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RESEARCH ARTICLE

Effects of Nonylphenol on Motion Kinetics, Acrosome and Mitochondrial Membrane Potential in Frozen-Thawed Bull Sperm [1]

Cevdet UĞUZ [1] Ömer VARIŞLI [2] Cansu AĞCA [3] Yüksel AĞCA [3]

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[1] Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Medical Biology and Genetics, TR-03200 Afyonkarahisar - TURKEY
[2] Harran University, Faculty of Veterinary Medicine, Department of Artificial Insemination, TR-63300 Şanlıurfa - TURKEY
[3] University of Missouri-Columbia, College of Veterinary Science, Department of Pathobiology, Columbia, MO 65211, USA

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Summary

Recent reports have shown that the Nonylphenol (NP), a well-known toxic environmental chemical has adverse effects on sperm in different species. Therefore, the current study was aimed to determine the deleterious effect of NP on frozen-thawed bull sperm. Sperm were exposed to different concentrations of NP (1, 10, 100, 250 and 500 µg NP/mL) dissolved either in DMSO or Ethanol and incubated for 4 h at 37°C. Sperm parameters were assessed at 0, 1st, 2nd, 3rd, and 4th h of incubation. Both percent motility and mitochondrial membrane potential (MMP) of sperm have decreased significantly ($P<0.05$) at dose level of ≥ 250 µg NP/mL. Acrosome reaction was induced ($P<0.05$) even at minimal dose concentrations of 1µg NP/mL ($P<0.05$). However, the percentage of acrosome reacted sperm increased ($P<0.05$) with increase in dose level. In conclusion, NP impairs the sperm characteristics by adversely affecting motion kinetics, mitochondrial membrane potential and inducing premature acrosome reaction in bovine.

Keywords: *Acrosome, Bull sperm, DNA, Flow cytometry, Mitochondria, Nonylphenol*

Nonylphenol'ün Dondurulmuş-Çözdürülmüş Boğa Sperma Hareket Kinetiğine, Akrozom ve Mitokondrial Membran Potansiyeline Etkisi

Özet

Son çalışmalar çevre toksini olarak bilinen Nonylpfenol (NP)'in, değişik türlerin sperm parametreleri üzerine zararlı etkisinin olduğunu göstermiştir. Bu çalışma NP'in dondurulmuş-çözdürülmüş boğa spermaası üzerine etkisinin tespit edilmesi amacı ile yapılmıştır. Sperma örnekleri Ethanol veya DMSO içerinde değişik yoğunlukta (1, 10, 100, 250 ve 500 µg/ml) çözdürülen NP ile 4 saat 37°C'de inkübe edildi. Sperm parametreleri inkübasyonun 0., 1., 2., 3. ve 4. saatlerinde ölçüldü. Hem motilite ve hem de mitokondrial membran potansiyeli 250 µg NP/ml ≥ dozlara sahip gruplarda önemli derecede düşmüştür ($P<0.05$). Minimal doz olan 1µg/ml NP'de akrozom reaksiyonunu indüklenmiştir ($P<0.05$), ancak dozun artmasına bağlı olarak akrozom reaksiyonu geçirmiş sperm oranında da artışlar olduğu tespit edilmiştir ($P<0.05$). Sonuç olarak NP; spermatozoa hareket kinetiği, mitokondrial membran potansiyeli ve akrozom reaksiyonu üzerine negatif etki yaparak sperma parametrelerine zarar verdiği saptanmıştır.

Anahtar sözcükler: *Akrozom, Boğa spermaası, DNA, Flow sitometri, Mitokondri, Nonylphenol,*

İletişim (Correspondence)
+90 272 2281312/133
cuguz@aku.edu.tr

INTRODUCTION

There has been a serious concern in the industrial world that many living organisms on earth, from lower vertebrates to mammals, are likely to be directly or indirectly exposed to environmental chemicals such as pesticides and other industrial chemicals used in livestock production [1-3]. Among these industrial chemicals alkyl-phenolpolyethoxylates (APEs), also called estrogenic environmental endocrine disrupters, are widely used as non-ionic surfactants and anti-oxidants in detergents, herbicides, pesticides, paints, plastic ware, emulsifiers [4] and intra vaginal spermicides [5]. About 650.000 metric tons of APEs is produced in a year around the world and it has been shown that 60% are accumulated in the streams, rivers, lakes, and seas [6]. APEs undergo biodegradation processes to give short side chain derivatives such as nonylphenol (NP), octylphenol (OP) and butylphenol (BP) in anaerobic conditions in water [7]. NP, one of the most abundant derivatives of APEs, has been demonstrated to stay in a biologically active state in the body longer natural estrogen [8]. NP has an estrogenic activity posing a potential danger to living organisms in various ways. For example, nonoxynol-9 consisting NP is used as an intravaginal spermicide and condom lubricant [3]. When administered to lab animals it is metabolized to free NP inhibiting testicular development and decreasing sperm motility, viability and sperm count [9-16].

