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Protective Antioxidant Effects of N-acetylcysteine on Testicular Tissue and Serum Testosterone in Paranonylphenol-Treated Mice (A Stereological Analysis)

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Abstract

Paranonilphenol (p-NP) is an environmental pollutant that affects the male reproductive system through induction of oxidative stress. The aim of this study was to evaluate the protective effects of N-acetylcysteine (NAC) as an antioxidant against impairment of testicular tissue caused by p-NP. In this study, 24 adult male NMRI mice were randomly divided into 4 groups (n=6 each groups): 1-Control, 2- p-NP (250 mg/kg/day), 3-NAC (150 mg/kg/day) and 4-p-NP+NAC. After 35 days of orally treatment a significant decrease in the diameter ($P<0.01$), germinal epithelium height ($P<0.04$), basement membrane thickness and volume of seminiferous tubules ($P<0.02$) and as well as a significant decrease in serum testosterone level ($P<0.04$) were observed. Also a significant decrease in the count of sertoli ($P<0.04$), leydig ($P<0.02$), spermatogonia ($P<0.04$), spermatocyte ($P<0.04$), round and long spermatid ($P<0.02$) cells in the p-NP group compared to other group were observed. In the p-NP group, atrophy, edema, reduction in lumens sperm density and vacuoled were observed. While in this group a significant increase in the interstitial tissue volume ($p<0.01$), level of testis MDA ($p<0.01$) and count of the positive-TUNEL cells ($p<0.01$) in the seminiferous tubules were observed. NAC can significantly compensate the parameters mentioned above in NAC +p-NP group compared to the p-NP group.

The findings of this study showed that NAC, as a potent antioxidant, can compensate the adverse effects of p-NP on testicular tissue.

Keywords: N-acetylcysteine; Paranonylphenol; Testicular tissue; Mice

Introduction

The paranonilphenol (p-NP) is known as an environmental pollutant which causes an endocrine disorder [1] and oxidative stress inducer [2]. Nonylphenol (NP) is found in fresh fruits, vegetables and rice. It is also widely used in the preparation of oily additives, resins, plastics, detergents, dyes, cosmetics and paper and also it is mainly released through industry, urban sewage and contaminated rivers [3,4].

Several lines of studies demonstrated p-NP toxicity on testis [1,5,6]. p-NP by inducing oxidative stress reduces the height of germinal epithelium, diameter and thickness of the seminiferous tubule base membrane as well as reduces the population of spermatogenic cells, sertoli and serum testosterone levels [6-8]. The p-NP disturbed the binding between sertoli and spermatogenic cells in testis and also changes the level of Connexin 43 and inhibits its phosphorylation that is activated by the protein kinase pathway [9]. And with the increase of the reactive oxygen species (ROS) [10] and weakening of the activities of antioxidant defense enzymes [7], this pollutant causes oxidative stress [11], followed by the death of germ cells [2]. Since p-NP induced oxidative stress in testis [11], the use of antioxidants can possibly prevent its toxic effects. Therefore, NAC was used as an antioxidant in this study. This antioxidant by preventing the expression of pre-apoptotic genes [12] and increasing glutathione levels [13] acts as a strong antioxidant. And also NAC by facilitating in the production and function of Nitric oxide has several medicinal effects [14]. Glutathione is one of the major antioxidants in the body, which has undesirable effects of free radical molecules, which play an effective role in cellular protection [15]. In the course of oxidative stress, glutathione concentration decreases [13]. The administration of NAC increases the concentration of intracellular glutathione and compensates for its deficiency [16]. In several studies, the role of NAC has been reported to reduce the cell death in spermatogenic and sertoli cells and also this pollutant has been shown to increase the cellular bioavailability by reducing lipid [17-19]. Regarding the induction of oxidative stress by p-NP and NAC as a potent antioxidant, the purpose of this study was to investigate if NAC, can compensate the adverse effects of p-NP on testicular tissue in mice.

