Reproductive Toxicity of Aluminum Oxide Nanoparticles and Zinc Oxide Nanoparticles in Male Rats

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ABSTRACT

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INTRODUCTION

Among various nanoparticles (NPs), aluminum oxide nanoparticles (Al_2O_3NPs) are one of the most important because of its promising technological applications. It is currently one of the two US market leaders for nanosized materials [1]. Based on these extensive commercial applications, they have drawn considerable attention as potential environmental

The rapid development of nanotechnology raises both interest and concern among researchers. Thus, this study investigated the reproductive toxicity of aluminum oxide nanoparticles (Al2O3NPs) and zinc oxide nanoparticles (ZnONPs) alone or their combination in male rats via testicular damage, spermatoxicity, DNA fragmentation, mitochondrial transcription factor A (mtTFA) gene, uncoupling protein 2 (UCP2) gene, tumor suppressor gene p53 (p53), cytokines, oxidative stress, sex hormones and testes histopathology. Animals were administered orally with Al2O3NPs (70 mg/ kg BW), ZnONPs (100 mg/kg BW) or their combination every day for 75 days. The obtained results represented that exposure to Al2O3NPs and ZnONPs or their combination caused marked DNA fragmentation and at gene expression level mtTFA gene was significantly suppressed. Semen characteristics, testosterone, TSH, antioxidant enzymes and reduced glutathione were significantly decreased. Whereas, free radicals, nitric oxide, UCP2 gene, p53, tumor necrosis factor-a, interliukin-6, FSH, LH, T3 and T4 were significantly increased. Moreover, testes histopathological examination and steroidogenesis enzymes were changed. In conclusion, the present study showed that the reproductive toxicity induced by the combination of Al2O3NPs with ZnONPs was more pronounced than each one.

Keywords: Aluminum Oxide Nanoparticles; Zinc Oxide Nanoparticles; Male Rats; Reproductive Toxicity; Mitochondrial Transcription Factor A; Uncoupling Protein 2; DNA Fragmentation; Semen Characteristics; Hormones; Oxidative Stress; Antioxidants; Cytokines; Histopathological Changes.

pollutants [2]. The genotoxicity observed with Al₂O₃NPs may be due to pro-inflammatory effects through a reactive oxygen species (ROS)-mediated mechanism [3]. Prabhakar et al. [4] demonstrated significant increase in myocardial malondialdehyde with significant reduction in myocardial SOD and CAT enzymes activities and GSH concentration in nano-alumina intoxicated rats suggesting enhanced oxidative

stress state in myocardium. Some articles reported micronuclei increase [5], DNA damage [5] and chromosomal aberrations [6] caused by AlCl₃NPs.

Also, zinc oxide nanoparticles (ZnONPs) are among the metal oxide nanoparticles and have received considerable attention. This is due to their low production cost, ability to form diverse structures and various biological applications including drug delivery, bio-imaging probes, cancer treatment, antibacterial and immunomodulatory agent [7]. Previous studies have shown the cytotoxic and pro-inflammatory effects of ZnONPs in different types of cells [8]. In vitro and in vivo studies have shown that ZnO nanoparticles caused membrane damage, inflammation, DNA damage, apoptosis and complex cell-cell and cell-matrix interactions and changes in some hormones in mammalian cells [9]. Adverse effects of ZnONPs can be mainly attributed to the generation of ROS leading to membrane damage, directly or indirectly [10]. There is scarce information about the possible reproductive toxicity of the aluminum oxide nanoparticles (Al₂O₂NPs) and zinc oxide nanoparticles (ZnONPs) alone or in combination despite of their current use in novel technology. Therefore, the potential risks on reproductive health should be investigated, especially in those who are occupationally exposed to Al₂O₃NPs and ZnONPs. To achieve this aim, we determined semen characteristics, DNA fragmentation, mitochondrial transcription factor A gene, uncoupling protein 2 gene, p53, cytokines, oxidative stress and sex hormones and testes histopathology in male rats.

MATERIALS AND METHODS

Tested compounds and doses

Al₂O₃NPs nanopowder (about 50 nm particle size) and ZnONPs nanopowder (about 100 nm particle size), were purchased from Sigma Aldrich Chemical Company (St. Louis, MO, USA). The dose of aluminum oxide nanoparticles was 70 mg/kg BW (aqueous suspension) and was chosen according to [11]. The dose of ZnONPs was 100 mg/kg BW (aqueous suspension) was chosen [12].

Animals and experimental groups

Forty Wistar male albino rats 4-5 months age and weighing 160-170g were used in the present study. Animals were housed in a stainless steel wire cages and given food and water *ad libitum*. Animals were maintained in a controlled atmosphere, a temperature of 25 ± 5 °C and 50-70% humidity. After two weeks of acclimation, animals were divided into four

equal groups as follows: a control group and 3 treated groups; group 2, 3 and 4 which were orally treated with Al₂O₃NPs (70 mg/kg BW), ZnONPs (100 mg/kg) and Al₂O₃NPs plus ZnONPs, respectively. Rats were orally administered their respective doses daily for 75 consecutive days.

