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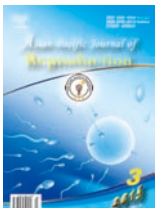

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In vitro assessment of ROS on motility of epididymal sperm of male rat exposed to intraperitoneal administration of nonylphenol

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ABSTRACT

Objective: To explore the mechanism by which nonylphenol (NP) interferes with male infertility through evaluation of its effects on epididymal sperm of adult male rats. **Methods:** Twenty four Sprague-Dawley (SD) rats were used as epididymal sperm donors. Previously rats were administrated with NP (0, 2, 10 and 50 mg/kg) body weight respectively in corn oil every forty-eight hours by intraperitoneal injection for 30 days. Computer assisted sperm analysis (CASA) was used to determine parameters of sperm. The sperm morphology examination was conducted with a high resolution microscope. **Results:** Results indicated that exposure to NP has no effect on body weight, while testes weights were significantly decreased. Computer assisted sperm analysis (CASA) showed significant decline in the percentage of motile spermatozoa (P<0.001), STR and LIN (P<0.01), significant increase in ALH (P<0.001), while significant decline in BCF (P<0.001) respectively. Plasma LDH was significantly increased while; plasma γ-GT activity was significantly decreased. H_2O_2 production and malondialdehyde (MDA) were significantly increased. The Plasma CAT, GSH-Px and SOD activities were significantly decreased. **Conclusions:** This concludes that NP leads oxidative stress in the epididymal sperm of rats. Moreover, NP can disrupt sperm motility and alterations in the sperm morphology.

1. Introduction

Male infertility has become a major medical problem as well as social stigma. The increasing rate of testicular cancer and the incidences of abnormalities in male and female reproductive tracts[1] during the last decades have been reported. Alkylphenol especially nonylphenol (NP) is widely used as lubricating oil additives, plasticizers and surface-active agents[2]. NP is a final metabolite of Nonylphenol polyetholate (NPE) and NP is more stable and persistent than NPE[3]. (NPE) is a non-ionic surfactant widely used as component of detergent, paints, herbicides, and many other synthetic products[4].

NP is one of the chemicals believed to cause endocrine disruption and affect sperm quality in mammals and to play biologically active for a longer period of time in the body than endogenous estrogens. However, with the developments of industry, large amount of NP have been discharged into water[5]. Hence it's distributed into aquatic environments such as rivers, lakes and seas through sewage treatment plants[6, 7]. Its ability to accumulate in the organs of aquatic species is considered a potential hazard for the reproductive system in humans and animals exposed to the food chain. Therefore, cytotoxicity effect of NP carried out in diverse organs, including those in the reproductive system has been reported in many studies[8-10]. The disruption of endocrine function by

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inhibiting between estrogen and the estrogen receptor[10, 11] is due to its characteristic weak estrogenic activity[12, 13]. Previous studies collectively suggest that these estrogenic compounds might cause a variety of negative reproductive outcomes, especially declines in sperm counts in men, remain extremely controversial[14].

Several environmental contaminants such NP can induce oxidative stress by generating ROS such as hydrogen peroxide (H_2O_2) and superoxide anion (O^{2-}), resulting in great damage to the male reproductive system, including altered steroidogenesis, disturbed testicular structure and decreased sperm number in the epididymis[15]. Moreover, it has been reported that NP administration increased ROS level and depressed the activity of antioxidant enzymes in rat epididymal sperms[16], testis[17] and testicular Sertoli cells[18, 19]. However, the cellular or biochemical mechanism by which NP causes reproductive toxicity has not been fully elucidated[8]. When produced in excessive amounts, the ROS stimulate DNA fragmentation and loss of sperm function associated with peroxidative damage to the mitochondrial and plasma membrane. Moreover, sperm plasma membrane, being rich in polyunsaturated fatty acids, is highly susceptible to ROS attack. At the same time, epididymis being its transit through caput to cauda region of epididymis and facilitates their maturation process[20]. The epididymis is known to play an important role in providing the microenvironment for sperm maturation and storage of sperm[21].

In the last decade the effect of ROS on the semen quality has been studied but not well established with excess production of ROS including motility and abnormal spermatozoa. Although semen analysis is routinely employed in the evaluation of the male infertility to predict the cause behind impaired fertility. Suitable sperm motility is precondition for fertilization of the ovum to take place. Thus an alteration in this time can provoke problems in such maturation as well as alter the number of gametes available for ejaculation[22]. Oxidative damage to spermatozoa is well known to be a potential cause of fertilization failure[23]. This study was designed to show if estrogens affect sperm function in vitro there could be important consequences for fertility in vivo. In our present study, we hypothesized that NP exposure in adult male rats would lead to oxidative stress by generating ROS that induce reproductive abnormalities. Thus, the purpose of this study was to evaluate the effects of NP on sperm motility parameters, morphological and biochemical marker changes related to oxidative stress in the serum plasma, and then propose a molecular pathway in order to explain the deleterious effects of this chemical on epididymal sperm quality.

