JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

IN VITRO ASSESSMENT OF EXPOSURE TO NONYLPHENOL ON VIABILITY OF BOVINE SPERMATOZOA

Jana Lukáčová, Tomáš Jambor, Norbert Lukáč*

Address(es): Ing. Jana Lukáčová,
Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Animal Physiology, Trieda A. Hlinku 2, 949 76 Nitra, Slovak Republic, phone number: +421-37-6414288.

*Corresponding author: jana312@gmail.com

ARTICLE INFO

Received 15. 10. 2013
Revised 21. 11. 2013
Accepted 8. 1. 2014
Published 1. 2. 2014

Regular article

OPEN ACCESS

ABSTRACT

Nonylphenol (NP) is a toxic xenobiotic compound classified as an endocrine disruptor that bioaccumulates in the body and causes endocrine disruption. NP can result in male reproductive dysfunction, altered testicular development and decreased male fertility. The target of this *in vitro* study was to determine the effect of NP as an endocrine disruptor on the viability of spermatozoa. 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) and 0.1% ethanol on the viability of bovine spermatozoa after 6 h of *in vitro* cultivation. The viability of bovine spermatozoa was detected by the MTT cytotoxicity assay. The viability in groups with NP dissolved in 0.1% DMSO was significantly (P<0.05) decreased at 1 µg/mL of NP and significantly decreased (P<0.001) at doses > 10 µg/mL of NP and was decreased significantly (P<0.001) in all experimental groups with NP dissolved in 0.1% ethanol. After 6 h of culture the MTT assay proved a negative effect of all NP doses on the cell viability. The lowest survival of spermatozoa was determined after the addition of 200 µg/mL of NP. The obtained data indicate that the negative effect of NP on the viability must be seriously considered in the case of exposure to NP in animals and humans.

Keywords: bovine spermatozoa, nonylphenol, viability, MTT assay

INTRODUCTION

The modern human life is dependent on a variety of man-made chemicals. Some of the chemicals and their degradation products in the environment can be hazardous to animal as well as human health **(Miyaura and Iwata, 2002)**. Currently, risk to human health is represented by different consumer products of a wide and heterogenous group of compounds, namely endocrine disruptors chemicals (EDCs) that can act at several sites, mimicking the occurrence of natural hormones, blocking their production or by inhibiting/stimulating the endocrine system **(White *et al*., 1994; Hansen *et al*., 1998; Sumpter and Johnosn, 2005; Hotchkiss *et al*., 2008)**.
EDCs can be divided to natural hormones normally occurring in humans or animals and plant derived metabolites. On the contrary, the second group includes various exogenous man-made substances such as synthetic hormones and drugs, industrial chemicals (organochloride pesticides, dioxins, polychloro biphenils (PCBs), polybromo biphenils (PBBs), and alkylphenols, parabens (found in cosmetics and other personal care products), manufactured industrial products such as plastic additives (bisphenol A, phthalates), antifouling paints and chemicals used in farm animal production **(Pinto *et al*., 2008; Diamanti-Kandarakis *et al*., 2009)**. A well known class of environmental endocrine disruptors is the group of compounds known as alkylphenolpolyethoxylates (APEs), which are non-ionic surfactants, consisting of branched-chain alkylphenol that can react with ethylene oxide, producing an ethoxylate chain **(White *et al*., 1994)**. They are used in variety of products, including detergents, emulsifiers, pesticides, herbicides, paints, cosmetics and plastic ware **(Nimrod and Benson, 1996)**. APEs undergo a biodegradation process to produce short side chain derivates such as nonylphenol, octylphenol and butylphenol in anaerobic conditions in water **(Montgomery-Brown and Reinhard, 2003)**.
Nonylphenol (NP) is one of the most abundant alkylphenolpolyethoxylates (APEs) derivates and can stay biologically active for a longer period of time in the body than endogenous estrogens **(Blackburn *et al*., 1999; Servos, 1999; Tapiero *et al*., 2002; Uguz *et al*., 2003; Ahel and Giger, 1993)**. Nonylphenol is a toxic xenobiotic compound classified as an endocrine disruptor capable of interfering with the hormonal system of numerous organisms **(Soares *et al*., 2008)** and is widely used in common cosmetic products and detergents **(Hughes *et al*., 2000)**. NP is hazardous to the health of human and animals, especially to male fertility **(Gong and Han, 2006)**. NP treatment resulted in great damage to the reproductive system, including disturbed testicular structure, low circulating testosterone, reduced testis size **(Nagao *et al*., 2001)**, altered steroidogenesis and decreased sperm count in the epididymis **(Tan *et al*., 2003)**. **Gong and Han (2006)** reported that NP induces apoptosis in rat testicular cells, particularly in Sertoli cells by inhibiting the Ca^{2+} pump in the endoplasmic reticulum.
The obective of this *in vitro* study was to analyze the effect of various concentrations of nonylphenol (NP) dissolved in either 0.1% dimethyl sulfoxide or in 0.1% ethanol on the viability of bovine spermatozoa.

