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

VPLYV NONYLFENOLU (NP) NA POHYBLIVOSŤ SPERMIÍ *IN VITRO*

Lukáčová J., Kňažická Z., Tvrdá E., Lukáč N., Massányi P.

Department of Animal Physiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic

E-mail: jana312@gmail.com

ABSTRACT

Our objective was to evaluate the dose- and time-dependent effect of nonylphenol (NP) (1, 10, 100 and 200 µg/mL) dissolved in 0.1% ethanol (ETOH) on the motility and progressive 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™ 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 the experimental groups were recorded. The lowest spermatozoa motility was found at doses > 100 µg/mL of NP in comparison to the control group. The data obtained from our study indicate that the exposure to high doses of NP has the negative effect on spermatozoa motility.

Key words: endocrine disruptors, 4-*n*-nonylphenol, bovine spermatozoa, CASA system

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

INTRODUCTION

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).

NP 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 profoundly impairs testicular function as evidenced by reduced testis size (Chitra *et al*., 2002), low circulating testosterone, disturbed testicular structure and suppressed spermatogenesis (Nagao *et al*., 2001; Tan *et al*., 2003; Cardinali *et al*., 2004).

NP influences an increasing incidence of testicular cancer during the past decades, as well as an increase in occurence of cryptorchidism and hypospodias (Carlsen *et al*., 1995; Toppari *et al*., 1996).

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 objective of this study was to determine the effect of various concentrations of nonylphenol dissolved in 0.1% ethanol (ETOH) during several time periods (0 h, 2 h, 4 h and 6 h) on the motility and progressive motility of bovine spermatozoa.

MATERIAL AND METHODS

Semen samples and *in vitro* culture

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, Grosotto, Italia), using a dilution ratio of 1:40, depending on the original spermatozoa concentration.

Spermatozoa were incubated with various concentrations of nonylphenol (4-*n*-NP; Fluka, Buchs, Switzerland) dissolved in 0.1% ethanol (ETOH, Sigma-Aldrich, Bratislava, Slovak Republic) (group A – 1; B – 10; C – 100; D – 200 µg/mL of NP). The control (Ctrl) group was cultured with physiological saline solution.

Spermatozoa were cultivated in the laboratory at room temperature (22-25°C). The control group (medium without NP) was 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™ 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). 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). 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% ETOH

Evaluation of the percentage of spermatozoa motility showed slightly decreased values in all doses of NP dissolved in 0.1% ETOH compared to the control (Ctrl) group during time 0 h of *in vitro* cultivation. The results are shown in the Table 1. In this time, the lowest spermatozoa motility was recorded 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%).

The decreased spermatozoa motility was found in all experimental groups in comparison with the control group after 2 h of cultivation 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.

Tab. 1 Bovine spermatozoa motility (MOT; %) exposed to NP dissolved in 0.1% ETOH in various time periods ***(Lukáčová et al., 2012)***

Groups	**Control Ctrl**	**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

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

An identical decrease of spermatozoa motility was determined also for the percentage of progressive motile spermatozoa during all time periods. The results are shown in the Table 2. In 0 h of cultivation, the lowest spermatozoa motility was found in the groups B and C ($P<0.05$) and the group D ($P<0.001$) using the doses of NP (10 µg/mL; 100 µg/mL; 200 µg/mL) in comparison with the control group.

The decreased progressive motility was also recorded in all experimental groups in comparison with the control group after 2 h of *in vitro* cultivation and significant difference ($P<0.05$) was found between the group D and the control group (76.83% versus 86.13%).

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

After 6 h of *in vitro* cultivation a significant decrease of progressive spermatozoa motility ($P<0.05$ and $P<0.001$) was found in the groups C and D.

Tab. 2 Bovine progressive spermatozoa motility (PROG; %) exposed to NP dissolved in 0.1% ETOH in various time periods

Groups	**Control Ctrl**	**1 A**	**10 B**	**100 C**	**200 D**
			µg/mL of NP		
Time 0					
x	90.31	88.85	86.59^{C}	84.48^{C}	83.21^{A}
minimum	87.28	80.32	52.30	66.66	62.21
maximum	93.46	92.56	97.62	94.76	92.30
S.D.	2.85	3.78	9.26	7.52	8.88
CV (%)	2.75	3.91	9.66	8.26	6.72
Time 2					
x	86.13	84.25	81.31	78.96	76.83^{C}
minimum	72.08	71.21	65.15	52.00	49.35
maximum	96.36	94.90	93.00	93.85	93.12
S.D.	4.70	6.45	6.26	9.31	12.62
CV (%)	11.36	7.86	16.34	29.54	20.47
Time 4					
x	80.73	79.91	73.54	71.25^{A}	67.85^{A}
minimum	55.66	60.20	53.02	48.33	44.21
maximum	93.45	91.04	87.98	91.31	84.94
S.D.	8.31	7.81	11.28	19.31	14.26
CV (%)	6.65	7.29	8.51	11.72	12.28
Time 6					
x	72.62	70.32	67.77	64.32^{C}	57.93^{A}
minimum	65.35	59.47	59.01	49.99	56.21
maximum	83.23	81.02	81.48	88.49	79.06
S.D.	5.75	16.40	14.35	10.43	10.84
CV (%)	7.71	16.79	12.62	13.20	10.76

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

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

The obtained data indicate, that NP has an inhibitory effect on spermatozoa motility. These findings of our experiment confirm the studies of **Uguz *et al.* (2008, 2009)** that examined the effect of NP (1, 10, 100, 250 and 500 µg/mL) on epididymal rat spermatozoa during various 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.

