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The Effects of Nonylphenol on Gamete Physiology in Bovine

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Abstract
Alkylphenol ethoxylates (APEOs) are used as non-ionic surfactants in variety of industrial, agricultiral and domestic products such as pesticides, detergents, paints and cosmetics. Therefore, these compounds can reach to humanbeing through foodchains. These endocrine disurpters also called Xenoestogen have estrogenic, carsinogenic and toxic effects. Nonylphenol (NP) exerts its estrogenic effects by binding to estrogen receptors. NP causes morphological and functional alterations in male and female genital tract and mammary glands. These alterations may induce infertility, mammary and prostate cancer. Therefore, the main purpose of this study is to determine the adverse effects of NP on sperm and oocytes. The effects environmentally relevant NP concentrations such as 0.01, 0.1, 1, 10 and 100 µg NP/ml were employed. NP mediated abnormalities in sperm DNA and oocyte maturation were inviestigated. Tunel assay was performed to determine the adverse effects of NP on sperm DNA, whereas the effects of NP on oocyte maturation in TCM-199 were tested. The present study demonstrated that 100 µg NP/ml concentration induced apoptosis by causing DNA breaks in bovine sperm cells. This study also showed that 100 µg NP/ml concentrations inhibits oocyte maturation. It is concluded that NP have adverse effects on the integrity of sperm DNA and oocyte maturation.

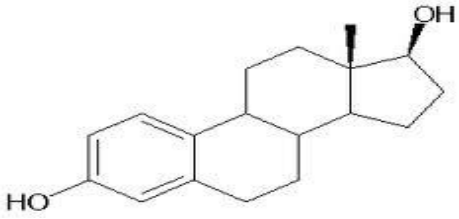
Keywords: Nonylphenol, sperm, oocyte, Tunel Assay, endocrine distruptors

INTRODUCTION

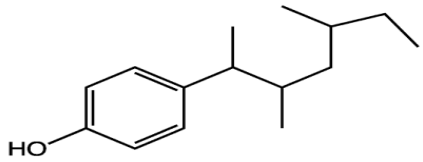
There are some chemicals like phenolics, alcohols, aldehydes, steroles, polyaromatic hydrocarbons, pesticidies, dioxins, alkylphenolic compounds, hormonal and anti-hormonal drugs, detergents, heavy metal compounds, polychlorinated biphenyl (PCB) and polybrominated biphenyl (PBB) causes environmental pollution [1, 2]. These environmental endocrine disrupters, referred to as "Xenoestrogens" have been shown to have hormone-like activity [3]. Xenoestrogens can mimic the effects of endogenous estrogen and thus have been implicated in disorders of the reproductive system [4]. Alkylphenol ethoxylates (APEs) belong to the group of nonionic surfactants that are widely used in the manufacturing of detergents, emulsifiers, pesticides, herbicides, insecticides, plastics, paints, cosmetics and many other synthetic products [5]. APEs are however not stable in the environment and can gradually lose the ethoxy units and eventually breakdown to alkylphenols. Compared with their main compounds, alkylphenols, especially nonylphenol and octylphenol, are more hydrophobic and more toxic. Numerous studies have demonstrated the endocrine disrupting properties of these chemicals [6-8]. Alkylphenols (APs) have been found as ubiquitous environmental pollutants with reproductive and developmental toxicity [9].

The annual production of APEs has been reported to reach 650,000 tons worldwide [10], in which nonylphenol ethoxylates (NPEs) account for about 80% [11]. Nonylphenol (NP), an estrogen mimicking compound is produced by biodegradation of alkylphenol ethoxylates [12]. NP was found to mimic the natural hormone 17β-estradiol by competing for the binding site of the estrogen receptor [13, 14], due to their structural similarity (Figure 1).

The main source of NP in surface waters (streams, rivers, lakes and estuaries), oceans and sediments appears to be closely related with the discharge of effluents from STW, proximity of industrialized/urban areas and other related anthropogenic activities such as storm water discharges and run-off [15-17]. The estrogenic pollutants 4-tert-octylphenol (OP), NP and bisphenol A (BPA) were determined in surface water samples from the Haihe River, Tianjin, China. Among the samples collected from 14 sampling sites, only one sample was found to have a relatively high concentration of BPA (8.30 µg/L) and NP (0.55µg/L) [18]. NP and butylphenol (BP) concentrations were measured and quantified in water, sediment, and the tissues of fishes collected in two rivers, Sakarya and Degirmendere Rivers, Turkey. Butylphenol (BP) were detected in sediment samples at one sampling stations of both rivers with 1.68 and 3.15 µg /g sediment, while NP were detected with the amount of 4.46 µg /g sediment in one sampling station in Degirmendere river [19].