Material and Methods

Animals and treatments

In this study, 24 adult male NMRI mice were purchased from Pasture's institute, Tehran, Iran. The animals were housed in plastic cages at 12 hour light/dark cycle, 24°C ± 2°C with water and food ad libitum. Animals were randomly divided into 4 groups (n=6 each group) including: 1-control, 2-p-NP (250 mg/kg/day, Sigma), 3-NAC (150 mg/kg/day, Sigma) and 4: p-NP (250 mg/kg/day)+NAC (150 mg/kg/day). Doses p-NP and NAC were selected according to studies Soleimani et al. [8] and Sotoudeh et al. [20], respectively. They were treated orally for all groups over a period of 35 days.

Estimating the shrinkage, the total volume of testis and Histopathology study

After this treatment, weighing the mice and then anesthetized with diethyl ether, and the right testicle was removed. The size of the testis was measured by the immersion method [8,21]. Then, the testicles were placed for a modified Davidson's fluid fixative for fixation. After fixation, the Orientator method was used to create isotropic uniform random sampling (IUR, Howard and Reed) [22]. The sections obtained from each testis were subjected to tissue processing (Leica) and placed in paraffin blocks, then paraffin molds prepared by microtome (Leitz 1512), sections of 5 and 20 µm thicknesses were stained with Heidenhain's Azan method [21]. Then, Histopathologic assessments were performed. As well as calculate shrinkage using trocar, randomly, three round segments from the testicular tissue sections of each mice were prepared and each of them measured two perpendicular diameters, then the mean radius and ultimately the area. The wrinkling caused by the processes of fixation, sapping and tissue paraffinization, the density and compaction generated by the blade function in the microtome. After staining, the area of the round pieces were re-measured and the volume shrinkage was calculated using the Formula-1 (Figure 1) [21,23]. According to the method of Soleimani et al. [8], by reducing the amount of wrinkling, the volume of Immersion method, the actual Testis volume was calculated.

1	$V_s = 1 - (AA/AB)^{1.5}$	**V_s**:Volume shrinkage. **AB** and **AA**, respectively, the area of the round parts was taken before and after staining.
2	H = Sv / Vv	height of the germinal epithelium **(H)**, surface density of the germinal epithelium **(Sv),** volume density of the germinal epithelium **(Vv)**
3	$Sv = 2 \times \frac{\sum_{t=1}^{n} Ii}{L/p \cdot \sum_{t=1}^{n} Pi}$	**ΣIi=** the count of collisions with the internal surface of the germinal epithelium, **ΣPi=** the count of points encountered with germinal epithelium, **L/p=** the length of the surface line grid on the actual scale of the tissue which is (214 µm).
4	TL= 8/3π × CM/SROL	**TL**: Harmonic mean Thickness layer. **π**: 3.14. **CM**: count of measurements. **SROL**: sum of the reciprocal of orthogonal intercept length.
5	$Nv = \frac{\sum_{i=1}^{n} Q_i}{h. \sum_{i=1}^{n} P_i . a/f}$	**ΣQi**: total count of cell types counted, **ΣPi**: is the sum of the points encountered with the selected fields, **h=** the height of the cut where the count is made, **a/f** = of the frame level was considered on the actual scale of the tissue.

Figure 1: Formulas used in the stereological methods. 1-Volume shrinkage. 2-Height of the germinal epithelium. 3-Surface density of the germinal epithelium. 4-Harmonic mean Thickness layer. 5-Density of cell types.

TUNEL assay

In the testis, apoptosis was assessed by fluorescent staining and the TUNEL method. To study morphological features of apoptosis, a combination of propidium iodide (PI, Sigma, USA, 10 µg/ml in PBS; 5 minutes at room temperature) and Hoechst 33342 (Sigma, USA, 5 µg/ml in PBS; 30 seconds at room temperature) was used. To use the TUNEL technique, a TUNEL Kit purchased from the Roche company (Germany) was used. The basis for the diagnosis in the TUNEL test is the detection of the fracture in the DNA by the Terminal deoxynucleotidyl transferase (TDT) enzyme and the dUTP marker, which marks free 3'-OH in the terminal end regions of the nucleic acids. TUNEL has the ability to detect two apoptotic cells with damaged DNA (Positive TUNEL) and none apoptotic cells with healthy DNA. The staining steps were performed according to the manufacturer's proposed protocol. In this study, thymus mice were used as a positive control (French et al.). In order to evaluate the number of positive TUNEL cells, a total of 20 seminiferous tubules were counted randomly in different groups by the Microscope Fluorescence (Olympus, IX70) [24].