The estrogenic effects of chemicals might emerge by (1) mimicking the effect of endogenous hormones, (2) antagonizing the effect of endogenous hormones, (3) disrupting the synthesis and metabolism of endogenous hormones or (4) disrupt the synthesis and metabolism of hormone receptors [3]. To date, the adverse effects of NP on many different species including mice [14,17], rats [13,18], quails [19] and aquatic species including fish, and sea urchins [1,10,11,20-23] have been reported. Cattle are the most widely raised livestock and bull sperm is used for artificial insemination worldwide. Thus, determining the effect of environmental pollutants such as NP on sperm is important for bulls' reproductive health.

Concentrations of NP in air, soil and water are not lethal to living organisms. However NP have been reported to accumulate in especially fat tissue up to 750 folds more than its environmental concentrations [20,21,24-26]. Numerous studies have shown that APEs, especially NP, is found in foodstuffs including fresh fruits vegetables and rice [6,27,28,29], human milk [30-32] aquatic as well as livestock products [22,23]. Kawaguchi et al.[33] demonstrated that feedstuffs used in animal nutrition might contain about 200 µg NP/kg. Thus APEs particularly NP may pose serious threat on the health, reproduction and fertility of terrestrial organisms through the intake of NP in foodstuffs. Therefore, the current study was aimed to determine the deleterious effect of NP on motion kinetics, acrosome reaction and mitochondrial membrane potential of frozen-thawed bull sperm.

MATERIAL and METHODS

Chemicals

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated.

Sperm Samples

Frozen sperm samples from Holstein bulls were thawed and diluted in HEPES buffered Tyrode's lactate (TL-HEPES) solution (~290 mOsm and pH 7.4) containing 3 mg/mL bovine serum albumin (BSA). For each treatment eight straws of frozen sperm samples were thawed and pooled in a 10 ml tube. Sperm samples were washed twice by centrifugation (275 x g) for 15 min each time, re-suspended in culture medium to 100 x 10^6 sperm/mL, and held at 20-25°C for not more than 1 h before incubations were initiated. Initial motility was determined by a computer-assisted sperm motility analysis (CASA) system (Hamilton Thorne Biosciences, Inc., M2030, Beverly, MA). Sperm samples having 80% or higher motility were used. The experiment was replicated three times.

Sperm Incubation with NP and Motility Assessment

To determine the effects of NP on sperm parameters in treatment groups, two different solvents, namely ethanol (ETOH) and dimethyl sulfoxide (DMSO), were used to dissolve and deliver NP (4-Nonylephenol with 99% purity) to the sperm within the medium. Both solvents were also tested alone for their ability to alter sperm quality. Aliquots of 0.5 mL with concentration of 100x10^6 sperm/mL were transferred in one set of 12x55 mm polypropylene tubes containing 0 (control), 1% ETOH (solvent control), 1 µg 17β-estradiol /mL, and 1, 10, 100, 250 and 500 NP/mL. Aliquots of 0.5 mL with concentration of 100x10^6 sperm/mL were transferred to another set of 12x55 mm propylene tubes containing 0 (control), 1% DMSO (solvent control), 1 µg 17β-estradiol /mL, and 1, 10, 100, 250 and 500 µg NP/mL. NP and 17β-estradiol used in the previous set were dissolved in ethanol, while NP and 17β-estradiol used in the latter set were dissolved in DMSO. While attaining the above final concentrations of NP or 17β-estradiol, neither ETOH nor DMSO exceeded the amount of 1%. Therefore, either 1% ETOH or DMSO was also added in the previous and latter set, respectively. All samples were then incubated for 4hr at 37ºC and 5% CO_2. At the end of incubation time point 1, 2, 3 and 4 h, sperm motility characteristics (*i.e.* total motility, progressive motility and average path velocity; VAP) were assessed and recorded for each treatment using CASA. Briefly, Aliquots of 10 µl bull sperm suspension were put onto a pre-warmed 10 µm deep Makler Counting Chamber (Sefi-Medical Instruments, Haifa, Israel) and sperm motility characteristics were determined by the

CASA system at 37°C. At least 6 fields were counted and the measurements were replicated 2 times for each donor. Minimums of about 200 cells were analyzed in 6 fields. Motility estimates were validated manually by the video playback option of the instrument. The setting parameters of measured sperm motion parameters for the CASA were: frames per second; the duration of the tracking time (1 sec), average path velocity cutoff (50 µm/sec) and low VAP cutoff (20 µm/sec).

