American Journal of Pharmacology and Toxicology 9 (1): 29-38, 2014
ISSN: 1557-4962

doi:10.3844/ajptsp.2014.29.38 Published Online 9 (1) 2014 (http://www.thescipub.com/ajpt.toc)

POTENTIAL AMELIORATIVE ROLE OF N-ACETYLCYSTEINE AGAINST TESTICULAR DYSFUNCTION INDUCED BY TITANIUM DIOXIDE IN MALE ALBINO RATS

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Received 2013-12-10; Revised 2013-12-22; Accepted 2013-12-31

ABSTRACT

In this study, we examined the ameliorative action of N-Acetylcysteine (NAC) against Titanium Dioxide (TiO2) induced testicular degeneration in albino rats. Adult male albino rats were given saline as a control group, TiO2 (1200 mg kg^{-1} BW), NAC (100 mg kg^{-1} BW) and co-treatment of NAC and TiO2 as a protective group for 3 months. Testicular tissues were extracted for changes in testicular gene expression and histopathology. Administration of TiO2 significantly increased mRNA expression of IL-6 and TNF-α that are normalized by NAC administration. TiO2 administration down regulated Glutathione-S-Transferase (GST) while increased B-cell Lymphoma2 (BcL2) expressions. Co-administration of rats by NAC together with TiO2 normalized changes in GST and BcL2 expression. Expression of steroidogenesis related genes [Androgen Binding Protein (ABR), 17β-Hydroxysteroid Dehydrogenase (17β-HSD), cytochrome P450 17A (CYP17α) and aromatase] showed down regulation in TiO2 administered groups and normalized when NAC given together with TiO2. Moreover, TiO2 induced toxicity in testes that accompanied by degeneration in seminiferous tubules with congestion, oedema and cell disruption that are partially normalized by co-administration of NAC with TiO2. In conclusion, the present findings confirmed the benficial effect of NAC to prevent apoptosis in spermatogenic and sertoli cells and testicular dysfunction induced by TiO2 in male albino rats.

Keywords: Titanium Dioxide, N-Acetylcysteine, Steroidogenesis Related Enzymes, Gene Expression, Testicular Dysfunction and Apoptosis

1. INTRODUCTION

Titanium Dioxide (TiO2) is widely used as an additive, in paints, in sunscreens and in cosmetic creams as well as in the environmental decontamination of air, water and soil by the destruction of pesticides (Fisher and Egerton, 2001; Kaida *et al*., 2004; Choi *et al*., 2006; Medina *et al*., 2007). The degree of cellular damage and oxidative stress of nanoparticles are related to the particle size and its chemical composition (Hoet *et al*.,

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2004). The smaller the particles of TiO2, the more reactivity, effectivity and toxicity (Oberdorster, 2006). Oxidative stress is one of the most important toxicity mechanisms in body tissues as in lung, kidney, testes, brain and spleen (Li *et al*., 2010; Sang *et al*., 2012). Higher doses of TiO2 nanoparticles (324-2592 mg kg^{-1} bw) are accumulated in spleen, liver, kidney, lung and testes (Chen *et al*., 2009). Upon exposure to TiO2, pathological lesions occur in the liver, spleen, kidneys, testes and brain (Shi *et al*., 2013; Jia *et al*., 2013). Consequently disruption in organ function occur and alteration in inflammatory and defensive mechanisms of body. Testes are rich in polyunsaturated fatty acids which is a main target of Reactive Oxygen Species (ROS). Recent studies have shown that ROS-mediated oxidative stress can cause cellular testes damage (Turner and Lysiak, 2008). ROS are scavenged by antioxidant defense systems including reduced glutathione.

As known, N-Acetylcysteine (NAC is an antioxidant and free radical scavenger, used extensively in conditional nutrient (Moschou *et al*., 2008). NAC acts as a cysteine donor (precursor of de novo GSH synthesis) and maintains or even increases the intracellular levels of glutathione, a tripeptide which protects cells from toxins and free radicals (Stanislawski *et al*., 2000; 2003; Van de Poll *et al*., 2006; Sadowska *et al*., 2007; Atkuri *et al*., 2007). NAC free thiol group is capable of interacting with electrophilic groups of ROS (Sen and Packer, 2000). It has a protective effect for a range of cell types, including cell death in testes induced by oxidative stress (Cay *et al*., 2006). Zafarullah *et al*. (2003) reported that cell growth and survival rate increased in response to ROS-induced injuries that lead to growth arrest and apoptosis. Thus, NAC can restore the imbalance between pro-oxidant and antioxidant systems during oxidative stress. NAC is safe and well tolerated when administered orally but has documented risks with intravenous administration (Dodd *et al*., 2008). Rarely, it can cause rashes, fever, headache, drowsiness, low blood pressure and liver problems in higher doses (Dodd *et al*., 2008).

