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Safe Use of Repeated Injection of 50nm Gold Nanorods on Reproductive Function in Male Albinorats: Histological, Morphometric, Hormonal and Oxidative Stress Parameters

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Abstract: *Gold nanoparticle have received a great focus todays. In this study, we investigated the effect of repeated administration of gold nanorods (AuNRs) on testicular function, sexual hormones and oxidative stress parameters. Twenty adult male albino rats were allocated into two equal groups each of ten rats. The first group received 1ml/kg saline I.P and the second group received 7.5µg/kg of AuNRs (50nm) I.P five consecutive days every month for two months. At the end of the second month, all rats from both groupswere sacrificed. Histopathological examination revealed no histopathological or morphometrical changes in testis,epididymis and seminal vecicle of AuNRs injected male rats. AuNRswere not detected by transmission electron microscope. Biochemical results detected that serum total and free testosterone significantly increased in AuNRs treated group in comparison with control group. Luteinizing hormone significantly decreased. Follicle stimulating hormone significantly increased. Cortisol hormone significantly decreased. Malondialdehyde significantly decreased while antioxidant enzyme as catalase, Glutathione peroxidase and superoxide dismutase were significantly increased in AuNRs in comparison with control group.We conclude that repeatedi.pinjecton of 7.5µg/kg 50 nmAuNRs five consecutive days each month for two months has not any testicular toxicity as well as it show antioxidant activity.*

Key words: *Repeated injection,AuNRs, Testicular function, sexual hormone, Cortisol, oxidative stress.*

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I. Introduction

Nanotechnology is an emerged and fast growing field of research with numerous applications in science and technology and manufacturing of new materials [1]. Nowadays, gold nanoparticles is one of the most widely used NPs in nanomedicine. Their easy way to synthesis, modification andstability make them promising NPs for biomedical applicationsincluding photothermal therapy, diagnosis, imaging, targeted therapy and gene delivery [2]. Among the gold nanoparticles is gold nanorods (AuNRs) which is the optimal photothermal therapy due to their surface plasmonresonance band at the near infra-red region [3], [4]. However, despite these advances toward potential therapeutic of AuNRs, a big concern has arisen on the possible toxicological effects of such molecules within biologic systems and the environment especially on reproduction[5]**.** The male reproductive system is known to be more sensitive to exogenous materials than other organ systems and has been classified as vulnerable in recent years[6].

Most of the adverse effects of NPs on male reproductive function are mainly due to modification of the testicular structure, impairment of spermatogenesis and alteration in the biosynthetic and catabolic pathways of testosterone[7]. Zakhidov et al[8]demonstrated that the gold nano-particle shows spermatotoxic effects and may impact mouse spermatogenesis. Behnammorshedi et al[9]observed degenerative changes in the seminiferous tubules after intraperitoneal injection of 0.5 cc gold nanoparticles at dose of 100ppm.In in vitro study**,** concludethat gold nanoparticles can reduce the sperm parameters such as motility and normal morphology and also increase the rate of sperm DNA damage. These deleterious effects were more obvious in maximum dose (200 µg/kg) and chronic phase (35days)[10]. Nanoparticles also caused an increase in oxygen free radicals, such as superoxidase and oxidation of molecules such as proteins[11]. In contrast with previous results, Lie et al [12]found no differences in testicular morphology between AuNPs-treated mice and controls.

The studies indicating the effects of gold nanoparticles on male reproductive system are very limited. Therefore, the aim of this work was to examine if gold nanorods can pass the blood testicular barrier after

repeated administration for two months, and could induce reproductive toxicity in male rats including histopathological changes, hormonal or oxidative stress parameters.

