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TOXIC EFFECTS OF NONYLPHENOL ON OXIDATIVE STRESS IN TESTES AND EPIDIDYMIS OF ADULT ALBINO RATS

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ABSTRACT

The present study is aimed to investigate the toxic effects of nonylphenol on oxidative stress in testes and epididymis of adult albino rats. Randomly 32 healthy adult male rats were divided into 4 groups and each group with 8 animals. First group served as control, second, third and fourth groups were administered with nonylphenol at doses of 1 µg/kg bw, 10µg/kg bw and 100 µg/kg bw every alternative day for 55 days. After the treatment, on 57th day of experiment, animals were sacrificed and collected the tissues. The indices of testes and epididymis were significantly ($p<0.05$) decreased in nonylphenol treated rats than controls. In the testicular and internal parts of epididymis total lipid peroxidation levels were significantly ($p<0.05$) increased, whereas the activity levels of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase levels were decreased significantly ($p<0.05$) in nonylphenol treated rats when compared to the control group. DNA fragmentation was increased significantly in a dose dependent manner when compared to control. The present study concluded that exposure of male albino rats to nonylphenol at low concentrations induces oxidative stress in testes and epididymis of adult rats.

KEYWORDS: **Nonylphenol, lipid peroxidation, Oxidative stress enzymes, DNA fragmentation, testes, Epididymis, rats.**

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INTRODUCTION

Worldwide there is an increase in the production of variety of man-made chemicals and their degradation products. Most of them are capable of interfering with the hormonal system of numerous organisms and showing adverse effects. Nonylphenol (NP) is one of the example; it originates from the degradation of alkylphenol polyethoxylate (APE)[1]. It is widely used as emulsifiers, lubricating oil additive[2], surfactant in industrial and household products, construction materials, vulcanized rubber[3] and spermicides[4]. Nonylphenol has been appeared in aquatic environment such as river water, drinking water and sewage sludge[5] and also in ambient air, soil and biota[6]. Nonylphenol is able to mimic the effects of estrogen and exhibits endocrine disruption and cause adverse reproductive effects in mammals[7-9]. Nonylphenol induce toxicity on reproductive system of mammals by affecting testicular growth[10], sperm production[11], epididymis weight, gonadal function[4,12] , reduced testis size[13], decreased testicular and epididymal size and alterations in reproductive hormone levels in adult male rats[14]. Nonylphenol exposure in rats reduces serum testosterone levels and testicular steroidogenic enzyme activity, disturbed testicular structure and suppressed spermatogenesis[15]. In a biological system under oxidative stress conditions, several oxygen radicals including superoxide anion (O_2^-), hydroxyl radical ('OH), hydrogen peroxide (H_2O_2), and peroxyl radicals are produced and cause damage in DNA. Because of harmful effects of reactive oxygen species (ROS), high accumulation of oxygen radicals leads to cellular injury but the variety of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione S transferase (GST), glutathione peroxidase (GSH-PX) release and eliminates ROS from cells. ROS oxidizes hydrogen atoms from unsaturated fatty acids to initiate the peroxidation membranes lipids[16]. Lipid peroxidation is associated with cellular injury and is commonly used as an indicator of oxidative damage in cells and tissues[17]. Various environmental contaminants can induce oxidative stress by generating reactive oxygen species[13]. Several earlier studies reported the adverse effects of oxidative stress in testes induced by nonylphenol administration in rats [6,18- 20]. The toxic behavior of nonylphenol induces oxidative stress and elevates ROS levels resulting imbalance between proxidant and antioxidant systems in the reproductive tissues resulting reduction in fertility in treated rats [6,13,21]. Though there are several reports on the adverse effects of nonylphenol on induction of oxidative stress there by reproductive abnormalities in male reproductive system is still inadequate. In fact, the nature and mechanism of nonylphenol induced oxidative stress on male reproductive system is not clear. Earlier most of studies selected high concentrations of nonylphenol with oral administration to report toxic effects on reproductive system in experimental animals. Therefore the present work is aimed to investigate the toxic effects of intraperitoneal administration of nonylphenol at low doses on oxidative stress in the testes and different parts of epididymis, such as caput, corpus and cauda of experimental rats. The caput and corpus of epididymis has been involved in sperm maturation and cauda epididymis is associated with sperm storage.