2. Materials and methods

2.1. Chemicals and reagents

Nonylphenol (NP) was purchased from (DR Co., Augsburg, Germany, purity: 98%). Corn oil was obtained from (Sigma-Aldrich, St. Louis, MO, USA). Sigma Chemical Co. (St Louis, MO) USA, Collagenase, Trypsin–EDTA were obtained from GIBCO (Grand Island, NY, USA), Sodium lauryl sulphate from SRL, Eosin stain, Hematoxylin stain, Orange G stain from HiMedia (Mumbai). LDH, γ -GT CAT, GSH-Px, H_2O_2, MDA and SOD assay kit (Jiancheng Bioengineering Ltd., Nanjing, China). All other chemicals are of analytical grade.

2.2. Animals and treatments

Twenty four healthy male Sprague-Dawley rats (50-days olds) were used as epididymal sperm donors purchased from the Tongji Medical College Animal Laboratory (Wuhan, China). Previously rats were gavaged with NP (0, 2, 10, 50 mg/kg) body weight respectively in corn oil every forty-eight hours by intra-peritoneal injection for 30 days. The rats were housed in accordance with the policies of the Ethical clearance for the use of animals in the study was obtained from the Institutional Animal Ethics Committee prior to the initiation of the study, and the experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals published by Ministry of Health of People's Republic of China (Approval ID: 2011-s2456). Rats were housed in conventional rat cages at (24±3) ℃, humidity (50 ± 5) % in a controlled light environment (12 h light: 12 h dark) and provided free access to water and standard rodent chow, and the weight of each animal was recorded every forty-eight hours for 30 days and any gross abnormality was noted.

2.3. Dose selection and preparation

The doses and time used for the present study were derived from published data[24, 25] and the results of our preliminary experiment. 4-NP was dissolved in corn oil to obtain the desired concentration of NP dose range, i.e., 0, 2, 10 and 5 mg/kg. An additional control group that had received only corn oil. Dose formulations were mixed well and stored in crystal bottles at 37 ℃ overnight and were subsequently kept at room temperature throughout the study. Solutions were mixed thoroughly before use.

2.4. Necropsy and organ collection

2.4.1. Preparation of serum

Twenty four hours after the last dose, the animals were sacrificed. The blood samples were collected from the retro-orbital sinus in heparinized tubes. Samples were centrifuged at 980 for 15 minutes and supernatant plasma was separated from the clot and stored at -80 ℃ until analysis.

2.4.2. Organ and sperm collection

All organs were removed cleaned from adhering fat and connective tissues and rapidly weighed and frozen until analyzed. The testes were quickly frozen at -80 ℃ for later use for biochemical assays, while epididymal sperm from individual rat were kept separately and

immediately to use for sperm analysis (CASA).

2.5. Assessment of oxidative stress

The CAT, GSH-Px, SOD, H_2O_2 and MDA Assay Kits were used (Jiancheng Bioengineering Ltd., Nanjing, China). All operation was done at 4 ℃. Protein concentrations were determined using a BCA kit (Beyotime Biotech Inc., China) that employed serum albumin as a standard.

CAT can decompose H_2O_2, the reaction solution with the absorbance at 240 nm decreased reaction time, CAT activity was calculated according to the rate of change in absorbance. One unit is defined as the degradation of 1 nmol H_2O_2 in the reaction system per mg protein per minute.

GSH-Px activities were assayed by quantifying the rate of oxidation of the reduced glutathione to the oxidized glutathione by H_2O_2. One unit of GSH-Px was defined as the amount that reduced the level of GSH by 1 μM in 1 min/mg protein; at 412 nm absorbance.

SOD activity in supernatant was determined by determining the reduction of nitro blue tetrazolium (NBT) by O_2^- produced from the xanthine-xanthineoxiase system. One unit of SOD was defined as the amount protein inhibits the rate of NBT reduction by 50%. Results were defined as U/mg protein.

H_2O_2 and the titanium dioxide over sulfuric acid to form a yellow complex, which has a characteristic absorption in 415 nm. Results were defined as μmol/mg protein.

MDA level were assessed to determinate the concentration of MDA, measuring thiobarbituric-acid (TBA) reacting substances at 53 nm. The level of MDA was expressed as nmol MDA per milligram protein.