Blood samples collection and tissue preparations

At the end of the 75th day of the experimental period, all animals of each group were anaesthetized with diethyl ether and sacrificed. Blood samples were collected by cardiac puncture from anaesthetized rats in test tubes containing heparin as an anticoagulant. Blood samples were centrifuged at 860 ×g for 20 min for the separation of plasma. Plasma was kept at -80 °C until analyses of the tested parameters. Testes were immediately removed, washed using chilled saline solution (0.9%), and removed the adhering fat and connective tissues. testes were minced and homogenized (10%, w/v), separately, in ice-cold sucrose buffer (0.25 M) in a Potter–Elvehjem type homogenizer. The homogenates were centrifuged at 10,000 ×g for 20 min at 4 °C, to pellet the cell debris and the supernatant was stored at -80 °C for the determination of tested parameters.

Body and organs weights

Initial and final body weights of male rats were recorded and subsequently body weight gain (g/75 days) was calculated. Body weight gain (g/75 days) = Final weight – Initial weight. The reproductive sex organs (testes, prostates and epididymis) were immediately removed, washed using chilled saline solution (0.9%), removed the adhering fat and connective tissues, dried on tissue papers and weighed.

Quantitative analysis of testicular gene expression of mitochondrial transcription factor A (mtTFA) and uncoupling protein 2 (UCP2) using quantitative real time reverse transcriptase- polymerase chain reaction (qRT-PCR)

Total RNA was isolated from testicular tissues using GF-1 Total RNA Extraction Kit (Vivantis, Malaysia) ViP rime PLUS One step Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) Green Master Mix (Vivantis, Malaysia) was used for the relative quantitative determination of the gene expression of mtTFA [13] and UCP2 [14] at mRNA level using GAPDH as an internal reference gene for validation of the extraction procedure and calculation of relative expression.

Assay of DNA fragmentation as a marker of cell death

Total DNA in the tissues was isolated using DNeasy kit (Qiagen, Germany) and the concentration and purity DNA were assessed using Nanodrop2000@ (Thermo Fisher Scientific, USA). Then DNA fragmentation, as a marker of cell death, was assayed [15].

ELISA measurements

Tumor suppressor gene p53, tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6) were assayed using Enzymelinked Immunosorbent Assay (ELISA) kitsin testes tissue homogenates according to the manufacturer instructions using the serial standard of each parameter.

Markers of oxidative stress

The process of lipid peroxidation resulted in the end product of malondialdehyde (MDA). The MDA was determined as thiobarbituric acid-reactive substances (TBARS) assay in which MDA was heated with thiobarbituric acid (TBA) at a low pH to produce a pink chromogen with a maximum absorbance at 532 nm. The level of TBARS was calculated from a standard curve constructed using serial concentration of tetramethoxypropane (TMP) [16]. The Griess reaction was used to determine the concentration of nitrite and nitrate as nitric oxide end products (NOx) in the deproteinized samples. The Griess reaction was supplemented with the reduction of nitrate to nitrite by NADPH-dependent nitrate reductase. The assay procedure consisted of two steps: the first required the diazotization of sulphanilic acid with nitrite ions followed by the second step of coupling this product with a diamine, resulting in a measurable pink metabolite 540 nm. The level of NOx was determined from the slope of the standard curve constructed using serial concentration of sodium nitrite. The total antioxidant capacity (TAC) and the activities of superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione S-transferase (GST) and catalase (CAT) in the tissue homogenates were measured using colorimetric kits (Biodiagnostic, Egypt) according to the manufacturer instructions and using specific standard for each parameter. Reduced glutathione content was assayed after protein precipitation using a metaphosphoric acid reagent. The assay was based on the oxidation of GSH by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to yield GSSG and 5-thio-2nitrobenzoic acid (TNB). The rate of TNB formation was assayed at 412 nm and was proportional to the GSH present in the sample. The rate of formation of TNB was monitored by recording the change in the absorbance. This was found to be

412 nm per minute (ΔA /min). The total glutathione content in the samples was determined from a GSH standard curve and the results were subsequently expressed as nmol/g tissue by dividing the concentration of glutathione in the sample by the weight (in grams) of tissue used to prepare the sample [17].

Determination of 17-ketosteroid reductase and 17β-hydroxysteroid dehydrogenase activities in testes

Streoidgenic 17-ketosteroid reductase enzyme activity (17-KSR; EC 1.1.1.64) and 17 β -hydroxysteroid dehydrogenase enzyme activity (17 β -HSD; EC 1.1.1.51) were estimated [18].

