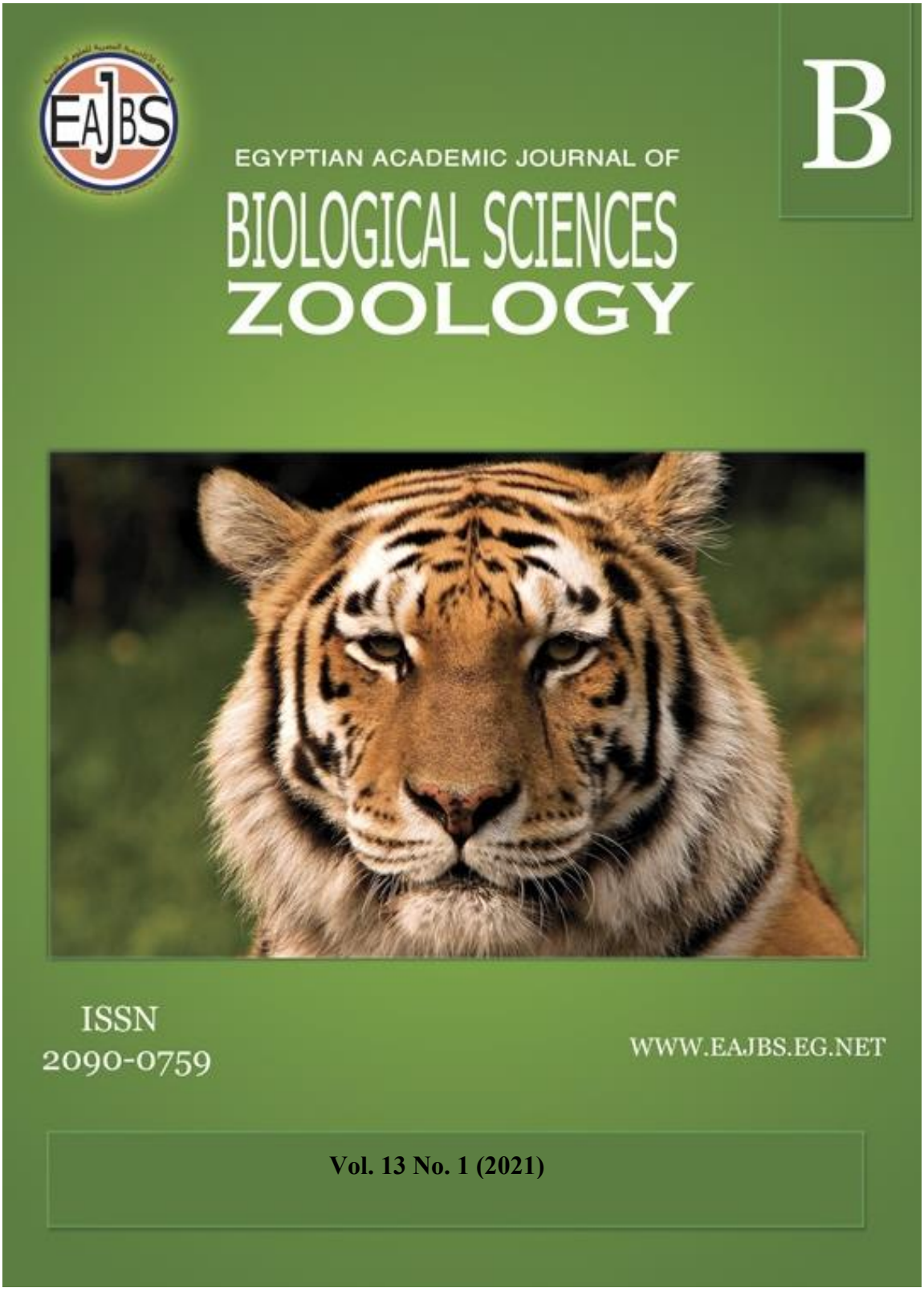

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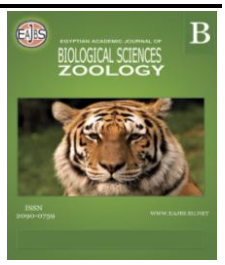

Histological and Biochemical Alterations in Testis Rats Treated with Chitosan Nanoparticles Against Hydroxyapatite Nanoparticles

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ABSTRACT

The objective of this study was to analyze the potential toxicity of the Hydroxyapatite. 60 male albino rats were treated with Hydroxyapatite for 30 days. Toxicity was monitored by quantitative analysis of the serum testosterone hormone level; follicle-stimulating hormone (FSH) level in serum, luteinizing hormone (LH) level in serum, prolactin hormone PRL) level in serum, AST activity and Alkaline phosphatase activity was measured and tests were collected for histopathological study. The results refer to a significant decrease in testosterone; FSH, LH and PRL while a significant increase in AST and ALP). Histopathology revealed moderate disturbance with an irregular cycle of spermatogenesis in seminiferous tubules, a marked decrease in the number of spermatogenic cells in the seminiferous tubules, a marked decrease in the number of sperms and Leydig cells. The administration of the Chitosan had beneficial and decrease side effects against the deleterious changes of Hydroxyapatite. In conclusion, results suggest a potential contribution of Hydroxyapatite to the etiology of somebody's diseases while Chitosan has beneficial effects as it tends to dampen Hydroxyapatite toxicity in rats.