II. Materials and Methods

2.1 Preparation of gold nanorods (AuNRs)

AuNRs solution was prepared using seed-growth approach according to [13]. Briefly, the reaction was carried out through the following steps: 1) **Seeding solution,** 5 mL of (5×10^{-4} M) $HAuCl_4$ is added to CTAB solution (5 mL, 2×10^{-1} M) with gentle shaking and an orange solution was obtained. 600 µL of (10^{-2} M) ice-cold $NaBH_4$ is injected at once to the above mixture. The color of the mixture is instantly turned from orange to reddish brown. 2) **Growth solution,** 300µL of (4×10^{-3} M) $AgNO_3$ is added to a mixture of CTAB (5 mL, 2×10^{-1} M). To this aqueous mixture, 70 µL of (7.8×10^{-2} M) ascorbic acid is added which results in changing the growth solution from orange to colorless. Finally, 12 µL of the seed solution is injected at once to the growth solution. The color of the growth solution changes slowly within 30-45 min to the reddish purple. 3) **Gold NanorodsPEGylation:** Thiol-terminated methoxypoly-(ethylene glycol) (mPEG-SH) (MW=5000) was purchased from Nanocs Co. The raw nanorod solution was centrifuged at 15000 rpmfor 20 min to pellet the nanorods, decanted, and then re-suspended to 10 ml of deionized water to remove excess CTAB. 0.05 g of mPEG-SH were added to the nanorod solution. The mixture sat overnight at room temperature, then was centrifuged, decanted, and re-suspended in deionized water twice totemperature, then was centrifuged, decanted, and re-suspended in deionized water twice to remove excess CTAB and mPEG-SH.

2.2 Experimental design

Animals were purchased, maintained, and handled using the protocols approved by Ethical Committee of the Faculty of Veterinary Medicine, Zagazig University. Twenty mature male albino rats (150 g bwt) were obtained from the animal house at Zagazig University and kept in a 12 hour/12 hour light/dark cycles. Rats were randomly divided into two equal groups (10 per each). Control group received 1ml/kg saline i.p, while the second (Experimental group, AuNRs group) received 7.5µg/kg AuNRs (50nm) i.pfor five consecutive days each month(total doses equal ten) for two months [14]. At the end of the second month, all rats from both groups were sacrificed by cervical dislocation.

2.3Assessment of histopathological changes using Light and TEM microscope

After scarification, for histopathological studies, testis and epididymis from control and AuNRs injected rats were dissected out and divided into two parts. The first part was fixed in Bouin‘s solution for 48 h,then dehydrated in 95% ethanol and then cleared in xylene before embedded in paraffin according to [15]. Microsections (4 µm) were prepared and stained with Haematoxylin and Eosin stain and were examined under light microscope.

The second part of testis was fixed in 2.5% glutaraldehyde solution for 6 h, dehydrated through an ethanol series (70% for 15 min, 90% for 15 min, and 100% for15 min twice) and embedded in Epon/Araldite resin (polymerization at 65 ° C for 15 h). Thin sections were cut (70 nm thick), placed on grids and stained for 1 min each with 4% uranyl acetate (1:1, acetone/water) and 0.2% Reynolds lead citrate (water), air-dried and imaged under an 100 kV accelerating voltageusing JEOL JEM 2010 TEM .

2.4 Morphometric evaluation of seminiferous tubules and epididymis

Ten sections of seminiferous tubules that were round or nearly round were measured for control and AuNRs treated rats at each time point. The tubular diameter was measured at ×400 magnification using image analyzer Leica (DMLB) and Leica Qwin software. Different spermatogenic cells including spermatogonia, spermatocytes, and spermatids were identified according to their distinct morphological characteristics and counted**.** The minor and major axes diameter of the seminiferous tubule, and the epithelial thickness was calculated from the spermatogenic cells on the basement membrane through the sidelines cells of the tubules lumen[16].

Around 20 sections of ductus difference, the tubular diameter and epithelial height of the ductus difference and number of speramtozoa were measured ×400 magnifications using. The diameter of the ductus difference was measured, epithelium height an across the minor and major axes, and the mean diameter obtained.

2.5 Blood Sample collection, hormonal and biochemical analyses

Blood samples were collected through the orbital sinus. Blood was centrifuged at 1000 g for 15 min and serum samples were separated and stored at -20°C for hormonal and blood biochemical analyses.