Cytokines are proteins secreted by most cell types (Feghali and Wright, 1997). They are pharmacologically active, exhibiting both beneficial and pathologic effects on the target cells. They are either pro or inflammatory or anti-inflammatory proteins secreted to maintain body defensive mechanism. Imbalanced expression of cytokines has been implicated in the progression of many diseases (Arend and Gabay, 2004). Their expression reflect the immune and health state of the body. In this study, we investigated the effects of NAC on anatase nano-TiO2 induced alterations in gene expression of steroidogenesis, reduced Glutathione (GST), testicular inflammatory and apoptotic activity (IL-6 and TNF-α and BcL2 respectively) and testes histopathology and possible protection by N-acetylcysteine in male albino rats. The enzymes of steroidogenesis examined are Androgen Binding Protein (ABR), 17β-hydroxysteroid dehydrogenase (17β-HSD), Cytochrome P450 17A (CYP17α) and aromatase.

2. MATERIALS AND METHODS

2.1. Materials and Animals

Ethidium bromide, agarose and TiO2 anatase form, particle size (25-70 nm) were purchased from Sigma Aldrich Co., MO, USA. The male albino rats were purchased from Egyptian Co for Experimental Animals Import, Helwan, Cairo, Egypt. Gum acacia and NAC were obtained from El-Nasr Co., Cairo, Egypt. Cytokines primers were from MACROGEN, Seoul, Korea. Twenty four male albino rats weighting 150-200 g were obtained from the Animal House in Faculty of Veterinary Medicine, Benha Universitiy. Rats were given free access to food and water with 12h/12h dark light cycle. All animals were left one week for adaptation. They were housed in separate well-ventilated cages, under standard conditions.

2.2. Experimental Design

Twenty four rats were divided into 4 groups (6 rats per group). Group I (control group), were kept under standard conditions, fed on balanced diet for 3 months. Group II (NAC treated group) received 100 mg kg^{-1} body weight NAC once daily by gastric gavage for 3 months. Group III (TiO2 treated group) received 1200 mg kg^{-1} body weight TiO2 by gavage (1/10 LD 50) in 1mL of 5% gum acacia solution as a solvent once daily for 3 months. The dose of TiO2 was used based on studies of (Wang *et al*., 2007). Group IV (NAC+TiO2) received 100 mg kg^{-1} body weight NAC one hour before TiO2 by gavage once daily for 3 months. At the end of the experiments, the rats were sacrificed. Blood and testes were taken for histopathology and gene expression.

2.3. RNA Extraction and cDNA Synthesis

For preparation of total RNA, testes (approximately 100 mg per sample) were collected

from rats, flash frozen in liquid nitrogen and subsequently stored at -70°C in 1 mL Qiazol (QIAGEN Inc., Valencia, CA). Frozen samples were homogenized using a Polytron 300 D homogenizer (Brinkman Instruments, Westbury, NY). Then 0.3 mL chloroform were added to the homogenate. The mixtures were shaken for 30 sec followed by centrifugation at 4°C and 12,500 rpm for 20 min. The supernatant layer was transferred to a new set of tubes and an equal volume of isopropanol was added to the samples, shacked for 15 sec and centrifuged at 4°C and 12,500 rpm for 15 min. The RNA pellets were washed with 70% ethanol, briefly dried up then, dissolved in Diethylpyrocarbonate (DEPC) water. The prepared RNA integrity was cheched by electrophoresis. RNA concentration and purity were determined spectrophotometrically at 260 nm. The ratio of the 260/280 optical density of all RNA samples was 1.7-1.9.

For synthesis of cDNA, mixture of 2 µg total RNA and 0.5 ng oligo dT primer in a total volume of 11 µL sterilized DEPC water was incubated in the PeX 0.5 thermal Cycler (Thermo Electronic Corporation, Milford, Ma) at 65°C for 10 min for denaturation. Then, 4 µL of 5X RT-buffer, 2 µL of 10 mM dNTPs and 100 U Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase (SibEnzyme Ltd. Ak, Novosibirsk, Russia) were added and the total volume was completed up to 20 µL by DEPC water. The mixture was then re-incubated in the thermal Cycler at 37°C for 1 h, then at 90°C for 10 min to inactivate the enzyme.