(a) 17β-estradiol



(b) NP

Figure 1. The compare of 17β-estradiol and NP [3].

The EU report also estimates NP exposure due to environmental sources to be 5 µg/kg/day, with 70% - 80% of this due to fish and shellfish consumption [20]. The daily intakes based on consumption studies in µg NP/ kgbody weight per day for high consumers in the baby category (0.23–0.65 µg NP/ kg body weight per day) were relatively high. This could lead to a higher risk especially for babies [21]. A recent Italian study found maximum exposures from human breast milk of 3.9 µg NP /kg/day [22].

NP is an endocrine disruptor with harmful effects including feminization and carcinogenesis onvarious organisms [23]. Xenoestrogens and the degradation products of these chemicals can be estrogenic, mutagenic, toxic and carcinogenic [2, 24]. Endocrine disrupters have substantial biological effects on prostate cancer devolopment and progression [25]. NP is an estrogenic compound that alters pS2, MUCl and ER gene expression in MCF-7 cells [26]. NP exposure affected expression of cell receptors and may damage specific secretory function of Sertoli TM4 cells, which may be associated with the male-specific reproductive toxicity of NP [27]. It has been reported that sublethal doses of NP were accumulating in the bodies of the fish and causing histopathological and biochemical changes in the livers of rainbow trout [28]. It is concluded that NP induces apoptosis in liver involving both mitochondria-dependent and Fas–Fas-L pathways and thereby, leading to hepatic damage in rats [29] and have the potential to promote rat lung carcinogenesis, possibly via a mechanism involving stimulation of cell proliferation and DNA damage caused by oxygen radicals [30]. Exposure to NP may lead to thyroid dysfunction. It may be a potential contributor to thyroid disruption [31]. It has been reported that the intact form of 4-NP at environmentally relevant concentrations can transfer across the human placenta albeit at a slow rate [32]. Maternal NP exposure level is associated with an increased risk of low neonatal weight. Primiparas are especially at risk, and the second trimester of pregnancy may be the critical stage of exposure [33].

Male Sprague-Dawley (SD) rats were exposed to NP by gavage at dosages of 0.125 and 250 mg/kg/day for 50 days. The sperm density of the head of epididymis and the testosterone level descended at 250 mg/kg/day [34]. To determine the effects of NP on epididymal rat spermin vitro, epididymal sperm samples from Sprague-Dawley rats were incubated in 1, 10, 100, 250 and 500µg/ml NP for 1, 2, 3, or 4 h. The results indicate that major mechanism of action of NP on rat sperm is by adversely affecting their acrosomal integrity. However, NP-induced impaired sperm motility, decreased mitochondrial membrane potential also it is likely to play an important role in destruction of sperm function [35-37]. The ovary weight, mean total volume of ovary and cortex, number of antral and graafian follicles and body weight were decreased significantly ($P<0.05$) in the p-NP treated rats compared to control and other groups, while the number of atretic follicles increase [38].

MATERIALS AND METHODS

Chemicals

NP was purchased from Aldrich (Southampton, UK); DMSO (dimethyl sulfoxide) (C_2H_6OS) and hydrogen peroxide (H_2O_2) were purchased fromMerck (Merck & Co., Inc., Darmstadt, Germany); Triton –X 100, PBS (Phosphate buffered saline), salin imidazole, polylysine and formaldehyde (10%) were purchased from Sigma(Sigma Chemical Co., St. Louis, MO, USA); Proteinaz –K, Zymed (Zymed, San Francisco, California, USA); TUNEL enzyme, TUNEL Label and TUNEL Dilution Buffer were purchased from Roche (Roche Diagnostics GmbH, Mannheim, Germany) and Mounting medium were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

NP Concentrations

DMSO was used as solvent solution. To determine the dose dependent effect of NP 0.01, 0.1, 1, 10, and 100 µg NP/ml were employed. The DMSO was 0.01% of total volume. The same amount of DMSO was also added into the solvent control group which does not contain NP.