Semen characteristics

The left epididymis was excised and placed in a warmed petri dish contain 0.2 ml of calcium and magnesium free Hank's solution at 37°C. The tissue was minced with scalpels for approximately 1 min and placed in a 37°C incubator for 15 min prior to determining sperm motility. The suspension was stirred, one drop was placed on a warmed microscope slide, and a 22x22 mm coverslip was placed over the droplet. At least 10 microscopic fields were observed at 400 x magnification using a phase-contrast microscope, and the percentage of motile sperm was recorded [19,20]. The coverslip was removed and the spermatozoa suspension was allowed to dry in air. The sample was stained with 1% eosin Y/5% nigrosin and examined at 400 x for morphological abnormalities. Three hundred spermatozoa from different fields were examined for each sample as described previously [19,20].

The right epididymis and specimens of right testis were frozen immediately after weighing. After thawing at room temperature, the whole epididymis and specimens of testis were homogenized in 0.5 ml of a solution of 0.9% NaCl containing 2% of Triton X-100. Ten strokes of a manual glass homogenizer were used for each sample. The testis and epididymis homogenates were diluted with 1.5 ml of the homogenization solution and spermatozoa were counted at 400 x magnification using a Neubauerhemocytometer. Three counts per sample were averaged [20]. Assessment of sperm count and abnormal spermatozoa was performed using an eosin-nigrosine blue staining mixture as described by Blom [21].

Determination of reproductive and thyroid hormones

Testosterone was determined by using Enzyme-linked Immunosorbent Assay (ELISA) kit for the quantitative measurement of testosterone in plasma (DRG international co., U.S.A). Follicle stimulating hormone (FSH) was assayed

by using ELISA kit for the determination of Follitropin in rat plasma (BIOCODE-HYCEL co. Rue E. Solvay, 101-103 - 4000 Liège - Belgium). Luteinizing hormone (LH) was determined by using ELISA kit for the quantitative determination of Rat LH concentrations in serum, plasma and other biological fluids (Elabscience Biotechnology Co., Ltd). Rat thyroid stimulating hormone (TSH) was assayed by using ELISA kit for the quantitative determination of ratTSH concentrations in plasma (CUSABIO BIOTECH CO., LTD.). Triiodothyronine (T3) was assayed by using ELISA Kit for the quantitative measurement of T3 in rat plasma (Abnova CO., KA 0925). Rat Thyroxine (T4) was assayed by using ELISA kit *in vitro* quantitative detection of rat in plasma (Biovision CO., U.S.A).

Histological section preparation of testes

Testes was obtained from rats, and immediately fixed in 10% formalin, and then treated with a conventional grade of alcohol and xylol, embedded in paraffin and sectioned at 4-6 µm thickness. The sections were stained with Haematoxylin and Eosin (H&E) stains and photographed on the PC screen using a light microscope (Olympus BH-2; Japan) with a digital color camera attachment (Sanyo VVC-6975 P; Japan) and dial indicator for studying the histopathological changes [22].

Statistical analysis

Results are reported as means \pm SE. Statistical analysis for all studied parameters was performed using the general linear model (GLM) produced by Statistical Analysis Systems Institute [23]. Duncan's New Multiple Range Test was used to test the significance of the differences between means [24]. Values of p<0.05 were considered statistically significant.

RESULTS

Initial and final body and sex organ weights

Data showed that there is no significant difference between control and all treated groups in the initial body weight. While, final body weight, body weight gain, testes and epididymis weights significantly decreased, and prostates weight significantly increased in aluminum oxide nanoparticles (Al₂O₃NPs), zinc oxide nanoparticles (ZnONPs) and their combination compared to control group. The final body weight, body weight gain, epididymis and prostates weights showed a similar pattern of change between Al₂O₃NPs and ZnONPs groups. It was pronounced that the effect of the combination was more than each of them alone **(Table 1)**.

Table 1: The body and organs weights of male rats treated with aluminum oxide nanoparticles (Al₂O₃NPs), zinc oxide nanoparticles (ZnONPs) and their combination

	Experimental groups						
Parameter	Control	Al ₂ O ₃ NPs	ZnONPs	Al ₂ O ₃ NPs+ ZnONPs			
I.B.W. (g)	169±3.17	167±2.38	170±2.24	168±2.91			
F.B.W. (g)	222±6.16ª	207±3.67 ^b	201±5.50 ^b	195±4.53 ^b			
B.W.G (g/75 days)	53.5±6.54ª	40.0±5.37 ^{ab}	30.5±5.40 ^b	27.0±5.12 ^b			
Testes weight (g)	2.78±0.06ª	2.55±0.07 ^b	2.14±0.10 ^c	2.09±0.07°			
Epididymis weight (g)	$2.85 \pm 0.08^{\text{a}}$	$2.29\pm0.06^{\text{b}}$	$2.20{\pm}0.10^{\rm bc}$	1.96± 0.10°			
Prostate weight (g)	0.59±0.03ª	0.65±0.02 ^{ab}	0.70±0.03 ^b	0.71±0.03 ^b			

Data presented as Mean±SE. For each parameter, the groups with different superscript letters are significantly differ, p<0.05. (IBW = Initial body weight, FBW = Final body weight, BWG= Body weight gain).