2.4. Semi-Quantitative PCR Analysis

Specific primers for tested genes (**Table 1**) were designed using Oligo-4 computer program and synthesized by Macrogen (Macrogen Company, GAsa-dong, Geumcheon-gu. Korea). PCR was conducted in a final volume of 25 µL consisting of 1 µL cDNA, 1 µL of 10 picomolar (pM) of each primer (forward and reverse) and 12.5 µL PCR master mix (Promega Corporation, Madison, WI) the volume was brought up to 25 using sterilized, deionized water. PCR was carried out using a PeX 0.5 thermal Cycler with the cycle sequence at 94°C for 5 min one cycle, followed by different cycles each of which consisted of denaturation at 94°C for one minute, minute with additional final extension at 72°C for 7 min. As a reference, expression of Glyceraldehyde-3-Phosphate Dehydrogenase (G3PDH) mRNA was detected by using specific primers (**Table 1**). PCR products were electrophorized on 1% agarose gel (Bio Basic INC. Konrad Cres, Markham Ontario), stained with ethidium bromide in TBE (Tris-Borate-EDTA) buffer. PCR products were visualized under UV light and photographed using gel documentation system. The intensities of the bands were quantified densitometrically using ImageJ software (http://imagej.en.softonic.com/).

2.5. Histopathological Examination

The testes of male albino rats were collected at the end of experiment from tested groups. The samples were fixed in Bouin's solution, then dehydrated in ascending grades of alcohols, cleared in xylene and embedded in paraffin. The samples were casted, then sliced into 5 µm in thickness and placed onto glass slides. The slides were stained by general and specific stains (Wilson and Gamble, 2008). PAS and Masson's Trichrome reactions were carried out based on protocols of Kiernan (1999) and Carson (1990) respectively.

2.6. Statistical Analysis

Results are expressed as means ± S.E of 5 different rats per each group. Statistical analysis was done using ANOVA and Fischer's post hoc test, with $p<0.05$ being considered as statistically significant.

Table 1. PCR conditions for rat cytokines and spematogenesis genes

Gene	Forward primer	Reverse primer	PCR cycles and Ann. Temp.
IL-6 (450bp)	5'-AGTTGCCTTCTTGGGACTGATGT-3'	5'-TGCTCTGAATGACTCTGGCTTTG-3'	30 cycles, 57°C 1 min
TNF-α (256 bp)	5'- CCACCACGCTCTTCTGTCTAC-3'	5'-ACCACCAGTTGGTTGTCTTTG-3'	30 cycles, 58°C 1 min
BcL2 (350 bp)	5' TCC ATT ATA AGC TGT CAC AGA GG -3'	5'-- GAA GAG TTC CTC CAC CAC C -3'	30 cycles, 55°C 1 min
GST (575 bp)	5'- GCTGGAGTGGAGTTTGAAGAA-3'	5'- GTCCTGACCACGTCAACATAG -3'	33 cycles, 55°C 1 min
ABP (195 bp)	5'- CCATTCCTCCTTTGAGTTTCGA-3'	5'- CAGCTCCACCCGGGTGC-3'	35 cycles, 65°C 45 sec
Aromatase (142 bp)	5'- GCCTGTCGTGGACTTGGT -3'	5'-GGTAAATTCATTGGGGTTGG -3'	33 cycles, 57°C 1 min
CYP17 α (138)bp	5'- GCTAACATTGACTCCAGCATTGG-3'	5'-GAAGCGCTCAGGCATAAACC-3'	33 cycles, 60°C 1 min
17β-HSD (114 bp)	5'- CTCTGGGCACTGCATCAC-3'	5'- CAAGTAACTCTGCGTGGGT-3'	33 cycles, 53°C 1 min
GAPDH (309 bp)	5'-AGATCCACAACGGATACATT-3	5-TCCCTCAAGATTGTCAGCAA-3'	25 cycles, 52°C 1 min

3. RESULTS

3.1. IL-6 and TNF-α Expression in Testes after TiO2 and NAC Administration in Male Albino Rats

Administration of TiO2 to rats for 3 months increased IL-6 and TNF-α expression in testes and their expression was normalized in rats administered NAC together with TiO2 (**Fig. 1a and b**). NAC administeration to rats alone has no effect on IL-6 and TNF-α expression.