Fluorescent Microscopic Evaluation of Acrosomal Integrity

At the end of 4 h exposure of bull sperm to various concentrations (0, 1, 10, 100, 250, and 500 µg/mL dissolved NP in either DMSO or ETOH), aliquots' of 3 µl samples from each treatment were smeared onto microscope slides to determine the effects of NP on the acrosomal status of bull sperm. Epifluorescent microscopy was used to assess acrosomal integrity after staining with Alexa Fluor-488-PNA (peanut agglutinin) conjugate (catalog no: L-21409, Molecular Probes, Eugene, OR, USA). The treated and control samples were smeared onto microscope slides and air-dried. The specimens were fixed with 99% methanol and kept at room temperature until fluorescence staining. For staining, slides were incubated with 20 µg/mL Alexa Fluor-488-PNA at 37°C for 30 min, washed with PBS, and analyzed by epifluorescent microscopy (Zeiss Axiophot, Germany, using a appropriate fluorescence filter). The images of stained sperm samples were classified into 2 groups: Sperm displaying strong and moderate bright fluorescence in the acrosomal region were considered to be intact, whereas sperm displaying weak, patchy, or no fluorescence in the acrosomal region were considered to be damaged. 100 sperm on each slide were evaluated to determine the proportion of sperm with intact acrosomes [13].

Flow Cytometric Analysis of Mitochondrial Membrane Potential

The lipophilic cationic probe JC-1 (Molecular Probes, Eugene, OR, USA) was used to assess the mitochondrial status of the sperm before and after treatments following the manufacturer's guidelines. Essentially, the fluorescence of JC-1 changes reversibly from green (monomeric status to orange (multimeric status) when mitochondrial membrane potential is high [34]. The sperm samples were exposed to NP for different time points (1 and 4 h) and concentrations 0 (control), 1, 10, 100, 250, and 500 µg/mL. The samples were then diluted with TL-HEPES and 300 µl were transferred to 12x55 mm polypropylene tubes. 3 µl of JC-1 stock solution (2 µM JC-1 in DMSO) were added and the tubes were incubated at 37°C in a humidified incubator with 5% CO_2 and 95% air for 30 min. At the end of each incubation period, the stained samples were brought to the Flow Cytometry Core Facility for analysis using a FACS analyzer flow cytometry (FACSCalibur, Becton-Dickinson, and San Hose CA). The generated data were examined using Cell Quest software (version 3.3) to determine the cells with high mitochondrial membrane potential (orange stained) using the appropriate gating parameters. Ten thousand spermatozoa from each treatment were used in the evaluation of mitochondrial membrane potential.

Flow Cytometric Analysis of Chromatin Integrity

The sperm samples were exposed to NP for different time points (1 and 4 h) and concentrations 0 (control), 1, 10, 100, 250, and 500 µg/mL. We used the exact method that was previously described for mouse [35], bull, deer and dog sperm [36] to assess chromatin stability using metachromatic staining with Acridine Orange (AO) (In vitrogene, Molecular Probes, Eugene, OR, USA). Essentially, AO dye is known to fluorescence in green when incorporated into intact double stranded DNA helix, and in red when incorporated into denatured DNA. Briefly, samples were diluted with TNE buffer (pH 7.4) into polypropylene tubes at a final sperm concentration of $30x10^6$ cells/mL. For analysis, samples kept in an ice water bath at 4°C were mixed with 400 µl of an acid-detergent solution (pH 1.4). Exactly 30 sec later, 1.20 mL of acridine orange staining solution (pH 6.0, 4°C) containing 6 µg/mL purified AO was added. The stained samples were analyzed at 30 min after AO staining. Samples were then analyzed on a FACS Calibur Flow Cytometer (Becton Dickinson Immunochemistry Systems; San Jose, CA, USA), equipped with standard optics and an argon laser tuned at 488 µm. The generated data were examined using Cell Quest software (version 3.3) to determine the cells with damaged chromatin using the appropriate gating parameters. Ten thousands spermatozoa from each treatment used in the evaluation of chromatin status.

Statistical Analysis

The data were analyzed by analysis of variance. MINITAB was used to analyze the data. The time effect, treatment effect, and their interaction were determined. Tukey's test was used to compare means using pair-wise comparison. For all statistical tests, α-value of 0.05 and 0.01 were chosen as the level of significance.

RESULTS

Bull sperm motility values (mean±SEM) after exposure to different concentrations of NP dissolved in either ethanol or in DMSO at the end of each incubation period are shown in *Table 1* and *Table 2*, respectively. The initial motility the sperm samples was 90±3%. Treatment of the sperm samples with1 µg 17β-estradiol/mL dissolved in ETOH or DMSO for 3 or 4 h did not have any effect on bull sperm motility ($P>0.05$). Sperm motility was not significantly affected ($P>0.05$) by 1 µg to 10 µg NP/mL concentrations. However, there was a significant decrease ($P<0.05$) in the sperm motility in 100, 250, and 500 µg NP/

Table 1. *Effects NP and 17β-E_2 diluted in ETOH on the motility of bull sperm*

Tablo 1. *ETOH içerisinde çözdürülen NP ve 17β-E_2'nin boğa sperma motilitesi üzerine etkisi*