MATERIALS AND METHODS

1. Maintenance of animals

Healthy rats of Wistar strain with a body weight ranging from 130–140g (60 days old) were purchased from authorized vendor (M/S Raghavendra Enterprises, Bangalore, India). All rats were housed in polypropylene cages (18" x 10" x 8") lined with sterilized paddy husk, and provided filtered tap water and rat food (purchased from HLL Animal Feed, Bangalore, India) *ad libitum* in an air-conditioned environment (25 ± 2°C) with a 12-hour light and 12-hour dark cycle. Experimental protocols were carried out at S.V. University, Tirupati in accordance with the guidelines approved by the institutional ethical committee (CPCSEA) (vide No. IAEC/No-438/01/a/CPCSEA).

2. Chemicals

Nonylphenol (CAS NO 46018, purity>98%) purchased from Sigma-Aldrich, USA was used as test chemical. The 2-thiobarbituric acid (TBA) and reduced glutathione (GSH), NADH, NADPH, NAD were purchased from Sigma Chemical Company (St Louis, Missouri, USA).

3. Experimental design

Adult male rats were divided into 4 groups of eight each. Animals in group 1 served as control, second, third and fourth groups were administered intraperitoneally with nonylphenol in 1, 10 and 100µg/kg bw respectively, at every alternative day for 55 days. At the end of the experiment, the animals were fasted overnight, on the 57th day, the rats were sacrificed by cervical dislocation. The essential tissues were dissected out, weighed for the nearest milligram immediately and washed using ice cold saline to remove the blood. Tissue somatic index (TSI) was calculated using the following formula:

TSI = weight of the tissue (g)/ body weight of the animal (g) X 100

Tissue was minced and homogenized (10 %w/v) in 0.025 M Tris- HCl buffer (pH 7.4), and centrifuged (3000 x g for 10 min). The resulting clear supernatant was used for various biochemical assays.

4. Biochemical assays

(i). Estimation of lipid peroxidation

Lipid peroxidation in testis and internal parts of epididymis was estimated spectrophotometrically by the method of Ohkawa (1979)[22] measuring thiobarbituric acid reactive substances (TBARS). A breakdown product of lipid peroxidation, thiobarbituric acid reactive substance was determined by the method of thiobarbituric acid reaction[22]. Briefly, the tissues were homogenized (10% W/V) in 1.15% KCL solution. Added 0.5 ml of saline (0.9% sodium chloride), 1.0 ml of (20% W/V) trichloroacetic acid (TCA) was added to 2.5 ml of homogenate. Samples were centrifuged for 20 minutes at 4000 x rpm, 0.25 ml of TBA reagent was added to 1.0 ml of supernatant, and samples were then incubated at 95°C for 1 h. An equal volume of n-butanol was added to the supernatant, after thorough mixing, the contents were centrifuged for 15 minutes at 4000 rpm. The organic layer was transferred into a clear tube and its absorbance was measured at 532 nm. The rate of lipid peroxidation was expressed as µ moles of malondialdehyde formed/gram wet weight of tissue. A standard curve was constructed with the known amount of malonaldehyde and the absorbance was measured at 532 nm.

(ii). Superoxide dismutase

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was determined by the method of Mishra and Fridovich [23]. SOD was assayed in the microsomal fraction according to its ability to inhibit the autooxidation of epinephrine at alkaline medium. Briefly, the testes and internal parts of epididymis were homogenized (10% W/V) in 50 mM ice-cold sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA. The homogenate was centrifuged at 105,000 rpm for 60 min. The supernatant (cytosol) fraction was used for the assay of enzyme activity. The reaction mixture contains 0.05 M carbonate buffer (pH 10.2), 30 mM epinephrine (freshly prepared) and the enzyme source. Changes in absorbance were recorded at 480 nm, measured at 10 seconds intervals for 1 minute in a spectrophotometer. The enzyme activity was expressed as Units/mg protein/min.