MATERIAL AND METHODS

Semen Samples and *In Vitro* Culture

Semen samples were obtained from 8 adult breeding bulls (Slovak Biological Services, Lužianky - 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 (sodium chloride 0.9%, w/v, Bieffe Medital, 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% dimethyl sulfoxide (Sigma-Aldrich, Bratislava, Slovak Republic) (group A – 1; B – 10; C – 100; D – 200 µg/mL of NP) and in 0.1% ethanol (DMSO, Sigma-Aldrich, Bratislava, Slovak Republic) (group A – 1; B – 10; C – 100; D – 200 µg/mL of NP). Spermatozoa were cultivated in the laboratory at room temperature (22-25°C). The control (Ctrl) group (medium without NP) was compared to the experimental groups (media exposed to different concentrations of NP).

Cytotoxicity Evaluation

The viability of the cells exposed to NP *in vitro* was evaluated by the metabolic activity (MTT) assay **(Mosmann, 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 (MTT) to purple formazan

particles by mitochondrial succinate dehydrogenase of intact mitochondria of living cells. The amount of formazan was measured spectrophotometerically. In brief, the cultured $3.0x10^6$ cells/mL in 96-well plates (NUNC, Denmark) were stained with MTT tetrazolium salt (Sigma, St. Louis, USA). MTT was dissolved in PBS (Dulbecco´s Phosphate Buffer Saline, Sigma, St. Louis, USA) at 5 mg mL and added to the cells (in 20 µL per well). After 1.5 h of incubation (37ºC), the cells and the formazan crystals were dissolved in 80 µL of isopropanol (2-propanol, p.a. CentralChem, Bratislava, Slovak Republic). Optical density was determined at a measuring wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Multiscan FC, ThermoFisher Scientific, Finland). The data were expressed in percentage of control (i.e., optical density of formazan from cells not exposed to NP).

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

Nonylphenol as an environment contaminant interferes with reproduction in fish **(Weber *et al*., 2002; Cardinali *et al*., 2004)** and mammals **(Nagao *et al*., 2001; Tan *et al*., 2003)**, adversely affect male reproduction **(Gong and Han, 2006)** and induces cell death in gonads and changes to other reproductive parameters.

Evaluation of Bovine Spermatozoa Viability

The viability of bovine spermatozoa detected by the MTT cytotoxicity assay in groups with NP dissolved in 0.1% dimethyl sulfoxide was significantly decreased in the groups A with 1 µg/mL (P<0.05) and in the groups B (10 µg/mL), C (100 µg/mL) and D (200 µg/mL) (P<0.001). Results are shown in the figure 1. The spermatozoa viability was significantly (P<0.001) decreased in all experimental groups with nonylphenol dissolved in 0.1% ethanol. Results are shown in the figure 2.
After 6 h of *in vitro* cultivation the MTT assay proved that all doses of NP have a negative effect on the cell viability. The lowest survival of spermatozoa was determined after the addition of 200 µg/mL of nonylphenol **(Lukac *et al*., 2013)**.