Biochemical markers including plasma lactate dehydrogenase (LDH) activity was measured using a-ketovaleric acid as the substrate[26] and γ-Glutamyltransferase (γ-GT) was measured by the method of[27].

2.6. Sperm count and motility using CASA system (CFT-9200)

Semen quality analysis was performed simultaneously using the CASA system (CFT-9200 computer-aided sperm and microorganism test and analysis system). The caudal epididymides sperm (from both sides) were dissected out, minced with surgical scissors in 2 mL of 0.9% physiological saline and then incubated at 37 ℃ and kept for 20 minutes to allow spermatozoa to leave the epididymal tubules. A 10 μL aliquot was pipetted and placed on slide to evaluate parameters of sperm. The percentage of motile spermatozoa, the percentage of progressive motility, DAP (distance average path, μm), DCL (distance curved line, μm) DSL (distance straight line, μm), VAP (velocity average path, μm/s), VCL (velocity curved line, μm/s), VSL (velocity straight line, μm/s), STR (straightness, VSL/VAP, %), LIN (linearity, VSL/VCL, %), WOB (wobble, VAP/VCL, %), ALH (amplitude of lateral head displacement, μm), and BCF (beat cross frequency, Hz) were evaluated according to the manufacturer's instructions.

2.7. Morphology and sperm normality criterion

A small amount of sperm suspension was smeared on to a slide using a pipette and fixed with methanol; after drying for 10 minutes, it was stained with 2% eosin for 1hour. Each of the stained slides was analyzed. The images were captured by a color light microscopy (Olympus IX-71, Tokyo, Japan) for high quality image production. Morphological evaluation was accomplished on a monitor screen and the total calculated magnification was (×400). The mains abnormalities, subjectively assessed, have been previously reported[28], we considered the sperm head, neck, midpiece and tail must be normal. The head should be oval in shape. Stained sperm samples were counted between 100 and 200 spermatozoa/animals and the percentage of normal sperm cells was calculated. It showed normal looking hook-shaped heads and the shape and thickness of the tail was thin uniform. Abnormal sperm cells included headless and hook less cells; amorphous shapes and forms; folded, short and double Y tail and other aberrations.

2.8. Statistical analysis

Results are presented as the (mean ± SD, n= 6) were compared by one way analysis of variance (ANOVA) followed by the Turkey-Kramer multiple comparison test using SPSS statistical package 17.0 (SPSS Inc, Chicago, IL, USA) and GraphPad PrismTM software version 5.0 (San Diego, USA). Pearson's correlation was used to evaluate the correlation among sperm motility parameters with plasma biomarkers using the same software. Semen quality analysis was performed simultaneously using the CASA system (CFT-9200 computer-aided sperm and microorganism test and analysis system). A P value less than 0.05 was taken as a criterion for statistically significant difference.

3. Results

3.1. Body and testis weight

No significant differences in body weight happed among the treated-group and control group during the experiment period, but final body weight of all animal were increased significantly, while a significant decrease in absolute and relative weights of testes has been observed (P<0.05 and P<0.01) (Table 1).

Table 1
Body weight (g) and selected absolute (g) and relative organ weight (mg/g) of male rats in control and treatment groups.

Parameters		Doses of NP (mg/kg b.wt)			
		Control	2 mg	10 mg	50 mg
Initial body weight		170.35±2.35	169.92±4.60	175.67±3.15	173.08±5.91
Final body weight		249.52±8.06	232.34±10.06	235.75±11.88	246.05±5.67
Weight gained		79.16±6.63	62.42±10.16	60.08± 3.95	72.95±9.57
Testis weight	Absolute	3.01±0.21	2.55±0.46*	2.46 ± 0.30*	2.34±0.34**
	Relative-to-body	12.07±0.91	10.94±1.77	10.46 ± 1.39*	9.52±1.38**

Data are presented as the mean ± S.D. (n =6). Statistical analysis (ANOVA) for differences from corresponding controls: *P<0.05; **P<0.01 versus control.

3.2. Effect of nonylphenol on rat sperm motility

Statistical outcome for the sperm motility parameters are shown in (Table 2). The statistical measurements showed significant decrease in the percentage of motile and progressive motile spermatozoa (P<0.05, P<0.01 and P<0.001 respectively) in dose related manner as compared to the corresponding controls; decline in the percentage of STR and LIN (P<0.05 and P<0.01) respectively, significant increase in ALH (P<0.05 and P<0.001) and significant decline in BCF (P<0.05, P<0.01 and P<0.001).