Estimating the volume of seminiferous tubules and interstitial tissue

According to the method of Soleimani et al. [21], in order to calculate the volume of seminiferous tubules volume and interstitial tissue volume, a microscope model (B × 41TE) was used with Olympus camera and software olysia using systematic random sampling method. An average of 5 fields of view of each 5 µm section was investigated by randomly placing a probe on each field. The total points encountered by the probe were selected with the entire field of view, and in the same way the points of contact with the tubes of seminiferous tubules and tissue The interstitial counting and volume density of each were calculated, then the total volume for each component is non-existent and each mice was indirectly estimated by multiplying the volume density in the total testis volume [8,21].

Estimating the length, diameter, height of the germinal epithelium and the basement membrane thickness of seminiferous tubules

According to the method of Soleimani et al. [21], the Formula-2 (Figure 1) was used to calculate the production of epithelium. In order to obtain the volume density of the germinal epithelium (Vv), the total count of contact points of the probe with the testicular tissue image was counted for each field of view, and then the total count of points encountered with germinal epithelium was counted and the total count of points encountered with testicular tissue was divided. Similarly, the volume density of germinal epithelium and then the surface density of the germinal epithelium (Sv) were calculated by the Formula-3 (Figure 1). A harmonic measurement method was used to calculate the average thickness of the base membrane. First, using a microscope equipped with a photo camera and Olisya with Objective software, 100 µm of all 5 µm of section were selected using a randomized, 25 field of view were taken using probe and motic image software were measured. Finally, using the Formula-4 (Figure 1), Harmonic mean thickness layer was obtained [8,21].

Estimating the count of leydig, sertoli, spermatogonia, spermatocyte, round and long spermatid cells

To calculate the length of seminiferous tubules, about 5 fields of view from each 5 µm section were selected using objective 10 and an average of 150 seminiferous tubules were counted in the testis of each mice using an unbiased counting frame. The longitudinal density of the seminiferous tubules was calculated and the longitudinal density was multiplied in the final volume of the testis to obtain the total length of the seminiferous tubules [8,21]. Optical dissector and special counting frame were used to calculate the count of cell types. For this purpose, 100-magnitude objective were randomly selected from all 20 µm sections of testicular tissue of each mice in the field of vision and evaluated using an unbiased counting frame and a microcator (ND 221 B, Heidenhain, Germany). The average count of 150 cells per mice was counted on average, and then the numerical density of cell types was calculated using the Formula-5 (Figure 5). Then, by multiplying the

obtained count from the Formula-5 in total testicular volume, the total count of cell types was obtained (Figure 2) [8].

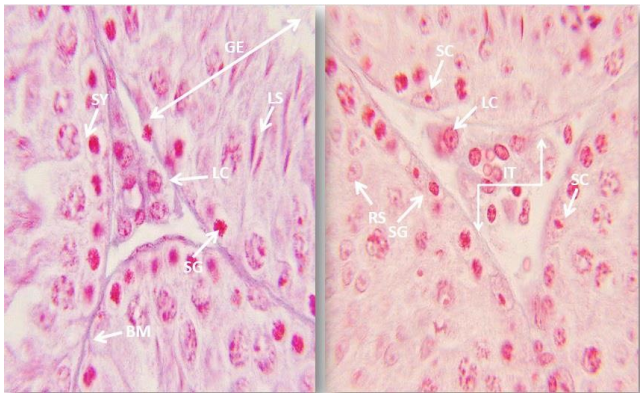

Figure 2: Image of testicular tissue in adult mice, the thickness of the incisions is 5 microns, the Heidenhain's Azan is staining and the magnification is 400X). (GE): Germinal epithelium, (IT): Interstitial tissue, (LC): Leydig cell, (SC): Sertoli cell, (SG): Spermatogonia, (SY): Spermatocyte, (LS): Long spermatid, (RS): Round spermatid, (BM): Base membrane.