INTRODUCTION

The reproductive system of male mammals mainly consists of the testis, ductus deferens and epididymis. The testis produces spermatozoa and the epididymis stores the spermatozoa and completes sperm maturation. While spermatogenesis is a complex process of multiphase, and activities of testicular marked enzymes, including sorbitol dehydrogenase `phosphatase (AKP), acid phosphatase (ACP), glucose-6-phosphate dehydrogenase (G-6PD), nitric oxide synthase (NOS), are related to this process in the testes **(**Fujisawa *et al.*, 2001). The primary functions of the testis, androgen production and gamete development, are regulated by the brain, e.g., hypothalamus and hypophysis via GnRH and gonadotropins. Importantly, the hypothalamo-hypophyseal circuit is subject to negative feedback regulation mediated by testicular factors. Testosterone inhibits the secretion of FSH and LH. For FSH, the protein hormone inhibin B plays an important role (Boepple *et al.*, 2008). Testosterone is essential to maintain normal testicular development and spermatogenesis, as well as secondary sexual characteristics. In testis, testosterone is produced by Leydig cells under the regulation of LH signaling (Zirkin and Tenover, 2012). Decreased testosterone levels are thought to arise from disturbed steroidogenesis in Leydig cells or reduced hypothalamic

GnRH or pituitary LH stimuli (Schlatt and Ehmcke, 2014). Leydig cells produce and secrete the most important male sexual hormone, testosterone. From the developmental, morphological and functional viewpoint, different types of cells can be distinguished: stem Leydig cells as founder cell, progenitor Leydig cells as a committed stem cell, fetal Leydig cells as a terminally differentiated cell in the fetus, and adult Leydig cells as the terminally differentiated Leydig cell (Ge and Hardy, 2007). In mammalian testes, Sertoli cells, the primary supportive cells of the seminiferous tubules, interact directly with germ cells to control their proliferation and differentiation (Kopera *et al.,* 2010). Thus, adequate Sertoli cell function is essential for spermatogenesis. Factors produced by Leydig cells have direct effects on Sertoli cells, stimulating their proliferation and the expression of several factors that may contribute to spermatogenesis (Lucas *et al.,* 2014). Leydig cells produce and secrete the most important male sexual hormone, testosterone. From the developmental, morphological and functional viewpoint different types of cells can be distinguished: stem Leydig cells as founder cell, progenitor Leydig cells as a committed stem cell, fetal Leydig cells as a terminally differentiated cell in the fetus, and adult Leydig cells as the terminally differentiated Leydig cell (Ge and Hardy 2007).

Hydroxyapatite nanoparticles: Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$(HAP)) is a bio ceramic material with a calcium to phosphorus ratio like to that of natural bone and teeth. Therefore, great clinical interest in its usage, since it is biodegradable, bioactive and biocompatible. It is currently used for bone graft substitutes, such as porous granules, block scaf-folds and coatings over metallic implants for bone regeneration (Nayar *et al.,* 2006). Particular physicochemical properties of the apatite structure allow it to form many different compositions, therefore allowing easy incorporation of ions in the crystal lattice (Morrissey *et al.,* 2005). To date, several methods of preparing HAP nanoparticles have been developed, including sol-gel, biomimetic deposition, electrodeposition, ultrasonic spray freeze-drying, spray dry, combustion synthesis and the wet chemical route (Venkatesan and Kim, 2015).

Investigations have revealed that nano-HAP powders possess improved densification and sinter ability due to increased surface area, which could ameliorate fracture toughness, and other mechanical properties (Abd El-Fattah *et al.,* 2014). In addition, nano-HAP, compared to HAP, showed a favourable effect on cell proliferation of human osteoblast-like cells *in vitro* and stimulated hard tissue regeneration *in vivo* (Zhou and Lee, 2011). Nevertheless, the exact role of nano-HAP in bone repair, especially the actual relationship between nano-HAP and important cells involved in bone regeneration, is far from being clarified, and the biocompatibility of nano-HAP is still a matter of debate (Shi *et al.,* 2009; Zhu *et al.,* 2013).