Treatment	n	Percent Motility (mean±SEM)			
		1 h	2 h	3 h	4 h
Control	3	$90\pm1^{a,*}$	$85\pm2^{a,*}$	$76\pm6^{a,*}$	$79\pm8^{a,*}$
ETOH	3	$91\pm2^{a,*}$	$83\pm3^{a,*}$	$78\pm6^{a,*}$	$74\pm8^{a,*}$
17β- Estradiol	3	$91\pm1^{a,*}$	$81\pm6^{a,*}$	$74\pm9^{a,*}$	$73\pm10^{a,*}$
1 µg NP/mL	3	$93\pm2^{a,*}$	$92\pm1^{a,*}$	$86\pm3^{a,*}$	$83\pm5^{a,*}$
10 µg NP/mL	3	$92\pm1^{a,*}$	$86\pm2^{a,*}$	$78\pm5^{a,*}$	$81\pm4^{a,*}$
100 µg NP/mL	3	$80\pm5^{a,*}$	$69\pm8^{a,*}$	$65\pm9^{a,*}$	$51\pm8^{a,**}$
250 µg NP/mL	3	$49\pm3^{a,**}$	$50\pm5^{a,**}$	$33\pm7^{a,**}$	$12\pm4^{a,***}$
500 µg NP/mL	3	$9\pm4^{a,***}$	$4\pm2^{a,***}$	$3\pm1^{a,***}$	$3\pm1^{a,****}$

*^{a,b} Different letters indicate significance in rows at (P<0.05); * Numbers indicate significance in columns at (P<0.05)*

Table 3. *Progressive motility and velocity values of bull sperm that were subjected to different NP concentrations dissolved in ETOH*

Tablo 3. *ETOH içerisinde çözdürülen değişik konsantrasyonlardaki NP'nin, boğa sperması prograsiv motilite ve hızına etkisi*

Treatment	n	Progressive Motility (%) (means ± SEM)		Velocity (VAP) mic/sec (means ± SEM)	
		1 h	4 h	1 h	4 h
Control	3	70 ± 7^{a}	54 ± 1^{ab}	105 ± 6^{a}	105 ± 5^{a}
ETOH	3	59 ± 3^{a}	62 ± 10^{a}	98 ± 5^{ab}	103 ± 13^{a}
17β- Estradiol	3	61 ± 6^{a}	51 ± 4^{ab}	97 ± 2^{ab}	79 ± 7^{b}
1 µg NP/mL	3	66 ± 7^{a}	58 ± 2^{a}	97 ± 3^{ab}	92 ± 6^{ab}
10 µg NP/mL	3	63 ± 9^{a}	55 ± 2^{ab}	98 ± 3^{ab}	86 ± 5^{ab}
100 µg NP/mL	3	53 ± 1^{ab}	38 ± 6^{b}	90 ± 1^{ab}	87 ± 4^{ab}
250 µg NP/mL	3	4 ± 1^{c}	0±0	39 ± 4^{c}	0±0
500 µg NP/mL	3	2 ± 2^{c}	0±0	40 ± 6^{c}	0±0

^{a,c} Different letters indicates significance at P<0.05

Table 2. *Effects NP and 17β-E_2 diluted in DMSO on the motility of bull sperm*

Tablo 2. *DMSO içerisinde çözdürülen NP ve 17β-E_2'nin boğa sperma motilitesi üzerine etkisi*

Treatment	n	Percent Motility (mean±SEM)			
		1 h	2 h	3 h	4 h
Control	3	$91\pm1^{a,*}$	$85\pm3^{a,*}$	$84\pm3^{a,*}$	$90\pm2^{a,*}$
DMSO	3	$92\pm1^{a,*}$	$83\pm3^{a,*}$	$80\pm4^{a,*}$	$77\pm7^{a,*}$
17β- Estradiol	3	$85\pm6^{a,*}$	$81\pm5^{a,*}$	$87\pm1^{a,*}$	$79\pm5^{a,*}$
1 µg NP/mL	3	$87\pm3^{a,*}$	$83\pm3^{a,*}$	$83\pm4^{a,*}$	$86\pm2^{a,*}$
10 µg NP/mL	3	$92\pm4^{a,*}$	$89\pm2^{a,*}$	$89\pm1^{a,*}$	$82\pm3^{a,*}$
100 µg NP/mL	3	$87\pm4^{a,*}$	$88\pm1^{a,*}$	$86\pm3^{a,*}$	$75\pm6^{a,*}$
250 µg NP/mL	3	$56\pm4^{a,**}$	$53\pm5^{a,**}$	$53\pm6^{a,**}$	$47\pm9^{a,**}$
500 µg NP/mL	3	$14\pm1^{a,***}$	$19\pm3^{a,***}$	$9\pm6^{a,***}$	$3\pm2^{a,***}$

*^{a,b} Different letters indicate significance in rows at (P<0.05); * Numbers indicate significance in columns at (P<0.05)*