For hormonal analysis, quantitative determination of total testosterone, free testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH) and cortisol were measured using enzyme immunoassay ELISA Kits

(BioCheck, Inc. Catalog No:BC-1115 for total testosterone, Diagnostics Biochem Canada Inc, USA, Catalog No: CAN-FTE-260 for free testosterone,Sino Gene Clon Biotech Co., Ltd for luteinizing hormone (LH, Catalog No:SG-20701), follicle stimulating hormone (FSH, SG-20702)and cortisol (Catalo No:SG-20892). The assay is based on a solid phase enzyme –linked immunosorbent assay with sensitivity of 0.05ng/ml, 0.018pg/ml, 2-40ng/L, 0.5-10IU/L and 0.6ng/ml for total testosterone, free testosterone, LH, FSH and cortisol, respectively.
For biochemical analysis, serum levels for oxidative stress parameters including malondialdehyde (MDA) as thiobarbituric acid-reactive substances were measured according to the method [17]. Catalase as an antioxidant enzyme was determined following the method [18]. Glutathione peroxidase assay was evaluated [19]. Superoxide dismutase was measured according toNishikimi et al[20].

2.6 Statistical analysis

Values are expressed as mean ± SEM (Standard error of Mean) and statistically analyzed using SPSS program, version 16 (SPSS Inc, Chicago, IL, USA). Data are analyzed by performing independent t-test. Differences were considered statistically significant when $P \leq 0.05$.

III. Results

3.1Body weight and clinical signs

The effect of repeatedi.p injection of AuNRs on body weight is presented in Table 1.Data showed thatrepeatedAuNRs treated group was healthy with normal water and feed intake and showed noweight loss or apparent toxicity in comparison with control group. No mortality were recorded along the duration of the experiment.

Table 1 Body weight variation between control and AuNRs injected male rats at the initial and the final time of experiment.

Table 1: Effect of repeatedi.p. injection of AuNRs (7.5µg/kg bwt) five consecutive days per month and for two months on body weight in male rats (Mean±SEM).

Group	Initialbodyweight/g	Final body weight/g
Control	130.70 ± 0.26	246.06 ± 2.01
AuNRs	131.23 ± 0.24	247.45 ± 2.35

3.2Histopathological examination of the testes and epididymis

Testicular and epididymal sections from control and AuNRs administration for consecutive days over a period of two months in male rats were compared to investigate the potential histolopathological alterations after repeatedAuNRs injection. Cellular structures of Sertoli cells, germinal cells and the intertubular spaces containing the Leyding cells were carefully examined to detect AuNRs, and noAuNRs were identified on the examined slides. Also, compared with control group (Fig. 1 A), testes in AuNRS injected rats were appeared well structurally preserved, without any histopathological alterations such as seminiferous tubule necrosis or histological disorganization at the level of germinal cells and spermatozoids, as well as the intertubular space or in the Leyding cells were observed in testis of AuNRs injected rats (Fig. 1B) versus the control group.In addition, in repeatedAuNRs injected rats, histological evaluation of the epididymal ducts showed normal well preserved histological structure including the pseudostratified epithelial lining and normal sperm density in their lumina(Fig. 1D) versus the control group (Fig. 1C).