Serum testosterone level test

In order to test the serum testosterone levels, blood samples were taken from the animal's heart, then centrifuging the blood samples for 10 minutes and at a rate of 3000 g. Serum testosterone concentrations were measured according to the DRG® Testosterone ELISA (EIA-1559). The findings were expressed in ng/ml.

Analysis of MDA level of testicular tissue

In order to evaluate the MDA level, the Dakdoky & Helal method is used briefly, the remainder of the left testicle was homogenized in a KCL solution of 1 to 10 by a low homogeneizer in 2 minutes. Then, it was mixed with a solution (TCA-TBA-HCL). The new solution was placed in a bain marie for 15 minutes, and after cooling, the micro tube was centrifuged for about 10 minutes at a rate of 1000 g. After completion of centrifuge, the microtip was dissolved and the absorbance was measured at 532 nm. Results were expressed in nanomol to gram of tissue [5].

Statistical analysis

Results were expressed as mean ± standard deviation (SD) for six animals per group. One-way analysis of variance (ANOVA) followed by Tukey's test was used to assess the statistical significance of data. $p<0.05$ was considered significant.

Results

Testis volume, seminiferous tubules volume, interstitial tissue volume and testis weight

In the p-NP group showed a significant decrease in the seminiferous tubules volume ($P<0.02$) as compared to other groups, while in the interstitial tissue volume in this group were significantly higher ($P<0.01$) than other groups. However, in the p-NP+NAC group seminiferous tubules volume showed a significant increase ($P<0.04$), also and interstitial tissue volume showed a significant decrease ($P<0.01$) as compared to the p-NP group. Also, there were testis volume and testis weight no significant difference ($p>0.05$) in different groups (Table 1).

Groups	Testis volume (mm^3)	Seminiferous tubules volume (mm^3)	Interstitial tissue volume (mm^3)	Testis weight (mg)
Control	81.88 ± 1.99^a	66.92 ± 1.21^a	14.95 ± 1.01^a	115 ± 40^a
p-NP	78.86 ± 2.11^a	66.75 ± 2.60^b	18.02 ± 1.74^b	113 ± 52^a
p-NP+NAC	81.29 ± 2.31^a	66.27 ± 1.69^a	15.00 ± 1.16^a	114 ± 23^a
NAC	83.88 ± 2.70^a	70.62 ± 3.01^a	13.25 ± 2.19^a	119 ± 33^a

Table 1: Comparison of testis volume (mm^3), seminiferous tubules volume (mm^3), interstitial tissue volume (mm^3) and testis weight (mg) in different groups in mice treated (for 35 days) whit para-nonylphenol (p-NP, 250 mg/kg/day) and N- acetylcysteine (NAC, 150 mg/kg/day), Date one present as mean ± SD, ANOVA, Tukey's test, the mean with the same superscripts do not differ significantly.

Length, diameter, basement membrane thickness and germinal epithelium height

In the p-NP group showed a significant decrease in the diameter of seminiferous tubules ($P<0.01$), thickness of basement membrane ($P<0.02$) and height of germinal epithelium ($P<0.04$) as compared to other groups. However, in the p-NP+NAC group, the parameters mentioned above showed a significant increase ($P<0.04$) as compared to p-NP group. Also, there was length of seminiferous tubules no significant difference ($p>0.05$) in different groups (Table 2).