Hydroxyapatite nanoparticles (HApNPs) have extensive applications in the field of medicine by virtue of its good biocompatibility and bioactivity (Kantharia *et al.,* 2014). It is known that the chemical formula and properties of synthetic hydroxyapatite are similar to those of the main inorganic constituent of bones and teeth. Hence, for some time so far this mineral has been widely applied as a biomaterial for orthopedic and dental applications for repairing or substituting hard tissues, and also as the drug delivery systems. Synthetic biomaterials composed of HA have been extensively investigated to construct artificial bone grafts purely made of HA or only as the surface coating by HA. However, apart from its bioactivity and biocompatibility, its mechanical strength and porous structure are also important when implanted into the human body (Huang *et al.,* 2011). Hydroxyapatite is the major inorganic constituent of the hard tissue of humans and animals. Due to its excellent biocompatibility and bioactivity, HAP has been widely employed in repairing hard tissue injury (Zhou and Lee, 2011). It is also used as a vehicle for drug, protein and gene delivery (Uskokovic and Uskokovic, 2011). However, some studies have reported that HAPNs exhibited significant cytotoxicity to some types of cancer cells including breast cancer cells (Meena *et al.,* 2012), osteosarcoma cells (Qing *et al.,* 2012), gastric cancer cells (Chen *et al.,* 2007) and glioma

cells (Xu *et al.,* 2012). The molecular mechanism of nano-HAP-induced apoptosis remains unclear, and there are few studies of nano-HAP-induced apoptosis in cancer cells. Chen *et al.* (2007) observed that nano-HAP significantly reduced cell viability and induced apoptosis in SGC-7901 cells that were characterized by hypodiploid DNA, morphological changes and DNA fragmentation. The increase in apoptosis was accompanied by increased expression of Bax, a pro-apoptotic protein, and decreased expression of Bcl-2, an anti-apoptotic protein. Additionally, the increase in apoptosis was correlated with a decrease in the mitochondrial membrane potential and release of cytochrome c from mitochondria into the cytosol. Furthermore, nano-HAP induced the activation of caspases (Zhu *et al.,* 2013), but did not activate caspase (Shi *et al.,* 2009). Z-VAD-fmk, a universal caspase inhibitor, dose-dependently inhibited nano-HAP-induced apoptosis.

Antioxidant nanoparticles: Antioxidants have received attention in recent years, due to their potential as prophylactic and therapeutic agents and more importantly their ability to fight oxidative stress. The main function of an antioxidant is to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) and convert them into less harmful or neutral products (Pellegrini *et al.,* 2003). Since orally delivered antioxidants are easily destroyed by acids and enzymes, only a small portion of consumed antioxidants gets absorbed, leading to low bioavailability and low concentrations at the target site (Souto *et al.,* 2013). Hence, there is an urgent need to develop effective methods for the efficient delivery of antioxidants to the required sites. Efforts have been done towards loading antioxidant molecules in advanced nano-particulate carriers, e.g., liposomes, polymeric nanoparticles (NPs), solid lipid NPs or self-emulsifying drug delivery systems (Watal *et al.,* 2013).

Chitosan: Chitosan (CN) is a naturally occurring copolymer obtained from deacetylation of chitin of crustacean shells, insects exoskeleton and is considered as dietary fiber due to indigestibility by digestive enzymes (Kohda *et al.,* 2012). CN has several functions in the fields of food preservation, microbial mitigation, biomedicinal and pharmaceutical products (Vinsova and Vavrikova, 2008) as well as its other biological activities such as its antioxidant properties (Chen *et al.,* 2003) and free radical scavenging activity *in vivo* and *ex vivo* (Huang *et al.,* 2005), antitumor activities in human liver (Kumar *et al.,* 2012), immuno-potentiating, antihypertensive in colon cancer (Nam et al., 2007), antifungal activities (Qin *et al.,* 2012), antiviral activity (Ai et al., 2012) and antimicrobial (Sun *et al.,* 2011). Abdel-Wahhab *et al.* (2015) and El- Denshary *et al.* (2015) reported that; chitosan nanoparticles (CNPs) show excellent activities compared to normal CN to promote the immune-enhancing effect, the antimicrobial and the anticancer activity.

Chitosan (CS), is a nontoxic copolymer consisting of β-(1, 4)-2- amino-2 deoxy-D-glucose (β1, 4-linked polymer of glucosamine) and lesser amounts of N-acetylglucosamine. It is a naturally occurring biodegradable and biocompatible cationic polysaccharide derived from the N-deacetylation of chitin which is the most abundant natural structure polysaccharide after cellulose. It can be found in the exoskeleton of crustaceans which can be obtained from the shell waste of the crab, shrimp and crawfish during processing industries and in fungal cell walls (Shahdat et al., 2007).