(iii). Catalase

The activity of catalase (CAT) (EC 1.11.1.6) was determined by based on its ability to decompose H_2O_2, using the method described by Chance and Machly [24]. The reaction mixture contains (2.5 ml, vol) mixer of 2.4 ml of 50 mM phosphate buffer (pH 7.0), 0.1 ml of tissue homogenate and 10 µl of 19 mM H_2O_2. Then the absorbance was read at 240 nm. CAT activity was expressed as µM of H_2O_2 consumed/min/mg protein.

(iv). Glutathione peroxidase

Glutathione peroxidase (GPx) activity (EC 1.11.1.9) was estimated by the method of Mohandas et al. [25]. Briefly, the reaction mixture contained 1.59 ml of 100 mM phosphate buffer (pH 7.6), 0.1 ml of 10mM EDTA, 0.1mL of 10 mM sodium azide, 0.1 ml glutathione reduced, 0.01 ml of 0.2 mM hydrogen peroxide and 0.1ml of enzyme source were immediately the contents were read at 340 nm in a spectrophotometer. The activity of GPx was expressed as nanomoles of NADPH oxidized/min/ mg protein.

(v). Glutathione Reductase

Glutathione Reductase (GR) (EC 1.6.4.2) utilizes NADPH to convert metabolized glutathione (GSSG) to the reduced and this was assayed by the method of Carlberg and Mannervik (1985)[26]. In brief, 1.75 ml of 100mM phosphate buffer (pH 7.6) and 0.1 ml of 10mM EDTA was taken in a test tube. To this, 0.05 ml of 20mM glutathione oxidised and 0.1 ml of 200mM NADPH were added and made up to 2.1 ml with water. To this solution, 0.1 ml of enzyme source was added and absorbance was read at 340 nm in spectrophotometer. The activity of GR was expressed as nanomoles of NADPH reduced/min/ mg protein. Protein content in the enzyme source was estimated by the method of Lowry et al. [27] using bovine serum albumin as standard.

(vi). DNA extraction

Testicular DNA was isolated according to the method of Gilbert et al. [28]. Thirty mg of testes tissue were taken from each group and washed twice with 1 ml TE buffer (20 ml of 1 M Tris (pH 8.0), 20 ml of 0.5 M EDTA, 100 ml of sterile distilled water). Tissues were homogenized and 300 µl of lysis buffer and 240 µl of10% SDS were added, vortexed gently and incubated overnight at 45°C in a water bath. Phenol 200 µl was added, shaken vigorously for 5 min, and centrifuged at 3000 rpm for 5 min. Supernatant was then pipetted out into a new tube, 200 µl phenol and 200 µl chloroform/isoamyl alcohol (24:1) were added, vortexed and centrifuged at 3000 rpm for 5 min. Supernatant were again pipetted out into a new tube and 25 µl of 3 M sodium acetate (pH 5.2) and 5 ml of ice cold 100% ethanol were added and the tubes were kept for overnight at -20°C and then centrifuged for 30 min. The solution was pipetted out gently to avoid disturbance to the DNA. DNA was washed in 70% ethanol and air dried. TE buffer 20-50 µl (10 mM Tris pH 8.0, 1 mM EDTA) and 2 µl RNase was added. Electrophoresis was performed using 1% agarose resolving gel in Tris borate EDTA buffer (TBE) containing 1µg ml^{-1} ethidium bromide. Five µg of total DNA per well was loaded. DNA ladder of 100 bp was loaded to identify the size of the DNA fragment. Electrophoresis was performed for 45 min at 100 V after separation of DNA the gel was viewed under gel doc system and photographed. Data were analyzed by one way analysis of variance (ANOVA) followed by Dunnett's test using a statistical software. Results were presented as mean ± S.D., $p<0.05$ were considered as statistically significant.

RESULTS

1. Tissue indice

Table 1 shows the effect of nonylphenol on organ weights in control and experimental rats. In nonylphenol treated rats weights of testes and internal parts of epididymis were significantly ($p<0.05$) decreased in group 3 and 4 when compared with controls and the

changes induced by nonylphenol were in dose dependent manner. No significant changes were observed in group 1 over the control rats.