3.3. Epididymal sperm morphological characteristics

The results of this study are summarized in the Table 3. Table 3 presents Mean ± SD number of spermatozoa and percentage of morphologically abnormal spermatozoa, which is significantly higher in treated-group with NP (P<0.05, P<0.01 and P <0.001 respectively) in dose related manner as compared to the corresponding controls (Figure 4).

3.4. Measurement of plasma LDH and γ -GT

Treatment with 2, 10 and 50 mg/kg/day of NP showed a significant increase in plasma LDH activity (36.26, 44.59 and 61.25 U/mg of protein respectively) (Figure 1A), while plasma γ -GT was significant decreased (2.03, 1.24 and 0.91 U/mg of protein respectively) in dose related manner as compared to the corresponding controls (Figure 1B).

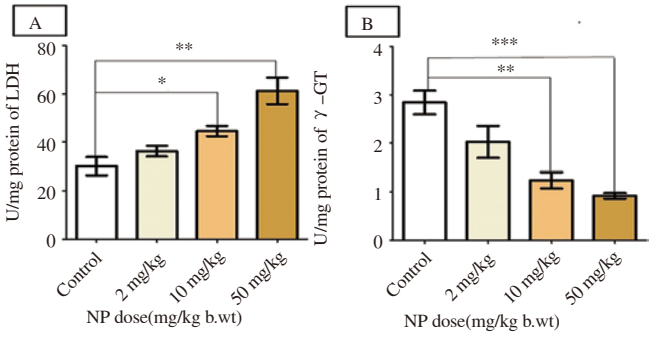

Figure 1. Effect of Nonylphenol (NP) on plasma LDH activities (U/mg of protein) (A) and γ -GT activities (U/mg of protein) (B) in male rat. NP was administered intraperitoneally (0, 2, 10 and 50 mg/kg) every forty-eight hours for 30 days. Data are presented as the mean ± S.D. (n= 6). Statistical analysis (ANOVA) for differences from corresponding controls: *P<0.05; **P<0.01; ***P<0.001 versus control.

Table 2
Summary statistics of epididymal sperm motility parameters.

Parameters	Treatments NP (mg/kg b.wt)			
	Control	2 mg	10 mg	50 mg
Motility (%)	51.71±1.59	42.38±5.53*	37.30 ± 0.84***	34.83 ± 3.18***
Progressive Motility (%)	28.01±0.94	23.42±4.09*	19.37±1.99**	18.81 ± 5.03**
DAP (μm)	20.88 ±1.56	23.09±1.44	21.01 ± 1.01	22.12 ± 1.50
DCL (μm)	31.17±4.31	35.90±1.37	36.08 ± 1.51	33.27 ± 3.32
DSL (μm)	16.79 ± 2.25	19.70±1.87	18.15 ± 1.94	17.95 ± 1.78
VAP (μm/s)	50.98 ±1.12	56.61±2.48	55.35 ± 1.45	50.88 ± 1.44
VCL (μm/s)	79.97±1.97	84.60±3.57	90.17 ± 3.24	81.01 ± 2.81
VSL (μm/s)	44.01±3.49	45.27±2.68	40.10 ± 1.09	40.15 ± 2.13
STR (%)	0.86±0.06	0.80 ± 0.08	0.73 ± 0.02**	0.79 ± 0.05*
LIN (%)	0.55±0.05	0.54±0.054	0.44 ± 0.02**	0.49 ± 0.027**
WOB (%)	0.64±0.02	0.67±0.02	0.61 ± 0.02	0.62 ± 0.03
ALH (μm)	4.23±0.41	5.67±0.77*	6.35 ± 0.44***	6.11 ± 0.28***
BCF (HZ)	21.91±1.51	16.93±1.11**	17.88 ± 0.76*	13.52 ± 0.69***

Data are presented as the mean ± S.D. (n =6). Statistical analysis (ANOVA) for differences from corresponding controls: *P<0.05; **P<0.01; ***P<0.001 versus control. DAP (distance average path, μm), DCL (distance curved line, μm) DSL (distance straight line, μm), VAP (velocity average path, μm/s), VCL (velocity curved line, μm/s), VSL (velocity straight line, μm/s), STR (straightness, VSL/VAP, %), LIN (linearity, VSL/VCL, %), WOB (wobble, VAP/VCL, %), ALH (amplitude of lateral head displacement, μm), and BCF (beat cross frequency, Hz).

3.5. Oxidative status

Treatment with 2, 10 and 50 mg/kg/day of NP significantly increased H_2O_2 production (1.24, 1.02 and 1.53μmol/mg protein respectively) and MDA (1.06, 1.22 and 1.49 nmol/mg protein respectively) in a dose-related manner in rat plasma as compared to the corresponding controls (Figure 2A and B respectively).

