

Nonylphenol (NP) is an environmental endocrine disruptor that has toxic, estrogenic and carcinogenic effects in fish, amphibians and mammals. NP can result in male reproductive dysfunction, altered testicular development, decreased male fertility and a decline of spermatozoa count. The target of this *in vitro* study was to determine the effect of NP on the spermatozoa motility. Specifically, we examined the dose- and time-dependent effect of nonylphenol (1, 10, 100 and 200 µg/mL) dissolved either in 0.1% dimethyl sulfoxide (DMSO) or 0.1% ethanol (ETOH) on the motility of bovine spermatozoa during several time periods (0 h, 2 h, 4 h and 6 h). The spermatozoa motility was determined by CASA (Computer Assisted Semen Analyzer) system using the Sperm Vision^{TM} program. The results showed a decreased spermatozoa motility in all experimental groups with the addition of NP. Significant differences ($P<0.001$ and $P<0.05$) between the control group and all experimental groups were recorded. The lowest motility of bovine spermatozoa was found at doses > 100 µg/mL of NP in comparison with the control group. The obtained data indicate that the exposure to high doses of NP has the negative effect on spermatozoa motility.

Keywords: endocrine disruptors, nonylphenol, spermatozoa motility, CASA

INTRODUCTION

Endocrine disruptors (EDs) are compounds that can interfere with and alter the homeostasis of the endocrine system. They can act at several sites, mimicking the occurrence of natural hormones, blocking their production and inhibiting or stimulating the endocrine system. EDs have in long-term adverse effects on human and animal health or their progeny. The effects also extend to the thyroid, nervous, immune system and metabolism in general **(White *et al*., 1994; Sumpter and Johnson, 2005; Hotchkiss *et al*., 2008)**.

Nonylphenol (NP) is a toxic xenobiotic compound classified as an endocrine disruptor capable of interfering with the hormonal system of numerous organisms. It originates principally from the degradation of nonylphenol ethoxylates **(Soares *et al*., 2008)**.

Nonylphenol is used in industrial and household detergents, cosmetic products and spermicides **(Gong and Han, 2006)**. NP can interfere with reproduction in fish, reptiles and mammals, and induce the cell death in gonads and changes to other reproductive parameters **(Nagao *et al*., 2001; Cardinali *et al*., 2004)**. NP poses a threat to the health and reproduction of mammalian species through direct intake of NP with various food products. NP is able to act as endocrine disruptor and cause adverse reproductive effects in mammals **(Hughes *et al*., 2000)**. NP can induce apoptosis in rat testicular cells, particularly in Sertoli cells by inhibiting the Ca^{2+} pump in the endoplasmic reticulum. Low micromolar concentration of NP induces testicular oxidative stress and cytotoxicity *in vitro* **(Gong *et al*., 2009)**.

The estrogenic effect of NP, such as induced expression of estrogen receptor (ER) and inhibiting estrogen binding to ER, might cause endocrine disruption **(Fekadu *et al*., 1999; Kwack *et al*., 2002)**. NP may affect Sertoli cell development and function, because males have much lower levels of estrogen than females **(Soto *et al*., 1991)**.

The objective of this study was to determine the effect of various concentrations of nonylphenol dissolved in 0.1% dimethyl sulfoxide (DMSO) and 0.1% ethanol (ETOH) during several time periods (0 h, 2 h, 4 h and 6 h) on the motility of 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. The semen was obtained on a regular collection schedule using an artificial vagina. 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 nonylphenol (4-*n*-NP; Fluka, Buchs, Switzerland) dissolved in 0.1% dimethyl sulfoxide (DMSO, Sigma-Aldrich Co.) (group A – 1; B – 10; C – 100; D – 200 µg/mL of NP) and in 0.1% ethanol (ETOH, Sigma-Aldrich Co) (group A – 1; B – 10; C – 100; D – 200 µg/mL of NP). The control spermatozoa (Ctrl) groups were cultured with 0.1% dimethyl sulfoxide (Ctrl 1) and 0.1% ethanol (Ctrl 2). Spermatozoa were cultivated in the laboratory at room temperature (22-25°C). The control groups (media without NP) were compared to the experimental groups (exposed to different concentrations of NP).

Computer-assisted semen analysis (CASA)

The motility analysis was carried out using a CASA (Computer Assisted Semen Analyzer) system – SpermVision^{TM} program (MiniTűb, Tiefenbach, Germany) with the Olympus BX 51 microscope (Olympus, 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, Izrael) and the percentage of motile spermatozoa (motility > 5 µm/s; MOT) was evaluated. This study was performed in ten replicates at each concentration (n = 10). At least 1000 spermatozoa were analyzed in each sample.