Chitosan nanoparticles: Nano chitosan is a bioactive and eco-friendly natural material with excellent physicochemical properties, and it is extensively used for formulating carriers in various fields including drug delivery, antibacterial and tissue engineering (Yue *et al.,* 2011). Additionally, in the textiles industry, nano chitosan is used for improving the strength and washability and to confer antibacterial effect (Huang *et al.,* 2009). Because of the high surface to volume ratio, polymeric NPs widely improve the loading capacity of the selected molecules. Chitosan is poorly soluble in water and highly soluble in acidic conditions, hence free amino groups contribute a positive charge on the entire chain. Polymeric chitosan-based nanoparticles can be prepared using many methods including ionic gelation, emulsion cross-

linking, spray drying and complex coacervation. Among these, the ionic gelation technique is fast and easy to carry out (Yien *et al.,* 2012).

MATERIALS AND METHODS

Animals:

Mature male Sprague Dawley albino rats averaged weights (150±10 g) (obtained from laboratory farms, Zoology Department, Faculty of Science, and Al-Azhar University, Egypt) were housed in stainless steel cages with water and food ad libitum, the temperature of 22±2ºC, humidity around 56% and 12 hrs light-dark cycle the rats were transferred to the animal house in Zoology Department, Faculty of Science, Al-Azhar University, Cairo.

Chemicals and Reagent:

Nano chitosan is purchased from Nanotech Egypt (Grenha *et al.,* 2005). With dose 140 mg/kg body weight/daily for 30 days. Nano Hydroxyapatite is purchased from Nanotech Egypt (Paz *et al.,* 2012). With doses 200 and 400 mg/kg body weight/daily for 30 days.

Experimental Design: -

60 male albino rats were randomly divided into 6 equal groups and labeled as groups 1,2,3,4,5 and 6 each group contains 10 rats, rats received all treatments daily via oral gavage tube along the period of the experiment, Group (1): Control rats, Group (2): Rats received Chitosan nanoparticles (CNPs) (140 mg/kg body weight/daily) for 30 days. Group (3): Rats received a low dose of hydroxyapatite nanoparticles (Ld HAP-NPs) injected intraperitoneally with 200 mg/kg body weight/daily) for 30 days. Group (4): Rats received a high dose of hydroxyapatite nanoparticles (Ld HAP-NPs) injected intraperitoneally with 400 mg/kg body weight/daily) for 30 days. Group (5): Rats received HAP-NPs were injected intraperitoneally with a low dose of hydroxyapatite nanoparticles (200 mg/kg body weight/daily) for 30 days and followed by treatment with chitosan nanoparticles (CNPs) (140 mg/kg body weight/daily) for successive 30 days. Group (6): Rats received HAP-NPs were injected intraperitoneally with a high dose of hydroxyapatite nanoparticles (400 mg/kg body weight/daily) for 30 days and followed by treatment with chitosan nanoparticles (CNPs) (140 mg/kg body weight/daily) for successive 30 days.

At the end of the experimental period, rats have fasted overnight. Rats from each group were weighed and euthanized with intraperitoneal injection with sodium pentobarbital and subjected to a complete necropsy.

Sample Collection:

The rats were anesthetized through ip injection of Thiopental Sodium (6 mg/kg) Harms and Ojeda (1974) on day 30 and blood samples were collected from all animals through retro-orbital venous plexus. Put into chilled non-heparinized tubes, serum was obtained by centrifugation at 3000 r.p.m for 10 minutes; sera were frozen at -20 oC for estimation of testes functions, and hormonal profile. Animals were sacrificed after 24 hours of the last treatment, the abdominal cavities were opened, testes were rapidly and carefully excised and all attached vessels and ligaments were trimmed off to work light microscope.

Biochemical Investigations:

Testosterone hormone level in serum was according to (Delacerda *et al*., 1973). Follicle-stimulating hormone level in serum was according to (Odell *et al*., 1981). Luteinizing hormone level in serum was according to (Odell *et al*., 1974). Prolactin hormone level in serum was according to (Duhau *et al.,* 1991). AST activity in serum was according to the method of Reitman and Frankel, (1957). Alkaline phosphatase activity in serum was according (Belfield and Goldberge, 1971).

Histopathological Examination:

The histopathology was carried out according to Bancroft and Stevens, (1990) using Harris Hematoxylin and eosin staining technique.

Statistical Analysis:

The analysis was done using the Statistical Package for the Social Sciences (SPSS software version 16) on a personal computer. Data were presented as the mean± standard error of the mean (SEM) and statistically analyzed by one-way ANOVA (Analysis of Variance) followed by Dunnett test. Dunnett test comparisons were performed to assess the significance of differences between groups. The criterion for statistical significance was set at $p<0.05$.