Testicular gene expression of mitochondrial transcription factor-A (mtTFA) and uncoupling protein 2

Data showed suppression of the testicular gene expression of mitochondrial transcription factor-A (mtTFA) in testes of rats treated with aluminum oxide nanoparticles (AI_2O_3NPs), zinc oxide nanoparticles (ZnONPs) and their combination by about 50%, 45% and 59% of control value, respectively **(Table 2)**. The testicular gene expression of uncoupling protein 2 (UCP2) showed significant induction in the rats treated with AI_2O_3NPs , ZnONPs and their combination by about 193%, 130% and 317% of control value, respectively **(Table 2)**. The treatment with the combination of AI_2O_3NPs plus ZnONPs showed the highest induction (more 3 folds than the control level).

DNA Fragmentation

The agarose gel electrophoresis of testicular genomic DNA showed very low or undetectable DNA laddering (DNA fragmentation) in the testes of the control rats (Fig. 1). The DNA intact band appears to be condensed near the application point with no DNA smearing suggesting no DNA fragmentation. On the other hand, the ZnO and Al₂O₃ nanoparticles-treatment alone or in combination resulted in massive DNA fragmentations which appear as DNA laddering (smearing).

Tissues levels of p53, TNF-α and IL-6

The rats treated with Al_2O_3NPs and ZnONPs alone or in combination showed significantly higher testes level of p53, TNF- α and IL-6 compared to control group. In addition, treatment with Al_2O_3NPs and ZnONPs in combination group showed significant increase in the level of p53 TNF- α and IL-6 compared to Al_2O_3NPs and ZnONPs groups (**Table 3**).

Semen characteristics and sex hormones

Data showed that Al₂O₃NPs, ZnONPs and their combination caused significant reduction in sperm motility and sperm count, and significant induction in abnormal sperm compared to the control group **(Table 4)**. Data showed significant decrease in the levels of testosterone and thyroid-Stimulating hormone (TSH), and significant increase in follicle stimulating hormone (FSH), luteinizing hormone (LH), tri-lodothyronine (T3) and Thyroxin (T4) levels in rats treated with Al₂O₃NPs, ZnONPs and their combination compared to the control group **(Table 5)**.

Antioxidant enzymes and free radicals

Treatment with Al_2O_3NPs , ZnONPs and their combination caused significant decrease in the activities of plasma and testes GPX, GST, CAT, SOD, GSH and TAC, and significant increase in TBARS and NO compared to control group. The effect of the combination of Al_2O_3NPs plus ZnONPs was more toxic than each other **(Tables 6-9)**.

Activities of steroidogenic enzymes

Data showed that treatment with Al_2O_3NPs , ZnONPs and their combination caused significant (P<0.05) increase in steroidogenic enzymes 17 β -hydroxysteroid dehydrogenase (17 β -HSD) activity and significant decrease in 17-ketosteroid reductase (17-KSR) comparison with the control group **(Table 10)**.

Table 2: The effect of aluminum oxide nanoparticles, zinc oxide nanoparticles and their combination on the testicular gene expression of mitochondrial transcription factor-A (mtTFA) and and uncoupling protein2(UCP2).

Experimental groups				
Control	Al ₂ O ₃ NPs	ZnONPs	Al ₂ O ₃ NPs + ZnONPs	
1.0± 0.05ª	0.5± 0.17 ^b (-50 %)	0.55 ± 0.02 ^b (-45 %)	0.41 ± 0.02 ^c (-59 %)	
1.0± 0.06ª	2.93 ± 0.29 ^b (193 %)	2.30 ± 0.27 ^b (130 %)	4.17 ± 0.18 ^c (317 %)	
	Control 1.0± 0.05 ^a 1.0± 0.06 ^a	Control Al_2O_3NPs 1.0± 0.05° 0.5± 0.17° 1.0± 0.06° (-50 %) 1.0± 0.06° (193 %)	Experimental groups Control Al_2O_3NPs ZnONPs 1.0±0.05 ^a 0.5±0.17 ^b 0.55±0.02 ^b 1.0±0.06 ^a 2.93±0.29 ^b (.45 %) 1.0±0.06 ^a 2.93±0.29 ^b 2.30±0.27 ^b (.193 %) (.130 %) (.130 %)	

Data presented as Mean \pm SE. For each parameter, the groups with different superscript letters are significantly differ, p<0.05. (Numbers between parentheses represent the percentage change from control value).