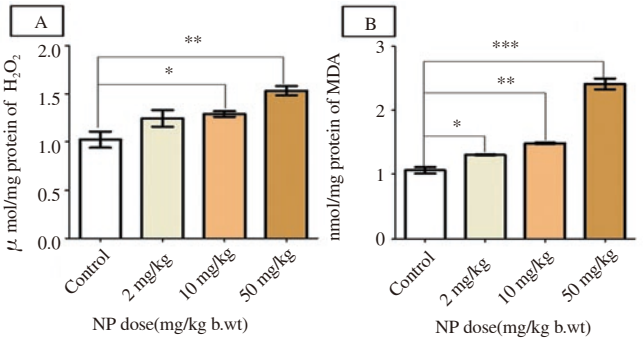

Figure 2. Effect of nonylphenol (NP) on H_2O_2 production (μmol/mg of protein) (A) and on MDA levels (nmol/mg of protein) (B) in serum plasma of male rat. NP was administered intraperitoneally (0, 2, 10 and 50 mg/kg) every forty-eight hours for 30 days. Data are presented as the mean±S.D. (n=6). Statistical analysis (ANOVA) for differences from corresponding controls: $^{*}P$<0.05; $^{**}P$<0.01; $^{***}P$<0.001 versus control.

3.6. Antioxidants status

Treatment with 2, 10 and 50 mg/kg/day of NP showed significant reduction in CAT (16.35, 10.69 and 7.78 U/mg protein respectively), GSH-Px (3.58, 1.89 and 1.38 U/mg protein respectively) and SOD (18.19, 11.36 and 7.31 U/mg protein respectively) activities in a dose-related manner in rat plasma as compared to the corresponding controls (Figure 3A, B and C respectively).

3.7. Correlation between LDH and γ-GT activity with sperm motility

Regression analysis showed significant negative correlation between percentage of motile sperm and progressive motile sperm with LDH activity (Pearson's rank correlation coefficient, r = -0.62, P = 0.0013; r = -0.44, P= 0.0295 respectively) Figure 5AB.

The scatterplot suggests a definite relationship between γ-GT activity and percentage motile sperm and progressive motile, with larger values of spermatozoa motility to be associated with larger values of γ-GT. There appears to be significant positive correlation the two-variables. We also note that there appears to be a linear relationship between the two-variable. (Pearson's rank correlation coefficient, r=0.76, P<0.0001; r = 0.74, P<0.0001 respectively) Figure 5CD.

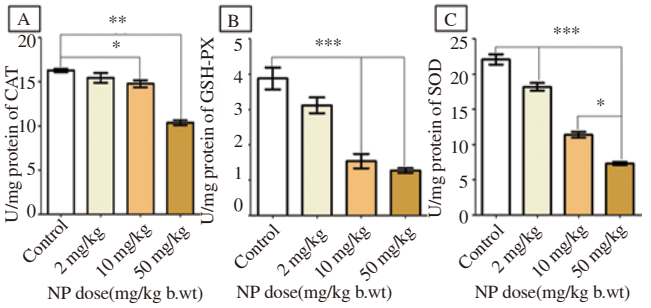

Figure 3. Effect of nonylphenol on CAT activities (U/mg of protein) (A), GSH-Px activities (U/mg of protein) (B) and SOD (U/mg of protein) (C) in serum plasma of male rat. NP was administered intraperitoneally (0, 2, 10 and 50 mg/kg) every forty-eight hours for 30 days. Data are presented as the mean ± S.D. (n = 6). Statistical analysis (ANOVA) for differences from corresponding controls: $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 versus control.

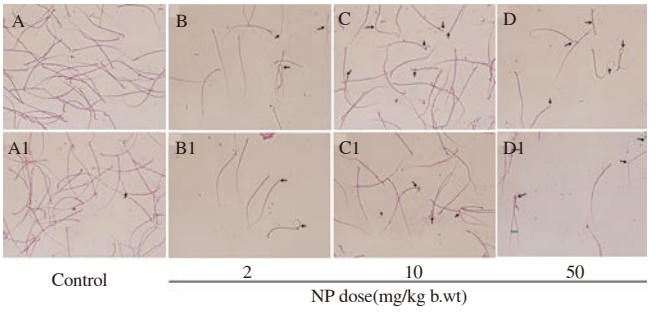

Figure 4. Photomicrograph (×400) of spermatozoa stained with 2% eosin. A1: bent tail; B: headless, B1: coiled tail, headless; C: pinhead, headless, flattened head, C1: bent neck, bent tail, coiled tail; D, D1: pinhead, flattened head, headless, bent tail, coiled tail and an unusual double tail indicates by green arrow. The arrow indicates abnormality of spermatozoa.