Sperm Sample

Fresh sperm were diluted to $50x10^6$ sperm/ml. Then, 0.5 ml sperm were added into the each tube. Then, sperm were incubated at 39ºC for 4 h. After incubation, each NP concentrations were diluted with PBS in ratio 1/2 and centrifuged at 5000 rpm for 5 min; the supernatant was then discarded. The spermatozoa pellet was resuspended in 1.5 ml PBS and centrifuged at 5000 rpm for 5 min. Supernatant was then discarded and spermatozoa pellet was resuspended to 0.5 ml with salin imidazol (cat no:10125). In brief, 5-10 µl spermatozoa was smeared on a glass slide and fixed with 10% formaldehyde for 20 min at room temperature. The slides were washed in PBS and stored at 4 ºC.

Tunel Assay

For the TUNEL technique, we used the In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). Upon removal from storage, samples were washed again in PBS (three times for 5 min each). They were then treated in a humidified chamber with proteinase K for 10 min at room temperature, washed with PBS, treated with H_2O_2 (6 ml H_2O_2 in 64 ml distilled water) for 10 min at room temperature and washed again with PBS. The slides were permeabilized with 0.1% Triton X-100 for 5 min on ice and washed again in PBS (three times for 5 min each). The permeabilized slides were incubated in the dark at 37 ºC for 1 h with the TUNEL reaction mixture, which contained terminal deoxynucleotidyl transferase (TdT) plus dUTP label. After labeling, samples were washed with PBS and analyzed immediately via fluorescence microscopy. Negative (omitting TdT from the

reaction mixture) and positive (using DNase I, 1 mg/ ml, for 10 min at room temperature) controls were included in each trial. At least 100 sperm were evaluated to determine the percentage of TUNELpositive sperm. Each microscopic field was evaluated first under fluorescence microscopy (40x magnification) to determine the number of reactive sperm and then under phase-contrast microscopy to determine the total number of sperm per field.

Bovine Oocyte

Bovine ovaries were obtained with a thermos bottle in 0.9% NaCl at 30±2 ºC from a slaughterhouse and used for aspiration of follicles 2–8 mm in diameter to collect oocytes. Only oocytes with several layers of cumulus cells and homogenously granulated cytoplasm were used in this study. Selected oocytes were washed three times in TL-HEPES. Tissue Culture Medium (TCM-199, Gibco/Invitrogen, Grand Island, NY) (4.5 ml) supplemented with 10% FCS(Gibco/Invitrogen) pyruvate (0.2mM), FSH (0.5µg/ml) (Sioux Biochemicals, Sioux City, IA), LH (5µg/ml) (Sioux Biochemicals), gentamycin (50 mg / ml) 25µg / ml final was used for maturating the oocytes. Ten oocytes were matured in 50µl drops covered with mineral oil for 18-22h at 39°C in 5% CO_2 in a humidified tissue culture incubator. After incubation the ratio of maturation was determined.

Statistical analyses

In this study, Chi Square test of independence statistic was used for the comparison of different treatments using SPSS (Version 17.0) in sperm experiments. The Chi Square statistic compares the tallies or counts of categorical responses between two (or more) independent groups. In this statistics technique the null hypothesis is that there is no difference in the distribution of cell counts to the outcome across comparison groups. In oocyte experiments, one-way ANOVA was performed using SPSS (Version 17.0). Tukey test was then used to determine the differences in experimental groups and Spearman corelation analysis was also used to determine the maturation percentage of oocytes in response to different dose of NP.

RESULTS

The Effects of NP on Bovine Spermatozoa

The results of Tunel assay were shown in figure 2. According to the results, the lowest apoptotic cell count was found in control groups as 33 (6.32%) and the highest apoptotic cell count was found in 100 µg NP/ml group as 58 (10.17%).

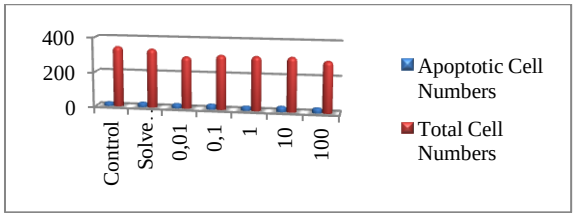


Figure 2.Total cell numbers and apoptotic cell number in NP experiment groups.

In Table 1, the results of chi square analysis is shown. As seen in table our H_o hypothesis (there is no differenceof apoptotic cell numbers between groups) is rejected. The significance is caused by the 100 µg NP/ml group, which contributed the most to the overall chi square test statistics.