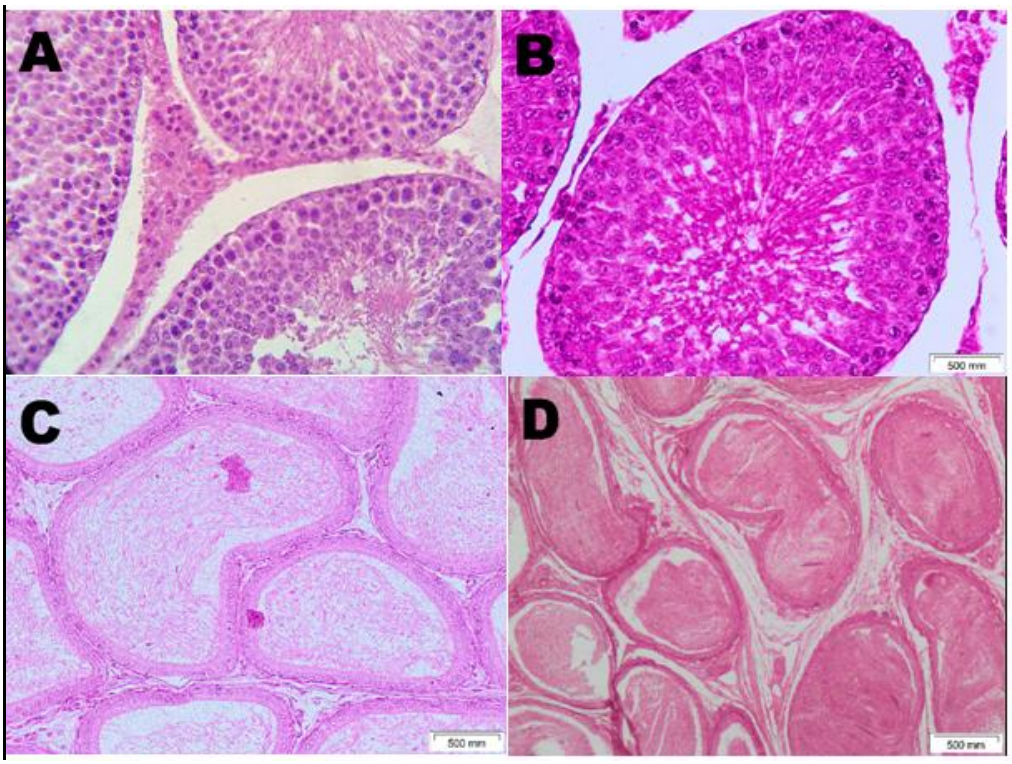


Fig. 1: Photomicrograph of cross-sections of H&E stained testis from control (A) and repeatedAuNRs injected groups (B, 400x). AuNRs injected group (B) showing normal histomorphological structure in comparison with control group. Epididymis in control group showed normal tubular structures with preserved epithelial lining, interstitial tissue and normal number of stored sperms(C). RepeatedAuNRs injection for five consecutive days per month over a period of two months showing normal epithelial lining and presence of abundant spermatozoa in the lumen ofepididymal ducts (D) versus control group(C) (H&E200x)

3.3Transmission electron microscope analysis

In the present study, AuNRswere not detected in testes and seminal vesicle by TEM. In comparison with control group, AuNRs treated groups showed seminiferous tubules with normal spermatogonia, spermatocytes, spermatids and spermatozoa and normal cytoplasmic organelles (Fig. 2).

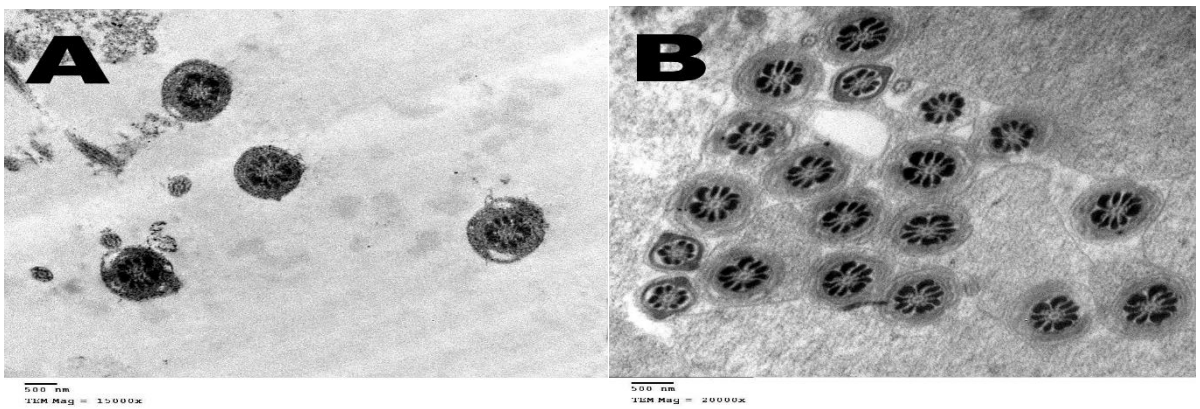


Fig. 2: Photomicrograph for testes from control group (A) showing normal sperm tail in control non treated animals; B) AuNRs treated group after repeated administration of AuNRsshowingnormal sperm tail.

3.4Morphometric Studies

The proportion of each cell type lining the seminiferous tubules seemed to be normal for control and AuNRs injected is presented in Table 2.Results indicated that the minor diameters of the seminiferous tubule and the height of epithelial lining of the seminiferous tubule, and average number of spermatogonias, spermatocytes, spermatids and spermatozoa were significantly ($P<0.05$) higher inrepeatedAuNRs injected male rats when compared with the control one.