RESULTS

In table (1) revealed that administration of rats with chitosan nanoparticles (CNPs) group (G2) has no significant effect on total testosterone, FSH and PRL concentration as compared to the control group (G1). A significantly decreased in low and high dose of hydroxyapatite nanoparticles (Ld HAP-NPs) group (G3) compared to the control group (G1). A significant increase in total testosterone, FSH and PRL concentration was detected in treated rats with a low dose of hydroxyapatite nanoparticles (Ld HAP+NPs) as compared to a high dose of hydroxyapatite nanoparticles (Hd HAP+NPs).

On the other hand; the treatment of rats treated with chitosan nanoparticles (CNPs) for 1 month after treatment rats with hydroxyapatite nanoparticles (G5&G6 respectively) has a significant increase in total testosterone, FSH and PRL concentration when compared to with hydroxyapatite nanoparticles group (G3&G4). On the other hand; a significant increase in rats treated with Ld HAP-NPs+CNPs group (G5) when compared with Hd HAP-NPs+CNPs group (G6).

In table (1) revealed that administration of rats with chitosan nanoparticles (CNPs) group (G2) has no significant effect on LH concentration as compared to the control group (G1). A significantly decreased in low and high dose of hydroxyapatite nanoparticles (Ld HAP-NPs) group (G3) compared to the control group (G1). No significant change in LH concentration was detected in treated rats with a low dose of hydroxyapatite nanoparticles (Ld HAP+NPs) as compared to a high dose of hydroxyapatite nanoparticles (Hd HAP+NPs). Treatment of rats treated with chitosan nanoparticles (CNPs) for 1 month after treatment rats with hydroxyapatite nanoparticles (G5&G6 respectively) has no significant change in LH concentration when compared to with hydroxyapatite nanoparticles group (G3&G4). Also, no significant change in rats treated with Ld HAP-NPs+CNPs group (G5) when compared with Hd HAP-NPs+CNPs group (G6).

In table (1) revealed that administration of rats with chitosan nanoparticles (CNPs) group (G2) has no significant effect on GOT and ALP activity as compared to the control group (G1). A significantly increased in low and high dose of hydroxyapatite nanoparticles (Ld HAP-NPs) group (G3) compared to the control group (G1). A significant decrease in GOT and ALP activity were detected in treated rats with low dose of hydroxyapatite nanoparticles (Ld HAP+NPs) as compared to a high dose of hydroxyapatite nanoparticles (Hd HAP+NPs).

On the other hand; the treatment of rats treated with chitosan nanoparticles (CNPs) for 1 month after treatment rats with hydroxyapatite nanoparticles (G5&G6 respectively) has a significant decrease in GOT and ALP activity when compared to with hydroxyapatite nanoparticles group (G3&G4). On the other hand; a significant decrease in rats treated with Ld HAP-NPs+CNPs group (G5) when compared with Hd HAP-NPs+CNPs group (G6).

Table 1: The changes in serum Testosterone, FSH, LH, PRL level, ASAT and ALP activities on the treatment of rats with hydroxyapatite nanoparticles and/or chitosan nanoparticles in different groups under study.

	days		Groups					
			Control(G1)	G2	G3	G4	G5	G6
Testosterone (ng/ml)	30	Mean ± S.E	3.20# ± 0.14	3.31# ± 0.27	2.27* ± 0.19	1.98* ± 0.21	3.01# ± 0.25	2.60# ± 0.18
FSH (mlU/mL)	30	Mean ± S.E	2.51# ± 0.35	2.63# ± 0.27	1.27* ± 0.16	1.14* ± 0.03	2.03#* ± 0.25	1.39* ± 0.20
LH (mlU/mL)	30	Mean ± S.E	1.13 ± 0.12	1.15 ± 0.09	1.08 ± 0.15	1.08 ± 0.09	1.11 ± 0.18	1.10 ± 0.10
PRL (ng/ml)	30	Mean ± S.E	0.598# ± 0.049	0.620# ± 0.042	0.445* ± 0.029	0.413* ± 0.035	0.497#* ± 0.019	0.471#* ± 0.022
ASAT (U/L)	30	Mean ± S.E	134.2# ± 7.49	124.6# ± 9.91	197.6* ± 13.20	209.4* ± 10.33	134.8# ± 9.84	152.8#* ± 12.65
ALP (U/L)	30	Mean ± S.E	142.1# ± 7.68	126.4# ± 12.66	198.0* ± 13.97	223.8* ± 10.41	136.6# ± 8.974	161.4#* ± 7.07
Each value represented means of 10 records ± S.E.								

Histopathology Results:

Histopathological study showed that the cycle of spermatogenesis is regular in all male rats in the control (G1) group (Fig. 1A). The structural components of the testis are the seminiferous tubules and interstitial cells (Leydig cells). Two types of cells are identified in rat seminiferous tubules, the Sertoli cells and the spermatogenic cells. Sertoli cells were found resting on the thin basal lamina (basement membrane) while the spermatogenic cells were arranged in many layers and identified as spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and finally mature spermatozoa (Fig. 1A). Testis sections in male rats treated with chitosan nanoparticles (CNPs) showed the normal structure of seminiferous with a regular cycle of spermatogenesis (Fig. 1B).