3.2. BcL2 and GST Expression in Testes after TiO2 and NAC Administration in Male Albino Rats

To test the antiapoptotic and antioxidant effect of NAC, we tested the expression of BcL2 and GST after administration of TiO2 or NAC either alone or together. **Figure 1c and d**, show that TiO2 increased BcL2 expression and decreased GST expression and administration of NAC together with TiO2 normalized the increase in BcL2 expression and the decrease in GST expression.

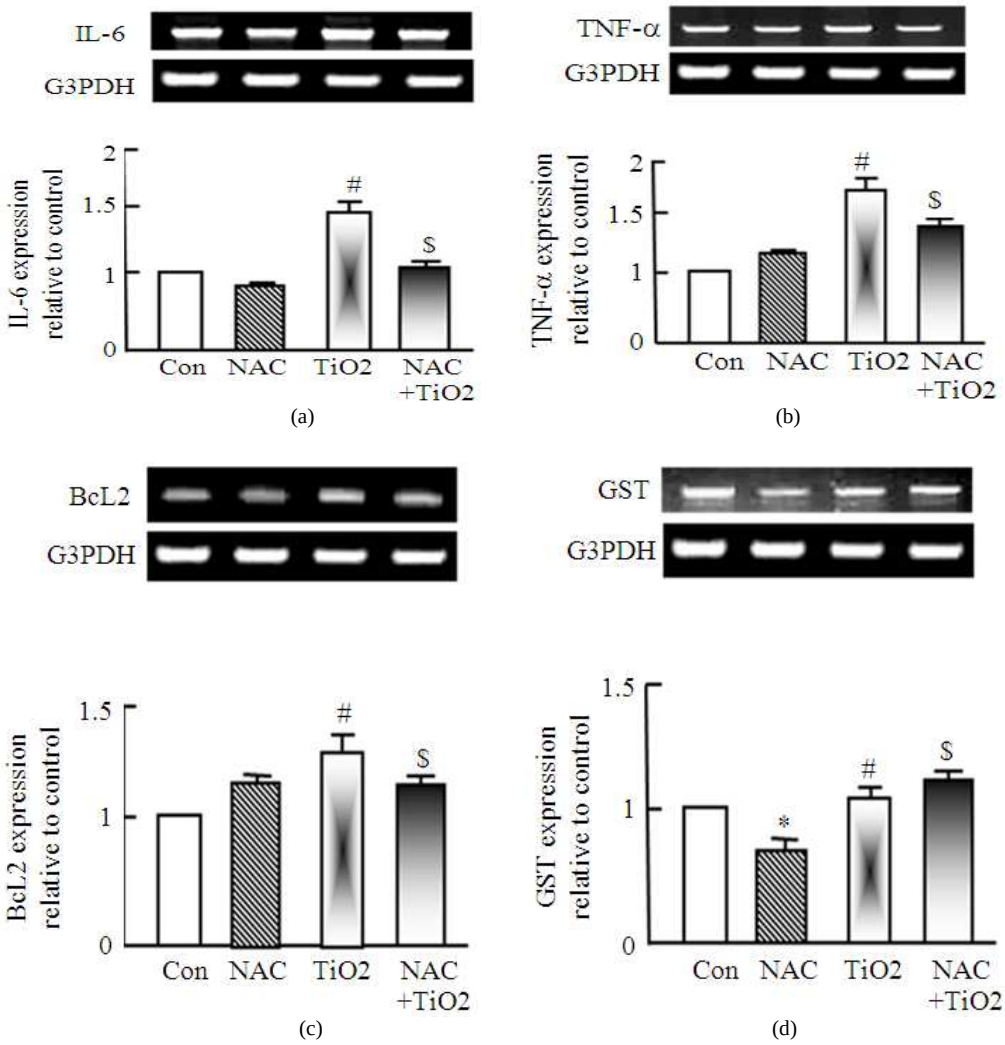

Fig. 1. RT-PCR analysis of IL-6, TNF-α, BcL2 and GST expression after administration of either NAC or TiO2 alone or together in Albino rats. NAC and TiO2 were administered for 3 months as described in materials and methods. RNA was extracted and reverse transcribed (3 µg) and RT-PCR analysis was carried out for of IL-6, TNF-α, BcL2 and GST genes. Densitometric analysis was carried for 3 different rats. *p<0.05 Vs control while # p<0.05 Vs NAC group and $ p<0.05 Vs TiO2