2. Lipid peroxidation levels

The changes in the level of LPO product in control and experimental rats are presented in figure 1. The levels of TBARS were significantly ($p<0.05$) increased in nonylphenol treated rats in dose dependent manner in testes and internal parts of epididymis.

3. ***Enzymatic antioxidants levels***

Activities of enzymatic antioxidants SOD, CAT, GPx and GR in testes (Table 2) of rats treated with nonylphenol were significantly ($p<0.05$) changed in a dose dependent manner when compared to control rats. The activity levels of SOD, CAT, GPx and GR were significantly ($p<0.05$) changed in caput, corpus and cauda epididymis in the nonylphenol treated rats in a dose dependent manner when compared to control rats (Table 3).

4. ***DNA fragmentation analysis***

DNA fragmentation was hardly visible in the control testes, while nonylphenol treatment at 1, 10 and 100 µg doses was detected. The band intensity of fragmented DNA was highest in the 100 µg treated group than other groups (Figure 2).

Table 1
***Effect of different doses of nonylphenol on reproductive tissue indices (W/W %) in* rats.**

Organ	Control	1µg	10µg	100µg
Testes	2.91 [a] ±0.37	2.89 [a] ±0.95 (-0.68)	2.23 [b] ±0.28 (-23.36)	2.11 [c] ±0.68 (-27.49)
Epididymis Caput	0.89 [a] ±0.07	0.85 [a] ±0.03 (-4.49)	0.76 [b] ±0.03 (-14.60)	0.71 [c] ±0.02 (-20.22)
Corpus	0.78 [a] ±0.06	0.74 [a] ±0.03 (-5.12)	0.36 [b] ±0.06 (-53.84)	0.33 [b] ±0.01 (-57.69)
Cauda	0.41 [a] ±0.05	0.37 [b] ±0.04 (-9.75)	0.29 [c] ±0.04 (-29.26)	0.21 [d] ±0.02 (-48.78)

Values are mean ± SD of eight individual observations.
Values in parentheses are percent change from control.
Values with same superscript in a row do not differ significantly from each other, p < 0.05.

Table 2
Effect of different doses of nonylphenol on antioxidant enzyme status in testes of rats

Antioxidant enzymes	Control	1µg	10µg	100µg
SOD(units/mg/min) (units/mg/min)	0.78 [a] ±1.18	0.74 [a] ±1.15 (-5.12)	0.70 [b] ±1.02 (-10.25)	0.65 [a] ±1.14 (-16.66)
CAT(nano mol H_2O_2 consumed/mg/min)	44.58 [a] ±3.19	42.81 [b] ±2.93 (-3.97)	39.65 [c] ±2.88 (-11.05)	35.14 [d] ±3.63 (-21.17)
Gpx (µmol NADPH oxidized/mg/min)	51.23 [a] ±6.97	50.07 [a] ±6.64 (-2.26)	48.32 [b] ±6.92 (-5.68)	43.06 [c] ±6.95 (-15.94)
Gr(µmol NADPH oxidized/mg/min)	75.43 [a] ±5.51	75.01 [a] ±5.34 (-0.55)	69.78 [b] ±5.22 (-7.49)	65.51 [c] ±5.62 (-13.15)

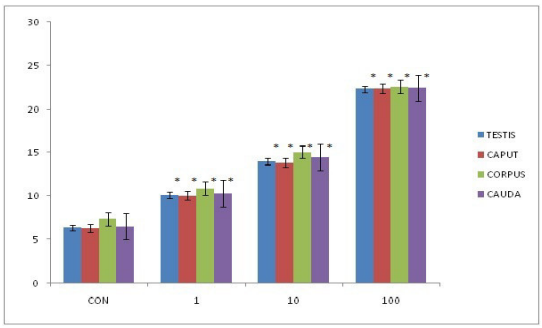
Values are mean ± SD of eight individual observations.
Values in parentheses are percent change from control.
Values with same superscript in a row do not differ significantly from each other p < 0.05.