Testis sections of treated rats with a low dose of hydroxyapatite nanoparticles (G3; Ld HAP-NPs) revealed mild disturbance with an irregular cycle of spermatogenesis in seminiferous tubules, a moderate decrease in the number of spermatogenic cells in the seminiferous tubules, mild vacuolar degenerative changes in the cytoplasm of the spermatogenic epithelium and in the Sertoli cells, marked decrease and abnormal distribution of spermatozoa were seen in the wide lumen of the seminiferous tubules and mild decrease in the numbers of Leydig cells (Fig. 1C). Testis sections of treated rats with a high dose of hydroxyapatite nanoparticles (G4; Hd HAP-NPs) revealed moderate disturbance with an irregular cycle of spermatogenesis in seminiferous tubules, a marked decrease in the number of spermatogenic cells in the seminiferous tubules, marked decrease in the number of sperms and Leydig cells (Fig. 1D).

Testis sections in Ld HAP-NPs+CNPs group (G5) shown good improvement with only mild degeneration seminiferous tubules and an increased in the sperm and Leydig cells numbers when compared with Ld HAP-NPs and group (Fig. 1E). On the other hand; testis sections in Hd HAP-NPs+CNPs (G6) revealed mild improvement in the seminiferous tubules, with moderate atrophy, degeneration in the cytoplasm, and mild to moderate increase in sperms and Leydig cells numbers when compared with Hd HAP-NPs and group (Fig. 1F).