Table 3: Testes levels of tumor suppressor p53 (p53), tumor necrosis factor- α (TNF- α) and interliukin-6 (IL-6) of male rats treated with aluminum oxide nanoparticles, zinc oxide nanoparticles and their combination.

	Experimental groups						
Parameter	Control Al ₂ O ₃ NPs ZnON		ZnONPs	Al ₂ O ₃ NPs+ ZnONPs			
p53 (ng/mg protein)	6.10 ± 0.40^{d}	10.50± 0.40°	11.98± 0.20 ^b	13.06± 0.14ª			
TNF-α(ng/g mtissue)	139± 1.22 ^d	331± 0.97⁵	317± 3.56°	401±0.88ª			
IL-6 (ng/gm tissue)	117± 0.88 ^d 195± 1.45 ^b 168± 2.85 ^c 267±						
Data presented as Mean±SE. For each parameter, the groups with different superscript letters are significantly differ, p<0.05.							
P53=Tumor supp	oressor, TNF-α = T	umor necrosis fa	actor-α, IL-6 = Int	erliukin-6			

 Table 4: Semen characteristics of male rats treated with aluminum oxide nanoparticles, zinc oxide nanoparticles and their combination.

	Experimental groups				
Parameter	Control	Al ₂ O ₃ NPs	ZnONPs	Al ₂ O ₃ NPs+ ZnONPs	
Sperm count (million/ml)	83.0±2.56ª	30.4±1.73°	42.3±2.18 ^b	25.20±1.40°	
Abnormal (%)	14.2±0.86°	36.0±1.77 ^b	35.4±1.87 ^b	42.0±2.13ª	
Motility (%) 87.2± 3.11 ^a 41.6± 2.16 ^b 39.6± 2.83 ^b 28.4± 0.68 ^c					
Data presented as Mean \pm SE. For each parameter, the groups with different superscript letters are significantly differ, <i>p</i> <0.05.					

 Table 5: Testosterone, follicle stimulating hormone (FSH), luteinizing hormone

 (LH), thyroid-Stimulating hormone (TSH), tri-lodothyronine (T3) and thyroxin (T4)

 of male rats treated with aluminum oxide nanoparticles, zinc oxide nanoparticles and their combination.

	Experimental groups					
Parameter	Control	Al ₂ O ₃ NPs	ZnONPs	Al ₂ O ₃ NPs+ ZnONPs		
Testosterone (ng/ml)	5.1±0.29ª	2.1±0.08 ^b	2.6±0.14 ^b	1.3±0.05°		
FSH (ng/ml)	0.69±0.04ª	1.51±0.03 ^b	1.59±0.05 ^b	1.91±0.06 ^c		
LH (ng/ml)	0.45± 0.03ª	0.94± 0.02 ^b	0.95± 0.04 ^b	1.30± 0.06°		
TSH (ng/ml)	4.10± 0.23ª	1.92± 0.06 ^b	1.66± 0.10 ^b	1.04± 0.06°		
T3 (ng/ml)	84.2±6.8°	152.8±5.4 ^b	133.8±8.8 ^b	178.0±4.9ª		
T4 (ng/ml)	0.93±0.06°	1.59±0.10 ^b	1.89±0.10 ^b	2.25±0.16ª		
Data presented as Mean±SE. For each parameter, the groups with different superscript letters are significantly differ, <i>p</i> <0.05.						

 Table 6: Plasma antioxidant enzymes of male rats treated with aluminum oxide nanoparticles (Al₂O₃NPs), zinc oxide nanoparticles (ZnONPs) and their combination.

Parameter	Experimental groups				
	Control	Al ₂ O ₃ NPs	ZnONPs	Al ₂ O ₃ NPs+ ZnONPs	
GPX (U/ml)	32.2±0.78ª	26.9±1.85 ^b	24.4±1.11 ^b	20.5±0.68°	
GST (µmole /hr)	0.64±0.04ª	0.42±0.02 ^b	0.36±0.01bc	0.29±0.02 ^c	
CAT (U/ml)	62.2±1.42ª	42.6±1.39 ^b	38.1±1.02 ^c	32.6±1.71 ^d	
SOD (U/ml)	1.23±0.05ª	0.87±0.04 ^b	0.93±0.02 ^b	0.55±0.02 ^c	
TAC (mM/L)	1.81±0.01ª	1.39±0.07 ^b	1.45±0.04 ^b	0.96±0.01°	

Table 7: Plasma reduced glutathione and free radicals of male rats treated with aluminum oxide nanoparticles (Al_2O_3NPs), zinc oxide nanoparticles (ZnONPs) and their combination.