Table 2: Morphometric analysis of long and minor axis of somniferous tubules in testes of control and after repeated administration AuNRs in male rats (Mean±SE)

Group	Diameter of seminiferous tubules (µm)		Epith. heights	Cell count			
	Long axis	Minor axis		Spermatogonia	Spermatocytes	Spermatid	Spermatozoa
Control	307.9±6.2	211.6±3.9	34.8±1.8	43.6±0.9	55.0±1.6	21.5±0.8	23.5±1.0
RepeatedAuNRs	302.5±3.7	246.4±1.0*	55.0±1.3*	63.6±2.4*	62.8±1.6*	80.2±2.2*	89.6±2.3*

*significantly differ from their respective controls at P<0.05.

Effect of repeated administration of AuNRs on long and minor axis of the epididymal ducts and number of spermatozoa is illustrated in Table 3. With regard to epididymis, AuNRs injected rats detected significant (P<0.05) increase in the diameter of major and minor axis of seminiferous tubules in comparison with control group. Morphometric studies of the height of the epithelial lining of epididymal ducts showed significant (P<0.05) increase in AuNRs injected rats versus the control group. In addition, the mean number of spermatozoa elicited significant (P<0.05) increase after repeated injection of AuNRs when compared with the control group.

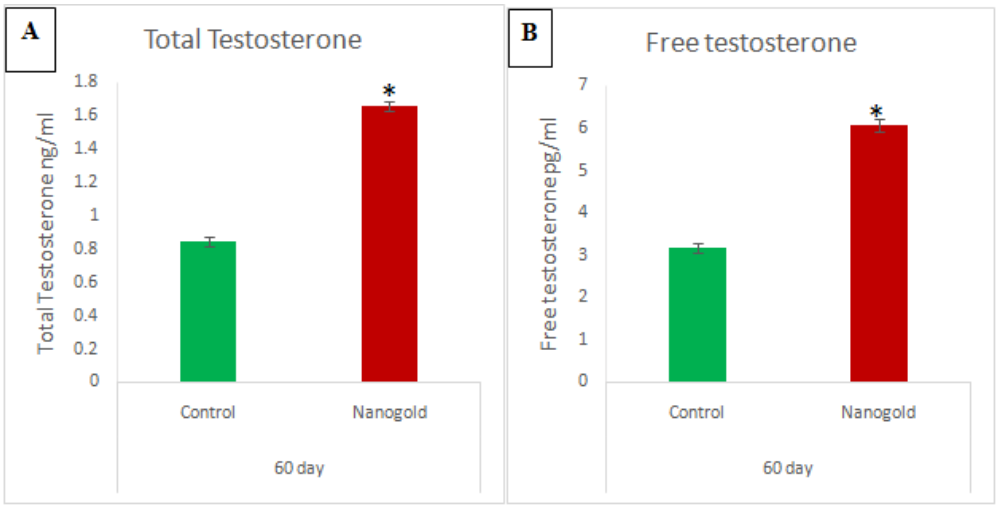
Table 3: Morphometric analysis of long and minor axis of the epididymal ducts and number of spermatozoa is illustrated in male rats in testes of control and after repeatedAuNRs injected in male rats (Mean±SE)

Group	Diameter of epididymalductus (µm)			spermatozoa
	Long axis	Short axis	Epith height	
Control	253.43±12.76	122.41±4.69	24.6±1.88	31.4±3.53
RepeatedAuNRs administration	281.305±3.7*	211.9475±3.6	35.73±0.6*	46.375±0.3*

*significantly differ from their respective controls at P<0.05.

3.5Effect of AuNRs on serum hormonal levels

Results indicated that AuNRs injection to male rats significantly (P≤0.05) increased serum total and free testosterone compared to control animals (**Fig 4A, B respectively**). Serum LH hormone recorded significant (P<0.05) decrease in AuNRs injected rats compared with the control group (**Fig 4C**). With regard to serum FSH hormone, AuNRs injected rats showed significant (P<0.05) increase versus the control group (**Fig 4D**). In contrast, serum cortisol level significantly (P<0.05) decreased in comparison with the control group (**Fig 5**).