Table 4. *Percent progressive motility and velocity values of bull sperm that were subjected to different NP concentrations dissolved in DMSO*

Tablo 4. *DMSO ile çözdürülen değişik konsantrasyonlardaki NP'nin, boğa sperması prograsiv motilite ve hızına etkisi*

Treatment	n	Progressive Motility (%) (means ± SEM)		Velocity (VAP) mic/sec (means ± SEM)	
		1 h	4 h	1 h	4 h
Control	3	65 ± 5^{a}	63 ± 5^{a}	108 ± 4^{a}	97 ± 1^{a}
DMSO	3	63 ± 8^{a}	53 ± 5^{ab}	105 ± 2^{a}	91 ± 2^{ab}
17β- Estradiol	3	58 ± 4^{ab}	50 ± 4^{ab}	96 ± 3^{ab}	99 ± 7^{ab}
1 µg NP/mL	3	54 ± 2^{ab}	61 ± 2^{a}	103 ± 1^{a}	96 ± 9^{a}
10 µg NP/mL	3	57 ± 5^{ab}	55 ± 6^{ab}	84 ± 9^{ab}	105 ± 2^{a}
100 µg NP/mL	3	60 ± 2^{ab}	53 ± 6^{ab}	104 ± 7^{a}	102 ± 5^{a}
250 µg NP/mL	3	46 ± 7^{abc}	29 ± 6^{bcd}	99 ± 7^{ab}	86 ± 9^{ab}
500 µg NP/mL	3	33 ± 15^{bcd}	25 ± 16^{bcd}	82 ± 10^{b}	41 ± 26^{c}

^{a,d} Different letters indicates significance at P<0.05

mL treatment at the end of all incubation time periods (*Table 1* and *Table 2*) in comparison to non-treated control. As shown in *Table 2*, bull sperm motility values was not significantly affected by 1, 10, and 100 µg NP/mL dissolved in DMSO (P>0.05), while it was significantly affected by 250 and 500 µg NP/mL dissolved in DMSO treatments (P<0.05). The progressive motility and velocity values in response to different concentrations are shown in *Table 3* and *Table 4*. Although there are gradual decreases in both progressive motility and velocity values in a dose and time dependent manner, significant decrease occur in concentrations over 100 µg NP/mL, especially in dissolved ETOH NP treated groups (P<0.05).

Treatment of sperm samples with 1 µg NP/mL dissolved in ETOH did not significantly affect the rate of acrosome defect *(Fig. 1)* in comparison to control (P>0.05), while 1 µg NP/mL dissolved in DMSO significantly increased the rate of acrosome defect in sperm samples *(Fig. 2)* compared to the control (P<0.05). Estradiol dissolved in both ETOH and DMSO as well as higher concentration of NP including 10, 100, 250, and 500 µg NP/mL dissolved either in ETOH or DMSO significantly induced bull sperm AR in comparison to both non-treated control and solvent control groups (P<0.05) (*Fig. 1* and *Fig. 2*).

Flow cytometric dot plots of bull sperm stained with JC-1 followed by exposure to different concentrations of NP dissolved in either ETOH or DMSO are shown in *Fig. 3*. This experiment was carried out for 1 or 4 h. MMP was significantly (*P*<0.05) reduced by incubation of bull sperm with 250 and 500 µg/mL NP dissolved in either ETOH *(Fig. 4)* or DMSO *(Fig. 5)*. Although it is not significant, the NP induced decrease in MMP appear to be higher in NP dissolved in ETOH group than NP dissolved in DMSO.

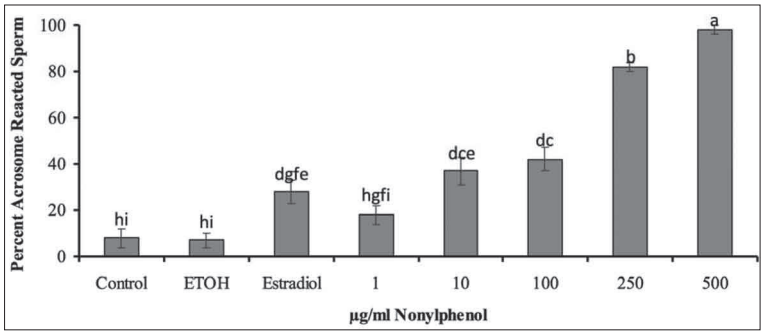
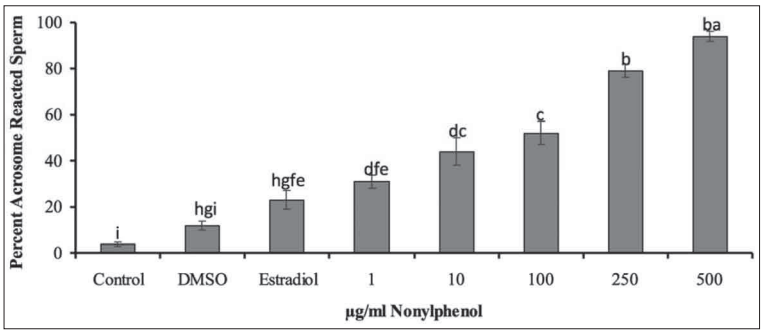