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.

Chitra *et al.* (2002) demonstrated, that nonylphenol administated at dose > 10 µg/kg for 45 days significantly decreased rat epididymal spermatozoa count.

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.

CONCLUSION

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

REFERENCES

Cardinali M., Maradonna F., Olivotto I., Bortoluzzi G., Mosconi G., Polzonetti-Magni A. M., Carnevali O. (2004): Temporary impairment of reproduction in freshwater teleost exposed to nonylphenol. Reproductive Toxicology, 18: 597-604.

Carlsen E., GIiwercman A., Keiding N., Skakkebaek N. E. (1995): Declining semen quality and increasing incidence of testicular cancer: is there a common cause? Environmental Health Perspectives, 103: 137-9.

de Jager C., Bornman M. S., van der Horst G. (1999): The effect of p-nonylphenol, an environmental toxicant with oestrogenic properties, on fertility potential in adult male rats. Andrologia, 31: 99-106.

El-Dakdoky M. H., Helal M. A. (2007): Reproductive toxicity of male mice after exposure to nonyphenol. Bulletin of Environmental Contamination and Toxicology, 79: 188-191.

Gong Y., Han X. D. (2006): Nonylphenol-induced oxidative stress and cytotoxicity in testicular Sertoli cells. Reproductive toxicology, 22: 623-630.

Gong, Y., Wu, J., Huang, Y., Shen, S., Han, X. D. (2009): Nonylphenol induces apoptosis in rat testicular Sertoli cells via endoplasmatic reticulum stress. Toxicology Letters, 186: 84-95.

Hughes P. J., McLellan H., Lowes D. A., Kahn S. Z., Bilmen J. G., Tovey S. C., Godfrey R. E., Michell R. H., Kirk C. J., Michelangeli F. (2000): Estrogenic alkylphenols induce cell death by inhibiting testis endoplasmic reticulum Ca(2+) pumps. Biochemical and Biophysical Research Communications, 277: 568-574.

Chitra K. C., Latchoumycandane C., Mathur P. P. (2002): Effect of nonylphenol on the antioxidant system in epididymal sperm of rats. Archives of toxicology, 76: 545-51.

Lukáčová J., Kňažická Z., Tvrdá E., Greń A., Lukáč N., Massányi P. (2012): The impact of nonylphenol (NP) on the spermatozoa motility *in vitro*. Journal of Microbiology, Biotechnology and Food Sciences, 1: 1551-1560.

Nagao T., Wada K., Marumo H., Yoshimura S., Ono H. 2001. Reproductive effects of nonylphenol in rats after gavage administration: a two-generation study. Reproductive toxicology, 15: 293-315.

Nimrod A. C., Benson W. H. (1996): Environmental Estrogenic Effects of Alkylphenol Ethoxylates. Critical Reviews in Toxicology, 26: 335-364.

Soares A., Guieysse B., Jefferson B., Cartmell E., Lester J. N. (2008): Nonylphenol in the environment: A critical review on occurrence, fate, toxicity and treatment in wastewater. Environment International, 34: 1033-1049.

Tan B.L., Kassim N.M., Mohd M.A. (2003): Assessment of pubertal development in juvenile male rats after sub-acute exposure to bisphenol and nonylphenol. Toxicology letters, 143: 261-270.

Toppari J., Larsen J. C., Christiansen P., Giwercman A., Grandjean P., Guillette L. J., Jr, Jégou B., Jensen T. K., Jouannet P., Keiding N., Leffers H., McLachlan J. A., Meyer O., Müller J., Rajpert-De Meyts E., Scheike T., Sharpe R., Sumpter J. S., Skakkebaek N. E. (1996): Male reproductive health and environmental xenoestrogens. Environmental health perspectives, 104(4): 741-803.

Uguz C., Varisli O., Agca C., Agca Y. (2008): 229 Effects of nonylphenol on motility, acrosomal integrity and mitochondrial membrane potential of epididymal rat sperm. Reproduction, Fertility and Development, 20: 194-194.

Uguz C., Varisli O., Agca C., Agca Y. (2009): Effects of nonylphenol on motility and subcellular elements of epididymal rat sperm. Reproductive toxicology, 28: 542-549.