Table 3
***Effect of different doses of nonylphenol on enzymatic antioxidants status in Internal parts of the epididymis* in rats**

Enzyme	Different parts in epididymis	Control	1µg	10µg	100µg
SOD (units/mg/min)	Caput	0.65±0.05	0.62 ^{ns} ±0.02 (-4.61)	0.56* ±0.08 (-13.84)	0.45* ±0.06 (-30.76)
	Corpus	0.73 ±0.08	0.69 ^{ns} ±0.05 (-5.47)	0.68 ^{ns} ±0.06 (-6.84)	0.63* ±0.07 (-13.69)
	cauda	0.65 ±0.05	0.63 ^{ns} ±0.01 (-3.07)	0.59* ±0.04 (-9.23)	0.46* ±0.03 (-29.23)
CAT (nano mol H_2O_2 consumed/ mg/min)	Caput	34.56 ±5.86	33.93 ^{ns} ±6.84 (-1.82)	28.35 ^{ns} ±2.58 (-17.96)	18.87* ±3.28 (-45.39)
	Corpus	35.83 $^{\pm}$ 5.24	33.61 ^{ns} ±1.63 (-6.19)	26.42* ±3.32 (-26.26)	21.86* ±1.79 (-38.98)
	Cauda	33.93 ±6.84	32.85 ^{ns} ±4.63 (-3.18)	25.62* ±3.17 (-24.49)	20.33* ±1.21 (-40.08)
Gpx(µmol NADPH oxidized/mg/min)	Caput	21.93 ±2.28	21.38 ^{ns} ±3.51 (-2.50)	19.83 ^{ns} ±1.68 (-9.57)	16.33* ±3.07 (-25.53)
	Corpus	23.25 ±1.92	21.17 ^{ns} ±1.54 (-8.94)	17.61* ±1.44 (-24.25)	15.95* ±2.22 (-31.39)
	cauda	23.13 ±1.77	21.25* ±0.93 (-8.12)	15.85* ±1.51 (-31.47)	12.85* ±0.68 (-44.44)
Gr(µmol NADPH oxidized/mg/min)	Caput	41.40 ±1.67	39.96 ^{ns} ±2.65 (-3.47)	28.00* ±1.27 (-32.36)	22.86* ±1.62 (-44.78)
	Corpus	29.16 ±1.00	25.53* ±0.60 (-12.44)	22.01* ±1.92 (-24.51)	18.40* ±0.73 (-36.89)
	Cauda	28.65 ±0.63	25.60* ±0.42 (-10.64)	24.46* ±0.47 (-14.62)	18.45* ±0.62 (-35.60)

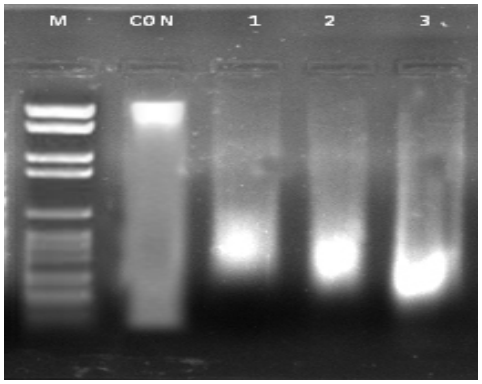
Values are mean ± SD of eight individual observations.
Values in parentheses are percent change from control.
Values with same superscript in a row do not differ significantly from each other. p < 0.05.

Figure 1
Effect of different doses of nonylphenol on lipid peroxidation (LPO) levels in the testes and internal parts of epididymis (µ moles/g tissue)in rats



Values are mean ± SD of eight individual observations.
Values with superscript on a bar differ significantly from controls, p < 0.05.

Figure 2
Evaluation of DNA fragmentation in testes of nonylphenol treated rats



Lane M: 100-bp DNA ladder; Lane con: Control shows intact DNA; Lane 1, 2, 3: shows Smear of DNA in the 1, 10 and 100 µg nonylphenol doses from rat testes respectively.

