ToxEHS

Ameliorative Effect of Zinc Oxide Nanoparticles on Nicotine Induced Testicular Dysfunction; Biochemical and Histological Study

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Abstract

Objective: The aim of this study was to evaluate potential impact of zinc oxide nanoparticle on the possible testicular toxicity induced by nicotine.
Methods: 24 adult male albino rats were divided into four groups; negative control group, zinc oxide nanoparticle group: rats were received 5 mg/kg/day zinc oxide nanoparticles orally, nicotine treated group: rats were administered nicotine (1 mg/kg/day) intraperitoneally and nicotine with zinc oxide nanoparticles: rats were treated with nicotine and zinc oxide nanoparticles daily for 28 days. Serum testosterone level, testicular malondialdehyde, SOD and catalase activities were measured. Testes were examined by light and electron microscopes as well.
Results: Significant decreases of serum testosterone level, testicular SOD and catalase activity and significant increase of testicular MDA in nicotine treated group when compared with control group were detected. Zinc oxide nanoparticles administration reversed these changes.
Conclusion: Zinc oxide nanoparticles ameliorated nicotine induced testicular dysfunction.

Keywords: Nicotine, Zinc oxide nanoparticle, Testicular dysfunction

Introduction

Nicotine is an alkaloid and it is the primary psychoactive chemical found in tobacco plants. Therefore cigarettes are very addictive. It was reported that tobacco smoke contains more than 4000 chemicals, more than 50 are known to be carcinogenic and at least 250 are known to be harmful[1]. Tobacco epidemic kills around six million people per year. So, it is considered one of the biggest public health threats[2]. During the past few decades, the rise in drug abuse and smoking had made negative effects on the reproductive system and glands beside its known detrimental effects on the vital organs such as the heart and brain[3].

A new prospect in harm reduction from tobacco use was represented by electronic cigarettes (e-cigarettes)[4]. However, studies are lacking in demonstration of e-cigarettes efficacy as harm reduction devices[5]. Compared to tobacco smoke, several laboratory studies have shown that e-cigarette aerosol contains reduced levels of toxicants but significant amounts of nicotine[6].

It was reported that after switching from tobacco to e-cigarettes, nicotine exposure remains unchanged, while exposure to selected carcinogens and toxicants is substantially reduced[7].

On the other hand, zinc (Zn) is the second most abundant trace element in the animal body. It can't be stored in the body and requires regular dietary intake to meet the physiological needs[8].

It is well known that Zn is an essential trace element required for germ cells maintenance, sperm motility regulation and spermatogenesis progression. Zn has a direct protective effect on human spermatozoa through protection against decreased motility and DNA fragmentation increase *in vitro*[9]. Zinc is found in high concentration in the testes and accessory sex glands so that it plays an important role in the reproductive system[10]. Compared to their corresponding bulk materials, nanoparticles are known materials of at least one dimension ≤ 100 nm, and this large surface-to-volume ratio results in unique characteristics. Zinc oxide nanoparti-

cles (ZnO NPs) are the new forms of materials with low toxicity and eminent biological properties that have notable ability to pass many barriers to efficient targeting of cells and molecules in many diseases[11].

Nanotechnology has revolutionized the commercial application of nano-sized minerals in the fields of medicine, electronics appliances, biological and pharmaceutical applications, engineering, information, environmental technology pigments, food and many more. Also, it has been used as a recent tool in the molecular and cellular biology, biotechnology, physiology, reproduction, mineral nutrition, pharmacology etc. in both human and animal models. Moreover, it could be used for pathogen detection. Also, nanotechnology is important for future animal husbandry and feeding in many economies depending on agriculture[12].

Zinc plays an important role in scavenging reactive oxygen because of its known antioxidative properties. Zn is important for sperm motility and viability. It is involved in lipid catabolism, and thus is the source of energy for motility of spermatozoa. Poor Zn nutrition may cause increased oxidative damage that may contribute to poor sperm quality and idiopathic male infertility[13].

Results

Biochemical Results

The results of this study have been appropriately summarized, tabulated and subjected to statistical analysis before completion and graphic illustration. ANOVA test was used in this study to compare between means of different groups.

As regards the groups; group I (-ve control), group II (ZnO NPs). There were a non significant differences ($p > 0.05$) between groups all over the period of the study by ANOVA (Table 1) as regard:

1. Serum testosterone level.
2. SOD and catalase activity in testicular tissues.
3. MDA in testicular tissues.

So the negative control group was chosen to be compared with the results of the treated groups; group III (nicotine) and group IV (nicotine + ZnO NPs).