Table 3

Effect of NP on epididymal sperm of male rats after intraperitoneal administration during 30 days.

Sperm Parameters	Treatments NP (mg/kg b.wt)			
	Control	2 mg	10 mg	50 mg
Epididymal spermatozoa number				
No. of spermatozoa (106/rat)	70.91 ± 6.35	66.23 ± 3.93	58.17 ± 2.49***	51.84 ± 6.24***
Total percentage of morphological abnormal sperm (%)	10.09 ± 1.57	18.50 ± 1.99 **	20.61 ± 2.59**	28.93 ± 3.19***
Abnormal head (%)	1.00 ± 0.47	2.10 ± 0.44**	4.15 ± 0.03***	7.10 ± 1.11***
Bent tail (%)	4.00 ± 0.65	9.05 ± 1.46**	11.20 ± 1.95***	12.40 ± 1.72***
Coiled tail (%)	2.00 ± 0.46	3.15 ± 0.62**	2.16 ± 0.38	3.20 ± 0.52***
Other defect (%)	3.09 ± 0.45	4.20 ± 0.62**	3.10 ± 0.37	6.23 ± 0.51***

Data are presented as the mean ± S.D. (n =6). Statistical analysis (ANOVA) for differences from corresponding controls: $^{*}P$<0.05; $^{**}P$<0.01; $^{***}P$<0.001 versus control.

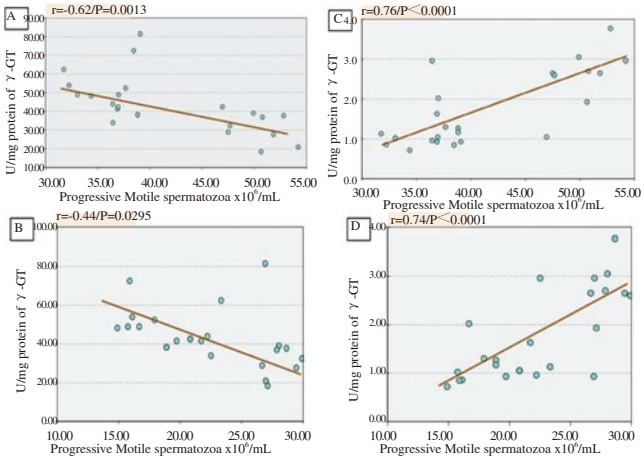

Figure 5. Correlation between serum plasma LDH activity with percentage motile sperms (A) and progressive motile sperms (B). Regression analysis is for total of case and control groups (r=-0.62, P=0.0013; r=-0.44, P= 0.0295 respectively). Correlation between serum plasma γ -GT activity with percentage motile sperms (C) and progressive motile sperms (D). Regression analysis is for total of case and control groups (r=0.76, P<0.0001; r=0.74, P<0.0001 respectively).

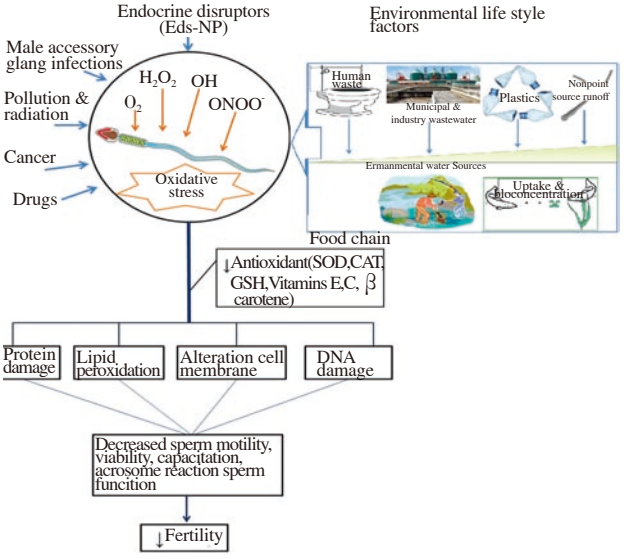

Figure 6. Proposed mechanism of increased production of ROS by abnormal spermatozoa motility[54].