Table 1. The effects of NP on sperm apoptosis (Chi square values of NP groups in sperm experiments)

Experimental Groups	Chi square values
Control	5.361[a]
Solvent Control	2.041[a]
0.01 µg NP /ml	0.396[a]
0.1 µg NP /ml	0.508[a]
1 µg NP /ml	0.278[a]
10 µg NP /ml	0.460[a]
100 µg NP /ml	7.444[b]
	16.488

Different letters indicate significance at P<0.05

The analysis has been redone by omitting the 100 µg NP/ml group. The result of the analysis showed that there is no difference of apoptotic cell numbers between the remaining groups. The present study demonstrated that 100 µg NP/ml concentration induced apoptosis by causing DNA breaks in bovine sperm cells.

The results of the TUNEL assay demonstrated that spermatozoa with DNA fragmentation exhibited green fluorescence in figure 3.

The Effects of NP to Oocyte Maturation

The rate of maturated oocytes was determined by observing polar cells at the end of the 18-22 hours of incubation in 39 ^oC, 5% CO_2 incubator. Maturated cell numbers and percentages at the end of incubation were given in Table 2.

Table 2. The Effects of NP to Oocyte Maturation

Experimental Groups	The number of the cell	The number of the matured cell	Maturation Percentage (%)
Control	30	21	70.00[a]
Solvent Control	30	20	66.66[a]
0.01 µg NP /ml	32	23	71.88[a]
0.1 µg NP /ml	31	21	67.74[a]
1 µg NP /ml	36	22	61.11[a]
10 µg NP /ml	30	18	60.00[a]
100 µg NP /ml	31	2	6.45[b]
ANOVA: F (9.477)=0.000 ; P<0.05			

Different letters indicate significance at P<0.05

As seen in Table 2, the lowest rate of maturation was observed in the 100 µg NP/ml group (6.45%). Maturity ratio of the other groups ranged between 60-70%.

Significant differences between the groups were observed (F (9.477) = 0.000, p <0.05) in statistical analysis. As a result of the Tukey analysis, which was done to determine the difference between the groups, maturation rate of 100 µg NP/ml concentration group (0.0645 ±0.24, p=0.00) was found significantly lower than the other groups.

After the correlation analysis which was done in order to determine the degree of NP amount's effect over maturation percentage, Spearman coefficient was found -0.793(p <0.05). It can be said that there is a very strong negative linear relationship between applied NP amount and maturation percentage.

After the incubation, the rate of matured oocytes was determined by observing polar cell (polar body) as shown in Figure 4.