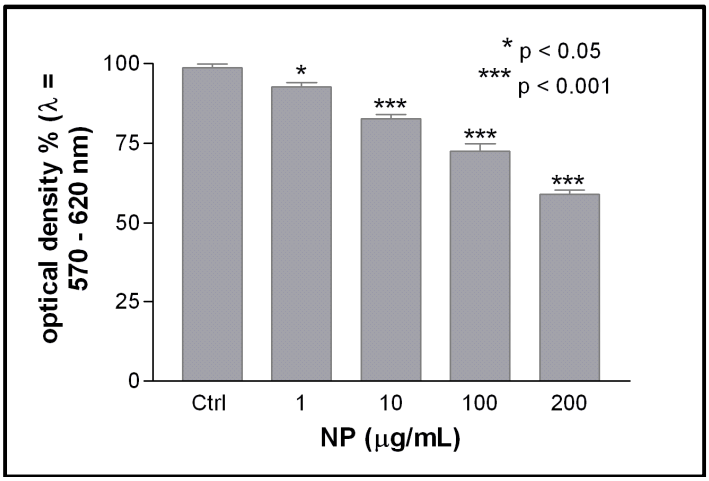

Figure 1 Effect of various concentrations of NP dissolved in 0.1% DMSO on the viability of bovine spermatozoa after 6 h.
Legend: Each bar represents the mean (± SD) optical density (λ = 570 - 620 nm) as percent of controls, which represented 100%. The control group was culture medium without NP administration. Group A – 1 µg/mL of NP; group B – 10 µg/mL of NP; group C – 100 µg/mL of NP; group D – 200 µg/mL of NP. The level of significance was set at P<0.001 and P<0.05. Statistical difference between the values of the control and treated spermatozoa in indicated by asterisks (Dunnett´s multiple comparison test). MTT assay.

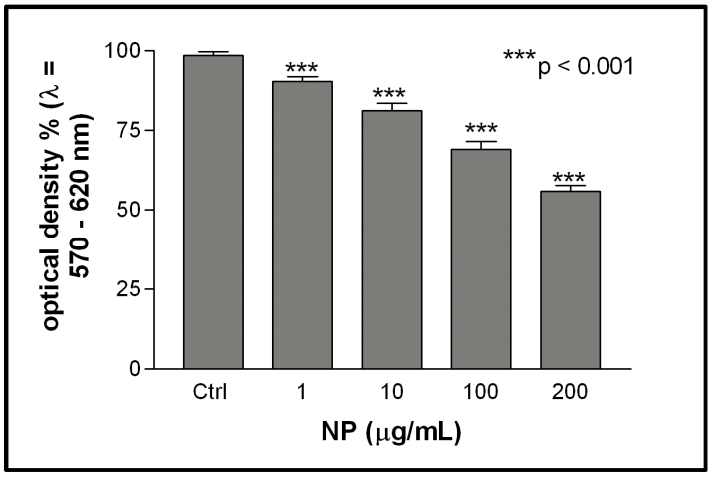

Figure 2 Effect of various concentrations of NP dissolved in 0.1% ethanol on the viability of bovine spermatozoa after 6 h.
Legend: Each bar represents the mean (± SD) optical density (λ = 570 - 620 nm) as percent of controls, which represented 100%. The control group was culture medium without NP administration. Group A – 1 µg/mL of NP; group B – 10 µg/mL of NP; group C – 100 µg/mL of NP; group D – 200 µg/mL of NP. The level of significance was set at P<0.001. Statistical difference between the values of the control and treated spermatozoa in indicated by asterisks (Dunnett´s multiple comparison test). MTT assay.