3.3. Expression of Steroidogenesis Regulating Enzymes in Testis of Albino Rats after TiO2 and NAC Administration

To test the effect of the pollutant on genes of testosterone biosynthesis, rats were treated with and/or without TiO2 in presence or absence of NAC. The expression of ABP, 17β-HSD, CYP17α and aromatase were examined using RT-PCR analysis. As seen in **Fig. 2a**, TiO2 induced significant decrease in ABP expression but in presence of NAC the suppression was normalized and increased. The down regulation in ABP expression was around 55% of normal expression. Also, NAC inhibited TiO2 induced 70% down regulation in aromatase expression that normalized in presence of NAC (**Fig. 2b**). Next, we examined the expression of CYP17α that plays a role as a rate-limiting enzyme in the biosynthesis of steroid hormones. TiO2 induced 43% down regulation in CYP17α expression in testis. Presence of NAC with TiO2 significantly normalized the expression of CYP17α (**Fig. 2c**). Next, we tested the expression of 17β-HSD, the enzyme responsible for conversion of androstenedione to testosterone.

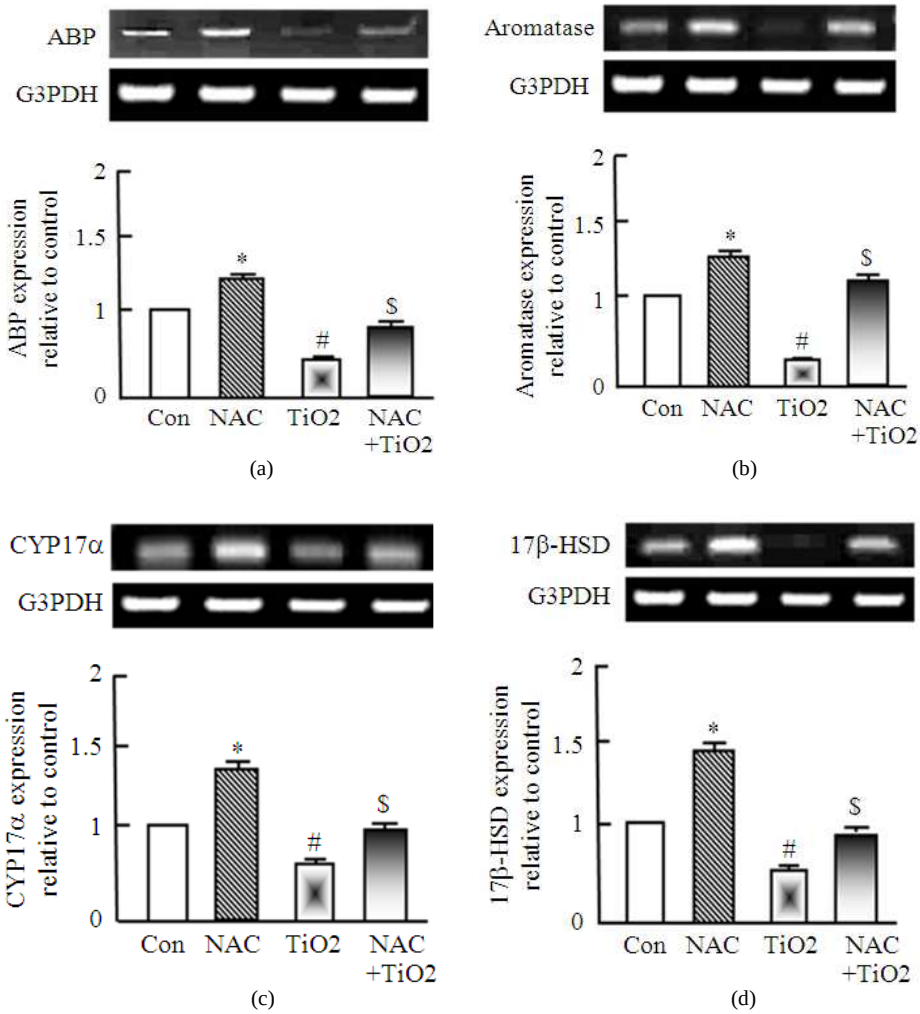

Fig. 2. RT-PCR analysis of steroidogensis related gene expression after administration of either NAC or TiO2 alone or together in Albino rats. NAC and TiO2 were administered for 3 months as described in materials and methods. RNA was extracted and reverse transcribed (3 µg) and RT-PCR analysis was carried out for of ABP, aromatase,CYP17α and 17β-HSD genes. Densitometric analysis was carried for 3 different rats. *p<0.05 Vs control while # p<0.05 Vs NAC group and $ p<0.05 Vs TiO