Groups	Length of seminiferous tubules (m)	Diameter of seminiferous tubules (µm)	Thickness of basement membrane (µm)	Height of germinal epithelium (µm)
Control	1.96 ± 0.58^a	54.10 ± 2.07^a	6.49 ± 0.22^{ab}	188.05 ± 2.05^{ab}
p-NP	1.89 ± 0.46^a	46.83 ± 1.89^b	6.16 ± 0.14^c	174.34 ± 3.19^c

p-P+NAC	1.91 ± 0.33[a]	53.88 ± 1.67[a]	6.44 ± 0.18[a]	184.78 ± 2.68[a]
NAC	2.02 ± 0.26[a]	57.63 ± 2.12[a]	6.75 ± 0.23[b]	191.61 ± 3.25[b]

Table 2: Comparison of length (m), diameter, basement membrane thickness and germinal epithelium height (μm) in different groups in mice treated (for 35 days) whit para-nonylphenol (p-NP,250 mg/kg/day) and N-acetylcysteine (NAC, 150 mg/kg/day), Date one present as mean ± SD, ANOVA, Tukey's test, the mean with the same superscripts do not differ significantly.

Spermatogonia, spermatocyte, long and round spermatid, sertoli, leydig and positive-TUNEL cells

In the p-NP group showed a significant decrease in the mean count of spermatogonia (P<0.04), spermatocyte (P<0.04), long spermatid (P<0.02), round spermatid (P<0.02), sertoli (P<0.04) and leydig (P<0.02) cells as compared to other groups. While in this group, there was a significant increase (P<0.01) in the positive-TUNEL compared to the other group. However, in the p-NP+NAC group, the parameters mentioned above were significantly improved compared to p-NP groups and are amount the control group (Table 3).

Groups	Spermatogonia (× 10^6)	Spermatocyte (× 10^6)	Long Spermatid (× 10^6)	Round Spermatid (× 10^6)	Sertoli cells (× 10^6)	Leydig cells (× 10^6)	positive-TUNEL cells (%)
Control	5.24 ± 0.35[a]	18.42 ± 0.67[a]	27.94 ± 2.15[a]	31.35 ± 1.64[ab]	2.99 ± 0.13[ab]	2.87 ± 0.19[a]	15.76 ± 1.55[a]
p-NP	4.45 ± 0.43[b]	16.25 ± 0.83[b]	21.71 ± 1.90[b]	26.05 ± 1.78[c]	2.61 ± 0.11[c]	2.52 ± 0.09[b]	24.18 ± 1.99[b]
p-NP+NAC	5.14 ± 0.29[a]	17.79 ± 0.68[a]	27.72 ± 1.94[a]	30.31 ± 1.89[b]	2.97 ± 0.17[b]	2.81 ± 0.18[a]	18.08 ± 2.08[a]
NAC	5.68 ± 0.47[a]	18.97 ± 0.89[a]	30.14 ± 2.32[a]	33.60 ± 1.22[a]	3. 03 ± 0.17[a]	3.02 ± 0.18[a]	14.82 ± 2.35[a]

Table 3: Comparison of the count of spermatogonia, spermatocyte, long and round spermatid, sertoli, leydig cells (× 10^6) and positive-TUNEL cells (%) in different groups in mice treated (for 35 days) whit para-nonylphenol (p-NP, 250 mg/kg/day) and N-acetylcysteine (NAC, 150 mg/kg/day), Date one present as mean ± SD, ANOVA, Tukey's test, the mean with the same superscripts do not differ significantly.

Serum testosterone

In the p-NP group, the level of serum testosterone was significantly (p<0.04) lower than the other group. In the p-NP+NAC group, the level of serum testosterone significant (P<0.05) increase as compared to the p-NP group (Figure 3).

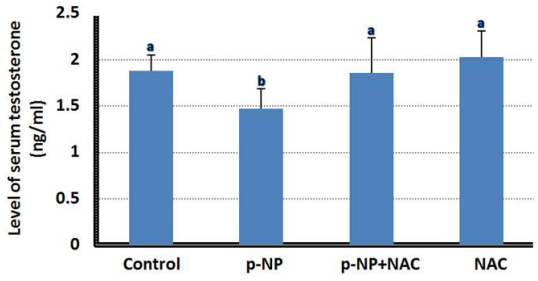

Figure 3: Comparison of serum testosterone level (p<0.04) in different groups 35 days after treated with para-nonylphenol (p-NP, 250 mg/kg/day) and N-acetylcysteine (NAC, 150 mg/kg/day). Date one present as mean ± SD, ANOVA, Tukey's test, the mean with the same letters do not differ significantly.