Mitochondria are important organelles in spermatozoa homeostasis and the activity of spermatozoa mitochondria is correlated with spermatozoa motility **(Gravance, 2000)**. Mitochondria are also the referential target of many toxic compounds **(Higgins and Rogers, 1974; Bragadin and Dell´Antone, 1996; Bragadin and Marton, 1997; Bragadin and Viola, 1997; Bragadin *et al*., 1998; Bragadin *et al*., 1999)** since damage to mitochondria which synthesize ATP gives rise to corresponding cell damage. **Bragadin *et al*. (1999)** found out that a low-level dose of nonylphenol inhibits ATP synthesis in mitochondria.
Apoptosis is a form of cell death by a characteristic set of morphological and biochemical changes. Apoptosis is recognized as an early cellular indicator of toxicity **(Weber et al., 2002)**. Nonylphenol induces apoptosis in rat testicular cells, particularly in Sertoli cells by inhibiting the Ca^{2+} pump in the endoplasmic reticulum **(Hughes *et al*., 2000)**. **Yao et al. (2007)** investigated the effects of NP on the mitochondrial membrane potencial and confirmed that the mitochondrial permeability transition was an important step in the induction of cellular apoptosis.
Gong *et al*. (2009) examined the effect of NP (0, 0.1, 1, 10, 20 and 30 µM) during time periods 6 h, 12 h and 24 h on viability of rat testicular Sertoli cells. Sertoli cell viability showed no significant variations between control and NP-treated groups after 6 and 12 h treatment. A significant decrease was reported after 24 h cultivation. In our *in vitro* study, we found that NP significantly affected the bovine spermatozoa viability after 6 h of cultivation. The lowest survival of spermatozoa was determined after the addition of 200 µg/mL of NP. Our data on spermatozoa viability are in accordance with the results described by **Gong and Han (2006)** who found out that NP significantly reduced the Sertoli cell viability at concentrations of 20 µM and higher after 12 and 24 h of cell cultivation.

CONCLUSION

Many authors investigated the toxic effects of nonylphenol on various types of cells and confirmed that nonylphenol detrimentally influences the viability of animal cells. Therefore the exposure to NP in animals and humans must be seriously observed. Our results support the idea that higher doses of NP (>100 µg/mL of NP) dissolved in 0.1% DMSO and in 0.1% ethanol negatively influence the bovine spermatozoa viability. We found out that all experimental concentrations of NP decreased the mitochondrial activity of spermatozoa after 6 h of cultivation. As mitochondria are substantial cell organelles their alterations lead to a loss of ATP production and subsequent cell death.

Acknowledgments: The research leading to these results has received funding from the European Community under project no 26220220180: Building Research Centre „AgroBioTech" and APVV-0304-12.

REFERENCES

AHEL, M., GIGER, W. 1993. Partitioning of alkylphenols and alkylphenol polyethoxylates between water and organic solvents. *Chemosphere*, 26, 1471-1478.