DISCUSSION

Testes and epididymis are important organs, where production and development of sperm takes place in male reproductive system and weight of these organs is a valuable index of reproductive health. In the present study, rats injected with nonylphenol significantly reduced the weights of testes and internal parts of epididymis (Table 1). Decreased weights of reproductive organs in animals treated with nonylphenol agree with earlier reports[10,13,14, 29, 30]. The reduced weights of organs in nonylphenol treated animals is caused by generation of free radicals in their respective tissues[13,17, 31- 33] i.e. oxidative stress may responsible for decrease in testis and epididymis weight and similar results were observed in the present study also. Nonylphenol exposure decreased the activity levels of anti-oxidant enzymes in dose dependent manner in the testes (Tables 2) and epididymis (caput, corpus and cauda) of the treated rats (Table 3). Under normal circumstances, equilibrium is maintained between oxidative stress of free radicals and anti-oxidative defense system in the tissues provided intake or biosynthesis of pro-oxidants, damaged biosynthesis of antioxidants or coupled of both induces oxidative stress [34,35]. In the present study, activity of SOD decreased in the testes and internal parts of epididymis of nonylphenol treated rats. The reduction in the activity of SOD causes a rise in the level of superoxide anion, which inactivates CAT activity [36]. The decreased activity of CAT in the testes and epididymis of nonylphenol treated animals may reflect the inability to eliminate H_2O_2 produced by the nonylphenol. At high concentrations CAT is the main scavenger of hydrogen peroxide [37]. GPx is also involved in the scavenging of hydrogen peroxide [38] and GR can directly interact with hydroxyl radical to detoxify them. In experimental rats decreased GPx and GR in the testes and epididymis may not able to remove free radicals generated by nonylphenol. In the present study the increase in lipid peroxidation in the testes and internal parts of epididymis (Fig. 1), could be due to the increase in the generation of free radicals in the nonylphenol treated rats. The oxidative stress generates reactive oxygen species that reacts with lipids causing peroxidation and eventually results in the release of products such as malondialdehyde, Hydrogen Peroxide and hydroxyl radicals. MDA being a useful indicator of oxidative damage in various diseases [39,40] are probably as the end product and an important molecular marker of lipid peroxidation. In the present study, increased MDA levels therefore indicate the oxidative stress induced by nonylphenol. These results are in agreement with earlier studies on the oxidative stress induced due to increased levels of lipid peroxidation in nonylphenol treated animals[13, 17, 21, 31]. In the present study administration of nonylphenol might have generated ROS in the affected tissues resulting decreased activities of antioxidant enzymes and concomitantly increased levels of lipid peroxidation thereby disturbed the balance between pro-oxidant and anti-oxidant systems in the treated rats [6, 17, 21, 33]. The decrease in the anti-oxidant enzymes in the present study clearly indicates the oxidative stress induced by nonylphenol. These results are in agreement with earlier studies on the oxidative stress induced due to reduced activity levels of anti-oxidant enzymes in nonylphenol treated animals [6,13,21] . The present study showed that Nonylphenol induced significant testicular DNA fragmentation in dose dependent manner compared to control group. However the DNA fragmentation was increased significantly at higher dose i.e. 100 µg treated animals compared to 1 and 10 µg treated rats. Nonylphenol produced oxidative stress significantly induced testicular DNA fragmentation, which is considered as a marker to detect cell apoptosis [41,42]. In earlier reports on the nonylphenol produced oxidative stress induces DNA fragmentation in some other cells to detect cell death such as PC12 cells [42], murine neural stem cells [43] and Jurkat cells [44]. Similar to earlier studies, in the present study also clears that oxidative stress is responsible for DNA damage in testes. The present results clearly indicated that exposure to nonylphenol at low doses induced oxidative stress with depressed activities of antioxidant enzymes. Adverse effects caused by nonylphenol on male reproductive system in adult rats (Authors unpublished data) might be due to oxidative stress.

CONCLUSION

The present study clearly reveals that the low doses of nonylphenol also induces oxidative stress by depressed activity of antioxidant in testis and epididymis and DNA damage in testes and are in agreement with generation of reactive oxygen species, created an imbalance between the pro-oxidant and anti-oxidant systems in the tissues of the treated rats which it leads to oxidative stress. The increased oxidative stress caused DNA damage in testes.

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