Fig 1. Effects of various concentrations of NP dissolved in ethanol on acrosomal integrity of epididymal bull sperm (P<0.05; different letters indicate significance, n =5)

Şekil 1. Ethanol içerisinde değişik konsantrasyonlarda hazırlanan NP'nin, boğa sperması akrozom bütünlüğüne etkisi (P<0.05; farklı harfler istatistiki farkı belirtir, n =5)

Fig 2. Effects of various concentrations of NP dissolved in DMSO on acrosomal integrity of epididymal bull sperm (P<0.05; different letters indicate significance, n =5)

Şekil 2. DMSO içerisinde değişik konsantrasyonlarda hazırlanan NP'nin, boğa sperması akrozom bütünlüğüne etkisi (P<0.05; farklı harfler istatistiki farkı belirtir, n =5)



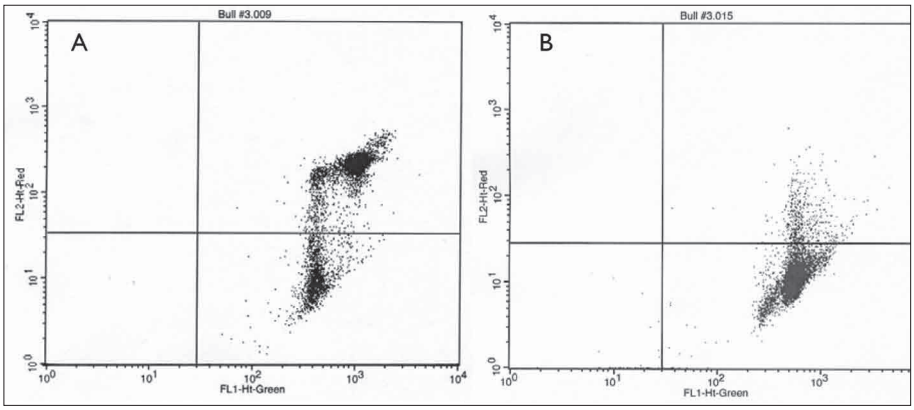


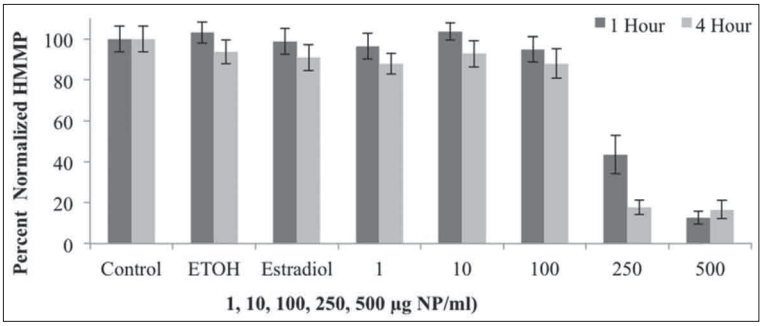
Fig 3. Representative flow cytometric dot plots to illustrate the effects of NP dissolved in ETOH or DMSO on bull sperm. Sperm cells in control or solvent control groups are shown in Panel A, while panel B shows the effects of 250 and 500 µg NP/ml dissolved in ETOH or DMSO on sperm cells

Şekil 3. ETOH ve DMSO içerisinde çözdürülen NP'nin, boğa sperması üzerine etkisinin flow sitometrik analiz ile gösterimi. Kontrol ve solvent kontrol grupları panel A'da gösterilmiştir. Panel B ise, ETOH veya DMSO içerisinde çözdürülen 250 ve 500 µg/ml oranlarındaki NP'nin, sperma üzerine etkisini göstermektedir

Fig 4. The effects of various concentrations of NP dissolved in ethanol on mitochondrial membrane potential of bull sperm (*P<0.05 vs. untreated control). HMMP: high mitochondrial membrane potential

1 h: 100% = 65.71, 4 h: 100% 63.38

Şekil 4. ETOH içerisinde değişik konsantrasyonlarda hazırlanan NP'nin, boğa sperması mitokondrial membran potansiyeline etkisi (*P<0.05, işlem uygulanmamış grup). HMMP: yüksek mitokondrial membran potansiyeli



In this experiment, we did not observe any NP induced impairment (P>0.05) of chromatin integrity of bull sperm regardless of NP concentration and duration of NP exposure based on AO staining and subsequent flow cytometric analysis (n=3).