The result of this study showed significant decrease of serum testosterone level, SOD and catalase activity in testicular tissues and significant increase of MDA in testicular tissues in nicotine treated group when compared with control group. While intake of zinc oxide nanoparticles can significantly prevent these changes in group IV (nicotine + ZnO NPs) (Table 2).

As regards the treated groups; group III (nicotine) and group IV (nicotine + ZnO NPs)

Histopathological Results

Groups I&II (-ve control group, ZnO NPs group)

Hematoxylin and eosin staining. Light microscope examination of sections of the testes revealed normal testicular histoarcticture in which the testicular parenchyma was formed of densely packed seminiferous tubules. They were lined by stratified germinal epithelium. Sperms were seen in their lumens. Between the seminiferous tubules, interstitial Leydig cells were present (Figures 1, 2).

Table 1. Statistical comparison among control groups (I & II) as regard serum testosterone levels, testicular catalase and SOD activity and testicular MDA along the period of the study by ANOVA test.

Parameter \ Group	Negative control group (I) Mean ± SD	Positive control group (II) Mean ± SD	F	P
Serum testesteron level (ng/mL)	6.51 ± 0.6	6.64 ± 0.5	0.166	> 0.05
SOD (U/L)	53.39 ± 6.79	54.9 ± 7.19	0.140	> 0.05
Catalase (mmol/L)	63.25 ± 4.60	63.55 ± 4.61	0.013	> 0.05
MDA (mmol/L)	95.23 ± 3.74	94.45 ± 4.09	0.119	> 0.05

SD = Standard Deviation, p > 0.05 = non significant, N.B: Number of rats = 6 rats for each group.

Table 2. Statistical comparison among the positive control, nicotine groups as regard SOD (U/L), and catalase (mmol/L) enzymatic activity and MDA (mmol/L) levels in testicular tissues by ANOVA test.

Parameter \ Group	Negative control group (I) Mean ± SD	Nicotine Mean ± SD	Nicotine + ZnO NPs	F	P
Serum testesteron level (ng/mL)	6.51 ± 0.6	3.95 ± 0.7	6.02 ± 0.5	30.214	< 0.0001***
SOD (U/L)	53.39 ± 6.79	33.35 ± 4.73	51.35 ± 6.45	19.888	< 0.0001***
Catalase (mmol/L)	63.25 ± 4.60	23.45 ± 2.065	60.25 ± 4.65	187.937	< 0.0001***
MDA (mmol/L)	95.23 ± 3.74	113.6 ± 7.94	97.95 ± 3.74	19.439	< 0.0001***

N: Number of sacrificed rats for each group was 6 rats, SD: Standard Deviation, ***: Highly-significant ($p < 0.0001$)

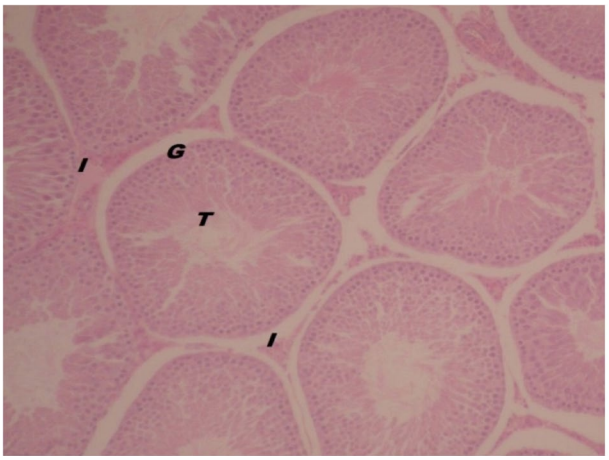

Figure 1. A photomicrograph of a section of testis of control group showing closely packed seminiferous tubules (T), lined by stratified germinal epithelium (G). Note narrow interstitial spaces (I) (H&E × 100).

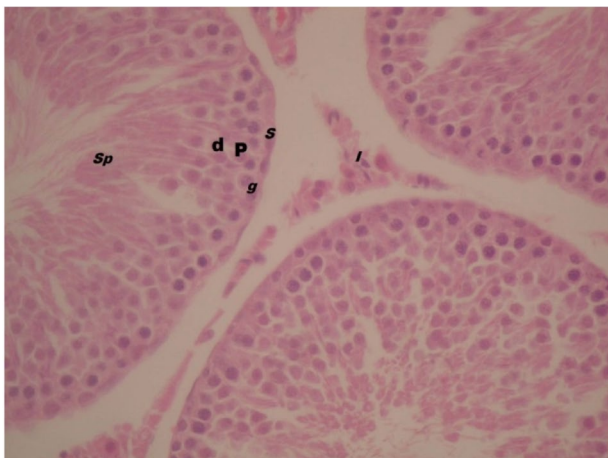

Figure 2. A photomicrograph of a section of testis of control group showing seminiferous tubules lined by spermatogonia (g), primary spermatocytes (p), spermatids (d), and Sertoli cells (S), with sperms (Sp) in their Lumina. Group of interstitial Leydig cells (I) in the interstitium can be seen (H&E × 400).