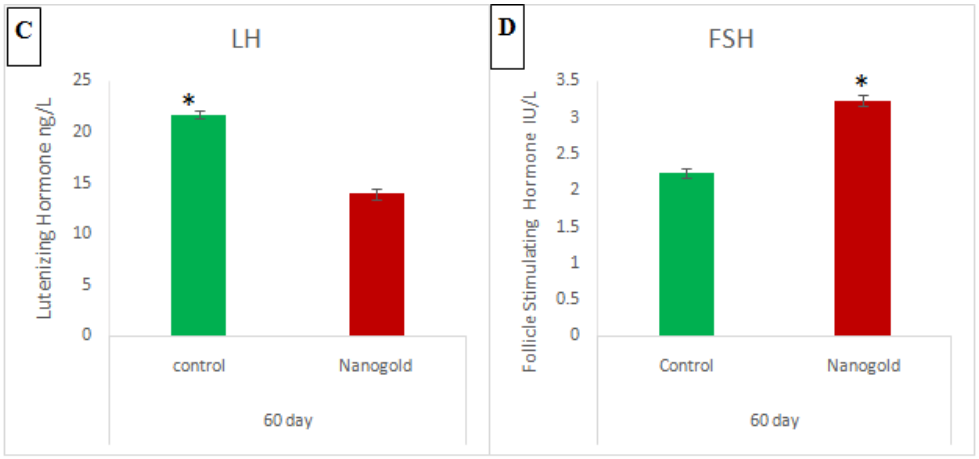


Fig3: Histograms demonstrating the effect of repeatedi.p. injection of 7.5µg/kg AuNRs five times each month for two months on serum total testosterone (A), free testosterone (B), luteinizing hormone (C) and follicle stimulating hormone (D) levels compared with the control group.* Significantly differ at $P \leq 0.05$

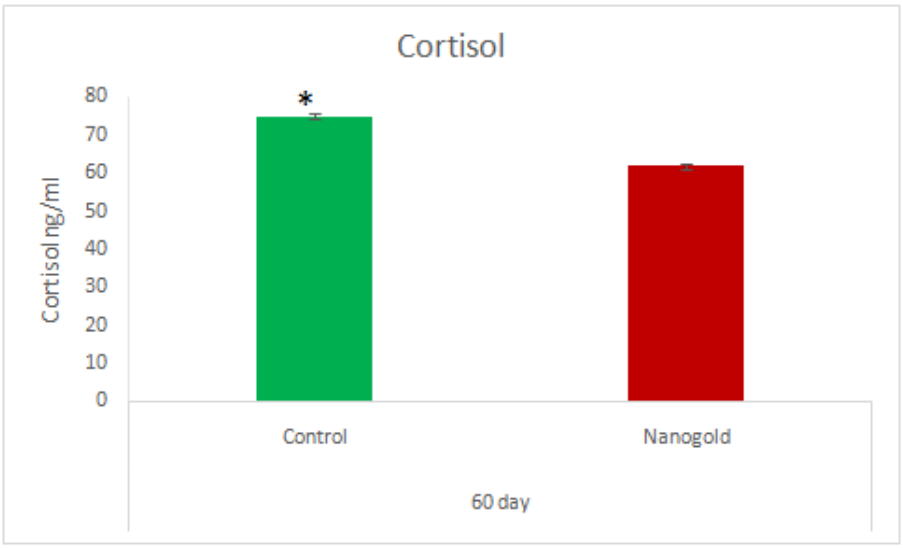


Fig4: Histogram representing the effect of repeatedi.p. injection of 7.5µg/kg AuNRs five times each month for two months on serum cortisol level. * Significantly differ at $P \leq 0.05$.

3.6Effect of AuNRs on oxidative stress parameters

Data presented in figure 5 illustrated changes in oxidative stress parameters in serum of control and repeated administration of AuNRs in male rats. Resulted revealed significant ($P \leq 0.05$) decrease in serum malondialdehyde as lipid peroxidase parameter in serum of AuNRs injected male rats when compared with the control animals (Fig5A). While, antioxidant enzymes as catalase, glutathione peroxidase and superoxide dismutase were significantly ($P<0.05$) increased after repeated administration of AuNRs group in comparisonwiththe control one (Fig5B,C andD, respectively).