MDA of testicular tissue

In the p-NP group, the level of testis MDA were significantly (P<0.02) higher than the other group. In the p-NP+NAC group, this parameter significant (P<0.05) decrease as compared to the p-NP group and is amount the control group (Figure 4).

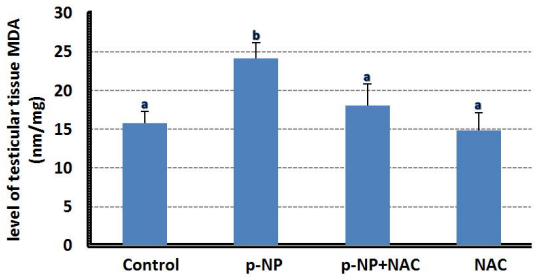

Figure 4: Comparison of testis MDA level (P<0.01) in different groups 35 days after treated with para-nonylphenol (p-NP, 250 mg/kg/day) and N-acetylcysteine (NAC, 150 mg/kg/day). Date one present as mean ± SD, ANOVA, Tukey's test, the mean with the same letters do not differ significantly.

Histopathology

Edema, atrophy and vacuoled in seminiferous tubules in the p-NP group were observed. Also, the reduction in lumens sperm density of the seminiferous tubes was lower than the other groups (Figure 5B). In the p-NP+NAC and NAC group, most seminiferous tubules were similar to the control group and tissue view compensated compared to the p-NP group (Figure 5C).

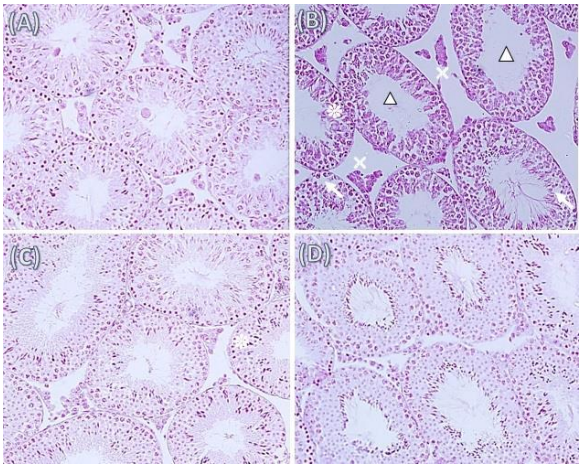

Figure 5: Microscopic Image of testicular tissue in adult mice in different groups treated with para-nonylphenol (250 mg/kg/day) and N-acetylcysteine (150 mg/kg/day, for 35 days, the thickness of the incisions is 5 microns, the Heidenhain's Azan is staining and the magnification is 200X). (A): Normal germinal epithelium arrangement in the control group (B): Irregular and decrease in epithelial Height (star), vacuoled (arrow), reduce sperm density in lumens (triangle) and increase Interstitial tissue in the para-nonylphenol group. (C): Normal germinal epithelium in the paranonylphenol+N-acetylcysteine group (star). (D): A normal and highly ordered germinal epithelium arrangement in the N-acetylcysteine group.

Discussion

The results of this study showed the induction of oxidative stress in mice treated with p-NP on testicular tissue followed by increase in lipid peroxidation and the induction of apoptosis in spermatogenic and leydig cells also decrease serum testosterone levels, that NAC could compensate the adverse effects of this pollutant. In the present study, p-NP exerted several histopathological changes in testis.