Electron microscopic examination. Electron microscopic examination of testes sections of this group showed parts of the seminiferous tubules surrounded by regular basement membrane and myoid cells. The tubules were lined by spermatogonia located directly above basal lamina with spherical or oval euchromatic nuclei. The spermatids were characterized by the presence of peripherally arranged mitochondria and large euchromatic nuclei with prominent nuclei. Section of sperms showed central axoneme surrounded with fibrous and mitochondrial sheathes (Figures 3, 4).

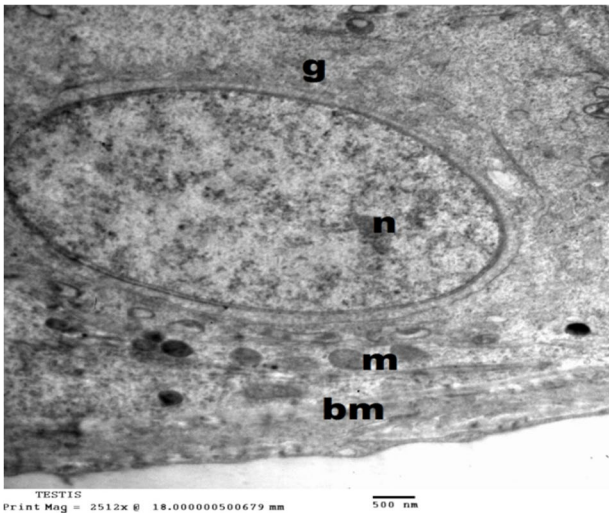

Figure 3. An electron micrograph of testis of control group showing a part of a seminiferous tubule surrounded by a regular basement membrane (bm). Spermatogonia (g) appear with oval euchromatic nucleus (n) and mitochondria (m) (TEM × 10000).

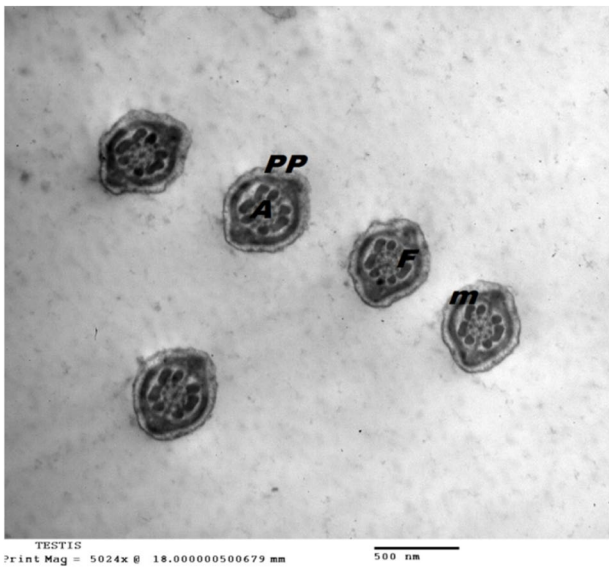

Figure 4. An electron micrograph of a section from testis of control group showing transverse sections in the principal piecfe (PP) of sperms. Observe the axoneme (A), fibrous sheath (F), and mitochondrial sheath (M) (TEM × 20000).

Group III (Nicotine treated group)

Hematoxylin and eosin staining. Seminiferous tubules were observed with irregular shapes and structures. Delumination of the tubules, vacuolization and numerous cells with dark stained nuclei and deep acidophilic cytoplasm were also seen (Figures 5, 6).

Electron microscopic examination. Electron micro-

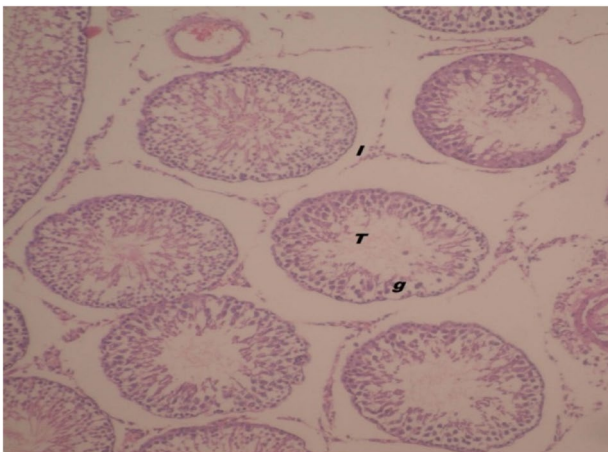

Figure 5. A photomicrograph of a section from testis of nicotine treated group showing many distorted seminiferous tubules (T) with vaculated germinal epithelium (g). The interstitial spaces are wide and contain few cells (I) (H&E × 100).