BRAGADIN, M., DELL´ANTONE, P. 1996. Mitochondrial bioenergetics as affected by cationic detergents. *Archives of Environmental Contamination and Toxicology*, 30, 280-284.
BLACJBURN, M.A., KIRBY, S.J., WALDOCK, M.J. 1999. Concentrations of alkyphenol polyethoxylates entering UK estuaries. Marine Pollution Bulletin, 38(2), 109–118.
BRAGADIN, M., MARTON, D. 1997. A proposal for a new mechanism of interaction of trialkyltin (TAT) compounds with mitochondria. *The Journal of Inorganic Biochemistry*, 68, 75-78.
BRAGADIN, M., VIOLA, E. R. 1997. Ni++ as a competitive inhibitor of calcium transport in mitochondria. In *The Journal of Inorganic Biochemistry*, 66, 227-229.
BRAGADIN, M., MARTON, D., MURGIA, M., RIZZOLLI, V., SCUTARI, G., DEANA, R. 1998. Interactions of trialkyllead compounds with rat liver mitochondria. *The Journal of Inorganic Biochemistry*, 69, 259-262.
BRAGADIN, M., PERIN, G., IERO, A., MANENTE, S., RIZZOLI, V., SCUTARI, G. 1999. An in vitro study on the toxic effects of nonylphenols (NP) in mitochondria. *Chemosphere*, 38, 1997-2001.
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.
DIAMANTI-KANDARAKIS, E., BOURGUIGNON, J. P., GIUDICE, L. C., HAUSER, R.,PRINS, G. S.,SOTO, A. M., ZOELLER, R. T., GORE, A. C. 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocrine reviews*, 30, 293-342.
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.
GRAVANCE, C. G., GARNER, D. L., BAUMBER, J., BALL, B. A. 2000. Assessment of equine sperm mitochondrial function using JC-1. *Theriogenology*, 53, 1691-1703.
HANSEN, P. D., DIZER, H., HOCK, B.,MARX, A., SHERRY, J., McMASTER, M., BLAISE, C. 1998. Vitellogenina – biomarker for endcrine disruptors. *TrAC Trends in Analytical Chemistry*, 17, 448-451.
HOTCHKISS, A. K., RIDER, C. V., BLYSTONE, C. R., WILSON, V. S., HARTIG, P. C., ANKLEY, G. T., FOSTER, P. M., GRAY, C. L., GRAY, L. E. 2008. Fifteen years after "Wingspread"--environmental endocrine disrupters and human and wildlife health: where we are today and where we need to go. *Toxicological Sciences*, 105, 235-259.
HIGGINS, E. S., ROGERS, K. S. 1974. Effects of cetyltrimethylammonium and dodecylnicotinamide halides on functional states of mitochondria. ,*Proceedings of the Society for Experimental Biology and Medicine*, 146, 85-91.
HUGHES, P. J.,McLELLAN, H., LOWES, D. A., KHAN, 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.
IWATA, M., ESHIMA, Y., KAGECHIKA, H. 2003. Retinoic acids exert direct effects on T cells to suppress Th1 development and enhance Th2 development via retinoic acid receptors. *International immunology*, 15, 1017-1025.
LUKAC, L., LUKACOVA, J., PINTO, B., KNAZICKA, Z., TVRDA, E., MASSANYI, P. 2013. The effect of nonylphenol on the motility and viability of bovine spermatozoa in vitro. Journal of environmental science and health. Part A, Toxic/hazardous substances & environmental engineering, 48(8), 973-979.
MIYAURA, H., IWATA, M. 2002. Direct and indirect inhibition of Th1 development by progesterone and glucocorticoids. *Journal of immunology*, 168, 1048-1094.
MONTGOMERY-BROWN, J., REINHARD, M. 2003. *Occurrence and behavior of alkylphenol* polyethoxylates in the environment. *Environmental Engineering Science*, *20, 471-478.*
MOSMANN, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. The Journal of Immunological Methods, 65(1-2), 55-63.
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*, 5, 293-315.
NIMROD, A. C., BENSON, W. H. 1996. Environmental Estrogenic Effects of Alkylphenol Ethoxylates. *Critical Reviews in Toxicology*, 26, 335-364.
PINTO, B., PICARD, D., REALI, D. 2004. A recombinant yeast strain as a short term bioassay to assess estrogen-like activity of xenobiotics. *Annali di igiene : medicina preventiva e di comunità*, 16, 579-585.
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.
SERVOS, M. R. 1999. Review of the aquatic toxicity, estrogenic responses and bioaccumulation of alkylphenols and alkylphenol polyethoxylates. *Water Quality Research Journal* of Canada, 34(1), 123-177.
SUMPTER, J.P., JOHNSON, A.C.2005. Lessons from Endocrine Disruption and Their Application to Other Issues Concerning Trace Organics in the Aquatic Environment. Environmental *Science* and *Technology,* 39(12), 4321-4332.
TAN, B. L., KASSIM, N. M., MOHD, M. A. 2003. *Assessment of pubertal development in juvenile male rats after sub-acute* exposure to bisphenol A and nonylphenol. *Toxicology letters*, 143, 261-270.
TAPIERO, H., BA, G.N., TEW, K.D. 2002. Estrogens and environmental estrogens. *Biomedicine & Pharmacotherapy,* 56, 36-44.
UGUZ, C., ISCAN, M., ERGÜVEN, A., ISGOR, B., TOGAN, I. 2003. The bioaccumulation of nonyphenol and its adverse effect on the liver of rainbow trout (Onchorynchus mykiss). *Environmental Research,* 92(3), 262-270.
WEBER, L. P. ,YIANNIS, K., HWANG, G. S., NIIMI, A. J., JANZ, D. M., METCALFE, C. D. 2002. Increased cellular apoptosis after chronic aqueous exposure to nonylphenol and quercetin in adult medaka (Oryzias latipes). *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, 131, 51-59.
WHITE, R., JOBLING, S., HOARE, S. A., SUMPTER, J. P., PARKER, M. G. 1994. Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology*, 135, 175-182.
YAO, G., LING, L., LUAN, J., YE, D., ZHU, P. 2007. Nonylphenol induces apoptosis of Jurkat cells by a caspase-8 dependent mechanism. *International Immunopharmacology*, 7, 444-453.