Parameter	Experimental groups				
	Control	Al ₂ O ₃ NPs	ZnONPs	Al ₂ O ₃ NPs+ ZnONPs	
GSH (nmol/ mg)	4.8±0.11ª	3.9±0.10 ^b	3.7±0.09 ^b	2.9±0.06 ^c	
TBARS (nmol/mg)	0.92±0.03°	1.34±0.07 ^b	1.26±0.08 ^b	1.61±0.10ª	
NO (u mol/L)	24.6±0.81°	44.1±1.67 ^b	45.5±1.84 ^b	54.1±2.71ª	
Data presented as Mean±SE. For each parameter, the groups with different super- script letters are significantly differ, p<0.05.					
GSH = reduced glutathione, TBARS = thiobarbituric acid-reactive substances, NO= nitric oxide.					

Table 8: Testes glutathione peroxidase, glutathione S-transferase, catalase, superoxide dismutase and total antioxidant capacity of male rats treated with aluminum oxide nanoparticles (Al_2O_3NPs), zinc oxide nanoparticles (ZnONPs) and their combination.

	Experimental groups					
Parameter	Control	Al ₂ O ₃ NPs	ZnONPs	Al ₂ O ₃ NPs+ ZnONPs		
GPX (U/g)	17.3±1.08ª	13.0±0.46 ^b	12.3±0.54 ^b	5.5±0.28°		
GST (µmole /hr)	2.04±0.03 ^a	1.69±0.04 ^b	1.74±0.03 ^b	1.32±0.08°		
CAT (U/g)	54.3±0.72ª	43.8±1.91 ^b	43.1±2.04 ^b	29.6±2.35°		
SOD (U/g)	13.3±0.25ª	9.7±0.33 ^b	9.3±0.64 ^b	6.7±0.46 ^{cc}		
TAC (mM/L)	1.7 ±0.04 ^a	1.2 ±0.04 ^b	1.3 ±0.06 ^b	0.9 ±0.07 ^c		
Data presented a	Data presented as Mean+SE. For each parameter, the groups with different					

superscript letters are significantly differ, p<0.05.

GPX=glutathione peroxidase, GST=glutathione S-transferase, CAT=catalase, SOD=superoxide dismutase, TAC= total antioxidant capacity

Table 9:Testes reduced glutathione and free radicals of male rats treated with aluminum oxide nanoparticles (Al_2O_3NPs), zinc oxide nanoparticles (ZnONPs) and their combination.

Parame-	Experimental groups					
ter	Control	Al ₂ O ₃ NPs	ZnONPs	Al ₂ O ₃ NPs+ ZnONPs		
GSH (u mol/g)	5.0±0.17ª	4.1±0.28 ^b	3.9±0.29 ^{bc}	3.3±0.21 ^c		
TBARS (n mol/g)	35.1±1.53°	50.9±0.46 ^b	52.7±0.99 ^b	59.1±2.33ª		
NO (umol/L)	52.6 ±1.65°	75.2 ±3.94 ^b	81.2 ±2.11 ^b	100.5 ± 1.56^{a}		
Data presented as Mean+SE. For each parameter, the groups with different						

superscript letters are significantly differ, p<0.05.

 $\mathsf{GSH}=$ reduced glutathione, <code>TBARS=</code> thiobarbituric acid-reactive substances, <code>NO=</code> nitric oxide.

Table 10: Testicular 17β-hydroxysteroid dehydrogenase (17β-HSD) and 17-ketosteroid reductase (17-KSR) of rats treated with aluminum oxide nanoparticles (Al₂O₂NPs), zinc oxide nanoparticles (ZnONPs) and their combination.

Paramotor	Experimental groups				
rarameter	Control	Al ₂ O ₃ NPs	ZnONPs	Al ₂ O ₃ NPs+ ZnONPs	
17β-HSD (U/ min/mg)	0.85±0.04 ^c	1.01±0.06 ^{bc}	1.13±0.03 ^b	1.44±0.09ª	
17-KSR (U/min/ mg)	16.4±0.69ª	12.7±0.60 ^b	10.3±0.51°	4.5±0.31 ^d	
Data presented as Mean+SE. For each parameter, the groups with different su-					

perscript letters are significantly differ, p<0.05.