Statistical analysis

Obtained data were statistically analyzed using PC program GraphPad Prism 3.02 (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) 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 AND DISCUSSION

NP is one of the most abundant alkylphenolpolyethoxylate derivates (APE) and can stay biologically active for a longer period of time in the body than endogenous estrogens **(Nimrod and Benson, 1996)**.

Many authors described the negative impact of nonylphenol on fish **(Tollefsen and Nilsen, 2008)**, amphibians **(Feng *et al.*, 2011)** and mammals. NP profoundly impairs testicular function as evidenced by reduced testis size **(de Jager *et al.* 1999)**, disturbed testicular structure and suppressed spermatogenesis **(Nagao *et al.*, 2001; Cardinali *et al.*, 2004)** and can induce Sertoli cell apoptosis **(Gong *et al.*, 2009)** and oxidative stress in rats **(Gong and Han, 2006).**

Evaluation of bovine spermatozoa motility exposed to NP dissolved in 0.1% DMSO

Evaluation of the percentage of spermatozoa motility showed slightly decreased values in all doses of NP dissolved in 0.1% DMSO compared to the control group 1 (Ctrl 1) during time 0 h of *in vitro* cultivation. In this time, the lowest spermatozoa motility was recorded in the groups C (P<0.05) and D (P<0.001) using the highest doses of NP (100 µg/mL; 200 µg/mL) in comparison with the control group (88.16% and 87.54% versus 92.47%). The results are shown in the Table 1.

The decreased spermatozoa motility was found in all experimental groups in comparison with the control group after 2 h of cultivation. The lowest spermatozoa motility was detected in the group D (P<0.001) with the highest dose of NP (81.07 ± 12.85%).

After 4 h and 6 h of *in vitro* cultivation a decrease of spermatozoa motility was recorded in all experimental groups in comparison to the control group.

Table 1 Bovine spermatozoa motility (MOT; %) exposed to NP dissolved in 0.1% DMSO in various time periods

Groups	**Control 1** **Ctrl 1**	**1** **A**	**10** **B**	**100** **C**	**200** **D**
			µg/mL of NP		
Time 0					
x	92.47	91.95	89.95	88.16^{C}	87.54^{A}
minimum	80.95	83.33	72.72	63.63	70.45
maximum	97.29	98.30	98.97	97.43	98.00
S.D.	3.83	4.31	6.33	7.15	6.64
CV (%)	4.14	4.69	7.03	8.11	7.60
Time 2					
x	87.37	87.15	84.32	82.71	81.07^{A}
minimum	75.00	73.68	60.86	77.50	53.44
maximum	95.65	94.62	95.74	93.42	96.05
S.D.	5.88	5.39	7.39	4.20	12.85
CV (%)	6.74	6.19	8.76	5.08	15.85
Time 4					
x	84.60	82.50	79.52	75.80	70.12^{A}
minimum	62.22	67.85	52.77	50.25	46.80
maximum	95.74	93.65	93.45	90.42	86.11
S.D.	8.44	6.64	10.45	8.32	13.57
CV (%)	9.97	8.05	13.14	10.98	19.35
Time 6					
x	77.41	73.25	71.48	67.36^{C}	62.39^{A}
minimum	58.62	53.33	51.16	50.00	38.46
maximum	89.41	89.55	90.69	87.68	86.76
S.D.	12.98	10.76	10.26	12.23	12.59
CV (%)	16.77	14.68	14.35	18.15	20.19

Legend: x – mean, SD – standard deviation, CV (%) – coefficient of variation

$^{A}P<0.001$; $^{B}P<0.01$; $^{C}P<0.05$

Evaluation of bovine spermatozoa motility exposed to NP dissolved in 0.1% ETOH

The initial bovine spermatozoa motility showed slightly decreased values in all groups of NP dissolved in 0.1% ETOH in comparison to NP dissolved in 0.1% DMSO. The average values of spermatozoa motility in all experimental groups were lower in comparison to the control group 2 (Ctrl 2) during time 0 h of *in vitro* cultivation. The results are shown in the Table 2. The lowest spermatozoa motility was also observed in the groups B and C ($P<0.05$) and the group D ($P<0.001$) with the doses of NP (10 µg/mL; 100 µg/mL; 200 µg/mL) in comparison to the control group (89.30%; 87.89% and 87.30% versus 92.26%).

High NP concentrations decreased the average motility values also after 2 h and significant differences ($P<0.05$ and $P<0.001$) were found between the groups C and D and the control group (81.80% and 79.70% versus 87.06%).

After 4 h of cultivation was found, that the spermatozoa motility was also significantly decreased in the groups B ($P<0.05$), C and D ($P<0.001$).

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 C and D.