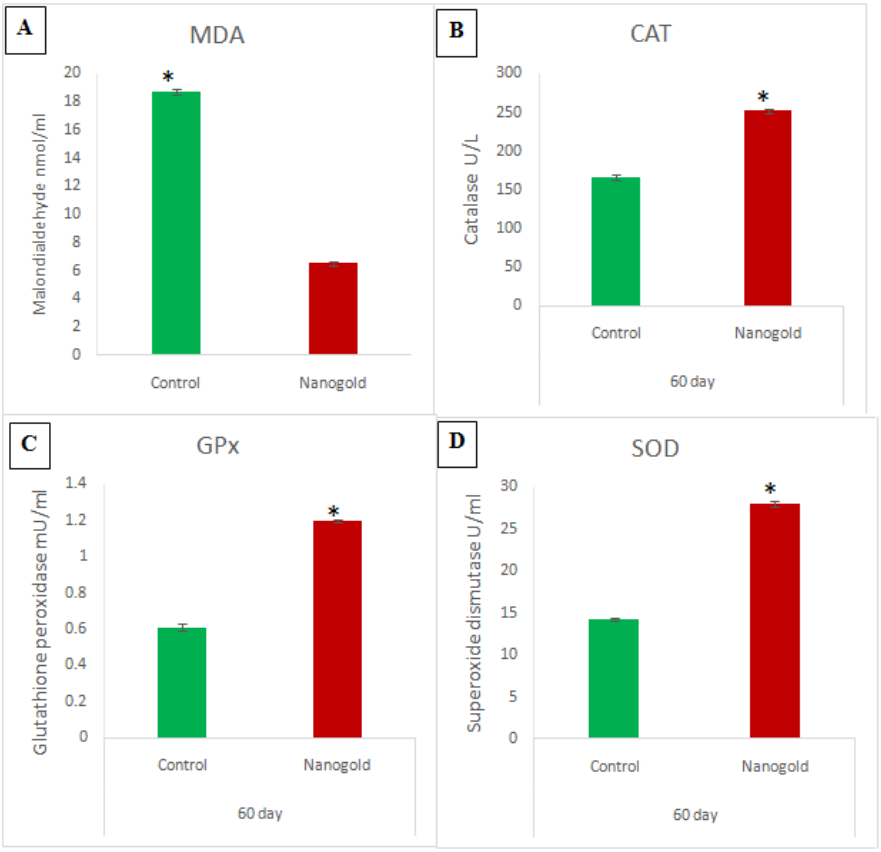


Fig 5: Effect of repeatedi.p. injection of AuNRs 7.5µg/kg bwtfive times each month for two months on serum malondialdehyde (A), catalase (B), glutathione peroxidase (C) and super oxide dismutase (D) levels versus the control group. Data are expressed as Mean ± S E and analyzed by independent t-test. * Significantly differ at $P \leq 0.05$.

IV. Discussion

Gold nanoparticles have many advantages over other metal nanoparticles due to their biocompatibility and non-cytotoxicity [21]. Therefore, it is necessary to evaluate the effect of repeatedi.p injection of AuNRs on male reproductive function by histopathology, morphometric analysis and determination of serum sex hormones and oxidative stress parameters.

In our work,repeated administration with AuNRsfor five consecutive days for two months did not elicit any clinical signs of toxicity such as fatigue or decrease in body weight. These findings are concomitant with previous results[22] ,[14]. Also, when male rabbits were received intravenous injection of AgNPs at 0.6 mg/kg (average size of 45 nm),AgNPs did not affect body weight, body temperature, or morphology of the testes [23].Similarly, using large animal models, Abdoon et al [24]found that intrarumoral injection of 50nm AuNRs did not affect body weight or induced and signs of toxicity in dogs and cats.

Furthermore, in this work, repeated injection of AuNRs for two months did not elicit any morphological or histopathological changes in the testes and epididymisof injected rats compared with control. These findings are in accordance with Li et al [12]who found no differences in testicular morphology between AuNPs-treated mice and controls, and there was no apparent increase in germ cell apoptosis between groups. No testicular histological abnormalities after intraperitoneal injection of pegylated gold nanoparticles in mice [25]. Regarding testicular bio-distribution, transmission electron microscope cannot detect 50 nm gold nanorods after repeated injection for two months. This study is in accordance withDe Jong et al [26] who demonstrated that 50-, 100-, and 250-nm particles were unable to reach testes. Previous biodistribution studies showed that small amounts of sub-micrometric particles reached the testes, depending on experimental conditions[27]. After intravenous injection of 80µg of 10-nm gold nanoparticles in rats, 55 ng/g was found in the testes, which means 1 out of 100,000 particles [26]. The size, shape, surface area, number, and clearance of GNPs play a key role in toxicity, and the distribution and accumulation of GNPs in the different rat organs may be mediated by dynamic protein binding and exchange**[**28].Nanoparticle size could be a key point for differences in testicular biodistribution [27]