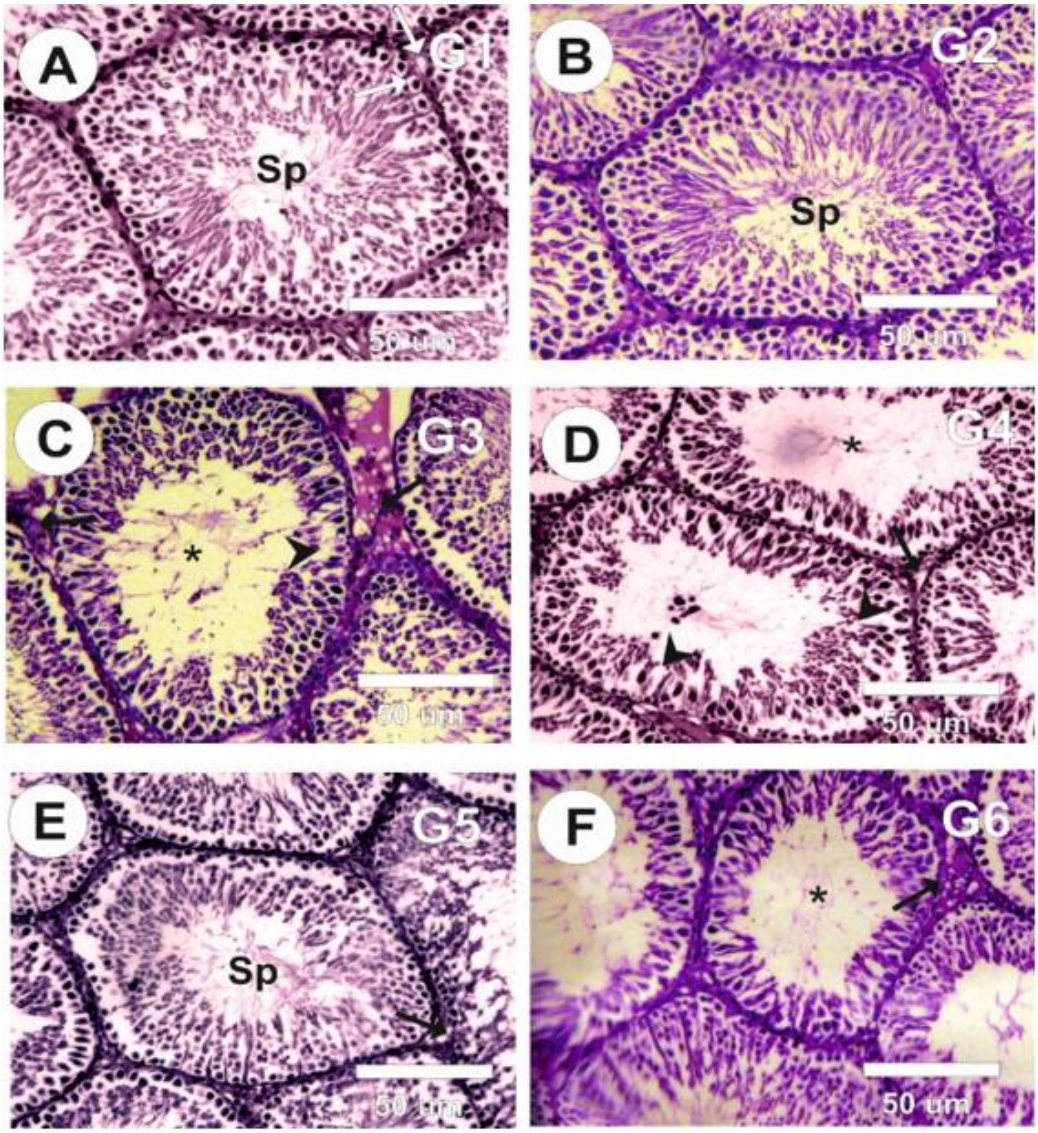

Fig.1: Photomicrographs of rat testis sections in the different groups stained with Haematoxylin & Eosin. **A&B**: Testes sections in control (G1) and chitosan nanoparticles (CNPs) (G2) groups showed a regular cycle of spermatogenesis in the seminiferous tubules and normal structure of interstitial cells (Leydig cells) and the lumen full with sperms (Sp). **C:** Testis sections in a low dose of hydroxyapatite nanoparticles (G3; Ld HAP-NPs) revealed a moderate decrease in the number of spermatogenic cells in the seminiferous tubules, mild vacuolar degenerative changes in the cytoplasm of the spermatogenic epithelium (arrowheads) and a marked decrease and abnormal distribution of spermatozoa (stars) was seen in the wide lumen of the seminiferous tubules and mild decrease in the numbers of Leydig cells (Black arrows). **D**: Testis sections in high dose of hydroxyapatite nanoparticles (G3; Hd HAP-NPs) revealed moderate disturbance with the irregular cycle of spermatogenesis in seminiferous tubules, a marked decrease in the number of spermatogenic cells in the seminiferous tubules, marked decrease in the number of sperms (stars) and Leydig cells (Black arrows). **E**: Testis sections Ld HAP-NPs+CNPs group (G5) shown mild degeneration seminiferous tubules and increased in the sperm (stars) and Leydig cells numbers (Black arrows). On the other hand; **F**: Testis sections in Hd HAP-NPs+CNPs (G6) revealed mild improvement in the seminiferous tubules, with moderate atrophy, degeneration in the cytoplasm, and mild to moderate increase in sperms and Leydig cells numbers (Black arrows).

DISCUSSION

Nanotechnology refers to a wide range of technologies that measure, manipulate, or incorporate materials and/or features with at least one dimension between approximately 1 and 100 nm. Despite the fact that there are a number of publications concerning the undesirable side effects of nanotechnology, the health and safety aspects of nanotechnology have lagged far behind its development.

Nanoparticles (NPs) usually ranging in dimension from 1-100 nanometers (nm) have properties unique from their bulk equivalent, with the decrease in the dimensions of the materials to the atomic level, their properties change, the nanoparticles possess unique Physico-chemical, optical and biological properties which can be manipulated suitably for desired applications (Wang *et al.,* 2011). In the current study; a significantly decreased the total testosterone, follicle-stimulating hormone (FSH) concentration, and luteinizing hormone (LH) levels in a low dose of hydroxyapatite nanoparticles (Ld HAP-NPs) and high dose of hydroxyapatite nanoparticles (Hd HAP+NPs) groups as compared to the control group.

Testicular oxidative stress causes a reduction in testosterone production, either because of the injury to the Leydig cells or to other endocrine structures like the anterior pituitary (Naughton *et al.,* 2001). Decreased Luteinizing hormones fail to stimulate Leydig cells to produce enough testosterone. Decreased follicle-stimulating hormone diminishes the release of androgen-binding protein (ABP) from the Sertoli cells, and thus, an overall decline in circulating testosterone occurs during severe oxidative stress. Decreased testosterone fails to regulate spermatogenesis properly to produce enough mature spermatozoa. It also fails to maintain the normal growth of accessory reproductive organs which play crucial roles in sperm maturation. As a prime regulator of male reproductive behavior, testosterone deficiency may lead to suppressed sexual behavior among men. Thus, by disrupting the endocrine reproductive functions, ROS may result in male infertility (Darbandi *et al.,* 2018)
Our results agree with Carlson *et al.* (2008) found that the nanoparticles can affect the leydig cell mitochondrial activity and thus reduce its secretion activity. In addition, nanoparticles also caused an increase in oxygen free radicals, such as superoxide and oxidation of molecules such as proteins, eventually reduce the leydig cell counts and decrease the production of testosterone, which is consistent with our findings. Dobashi *et al.* (2001) observed inhibition of LH-stimulated steroidogenesis by nitrogen oxide (NO) in Leydig cells. The stress-induced testicular NO also caused the decrease of steroidogenic enzyme activities (Kostic *et al.,* 2000). The results obtained by Guo et al. (2005) suggested that excessive NO compounds might directly inhibit the main second messenger cAMP that mediates gonadotropin action in the conversion of cholesterol to pregenolone in Leydig cell steroidogenesis, thus less testosterone was produced.