4. Discussion

Slower mobility of spermatozoa is mostly due to some deformity of the sperm whose of which are unable to move. It can be caused by a disorder in the production of sperm, and as other abnormalities as an endocrine disruptor. Nonylphenol (NP) is a widespread aquatic contaminant that can accumulate in fish[6]. Which increases the risk of exposure of men who live in urban areas and have a fish based diet (probably from contaminated waters)[29]. Several studies have reported NP as hazardous to the health of human and animals, particularly to male reproduction[14, 30, 31]. The present study demonstrated that NP was disrupting the epididymal sperm quality. NP treatment groups resulted in decrease of epididymal sperm motility parameters and increases the abnormal sperm morphology.

Body weight of the treated animals did not show significant changes, suggesting that the metabolic condition of the animals was within normal range. In our study, the body weight gain of NP-treated groups is less than that of the control group, but there was no significant difference between the four groups. On the other hand, the decreases of the absolute and relative weight of testes in NP-treated groups suggested may be due to inhibition of spermatogenesis[30], or the inhibition of steroidogenic enzyme activity[32]. This observation is also consistent with other reports[11,16]. On the other hand, study has shown depressed levels of testosterone (T) in male and female of Clarias gariepinus after exposure to 4-NP and this is similar to the results of studies on the effects of wastewater treatment plants (WWTP) which contains NP and NPEs in Carp[34,35]. NP could inhibit the activity of 17 alpha-hydroxylase enzymes, which are involved in testosterone synthesis[36].

Numerous biochemical studies were carried out on normal and abnormal semen qualities in order to elucidate factors that contribute to male infertility. LDH, as the main enzyme for glucose metabolism in testis spermatogenic cells, is related to the mature of seminiferous epithelium and sperm activity and could be used as an important indicator of spermatogenesis[37]. In our present study, the increase in activity of plasma LDH observed in NP treated groups suggests that NP exposure induce deterioration of germinal epithelium[38]. LDH is also known as biomarker of cell integrity[39]. Its increase in plasma has been markedly correlated with chronic oxidative stress, cardiotoxicity, testis carcinoma and many inflammatory conditions[40]. On the other hand, study shows that LDHC plays crucial role in the processes of glycolysis and ATP production in sperm flagellum that are required for male fertility and sperm function, and also is the major isozyme in male germ cells[41]. In addition, our data showed a significant decline in the percentage motile and progressive motile spermatozoa, and significant decrease in STR, LIN and BCF. These results confirm the negative correlation between percentage of motile sperm and progressive motile sperm with LDH activity and the positive correlation between γ -GT activity and percentage motile sperm and progressive motile, and supporting the hypothesis that environmental NP exposures may promote significantly reduced sperm motility. Suggesting that NP exposure can impair sperm motility, this finding may confirm previous study[14]. In other hand, study demonstrated that 750 ng/L NP is capable of impairing the motility of rosy barb (Punctuis conchonis[42] and japanese medaka sperm[43].

γ -GT, is a heterodimeric integral membrane glycoprotein that uniquely cleaves the γ bond between glutamate and cysteine to yield the dipeotide sys-gly. And is one enzyme that is critical to maintain intracellular levels of GSH within the epididymis is essential for the protection of spermatozoa against ROS and xenobiotics[44], highlighting the role of γ -GT in regulating antioxidant capacity, especially to the synthesis of the antioxidant GSH. γ -GT is a ubiquitous enzyme present in many tissues, including the testis, epididymis, and seminal vesicle. However, our data showed decrease in activity of plasma γ -GT observed in NP treated groups. It remains to be established what stage is critically affected in sperm function in the complete absence of γ -GT and decreased intracellular cysteine levels[45]. However, ROS are produced as a

by-product of γ -GT catalyzed GSH cleavage[46, 47], and thus, γ -GT activity can serve as an oxidant source. In addition, some of the defects in sperm function in human patients with dyspermia resulting from varicocele or genital tract infection can be improved by systemic supplementation of GSH[48]. Intracellular GSH levels are also known to be important for direct protection against oxidative injury to the testis, epididymis, and sperm[49].

Among the various viability characteristics of the rat sperm include, motility, sperm morphology, sperm mitochondrial integrity, ROS, LDH, γ -GT. NP reduced the epididymal sperm motility which may be due to the elevated level of ROS. Normal levels of ROS are required for sperm physiology, but excessive levels of ROS leads to excessive lipid peroxidation (LPO), DNA damage and sperm deterioration[50]. The generation of ROS can be exacerbated by environmental life style factors, pollution, radiation, and infectious diseases[51]. The mitochondria system is the major source of ROS in spermatozoa from infertile men. Spermatozoa may generate ROS in two ways: 1-NADPH-oxidase system at the level of the sperm plasma membrane and NADH-dependent oxido-reductase at the level of mitochondria[52]. The increase production of ROS by NP may be associated with mitochondrial and/or endoplasmic reticulum stress, and decreased sperm motility, defective acrosome reaction, and loss fertility[53]. This event may result in cascade of episode including LPO of sperm plasma membrane that ultimately affects an axonal protein phosphorylation and sperm immobilization[54] (Figure 6).