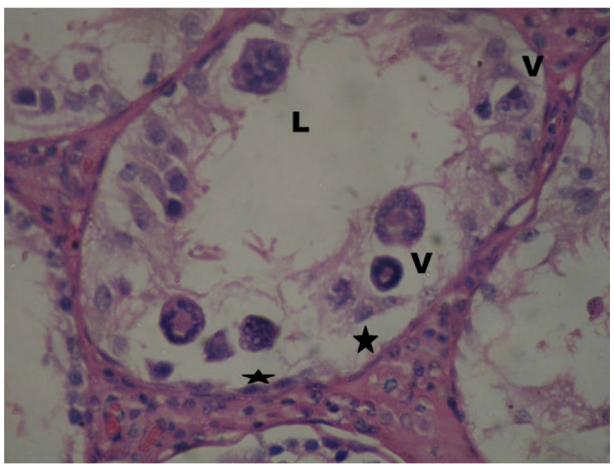

Figure 6. A higher magnification of previous figure from testis of nicotine treated group showing seminiferous tubule containing multiple vacuoles (v). Deluminzation of the tubules (L). The tubule contains separated germ cells with areas of absence of spermatogenic cells (*) (H&E × 400).

scopic examination of ultrathin sections showed the fine structure of different cells lining the seminiferous tubules. Sertoli cell have hardly seen heterohomatic nucleus with electron dense cytoplasm contain multiple vacuols. Wide separations between the germ cells were seen. Spermatocytes were with vacuolated cytoplasm and wide intercellular spaces between them. Cross sections in the mid pieces of sperms showed markedly affected mitochondrial and fibrous sheath surrounded by abundant cytoplasm (Figures 7, 8).

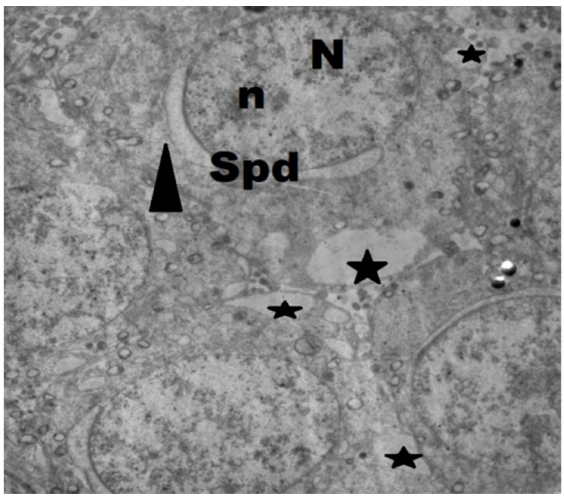

Figure 7. An electron micrograph of testis of nicotine treated group showing a group of spermatids (Spd) with wide separations between them (black arrow). Heterochromatic nucleus (N). Multiple vacuoles were observed (*) (TEM × 4000).

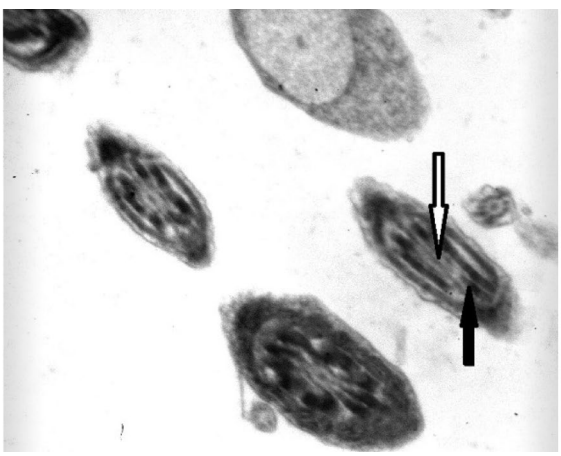

Figure 8. An electron micrograph of a section from testis of nicotine treated group showing marked distortion of the central axoneme in principle and end pieces of sperm (white arrows), distorted fibrous and mitochondrial sheathes (black arrows) (TEM × 20000).

Group IV (nicotine + ZnO NPs treated group)

Hematoxylin and eosin staining. The testis showed near normal histological structure. The seminiferous tubules lumen was patent, and some showed aggregation of sperms. Profiles of seminiferous separated by narrow interstitial spaces were noticed (Figures 9, 10).