Table 2 Bovine spermatozoa motility (MOT; %) exposed to NP dissolved in 0.1% ETOH in various time periods

Groups	**Control 2** **Ctrl 2**	**1** **A**	**10** **B**	**100** **C**	**200** **D**
			µg/mL of NP		
Time 0					
x	92.26	91.91	89.30^{C}	87.89^{C}	87.30^{A}
minimum	88.16	80.82	75.75	66.66	71.24
maximum	97.14	98.26	98.36	97.67	93.39
S.D.	2.50	3.88	5.69	7.66	4.05
CV (%)	2.72	4.23	6.38	8.72	4.64
Time 2					
x	87.06	86.63	83.17	81.80^{C}	79.70^{A}
minimum	75.00	74.41	61.70	65.21	52.63
maximum	95.65	95.00	98.96	95.74	88.88
S.D.	5.79	5.59	8.91	8.84	10.72
CV (%)	6.65	6.45	10.71	10.80	13.45
Time 4					
x	83.37	82.19	76.63^{C}	73.25^{A}	69.18^{A}
minimum	65.38	69.56	55.26	45.00	41.17
maximum	93.75	92.13	93.47	89.41	85.40
S.D.	8.37	5.88	11.60	14.47	13.91
CV (%)	10.04	7.15	15.13	19.75	20.10
Time 6					
x	74.74	72.19	69.23	66.56^{C}	60.76^{A}
minimum	52.94	47.82	42.85	36.66	50.00
maximum	89.04	93.02	83.52	87.27	77.61
S.D.	9.49	11.83	11.14	13.02	8.41
CV (%)	12.70	16.39	16.09	19.56	13.84

Legend: x – mean, SD – standard deviation, CV (%) – coefficient of variation

$^{A}P<0.001$; $^{B}P<0.01$; $^{C}P<0.05$

DMSO and ethanol are solvents, which are used for the dissolution of organic substances in biochemistry and cell biology **(Kelava and Cavar, 2011)**. The data obtained from this *in vitro* study indicate, that NP dissolved in ETOH has a higher inhibitory effect on spermatozoa motility than NP dissolved in DMSO. This indicates that a combination of NP and ETOH have a higher toxic effect than a combination of NP and DMSO. These findings of our experiment confirm the studies of **Uguz *et al.* (2009)** that examined the effect of NP (1, 10, 100, 250 and 500 µg/mL) on epididymal rat spermatozoa during different time periods (0 h, 1 h, 2 h, 3 h and 4 h) and recorded that exposure to a dose > 250 µg/mL of NP has detrimental effect on motility of rat spermatozoa. Our experiment indicates similar results at doses 200 µg/mL of nonylphenol on the motility of bovine spermatozoa.

De Jager *et al.* (1999) tested the influence of NP at doses 100, 250 and 400 mg/kg and reported that the dose 250 mg/kg of NP had effect on spermatogenesis, while the dose 400 mg/kg of NP impaired testicular mass and sperm count in adult rats. **Chitra *et al.* (2002)** demonstrated, that nonylphenol administated at dose > 10 µg/kg for 45 days significantly decreased rat epididymal spermatozoa count. **Dakdoky and El-Helal (2007)** found that a daily dose of 21.25 mg/kg of NP decreased significantly mouse spermatozoa motility for 35 days. **Uguz *et al.* (2008)** examined the impact of various concentrations of NP (1, 10, 100, 250, 500 and 1000 µg/mL) during several time periods (1 h, 2 h, 3 h and 4 h) at 37°C with 5% CO_2 in air and found out that exposure of rat sperm to a doses > 250 µg NP/mL has the negative effect on the motility of rat spermatozoa.

Octylphenol (OP), another metabolite of alkylphenolpolyethoxylates, is commonly found in industrial processing and in household and institutional cleaning products **(Ying *et al.*, 2002; Qian *et al.*, 2006)** and can mimic naturally produced estrogen by interacting with estrogen receptors similarly like nonylphenol **(Blake and Ashiru, 1997)**. **Bian *et al.* (2006)** demonstrated that the daily dose 150 mg/kg of OP significantly lowered the spermatozoa motility and raising the dose to 450 mg/kg/d of OP also significantly decreased testicular spermatozoa count and a daily sperm production.

CONCLUSION

The data obtained from this *in vitro* study describe the dose- and time-dependent effect of NP on the motility of bovine spermatozoa. Our results support the idea, that higher doses of NP (>100 µg NP/ml) dissolved in 0.1% DMSO and in 0.1% ETOH negatively influence the spermatozoa motility. Probably, these concentrations lead to the reproductive toxicity. The

obtained can contribute to better understanding the mechanism of NP action not only on the spermatozoa count and motility but also on the male reproductive system.

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