In the current study; treatment of rats with chitosan nanoparticles (CNPs) for 1 month after treatment rats with a low and high dose of hydroxyapatite nanoparticles (G5&G6 respectively) has significantly increased on total serum total testosterone, follicle-stimulating hormone (FSH) concentration and in luteinizing hormone (LH) levels when compared with hydroxyapatite nanoparticles group (G3&G4). In contrast, treatment of rats with chitosan nanoparticles (CNPs) group has no significant effect on total testosterone, follicle-stimulating hormone (FSH) concentration and luteinizing hormone (LH) concentrations compared to the control group.

The findings of this study showed significant elevation in serum alkaline phosphatase in rat groups intoxicated by hydroxyapatite nanoparticles. And, this study showed the elevation levels of serum aspartate aminotransferase (AST) under the effect of hydroxyapatite nanoparticles as marker cytotoxicity in groups In the current study; a significant increase in the levels of GOT and ALP in low and high dose of hydroxyapatite

nanoparticles (Ld HAP-NPs & Hd HAP-NPs) group compared to the control groups. On the other hand; a significant increase in the levels of serum GOT and ALP was detected in treated rats with a low dose of hydroxyapatite nanoparticles as compared to a high dose of hydroxyapatite nanoparticles. Treatment of rats with chitosan nanoparticles (CNPs) for 1 month after the treatment with a low or high dose of hydroxyapatite nanoparticles (as in G5&G6 respectively) has a significant decrease in the levels of GOT and ALP when compared with hydroxyapatite nanoparticles group (G3&G4).

Our results agree with Liu *et al*. (2005) who found that the intravenous injection of rod-shaped NHA results in acute increases in ALT, AST, BUN and ALP in rabbits. The intraperitoneally injected rod-shaped HANP results in no changes in AST, ALT and BUN in the serum of rats, but produces apoptosis in the liver cells and renal tubular epithelial cells. However, they were able to detect that the Wang *et al.* (2017) reported that; hydroxyapatite nanoparticles-Chitosan composite induced the significant elevation of liver and kidney functions in the serum of rats as well as the apoptosis in the liver and kidney tissues with no inflammation and necrosis at eight weeks of exposure by intraperitoneal injection.

Histological Examinations:

In this study; histopathological results revealed that testis sections in male rats in the chitosan nanoparticles (CNPs) showed the normal structure of seminiferous with a regular cycle of spermatogenesis. Testis sections of the low or high dose of hydroxyapatite nanoparticles intoxicated group revealed disturbance and not the regular cycle of spermatogenesis in seminiferous tubules, a significant decrease in the number of spermatogenic cells in the seminiferous tubules, marked thickened in the basement membrane, moderate vacuolar degenerative changes in the cytoplasm of the spermatogenic epithelium and in the Sertoli cells, decrease and abnormal distribution of spermatozoa was seen in the lumen of the seminiferous tubules and a significant decrease in the numbers of Leydig cells.

Testis sections in Ld HAP-NPs+CNPs group (G5) shown good improvement with only mild degeneration seminiferous tubules and an increased in the sperm and Leydig cells numbers when compared with Ld HAP-NPs and group. On the other hand; testis sections in Hd HAP-NPs+CNPs (G6) revealed mild improvement in the seminiferous tubules, with moderate atrophy, degeneration in the cytoplasm, and mild to moderate increase in sperms and Leydig cells numbers when compared with Hd HAP-NPs and group. That result was in accordance with Brohi *et al.* (2017) who concluded in their study that the use of hydroxyapatite led to a reduction in the productive functions of the testes proved by decreased both hormonal levels and spermatic count with histopathological changes of testicular tissue. Exposure to nanoparticles also affects the male reproductive system, including an impact on spermatogenesis from the point where it begins in the seminiferous tubules of the testes.

Conclusions

It is clear that HAP-NPs induced reproductive toxicity through; Over the generation of reactive oxygen species and TSH, FSH, LH, and Prolactine, Reduction in testes antioxidant enzymes (GST, SOD), and reduced glutathione (GSH). Showed induction in plasma levels of Na, K+, CL- and a significant increase in plasma ALP, GOTand TBARS, testes biochemical parameters. The presence of chitosan alone or/ in combination with HAP-NPs showed improvement in all of the above parameters in testes. Administration of Cs-NPs along with HAPNPs alleviated its possible reproductive toxicity.

Ethics Approval

All animals in this study were conducted under the criteria of the investigations and Ethics Committee of the Community Laws governing the use of experimental animals.

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