Electron microscopic examination. Parts of the seminiferous tubules were seen with their cytoplasm contained euchromatic nuclei and electron dense bodies. The spermatids were characterized by the presence of

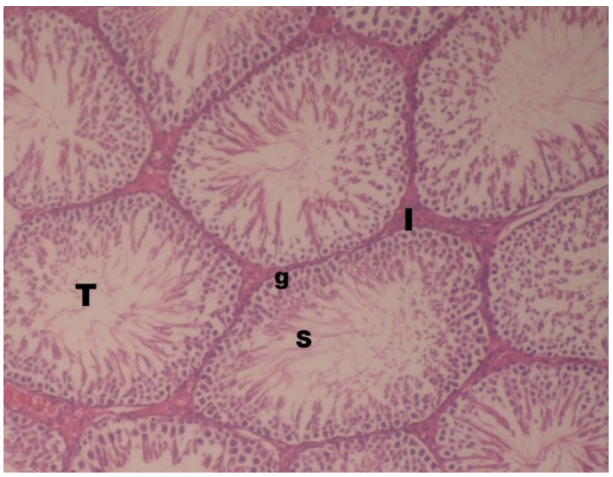

Figure 9. A photomicrograph of a section of testis of nicotine and ZnO NPs group showing closely packed seminiferous tubules (T), lined by stratified germinal epithelium (g) with sperms seen in their luminae (S), and narrow interstitial spaces in between the tubules (I) (H&E × 100).

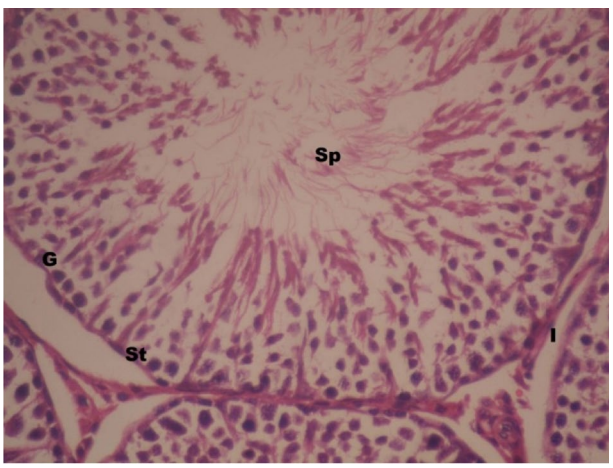

Figure 10. A photomicrograph of a section of testis of nicotine and ZnO NPs group showing seminiferous tubule lined by spermatogonia (G) and Sertoli cells (St), with sperms (Sp) in its Lumen. Narrow interstitium (I) between tubules can be observed (H&E × 400).

peripherally arranged mitochondria and large euchromatic nuclei with prominent nuclei (Figures 11, 12).

Discussion

Smoking is considered a universal phenomenon, even though it is well known to the public about its detrimental impacts on health and fertility[14]. Worldwide, about 30 million infertile males present especially in Africa and

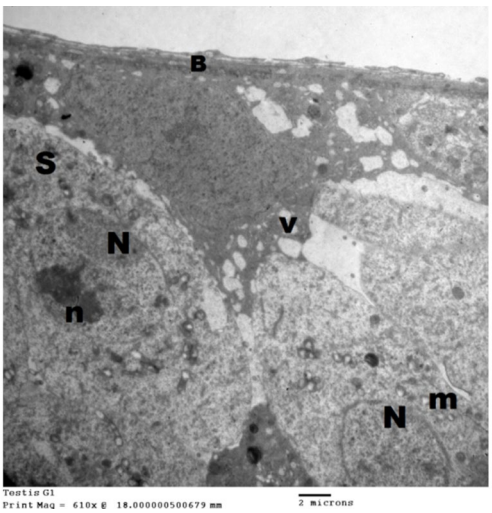

Figure 11. An electron micrograph of a section from testis of nicotine and ZnO NPs group showing regular basement membrane (B), Spermatogonias (S) with less vaculations (v) and euchromatic nucleus (N) and prominent nucleolus (n). Note, less separations between the germ cells (TEM × 5000).