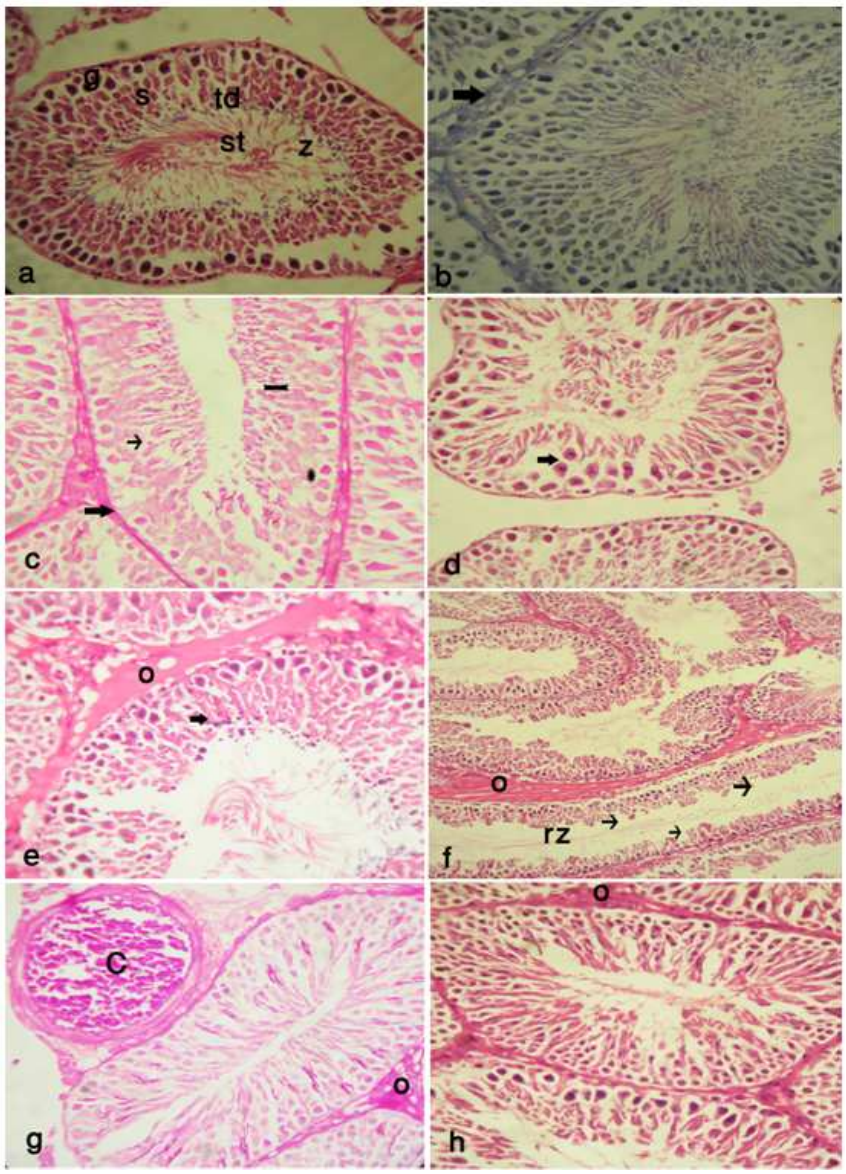

Fig. 3. Photomicrograph of testes of control, TiO2 and NAC administred rats. In (a), testes characterized by well organized seminiferous tubules (st), which consisted of spermatogonium (g), primary and secondary spermatocytes (s), spermatid(td) and spermatozoa (z); H&E .X40. In (b), fine collagen fibres with blood vesseles represent the interstitial CT between the seminiferous tubules (arrow). Masson trichrome X40. In (c), the rat testes showed positive PAS reaction in the basement membrane (thick arrow), sertoli cells (*) and spermatozoa (—),while faint PAS reaction was detected in the spermatogenic cells (arrow) PAS X 40. In (d). The testes of the Tio2 administerd group showed appotosis of sertoli cells (arrow) H&EX 40. In (e), numerous interstitial oedema spreaded between the seminiferous tubules (o) and appotosis of germinal layers were also detected (arrow), H&E X40. In (f), some seminiferous tubules showed decrease in the thickness of the germinal layer (arrows) with rare sperms (rz) and interstitial oedema (o) H&E X10. In (g), The testes in NAC and Tio2 administered rats showed partial improvement in the testicular cells while the congestion of the testicular blood vessels (C) and mild oedema (o) still persist PAS X40. In (h), the interstitial oedema was diminished in most of the seminiferous tubules while mild oedema still located in some parts of the interstital CT (o) and the general architecture of the testes was restored, H&EX40.