Meanwhile, administration of AuNRs for two months was accompanied by significant increase in morphometric analysis of testicular function including higher in testicular function when compared with the control group. Also, the major, minor axis of epididymal ducts and height of the lining epithelium were higher in AuNRa group than the control one. According to our knowledge, this is the first study on the morphometric changes in testicular function after AuNRs injection. These changes in testicular function could be attributed to the significantly higher free, total testosterone and FSHand lower LH levels in AuNRs injected rats when compared with control one. These results go hand in hand with Li et al [12]who intravenously inject 45mg/kg NH2@Au-NP at 48h intervals for 5 days and detected significant increase in testosterone levels. Also,Yoshida et al [29] reported that male mice intratracheally administered (0.1 mg/mouse for 10 times every week) with three sizes (14 and 56 nm) of carbon black nanoparticles showed elevated serum testosterone levels (not for nanoparticles with the size of 95 nm). It is well documented that testosterone is essential for male fertility and the maintenance of spermatogenesis [30]. Testosterone also has been shown to rapidly activate a series of kinases in Sertoli cells that are known to regulate spermatogenesis[31]. In contrast, chronic administration of gold nanoparticles has a deleterious effect on sperm parameters such as motility and normal morphology and secondly affect sperm chromatin remodeling and cause the increase instability of chromatin and also increase the rate of sperm DNA damage [10]. In addition, Carlson et al[11]found that silver nanoparticles can affect the leydig cell mitochondrial activity and thus reduce its secretion activity. These differences may be related to the type, shape, dose, or the size of the gold nanoparticles used, the duration of exposure and route of administration that may be responsible for the large variation in the hormones profile.

Cortisol is released in response to stress and low blood-glucose concentration[32]**.**In the current work, repeated administration of AuNRs over two months significantly decrease serum cortisol levels when compared with control animals.These changes in cortisol levels were associated by a significant decrease in serum malondialdehyde as lipid peroxidase parameter and significant and significant increase in antioxidant enzymes catalase, glutathione peroxidase and superoxide dismutasevalues when compared with control one. This finding indicate that gold nanorods has an "anti-stress" effect by lowering serum cortisol hormone.Similarly, Elshaer et al [33]reported that CeO_2NPs (at 0.5 or 5 mg/kg/week, intraperitoneally for five weeks) caused a significant reduction in cortisol hormone.In concomitant with this results,13-nm and 50-nm AuNPs showed their antioxidant effect by increasing the level of antioxidant enzyme catalase,which was considered as a primary antioxidant responsible for the direct elimination of ROS generated and attenuated the production of MDA [34].AuNPs has an anti-oxidative agent, by inhibiting the formation of ROS, scavenging free radicals; thus increasing the anti-oxidant defense enzymes[35]**.** In addition, gold nanoparticles demonstrated beneficial effects on the muscle healing process, such as reducing the production of ROS, balancing increased antioxidative enzyme activities, and reducing expressions of pro-inflammatory molecules[36].In contrast,intraperitoneal administration of 10 nm GNPs for exposure duration of 3 and 7 days resulted in significant increase in malondialdehyde (MDA: lipid peroxidation) values and significant decrease in superoxide dismutase (SOD) in rat liver, lung, heart, and kidney compared with the control animals[28]**.** Also, Khan et al[37]reportedsignificant increases in MDA levels in the liver of rats treated with gold nanoparticles 50 µl of 10 nm sized, i.p daily for 3 and 7 days. This contradictory could be related to the particles size.

V. Conclusion

We conclude that repeated administration of 7.5 µg/kg five consecutive days each month for two months is safe on the reproductive function. In addition, it has antioxidant effect

Compliance with ethical standards

Ethics approval

All the procedures performed in the current study were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996) and approved by ZU-IACUC (2/F/27/2018).

Conflict of interest

The authors declare that they have no conflict of interest.

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