In this study, a decrease in diameter, height, base membrane thickness and volume of the seminiferous tubules and also an increase in the volume of interstitial tissue were observed, which is consistent with the research by Soleimani et al. [8] and Soleimani et al. [21] with orally treated at a dose of 250 mg/kg/day. Also in this study, there were decrease in the total count of leydig, sertoli, spermatogonia, spermatocyte, and long and round spermatid, as well as a decrease in serum testosterone level in p-NP group, which These findings are consistent with previous studies [8,25,26]. NP can damage the linkage of Gap junction by reducing the expression of connexin 43 protein, causing a defect and apoptosis in sertoli and spermatogenic cells that may be a reason to reduction in epithelial height and tubule diameter [7,27], as well as disruption of the blood-testicle barrier and provides tissue edema [6]. On the other hand, this pollutant by induces oxidative stress [3,10]. Stop the B type spermatogonia in the G1 stage of mitosis, because the product of the *XPB1* gene (whose promoter is activated by oxidative stress), inhibits the expression of cyclin 1 protein, which is one of the necessary factors for mitosis [2,28]. Therefore, stopping cell division in the epithelium of the germinal layer will reduce spermatogenesis and sperm density in lumens and also reduce the height of germinal epithelium and seminiferous tubules diameter, in addition to p-NP by increasing ROS [2]. Activation of Bax and the release of C cytochrome from mitochondria, which resulted in activation of the Apaf1/Caspase-9 and Caspase-4 complexes [11]. The activation of caspase cascade results in the apoptosis in germinal and sertoli cells [29]. As a result of the reduced population of germinal and leydig cells. Considering that base membrane compositions include laminin, collagen types, heparin sulfate and proteoglycan that sertoli and myoid cells are responsible for synthesis them, disruption of these cells by p-NP reduces the composition of the base membrane and thus reduces its thickness [30].

NP has weak estrogenic activity and affects estrogen receptor and also induces male infertility *via* a negative impact on spermatogenesis [31]. Other results from this study was the reduction of serum testosterone level by p-NP, which is consistent with the results of previous studies [7,11]. The p-NP can reduce the biosynthesis of testosterone by inhibiting activity of the 17α-HSD enzymes and the cAMP pathway of Leydig cells [32]. p-NP disrupts Leydig and Sirtouli cells [33,34]. Since the function of the sertoli and leydig cells is interdependent [35], Thus, the disruption and decrease in both cells can lead to decreased testosterone production. In addition to testosterone directly or indirectly, affects in the inter-tissue and lymphatic space and causes an interstitial tissue and edema [36].

Several studies have shown that p-NP can exert adverse effects on cells and tissues through oxidative stress by increasing lipid peroxidation [5,11] and induction of apoptosis [2,5]. Therefore, it is reasonable to assume that in the present study testis histopathology and the reduction of testosterone induced by p-NP have been exerted through oxidative stress. To test this hypothesis, MDA (important indicator of lipid peroxidation) as an oxidative stress indicator and TUNEL test as an indicator of apoptosis in the testis were evaluated. Our results showed that mice treated with p-NP significantly increased MDA and positive-TUNEL in testis, which is in agreement with past studies [2,5,11].

If oxidative stress is responsible for the adverse effects of p-NP, make use of an antioxidant can compensate the toxic effects of p-NP. In the present study, the application NAC in the NAC+p-NP group not only compensated for histopathological changes in the testis and the level of serum testosterone, but also reversed the level of MDA and count of positive-TUNEL. NAC is known as a potent antioxidant [10,16]. Therefore, in the present study, this antioxidant was able to compensate the adverse effects of p-NP. NAC may do this, through directly by preventing the expression of pre- apoptotic genes [12] and indirectly by increasing glutathione levels [13]. In addition, NAC inhibits the activity of c-Jun N-terminal kinase, MAP kinase p 38, SAPK/INK, c-fos pathway and NF-κB, which can regulate many anti-apoptotic inflammation and genes, thus prevents cell death [12]. Several studies have reported the protective effect of this antioxidant in testicular tissue, testosterone and MDA level and also apoptosis against to various toxins [10,37,38].

Conclusion

Therefore, considering the findings of this study, NAC as a strong antioxidant by enhance antioxidant, defense system followed by reducing the oxidative stress and also preserving the level of testosterone, will have a protective effect on testicular tissue, thus NAC administration to improve the harmful effects produced by p-NP is recommended.

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