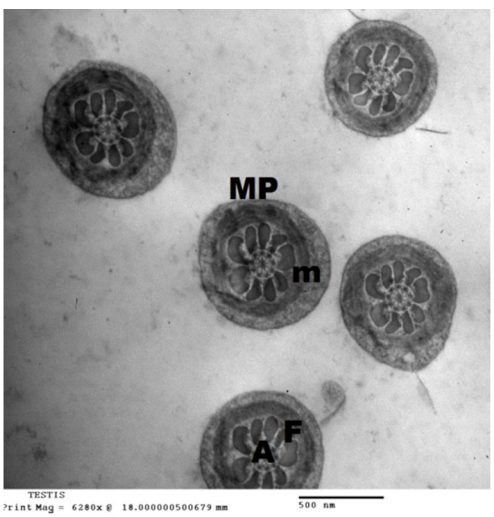

Figure 12. An electron micrograph of a section from testis of nicotine and ZnO NPs group showing transverse sections in the mid piece (MP) of sperms. Observe the axoneme (A), intact fibrous sheath (F) and mitochondrial sheath (M) (TEM × 20000).

Eastern Europe[15].

It was explained that the testes are sensitive to cigarette smoke and nicotine. Long-term exposure to it suppresses spermatogenesis and alters the morphology of spermatogenic and sertoli cells with sperm production affection, so leading to male infertility directly. It was reported that consumption of tobacco has a significant role in developing an infertile male population by exhi-

biting a strong negative effect on semen quality[16]. Also, it was stated that nicotine, the active alkaloid in tobacco, created its testicular dysfunction through testosterone synthesis affection[17]. Nicotine is known to promote cholesterol, triglycerides, phospholipids and free fatty acids synthesis in the testes and boost peroxidative damage. Due to its cumulative effects on DNA damage, oxidative stress and apoptosis, nicotine also corrupt function of sperms[18].

Added to ordinary smoking, nicotine replacement therapy has been excessively used[19]. Transdermal patch contains a dose of nicotine nearly similar to nicotine consumed by a light smoker. This is because nicotine transdermal patch and nasal spray contain a dose similar to the nicotine in 16 cigarettes smoking[20].

Zinc oxide (ZnO) is an inorganic material that is extensively used. Years ago, it was recorded as a Generally Recognized as Safe material (GRAS) by the Food and Drug Administration agency and it is actually used as a food additive[21].

Every year, zinc oxide nanoparticles (ZnO NPs) manufacturing rises. They are used in varied industrial areas as chemical fibers, electronics, medical diagnosis, sunscreens, cosmetics, dyes, paints, pigments, rubber, ceramics, catalysts, antibiotics, food products, tooth pastes and personal care products. It has been reported that ZnO NPs were incorporated in polymeric matrices to provide antimicrobial activity as it provides antimicrobial, antifungal and anticorrosive properties[22,23].

However, it is debatable whether ZnO NPs are safe or hazardous. Many studies considered that Zn is a low toxic compound, as it is an essential trace element in the body and is commonly added as a nutritional supplement[24].

On the other hand, other studies demonstrated the toxic impact of ZnO NPs *in vitro* like cytotoxicity and genotoxicity[25]. Thus, Nanoparticle Technology is considered a double-edged sword on living cells.

In this study, the results showed significant decrease of Serum testosterone level in nicotine treated group when compared with control groups. And this decrease was reserved by intake of zinc oxide nanoparticle.

This result was in parallel with the outcomes of previous study which proved that nicotine administration significantly decreased testosterone[26].

Tobacco consumption has been documented as an endocrine disruptor on the male hormone profile, specifically on LH, testosterone, and prolactin levels[27]. This might have been attributed to the disruption of testicular cyto-architecture by nicotine and its adverse effect on Leydig cell number and functioning leading to decrease serum testosterone level which secreted by Leydig cells[26].

Also the result of this study showed disturbance of oxidative markers in testicular tissues which reserved by intake of zinc oxide nanoparticles. This result are in line with previous studies which reported a significant decrease in testicular glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase with a significant increase in testicular lipid peroxidation and nitric oxide level when compared with the control[28].

Nicotine has been documented to alter the oxidant and antioxidant balance in rat in a dose and time dependent manner and alters lipid peroxidation and antioxidant enzyme in plasma of rats[29].

The inhibition of testicular SOD activity might also be due to either hyperglycemia as glucose induced oxidative stress in different tissues and/or due to loss of enzyme cofactors as copper and zinc[30].

Administration of nicotine significantly decreased CAT activity in the testes. The primary role of CAT is to scavenge H_2O_2 that has been generated by free radicals[31].

Nicotine treated rats showed an elevation in MDA level when compared with the control group. An increased MDA concentration might be due to decrease the production of antioxidants in the nicotine treated rats' tissues thereby shifting the delicate balance in favor of ROS thus leading to pathologic damage to sperm cells and loss of its function[32].