Normal motility is indicative of normal development of spermatozoa of axoneme during spermatogenesis in the testis, a normal maturation process in the epididymis, and normal seminal plasma constituents[55]. Moreover, increased poor sperm morphology has been reported to reduce successful fertilization rates with increased miscarriages even after successful embryo transfer[56]. In our study, we found that (18.50, 20.61 and 28.93% respectively) in a dose-related manner as compared to the corresponding controls sperm had abnormal morphology. The most predominant abnormality was abnormal head (flattened head, headless, pinhead) bent tail, coiled tail and other defect and we provided a clear picture of this overlap. Percentage normal morphology is an essential characteristic for *in vivo* and *in vitro* fertilization[57], and the best predictor among all of the sperm characteristics. Abnormal forms are merely defined on the basis of atypical forms, not identified by the cellular basis of their functional incapacity because of technical limitations of light microscopy. The flagella abnormality is associated in motility disorders[58] and is responsible for most cases of severe asthenozoospermia.

Sperm motility depends on mitochondrial functions and thus spermatozoa having adequate mitochondrial integrity (i.e. high MMP) should have robust motility. Previous studies suggested that MMP of human spermatozoa determined by JC-1 staining well correlated with their progressive motility[59]. Mitochondrial damage during chemical exposure could be one of the major reasons for reduced sperm motility[60]. Mitochondrial dysfunction can result in the formation of ROS. Alteration in the mitochondria membrane potential ($\Delta\psi m$) is known to be a major cause of precipitation of apoptosis in many cell types[61].

Oxidation-reduction (redox) reactions are important to the regulation of metabolic functions of the cell and in the protection of cellular components against oxidative damage. In this study, the level of H_2O_2 and MDA were increased in the plasma of NP-treated rats. However, H_2O_2 is known to be key agents causing cytotoxicity in spermatozoa to produce oxidative stress by decreasing the enzymatic defenses[62]. In addition, MDA is one of the major end products of lipid peroxidation, especially the polyunsaturated fatty acid[63]. The sperm cell membrane contains high levels of polyunsaturated fatty acids that yield considerable fluidity, necessary to allow the cell to fuse the oocyte[64]. LPO in spermatozoa results in decreased membrane fluidity and sperm motility, and a reduced capacity for fertilization[65].

Post-testicular maturation of spermatozoa taking place in the epididymis, and the immotile and immature spermatozoa released from the testes acquire progressive motility and fertilizing ability during their transit through the long epididymal duct. The antioxidant defense system protects the sperm during this transit and helps their maturation[20]. This research also shows that the variation of ROS and LPO is accompanied by concomitant depletion in the activities of antioxidant enzymes namely CAT, GSH-Px and SOD. CAT and GSH-Px have been considered the primary scavengers of H_2O_2[20], SOD can catalyse decomposition of superoxide radicals to produce H_2O_2. However, absence of adequate CAT or GSH-Px activity to degrade H_2O_2, more H_2O_2 could be converted to toxic hydroxyl radicals and may contribute to the oxidative stress of NP which may contribute to serious damage of sperm mitochondrial membranes. Decrease in the activity of GSH-Px indicates either reduced synthesis, elevated degradation or inactivation of the enzymes. Furthermore, SOD specifically metabolizes oxygen-free radicals and is believed to be the first and one of the most important lines of antioxidant enzyme defense systems against ROS. The antioxidant enzyme CAT protects SOD against inactivation by H_2O_2. Reciprocally, the SOD protects CAT against inhibition by superoxide anion[66]. The reduction in CAT activity reflects the inability of spermatozoa to eliminate H_2O_2 produced by NP[16]. The reversibility of the sperm motility by these ROS scavengers suggests that NP induced production of ROS may be related to the decreased motility caused by LPO.

In conclusion, our results demonstrate that exposure of NP reveal decrease in testes weights, decreased epididymal sperm motility and affect sperm morphology. The reduction in the activities of antioxidant enzymes and increase in H_2O_2 and LPO elicit that NP disrupts the prooxidant and antioxidant balance. This leads to excessive generation of ROS, thus causing oxidative stress in epididymal sperm of rat. Furthermore, the reduction in epididymal sperm motility may affect sperm quality and fertility capacity. This concludes that the exposure of male rats to repeated dose of NP caused sperm motility disruption and many alterations in the sperm morphology, indicating the estrogenic effect of this chemical pollutant.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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