Histopathology

Histological examination of adult male rat testicular tissues of the different studied groups. Rat testes of control group showed regular cross sections of seminiferous tubules with clusters of interstitial cells of Leydig in between. The tubules are lined by regular stratified layers of spermatogenic cells with different stages of spermatogenesis, including spermatogonia, spermatocytes and spermatids. Spermatozoa are seen filling the Lumina of the tubules. Sertoli cells are seen resting on the basement membrane (**Figure 2a & 2b**). **Figure 3a & 3b** showed the changes in rats treated with Al₂O₃NPs for which showed of Al2O3NPs treated rat testis showing distorted seminiferous tubules with loosely arranged detached spermatogenic cells, Most of the spermatogenic cells are with pyknotic nuclei and falling into the seminiferous tubules lumina. Notice the wide interstitial spaces in between the tubules containing few darkly stained Leydig cells. Figure 4a & b showed the changes in rats treated with ZnONPs which showed of ZnONPs treated rat testis showing seminiferous tubules with irregular shapes and wide empty lumina. Decrease height of the lining epithelium and many spermatogenic cells with pyknotic nuclei are noticed. The Leydig cells are almost absent with wide interstitial spaces. The changes in testes histology of rats treated with Al₂O₃NPs plus ZnONPs were presented in Figure 5a & b which showed that testis of treated rats showed many severely distorted seminiferous tubules with disruption and irregularity of their basement membrane. Other tubules are illustrating focal losses of the germinal epithelium and the disturbed organization of the spermatogenic cells Notice the wide interstitial spaces in between some tubules while others are showing obliterated spaces containing few darkly stained Leydig cells.



Figure 1: Stained agarose gel of testicular genomic DNA demonstrating apoptotic and necrotic cell deaths induced nanoparticles. lane 1 and 2: control, lane 3 and 4: ZnO nanoparticles treated rats, lane 5 and 6: Al₂O₃ nanoparticles treated rats, lanes 7 and 8: ZnO plus Al₂O₃ treated rats.

DISCUSSION

Fernandez et al. [25] found that determination of body weight supplies significant information on possible implications for the health of the organism and the toxicity of a compound. Also, the determination of absolute and relative weights of testes, epididymis, seminal vesicle and prostate can be utilized in the evaluation of risks from adverse effects on male reproductive apparatus. Body and organs weights are sensitive indicators of toxic chemicals and this is in agreement with the current study showed that exposure to Al₂O₃NPs, ZnONPs and their combination caused alterations in the body and sex organs weights. The effects of these nanoparticles may be referring to their toxicity on body and sex organs weights. The present study showed deterioration in sperm



Figure 2 A, B: Light micrographs of testis of control group which showed regular cross sections of seminiferous tubules (T) with clusters of interstitial cells of Leydig (L) in between. The tubules are lined by regular stratified layers of spermatogenic cells with different stages of spermatogenesis, including spermatogonia (Sg), spermatocytes (Sp) and spermatids (Sd). Spermatozoa (Sz) are seen filling the Lumina of the tubules. Sertoli cells (St) are seen resting on the basement membrane (B).



Figure 4 A, B: Light micrographs of testis of rats treated with ZnONPs which showed seminiferous tubules with irregular shapes and wide empty lumina (R). Decrease height of the lining epithelium and many spermatogenic cells with pyknotic nuclei are noticed (Y). The Leydig cells are almost absent with wide interstitial spaces (G).

characteristics after exposure to Al₂O₃NPs, ZnONPs and their combination. Also, Sullivan et al. [26] reported that reduction in the number of spermatids in the testis and per gram testis and in daily sperm production was found.

Mitochondrial transcription factor A (mtTFA) is a key transcription factor. During spermatogenesis in the mouse, mtTFA is expressed up to the late spermatocyte and early spermatid stage [27]. Therefore, it is important to determine mtTFA in the present study to know if the tested nanoparticles have effects on mitochondrial transcription factor A (mtTFA), as a transcription factor is a multi-functional protein plays an important role in the maintenance of mitochondrial DNA (mtDNA) integrity, replication and transcription [28]. The obtained data showed that Al₂O₃NPs, ZnONPs and their combination caused significant decline in mtTFA by 50%, 45% and 59%, respectively (**Table 2**) and hence affect



Figure 3 A, B: Light micrographs of testis of rates treated with Al2O3NPs which showed distorted seminiferous tubules with loosely arranged detached spermatogenic cells (R), Most of the spermatogenic cells are with pyknotic nuclei (X) and falling into the seminiferous tubules lumina. Notice the wide interstitial spaces in between the tubules containing few darkly stained Leydig cells (G).



Figure 5 A, B, C: Light micrographs of testis of the combination of Al2O3NPs with ZnO3NPs which showed many severely distorted seminiferous tubules (M) with disruption and irregularity of their basement membrane (W). Other tubules are illustrating focal losses of the germinal epithelium and the disturbed organization of the spermatogenic cells (P) Notice the wide interstitial spaces in between some tubules (G) while others are showing obliterated spaces containing few darkly stained Leydig cells (Z).

the mitochondrial DNA (mtDNA) integrity, replication and transcription [28]. In line with this assumption it was documented that, ZnO nanoparticles induce mitochondrial dysfunction, morphological modification, and apoptosis [29].