As seen in **Fig. 2d**, 17β-HSD expression is down regulated in TiO2 administered rats and NAC normalized its expression. The overall effect of NAC alone as seen in **Fig. 2** is stimulatory for spermatogenesis related gene expression.

3.4. Histopathological Findings in Testes of Albino Rats after TiO2 and NAC Administration

The testes of the control group were consisted of seminiferous tubules surrounded by interstitial connective tissue (CT). The seminiferous tubules lined by spermatogonium, primary and secondary spermatocytes, spermatid and spermatozoa. Pyramidal cells were located between the spermatogenic cells (Sertoli cells). Numerous interstitial cells (Leydig cells) were located in the interstital CT (**Fig. 3a**). The interstial CT mainly consisted of fine collagen fibers. Blood vessels were located between the interstitial CT in the testes of the NAC group (**Fig. 3b**). Positive PAS reaction was noticed in the basement membrane , sertoli cells and spermatozoa (**Fig. 3c**), while faint PAS reaction was detected in the spermatogenic cells. The testes of the TiO2 administerd group showed apoptosis of sertoli cells (**Fig. 3d**), numerous interstitial oedema spreaded between the seminiferous tubules and apoptosis of germinal layers were also detected (**Fig. 3e**), some seminiferous tubules showed decrese in the thickness of the germinal layer with rare sperms (**Fig. 3f**). The testes in NAC and TiO2 administered rats showed partial improvement in the testicular cells while the congestion of the testicular blood vessels and mild oedema still persist (**Fig. 3g**), the interstitial oedema was diminished in most of the seminiferous tubules while mild oedema still located in some parts of the interstital CT and the general architecture of the testes were restored (**Fig. 3h**).

4. DISCUSSION

In this study, we have shown the importance of NAC on testes function and its antiapoptotic and anti-inflammatory effect and its role in protection of testes. As known, NAC and L-carnitine are highly concentrated in the epididymis and spermatozoa, where it may serve as an intra-mitochondrial vehicle for the acyl group in the form of acyl CoA that acts as a substrate for the oxidation process to produce energy for sperm respiration and motility (Kanter *et al*., 2010). Accumulation of TiO2 in testes down regulated genes responsible for cholesterol transport and steroidogenesis including ABP, P450scc, 17β-HSD, CYP-17α and aromatase. Expression of ABP, CYP17α and 17β-HSD were significantly decreased in rats following exposure to TiO2 for 3 months. These genes play important roles in testosterone biosynthesis, for example, Progesterone is then converted to 17α-hydroxyprogesterone and androstenedione by CYP17α. Finally androstenedione is converted to testosterone by 17β-HSD. The decrease in gene expression of CYP17α and 17β-HSD indicates a possible contribution of these genes to the decrease in serum testosterone of male rats.

Apoptosis is a programmed cell death, it affects cell function and viability. BcL2 is known as apoptotic biomarker. Administration of TiO2 for 3 months up-regulated BcL2 expression and NAC down regulate it confirming antiapoptotic activity of NAC as reported by ours and findings of (Aggarwal *et al*., 2010). Germ cell apoptosis has been reported by several investigators to play an important role in normal testicular physiology (Modi *et al*., 2003). It is required for normal spermatogenesis and is believed to ensure cellular homeostasis and maintain the fine balance between germ cells and Sertoli cells (Said *et al*., 2004). Also, apoptosis is a key regulator of spermatogenesis in normal and pathological states (Kerr, 1992). High rates of apoptosis have been reported in testicular biopsies from infertile men with various degree of testicular insufficiency (Juriscova *et al*., 1999). Sensitivity to factors induced apoptosis was higher in the spermatogonia and spermatocytes, while the Sertoli and Leydig cells were comparatively resistant (Kanter *et al*., 2010). Such changes was ameliorated and normalized by NAC administration.