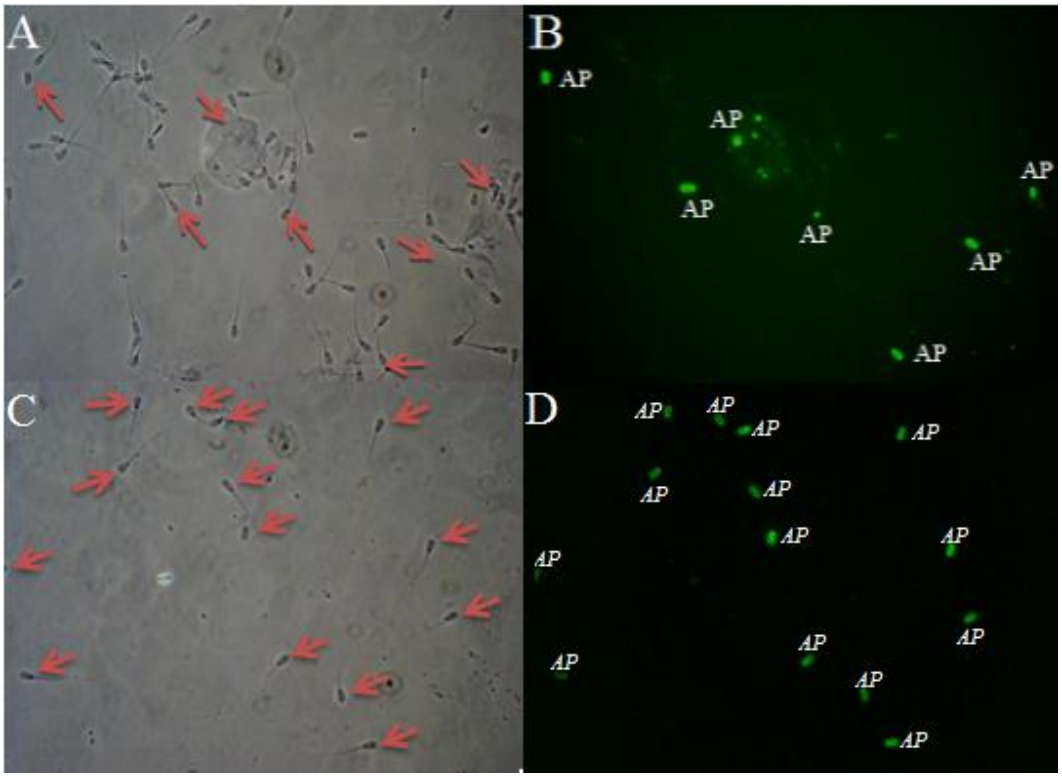


Figure 3. Micrographic presentation of NP effects on sperm apoptosis; (A) 100 µg NP/ml applied spermatozoa under phase contrast microscopy (B) 100 µg NP/ml applied spermatozoa under fluorescence microscopy (C) DNase applied spermatozoa under phase contrast microscopy (D) DNase applied spermatozoa under fluorescence microscopy (X 40).

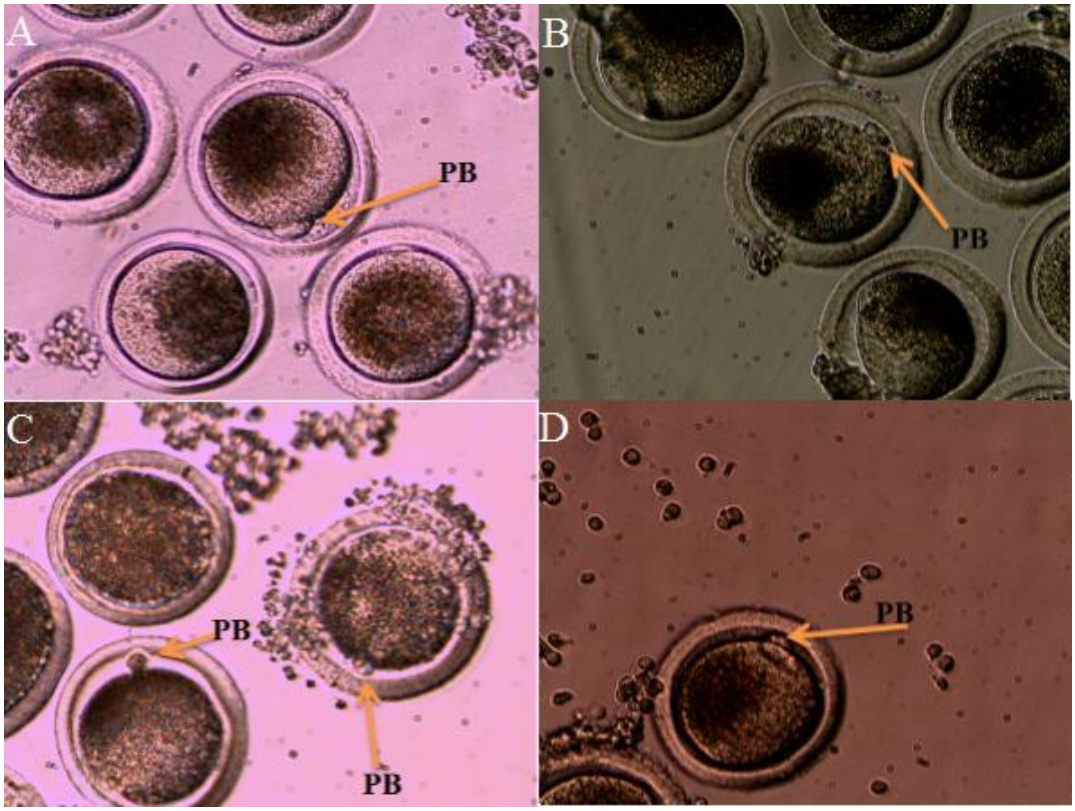


Figure 4. Micrographic presentation of NP effects on oocyte maturation**;** (A) Microscopic images of polar body (PB) in NP control group (B) Microscopic images of PB in 0.01 µgNP/ ml group (C) Microscopic images of PB in 10µgNP/ml group and (D) Microscopic images of PB in 100µgNP/ml group (X40).