40X

In the present study, the enhanced expression of UCP2 in testicular tissues of rats treated with nanoparticles may facilitate the proton leak that may act as a possible control mechanism for mitochondrial ROS production in the testis, with a mild uncoupling leading to a reduction in mitochondrial membrane potential, which in turn reduces ROS production. However this defense mechanism may have a serious metabolic consequence as the protein leak decrease the metabolic efficiency of the mitochondria through uncoupling the oxidation from phosphorylation leading to decreased ATP production. The later effect may participate directly and/or indirectly in the apoptotic and necrotic cell death. In line with

the present study, it was documented that, ZnO nanoparticle had a strong deleterious effect of mitochondrial function [30]. The mechanism by which nanoparticles induce the gene expression of UCP2 is unclear however we can postulate that it may a direct consequence of oxidative stress and increased free radicals and lipid peroxide which found in the present data **(Tables 6-9)**.

Testicular genomic DNA showed very low or undetectable DNA laddering (DNA fragmentation) in the testes of the control rats (Figure 1). Damage to DNA is a fundamental example of cellular toxicity, and it is critical to assess such damage for any nanoparticle that is likely to come in contact with humans, given that damage to DNA is highly correlated with an increased risk of cancer. Oguma et al. [31] reported that the major function of p53 tumor suppressor is to curtail proliferation of abnormal cells. Their data suggest that low amount of ZnONPs (50 mM) is enough to trigger activation of p53 which in turn drives the expression of important downstream antioxidant genes. They observed upregulation of p53 and its associated genes after low concentration of H₂O₂ treatment. The present study showed enhancement in tumor suppressor p53 (p53), tumor necrosis factor-a (TNF-a) and interliukin-6 (IL-6) of male rats treated with aluminum oxide nanoparticles, zinc oxide nanoparticles and their combination. Park et al. [32] demonstrated that Al₂O₂NPs for caused significant increase in inflammatory cytokines IL-6. Also, Rodrigo et al. [33] reported that aluminum nanoparticles have been shown to initiate inflammatory events in macrophages, including secretion of pro-inflammatory cytokines.

The present study showed that exposure to nanoparticles caused deteriorations in semen characteristics and imbalance in the reproductive hormones (Testosterone, FSH, LH) and thyroid hormones (TSH, T3 and T4) and this may be due to the increase the levels of free radicals and nitric oxide in testes and the reduction in the antioxidants (Tables 6-9). Dadong et al. [34] demonstrated that ZnO nanoparticles could decrease mitochondrial membrane potential, increase the production of oxygen free radicals (ROS) and lead to the over-expression of genes that activate caspase 12 in RGC-5 cells. Because the increase of oxygen free radicals lead to the production of oxidative stress in endoplasmic reticulum membrane and induce apoptosis and necrosis by expression of caspase 12 and finally cause cell death. ROS and membrane lipoperoxidation of Sertoli cells and the damage in Sertoli and leydig cells lead to imbalance in FSH, LH and test hormones. Naher et al. [35] showed an association with low antioxidant level in the semen and bad quality of spermatozoa of the infertile subjects. Also, decreased antioxidant level in the seminal fluid has a negative impact on semen quality. In addition, Aitken et al. [36] reported that infertile males who that produce high levels of ROS have a five-fold less chance of initiating a pregnancy than infertile males who produce low levels of ROS. The increase in TBARS can bring negative effects on motility, sperm–oocyte fusion, and induces midpiece abnormalities [37].

Shirvani et al. [38] reported that ZnO nanoparticles have impact on Sertoli cells, occurrence of vacuolation phenomenon, apoptosis and reduction in various cell lines including spermatogonia, round and elongated spermatids. According to Bockelheide et al. [39], the presence of vacuoles in the cytoplasm of Sertoli cells indicate direct harm of nanoparticles on these cells, such that one of the obvious tissue damage signs can be the presence of vacuoles in Sertoli cells cytoplasm and between cell lines of seminiferous tubules. Yoshida et al. [40] found degeneration in Leydig cell and damage in the seminiferous tubules due to exposure to nanoparticles. Increase in the activity of testicular 17β-HSD and decrease in the activity of 17-KSR in the present study may lead to decrease of testosterone which may explain the reduction of serum testosterone levels. One of the indicators of the chemical toxicity on reproductive system is the decreased level of testosterone [40].

CONCLUSION

In conclusion, the obtained results revealed adverse effects of Al2O3NPs and ZnONPs alone and or their combination on the testicular architecture and caused fertility problems through different pathways including; changing gene expression of the proteins involved in the mitochondrial biogenesis and function (mtTFA and UCP2), induction of oxidative stress, lipid peroxidation, nitric oxide production, apoptotic and necrotic cell death pathways in the testicular tissues. Also, induction of inflammatory pathway through enhancing production of Tumor necrosis factor α and interleukin-6, disturbing production of tumor suppressor p53, sex hormones imbalance, and histopathological changes. Also, the present study showed that the reproductive toxicity of the combination of Al2O3NPs plus ZnONPs was more pronounced than each one.

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