These results agree with a previous research which studied that chronic nicotine treatment even in low doses of 0.6 mg/kg can cause oxidative damage to the testes and the prostate and has obvious detrimental effect on sperm characteristics[20].

Light microscopic examination of nicotine treated group tesets showed seminiferous tubules observed with irregular shapes and structures. Deluminzation of the tubules, vacuolization and numerous cells with dark stained nuclei and deep acidophilic cytoplasm are also seen. While electron microscopic examination of ultrathin sections showed the fine structure of different cells lining the seminiferous tubules. Sertoli cell have hardly seen heterochromatic nucleus with electron dense cytoplasm containing multiple vacuoles. Wide separations between the germ cells were seen. Spermatocytes with vaculated cytoplasm and wide intercellular spaces between them. Cross sections in the mid pieces of sperms showed markedly affected mitochondrial and fibrous sheath surrounded by abundant cytoplasm. These changes were reserved with use of ZnO NPs in nicotine and ZnO NPs treated group.

Previous study showed that the testes exposed to nicotine, the ultrastructure was markedly different. There was thickening of the tunica propria, caused by an increase in collagen fibres under the irregular basal lamina. The myoid cells were separated by more collagen fibres. Junctional specializations between the Sertoli

cells were degenerated; the Sertoli cells had numerous polymorphic mitochondria with irregular cristae and an electron-dense matrix. There were malformed nuclei showing condensed chromatin. The germ cells were degenerated; the most conspicuous anomalies were in the spermatids, but there were also changes in the other cell types. Spermatids were dissociated, and had an irregularly outlined nucleus and poorly condensed chromatin. Acrosomes were irregular and abnormally configured In the middle portion of the spermatids, there was retention of an excessively large cytoplasmic droplet in most of the cells. In the cytoplasm there was an accumulation of electron-dense lipid droplets. The cytoplasm also contained abundant coarse mitochondria with degenerated cristae. There were some degenerative spermatids of irregular shape and with no nucleus. Mitochondrial cristae were lost and Golgi cisternae with acrosome-like morphology were full of electron-dense material[33]. Other studies showed reduction in percentage of tubules with severe edema in interstitial connective tissue, decreased Leydig cells distribution, and diminished tubular diameter as well as reduced spermatid volume. More analyses showed tubular depletion and germinal epithelium dissociation[34].

Zinc, the trace element, has antioxidant effects through competing with pro-oxidant metals such as iron and copper for binding sites and decreasing their ability for free-radical production and protecting-SH groups. It was also reported that zinc deficiency increases the risk of ROS-induced damage in the testes, blood, and liver of rats[35]. Also, it was proved the antioxidant property of zinc oxide nanoparticle[36].

According to a past research, there were protective effects of ZnO NPs on cyclophosphamide induced reproductive system damage[37].

Multiple researches had detected the reproductive and antioxidant function of zinc. Zinc plays an important role in the physiology of spermatozoa and spermatogenesis because the zinc levels are very high in the male reproductive system and seminal fluid. It has been documented that zinc depletion causes atrophy of the seminiferous tubules and failure of spermatogenesis in rats[38,39].

It was stated that zinc decreases lipid peroxidation by changing the membrane-bound site of redox-active metals such as copper and iron. Also, by triggering metallothionein synthesis, zinc has an indirect antioxidant effect. In addition, intracellular and extracellular Zn-Cu superoxide dismutase function as two key enzymes of the antioxidant system[40].

Materials and Methods

Chemicals

Nicotine ($C_{10}H_{14}N_2$) solution was purchased from Sigma Aldrich Co. and a dose of 1 mg/kg were prepared by normal saline.

Zinc oxide nanoparticles: ZnO NPs (Cas no.1314-13-2) dispersion was purchased from Sigma-Aldrich (Steinheim, Germany). Their size is <40 nm and their concentration is 20 wt% in H_2O. The density is 1.7 g/mL $\pm$ 0.1 g/mL at 25°C and pH is 7.5 $\pm$ 1.5 (for aqueous systems). The particle hydrodynamic diameter is <100 nm by using technique of dynamic light scattering. The suspension of ZnONPs was made using deionized water, and a bath of cold water was used to minimize particles heating. Then, the suspension was sonicated for 20 min in a bath sonicator (Model Julabo Labortechnik GMBH Germany) and was whirled for 1 min before every administration. To define their morphology and size, the sample was dissolved in ethyl alcohol, and the dispersed solution was dropped on a copper net. After that, they were examined using transmission electron microscope (TEM, JEOL 1010, Tachikawa, Tokyo, Japan) in the Mycology and Regional Biotechnology Center, Al-Azhar University, Cairo.