DISCUSSION

Alkylphenolpolyethoxylates (APEs) are known estrogenic environmental endocrine disrupter. One of the derivatives of APEs called NP has been shown to be widely found in fisheries and livestock products [14,29]. It has also been reported to be present in drink water and in foods [4,6,27]. Mothers consuming fish three times had NP in their milk with an amount of 32 ng/mL [29]. Therefore, it has been a serious concern for the world that many living organisms from lower vertebrates to mammals are likely to be directly or indirectly exposed to

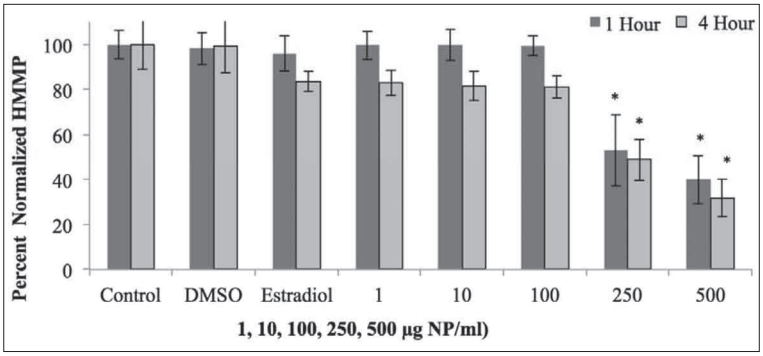


Fig 5. Effects of different concentrations of NP dissolved in DMSO on mitochondrial membrane potential of bull sperm (*P<0.05 vs. untreated control: n=3). HMMP: high mitochondrial membrane potential

1 h: 65.71, 4 h: 63.38

Şekil 5. DMSO içerisinde, değişik konsantrasyonlarda hazırlanan NP'nin, boğa sperması mitokondrial membran potansiyeline etkisi (*P<0.05, işlem uygulanmamış grup; n=3) HMMP: yüksek mitokondrial membran potansiyeli

various endocrine-disrupting substances present in the environment.

These contaminants could cause a number of reproductive health problems in exposed organisms including human, livestocks and aquatic species [1,2]. In vivo estrogenic activity of NP is much more potent than that of in vitro estrogenic activity of NP [37]. Exposure of the general population to NP is unavoidable because of the presence of the agent in the environment [17]. The effects of NP on livestocks have been described such as disruption in testicular development, decrease in sperm motility, viability, decrease in sperm count which eventually led to the decrease in male fertility [12-14,38]. Adverse effects of NP on mice [14,17], rat [13,18] and aquatic species [1,10,11,23,39] have also been reported.

Lukac et al.[16] reported that NP causes significant decrease in the viability and motility of bull sperm. Our results are in parallel to these findings. Indeed, motility analysis is most commonly used to evaluate semen quality. However, this assay alone is not enough to determine the fertilizing capacity of semen samples. Therefore, we examined the effects of NP on acrosome reaction and mitochondrial membrane potential in addition to sperm motion kinetics. It is possible to simultaneously measure multiple sperm parameters, including cell viability, acrosomal integrity and mitochondrial function. All these evaluation parameters help us to accurately describe the ability of spermatozoon fertilization capacity [40].

The present study showed that the motility of bull sperm was significantly (P<0.05) affected when they were exposed to ≥250 µg NP/mL solved in ETOH (*Table 1* and *Table 2*) or DMSO. As shown in *Table 3*, when sperm samples were exposed to NP in the presence of ETOH, the progressive motility and velocity of sperm were significantly decreased after treatment with 250 µg NP/mL (P<0.05). These results are in accordance with results obtained by Lukac et al.[16] in terms of NP mediated decrease in motility of bull sperm. However, we observed the NP mediated inhibition at a much higher concentration than used by Lukac et al.[16]. Inhibition of bull sperm motility by NP correlated with a decrease in the MMP (*Table 1, Table 2,* and *Fig. 3*).

Estrogens play an important role in the development and regulation of the male reproductive system. Non-nuclear receptors for estradiol are present on sperm plasma membrane [41]. It is well known that E2 and environmental estrogens can significantly stimulate mammalian sperm capacitation and acrosome reactions [37,41]. This research showed that NP-induced alterations in acrosomal status appeared to be one of the most sensitive indicators of NP exposure and significant acrosomal reaction of bull sperm started at NP exposures ≥1 µg NP/mL solved both in ETOH and DMSO. In addition, although motility parameters and mitochondrial integrity of sperm were affected at NP concentrations as low as 100 NP µg/mL and 250 µg NP/mL, respectively; acrosomal integrity was significantly reduced at even 1 µg NP/mL (*Fig. 1* and *Fig. 2*). These results support the sensitivity of acrosomal status as an indicator of toxic insult to sperm. This study on bull sperm is in agreement with previous studies which showed NP-induced sperm capacitation and subsequent acrosomal reaction in human and mouse sperm due to increase in intracellular level of cAMP [37,42]. Also, Adeoya-Osiguwa et al.[37] reported that E2 and environmental estrogens such as genistein (Gen), 8- prenylnaringenin (8-PN) and nonylphenol (NP) significantly affect the function of mature sperm by stimulating capacitation and fertilizing ability, compared with untreated controls. NP mediated capacitation or acrosome reaction is not considered an advantage for successful fertilization. On the contrary, it may be a disadvantage for sperm to fertilize an egg since it may cause premature acrosome reaction impairing sperm ability to reach the egg.