DISCUSSION

About researches with NP; Han et al. [34], applied 0.125 and 250 mg/kg/day of NP for 50 days on male Sprague-Dawley rats and according to Tunel result, NP increased dose-dependent apoptosis in testicular cells. Some other studies have also indicated that NP induces apoptosis in testicular cells [39-41]. Wang et al. [40] reported that, apoptosis in male rats' Sertoli cells which exposed to NP, increased with increasing concentrations of NP. Gong et al. [42], indicated that NP can form ER stress which plays an important role on apoptosis on Sertoli cells. Bennetts et al. [43], reported that the NP harms DNA integrity of Human Sperms and thus can cause miscarriages. Uğuz et al. [44], found in their study on rat sperm that, NP's >250 µg/ml concentration has negative effects on the mobility of sperm. Bian et al. [45], indicated in their study on male rat's sperms and OF, which can be connected to estrogen receptor by mimicking estrogen like NP, that daily 150 mg / kg of OF reduces the mobility of sperm cells and 450 mg/kg/day dose reduces sperm count in testes and daily sperm production. It was identified that estrogenic NP in 100 ppb concentration increases apoptosis six times in sertoli cells and leydig-homologous cells in mature man (Oryziaslatipes) spermatocytes [41]. In this study, it is observed that as NP intensity increases, the rate of apoptosis which causes bovine Sperm DNA breakage increases as well (Fig. 2). As a result of Chi Square test, the rate apoptosis in 100 µg NP/ml group is found statistically significant ($P<0.05$).

Michelangeli et al. [46], found that AF causes abnormal increases on Ca+2 intracellular level and cell death by depolarizing mitochondria in TM4 Sertoli cells (sperm maturation cells). Arslan et al. [47], left sea urchins' sperm and eggs exposed to NP (0.937- 18.74µg/L) and OF (5-160 µg/L) and as a result a reduction close to 20% occurred in success of fertilization and skeletal deformities increased in larvae formed by contaminated sperms. Yang et al. [48], examined effects of pre-reproductive NP exposure on reproductive system of Zebra fish, and found that 50 µg/L of NP causes reproduction weakening, slowing down CAT D activity, eggshell thickness reduction (23.6%) and increasing the ratio of disfigurement. Karadeniz et al. [49], carried out NP's electrochemical detection and monitoring interaction between NP and DNA by using single-use grapfite sensor technologies and DPV technique. Consequently NP -induced DNA damage occurred by NP – induced DNA oxidation especially guanine oxidation. Yucedag et al. [50] showed that nonylphenol has negative effects on hearing function in rats but the effects do not seem to be dose-dependent. Lukacova et al. [51], investigated NP's dose and time-dependent effect in their study on bovine sperm cells' mobility. The results support the claim that high doses of NP (greater than 100 µg/ml) affect mobility of sperm cells negatively. These concentrations probably have toxic effects on the reproductive physiology. In our study, dosage of >100 µg NP/ml has apoptotic effect; this results are parallel with literature.

When the data obtained from our experiments about NP's effect on cattle oocytes' maturation analyzed, it was seen that the lowest maturation rate was observed in 100 µg NP/ml applied group (6.45%). Rate of maturation varies between 60-70% in the other groups (Table 2). After statistical analysis, significant difference was found between the groups ($P< 0.05$). As a result of the Tukey analysis, which was done in order to determine the difference between the groups, it was identified that statistically the maturation rate of the 100 mg NP / ml concentration group was significantly lower than other groups.As a result of correlation analysis, which was done In order to determine the degree of applied NP amount's effect on maturation percentage, Spearman's coefficient was found "-0.793" ($P<0.05$). Thus it can be said that there is a very strong negative linear correlation between applied NP amount and maturation percentage.

In their research about PCB's (which is another endocrine system disruptor) effect on in vitro maturation of pig oocytes, Brevini et al. [52], indicated that, this substance diminishes the development ability of oocytes and PCB accumulation on pig organisms has harmful effect on breeding efficiency. Hwang et al. [53] stated that, NP and DES's had estrogenic effect on T.Obscuruss's oocyte maturation. Also they found that, NP and DES have different sensitivities according to oocyte development stages and DES's estrogenic effect is larger than NP. Pocar et al. [54] identified that OP has impact on in vitro cultured cattle oocyte maturation and their subsequent developmental competence. Also, they found that these effects were dose-related and the oocyte maturation is blocked by high concentrations of OP (1 and 0.1 mg / ml). It was observed that at these concentrations, nuclear maturation disorders increased and small percentage of oocyte carried out the first meiotic division. As a result of their research on Atlantic cod fish; Kortner and Arukwe [55] determined that NP has a negative impact on maturation and oocyte growth in teleosts and has unusually effects like creating hormonal imbalance and disturbing steroid formation.

In conclusion, this study showed that 100 µg NP/ml concentration induced apoptosis by causing DNA breaks in bovine sperm cells. This study also showed that 100 µg NP/ml concentration inhibits oocyte maturation. It is concluded that NP have adverse effects on the integrity of sperm DNA and oocyte maturation.

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