Animals

The study was done on 24 healthy adult male albino rats weighing 150-200 g. Rats were obtained from the animal house of Zagazig Faculty of Medicine. The rats received balanced food rich in all stuffs necessary to maintain their health before and during drug administration. It consisted of bread, barley and milk. Water was offered in separate clean containers. The experimental work was done according to the guiding principles for the use and care of experimental animals.

Experimental Design

24 Rats were randomly and equally divided into four equal groups; 6 rats/group: Group I (negative control group): No intervention was done to rats in this group for adjusting the basic parameters, and allowed drinking water *ad libitum*. Group II (ZnO NPs group): Rats were received 5 mg/kg/day ZnO NPs orally[41]. Group III (nicotine treated group): The group was administered nicotine (1 mg/kg/day) interaperitoneally (IP) for 28 days. Group IV (nicotine and ZnO NPs): The group was administered nicotine (1 mg/kg) interaperitoneally (IP) and 5 mg/kg ZnO NPs orally once daily for 28 days[41].

Twenty-four hours after the end of experimental duration, the rats were subjected to sampling of blood and testis tissue as the follows:

1) Collection of blood samples; Under light ether anesthesia, venous samples from the retro-orbital plexus were obtained by capillary glass tubes to measure: serum testosterone level.
2) Collection of testes samples; testes was dissected; one for estimating superoxide dismutase (SOD),

catalase activities in testis tissue and malondialdehyde (MDA). The other was sent for histopathological examination (light and electron microscope).

Methods

Measurement of Serum Testosterone Level

The Testosterone Rat/Mouse ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding[42]. An unknown amount of testosterone present in the sample and a defined amount of testosterone conjugated to horseradish peroxidase compete for the binding sites of testosterone antiserum coated to the wells of a microplate. After one-hour incubation on a shaker the microplate is washed four times. After addition of the substrate solution the concentration of testosterone is inversely proportional to the optical density measured[44].

Testicular SOD and Catalase Activities

Tissues were perfused in 0.9% NaCl containing 0.16 mg/mL heparin. Tissues was washed and minced in ice-cold 0.25 M sucrose, then homogenized, diluted and centrifuged at 4000 rpm and 4°C for two minutes. The supernatant was used to measure SOD[43]. According to pamphlet of Bio-diagnostic kits using calorimetric method, catalase activity was assayed[44].

Testicular Malondialdehyde (MDA) Level

The dissected tissues were put in petri dishes. After washing the tissues with physiological saline (0.9% NaCl), samples were kept at −87°C until analyzed. The tissues were homogenized for 5 min in 50 mM ice cold KH_2PO_4 buffer solution (pH 7.0) 0.5 g tissue + 5 mL buffer solution) using a glass-porcelain homogenizer and then centrifuged at 7000 × g for 15 min. All processes were carried out at 4°C. Supernatants were used to determine the MDA concentration[45]. MDA was assayed colorimetrically[46].

Histopathological Changes; Testis was Examined by:

Light microscopic examination. The testis was fixed in 10% formalin saline then the tissues were embedded in blocks of paraffin and 5 µm. thicknesses were prepared. These sections were subjected to Hematoxylin and Eosin (H&E) stains[47]. Then examined by light microscope (400 ×).

Electron microscope examination. Immediately after dissection, minute specimens were rinsed in 0.1 M phosphate buffer pH 7.2 (PB) to remove blood from the surface. Testis tissue greater than 2 cm long were cut into smaller pieces of approximately 3 × 3 mm and fixed in 3 percent glutaraldehyde, buffered with phosphate buffer for 3 hours. It was rinsed twice with phosphate buffer for 10 minutes per rinse. The tissues were then fixed in 2 percent aqueous osmium tetroxide for 2 hrs. and rinsed in 3 changes of distilled water for 10 minutes. Each dehydration was accomplished by immersion in a graded series of ethanol solutions of 25, 50, 75, 95 and 100 percent. Infiltration with propylene oxide and embedding with increasing concentrations of propylene oxide followed by dehydration were carried. Thin sections (600 nm) were obtained by use of Ultra microtome and were placed on a copper 200 mesh grid. They were stained with uranyl acetate and lead citrate and examined with GEOL-TEM1010electron microscope[48].

Conclusion

Taking the previous findings together, it can be concluded that nicotine induced testicular dysfunction which was ameliorated by co-administration of zinc oxide nanoparticles.

Conflict of Interest

Abeer R. H. Mahmoud declares that she has no conflicts of interest. Nashwa M. M. Shalaby declares that she has no conflicts of interest.

Ethical Approval

The experimental work was done according to the basic instructions advised through the Institutional Research Board for the use and care of experimental animals.

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