Sperm motility depends on mitochondrial function and thus sperm having adequate mitochondrial integrity (*i.e.,* high MMP) should have robust motility. Previous studies suggested that MMP of bovine sperm determined by JC-1 staining correlated well with their progressive motility [43]. Uguz et al.[13] reported that NP has adverse effects on progressive sperm motility and MMP in rats. In the present study, MMP of bull sperm responded to exposures to various concentrations of NP in a similar fashion as progressive motility. This may suggest that mitochondrial damage during chemical exposure could be one of the major reasons for reduced sperm motility. Bull sperm with normal mitochondrial integrity significantly declined

following exposure to 250 and 500 µg/NP. The amount of sperm with high MMP correlated well with the sperm having high percentages of progressive motility. This suggests that NP causes a decrease in motility by causing decrease in MMP in both rat and bull sperm. However, there are some differences in terms of NP-mediated damage between bull and rat sperm. Rat perm motility is more susceptible to NP exposure than that of bull sperm. For example, over 250 µg NP/mL concentrations have lethal effect on rat sperm [13]. The present study showed that 500 µg NP/mL concentrations were not lethal to bull sperm. This indicates that although NP has toxic effects on sperm, this effect is dose and time dependent in different species.

Although there was a dose-dependent adverse effect of NP on motility and mitochondrial and acrosomal integrity in bull, this study did not reveal any adverse effect of NP on sperm chromatin integrity of either species, up to 4 h of exposure in vitro. Han et al.[44] reported that the rats receiving NP by gavage at 250 mg/kg/day showed decrease in epididymal sperm density as well as the serum testosterone level. It has also been shown that the number of apoptotic cells in seminiferous tubules of treated rats was increased in a dose dependent manner. Similarly, Dobrzyńska [17] reported that eight-week exposure to NP and X-ray/NP combination reduced sperm counts while it increased the percent of abnormal spermatozoa and DNA damage in gametes in mice. Karadeniz et al.[45] reported that increasing concentration of NP dissolved in ethanol cause guanine oxidation in DNA determined by carbon nano-tube electrode. It has been suggested that base oxidation may lead to mutation in DNA [46]. However, Bennetts et al.[47] reported that 17β-estradiol, NP or Bisphenol A treated human spermatozoa did not show any DNA damage. It has also been shown that NP does not cause any damage in rat sperm DNA [13,48]. The results of the study confirm the findings of the above studies [13,47,48] that NP did not cause any damage in bull sperm DNA. Mammalian spermatogenesis is a dynamic process, during which the structure of sperm chromatin undergoes extensive alterations during the male germ cell development. These alterations are facilitated by a series of meiotic and mitotic events and the addition of protamines, ultimately leading to formation of tightly packed sperm chromatin [49]. Therefore, it is likely that mature sperm DNA are more resistant against mutagens than that of those early male germ cells (*i.e.* spermatogonia) [35].

NP is a lipophilic substance; therefore, it does not dissolve in water [24]. Dimethyl sulfoxide (DMSO) or ethanol (ETOH) is required to dissolve NP. In the present study, the solvents alone or in combination with NP were also tested for their ability to alter the effects of NP on sperm quality. NP appeared to be more detrimental when dissolved in ETOH than when dissolved in DMSO. For instance, bull sperm progressive motility was very low after exposure to 250 and 500 µg NP/mL in ETOH compared to DMSO (*Table 1* and *Table 2*). As expected, the corresponding velocity values also showed a similar pattern as determined for progressive motility (*Table 3* and *Table 4*). The velocity of sperm was significantly reduced after exposure to 500 µg NP/mL in DMSO, while the same effect on velocity was observed after exposure to 250 µg NP/mL solved in ETOH ($P<0.05$). The reduction in total motility was only significant after exposures to 250 and 500 µg NP/mL ($P<0.05$). The observed differences between NP dissolved in ETOH and that dissolved in DMSO may depend on solubility or bioavailability effects and ETOH-associated chemical alteration of NP into a more toxic compound. In conclusion, we have shown that NP alone is capable of inducing damage in sperm and that ETOH enhances the damaging effects of NP on bull sperm. However, the assessment of only one or two sperm parameters might not exactly predict the full effect of a xenobiotic on the fertility. For example, as mentioned above, although motility assessment is a powerful tool for evaluating fertility, chemical exposure can also cause impairment to other cellular elements, such as the acrosome or mitochondria which may result in fertilization failure or abnormal embryonic development. Although bull sperm is more resistant against NP mediated damage, the sperm parameters evaluated in this study showed that NP significantly impairs bull sperm function that may severely lower the fertilizing capacity of bull sperm.

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