In this study, TiO2 induced both oxidative stress that indicated by the decrease in reduced glutathione and inflammatory cytokines (IL-6 and TNF-α). It has been shown that NAC ameliorated the decrease in GST and lipid peroxidation induced by diazion (Oksay *et al*., 2013). Moreover, the changes in cytokines expression induced by TiO2 are ameliorated by NAC administration. NAC acts as antioxidant that stimulate GSH synthesis (Dodd *et al*., 2008) and is confirmed in this study by up-regulation of testicular GST expression in TiO2 administered rats. So NAC can restore the imbalance between pro-oxidant and antioxidant systems during oxidative stress and that are coincided with our findings. In testes, NAC serve as an important intracellular cofactor for the transport of activated long-chain fatty acids into the mitochondrial matrix, thereby facilitating oxidation and enhancing energy production. In the epididymis, free antioxidants are taken up from the

circulating plasma and actively transported through the epithelial cells into the epididymal plasma, using a specific carrier under the regulation of androgens (Enomoto *et al*., 2002). Moreover, antioxidants especially L-carnitine and NAC act as a factor capable of protecting the heart against ischemia/perfusion injury leading to myocyte death (Shug *et al*., 1991).

The protective effect of NAC on testicular damage in rat varicocele, testes torsion models and tetracycline-induced toxicity were shown in studied of (Cay *et al*., 2006; Payabvash *et al*., 2007; Farombi *et al*., 2008; Aktas *et al*., 2010; Duarte *et al*., 2010). They showed that NAC had good effects on testicular dysfunction but they did not examine testicular gene alterations that examined in our study. Toxicity induction by tetracyclins increased testicular Malondialdehyde (MDA) levels and decreased superoxide dismutase, catalase, GSH and glutathione-S-Transferase (GST) levels. NAC significantly attenuated these effects as reported in our findings (Farombi *et al*., 2008). Moreover, increased doses of TiO2 exhibited sever pathological changes, including rare sperm, sperm breakages, rarefaction of Sertoli cell and Sertoli cell apoptosis, necrosis of the seminiferous tubules, decreased germinative layer thickness, vaculation and irregular arrangement of sertoli cells of the seminiferous tubules (Gao *et al*., 2013).

Numerous studies have unequivocally shown that TiO2 exposure can migrate through different routes and accumulate in various organs (Chen *et al*., 2009; Shi *et al*., 2013; Jia *et al*., 2013), which, in turn cause oxidative stress, inflammation and apoptosis resulting in organ injury and failure. Dysfunction of the testes in turn affect reproduction and affects the function of the reproductive system (Jia *et al*., 2013). The cytochrome P-450 aromatase enzyme, the rate-limiting enzyme in estradiol synthesis, catalyzes the conversion of testosterone to estradiol. The present study demonstrated that aromatase mRNA levels decreased in TiO2 administered rats and NAC normalized aromatase expression in a way to control estradiol biosynthesis (Shi *et al*., 2007). It has been shown that NAC attenuates diazinon induced oxidative stress in rat testes (Oksay *et al*., 2013). Michael *et al*. (2010) reported that NAC supplementation improved semen quality and canine fertility.

ABP is a glycoprotein secreted by Sertoli cells in the seminiferous tubules. It binds specifically to testosterone, dihydrotestosterone and 17-beta-estradiol. The higher levels of these hormones enable spermatogenesis in the seminiferous tubules and sperm maturation in the epididymis (Patrao *et al*., 2009). So, NAC probably acts to enforce spermatogenesis through increase in ABP expression. Moreover, aromatase, or estrogen synthase, is an enzyme responsible for a key step in the biosynthesis of estrogens. It is a member of the cytochrome P450 superfamily that catalyze many reactions involved in steroidogenesis. In particular, aromatase is responsible for the aromatization of androgens into estrogens (Lu *et al*., 2010). In our results TiO2 inhibited ABP, 17β-HSD, CYP17α and aromatase expression while NAC has significant upregulation of steroidogenesis gene expression. Moreover, NAC induced partial improvement in the testicular dysfunction, germinal layer apoptosis and diminished the interstitial oedema in most of the seminiferous tubules.

5. CONCLUSION

The findings of this study confirmed that co-administration of NAC inhibited harmful effects of TiO2 that disrupted testicular function of male albino rats. NAC has antioxidant and antiapoptotic functions. Moreover, it improved steroidogenesis related gene expression which is ameliorated by TiO2 administration. NAC is a good health supplement for protection of male fertility from environmental toxic biohazards.

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