Evaluation of histopathological and ultrastructural changes in the testicular cells of Wistar rats post chronic exposure to gold nanoparticles

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Gold nanoparticles (GNP) have numerous therapeutic potentials due to their ability to cross blood barriers. However, limited data is available showing GNPs crossing the blood testicular barrier. Here we report results of chronic exposure (90 days) to GNPs ranging in size 5 to 20 nm in male Wistar rats. Histopathological and transmission electron microscopy (TEM) analysis show GNPs distributed and accumulated in majority of the testicular tissues. This shows the ability of GNPs of specific sizes to cross the blood testicular barrier effectively, indicating possible insignificant toxicity to spermatogenesis process due to chronic exposure. Thus, GNPs of smaller size can possibly be used for various therapeutic and diagnostic purposes.

Keywords: Gold nanoparticles (GNP), histopathology, blood testicular barrier, Wistar rats

Introduction

Advancements in the field of nanotechnology have helped usage of nanoparticles in various fields of medicine including diagnosis¹, imaging² and therapy³. Nanoparticles (NPs) are easy to synthesize and their physical properties (size and shape) can also be altered easily. Moreover, NPs can be tagged with specific ligands (drugs, antibodies or aptamers) for enhanced targeting applications⁴-⁶. Although the potential of nanoparticles in the field of medicine is promising, the lack of documented evidence of toxicological effects of these nanoparticles is concerning⁷,⁸. It is therefore of critical interest to study the effects of nanomaterials on animal systems, their patterns of bioaccumulation in various organs (including reproductive organs) and subsequent biological consequences. Among the various classes of nanoparticles gold based ones attract considerable attention because of their unique properties pertaining to therapeutic potential and drug delivery mechanisms⁹. Ayurveda, the ancient Indian system medicine has been using suvarnabhasmas, allegedly containing Au-nanoparticles, as a potent drug in treating many diseases¹⁰. By altering the size and shape of gold nanoparticles (GNPs) their plasmonic resonance can be shifted to the near infrared spectrum¹¹, at which the biological materials have low absorption (optical therapeutic window). These GNPs altered for absorption spectrum have high tissue penetration and can be used in imaging and cancer therapies¹². Decreasing the size of GNPs increases the surface to volume ratio which manifests novel changes in physical and chemical properties of these NPs. Moreover, the decrease in size is expected to affect the GNP’s interaction with the cells and tissues. Size-dependent distributions of GNPs in various organs have been studied in vivo¹²-¹⁷. Although, previous studies in mice based on oral administration of GNPs have documented the inverse correlation between size of the particles and absorption or distribution of GNPs in various tissues and organs, research on toxicity of GNPs are scant and more specifically on reproductive toxicity. There are only a few studies documenting the individual toxicity of GNP in vivo, therefore it would be of interest to evaluate the toxicity of gold nanoparticles¹⁸. Currently, there are a few data available regarding the accumulation of nanoparticles in vivo after their repeated administration. Here, we report the effect of chronic exposure of GNPs in rat testicular tissue. We studied the ability of
GNPs to cross blood testes barrier by histopathological and ultra structural analysis transmission electron microscopy (TEM). For the first time we present here the GNP accumulation in testicular tissues and their proliferation following chronic exposure.

Materials and Methods
Gold nanoparticles (GNPs) were freshly prepared and confirmed by spectrophotometer. GNPs with average diameter of about 15 nm were chemically synthesized in the Department of Biotechnology, MGM Institute of Health Sciences, Navi Mumbai, India\textsuperscript{19,20}. All the reagents were used of analytical reagent grade. To determine the size, shape, and aggregation state, GNPs were analysed by TEM and UV–Visible Spectrophotometer. The kinetics of particle development was followed at $\lambda = 519$ nm. UV–visible spectroscopy using 1 cm quartz cuvette on a Thermo-Scientific, Evolution 201 series spectrophotometer. TEM was used to characterize the particles. Sample was prepared by placing a drop of solution containing nanoparticles on a carbon-coated grid. Transmission electron microscopy (Tecnai G\textsuperscript{2}, 120kV TEM – FEI) at a voltage of 120 kV.

Animals and Treatment Groups
Ten to twelve weeks old adult male Wistar rats, weighing approximately 150 ± 5 gm, were obtained from the Haffkin Institute, Mumbai, India. The animals were housed in humidity- and temperature-controlled ventilated cages on a 12 h day/night cycle, with free access to standard laboratory food and tap water. The animals were randomly divided into 2 groups of 8 animals each. One group served as control and received ordinary drinking water. Experimental group was administered GNPs orally at a repeated dosing of 20 $\mu$g/g for duration of 90 days. For oral administration of the dose of nanoparticles gavage was prepared by bending the tip of the metal tube and coating it with silicone. Animal experiments were carried out with the approval of Institutional Animal Ethics Committee of the MGM Medical College, Navi Mumbai. Rats were sacrificed 12–16 h after the drug administration on the final day of drug administration. The right and left testes were removed, weighed and fixed in 10% formalin for histological examination.

Histopathology
Rats were sacrificed by cervical dislocation, and the testes were removed and fixed in 10% formalin solution. Histopathological analysis was performed as explained previously by Thakur et al\textsuperscript{20}.

Ultrastructural Analysis
Ultrastructural analysis of testes for presence or absence of GNP’s was performed using TEM as explained previously by Thakur et al\textsuperscript{20}.

Results

Synthesis and Characterization of GNPs
GNPs of average size of 15 nm were synthesized by reduction of H\textsubscript{2}AuCl\textsubscript{4} with sodium citrate according with the procedure described by Turkervich et al\textsuperscript{19} the solution turn bright red after the addition of 2 ml of sodium citrate(Fig. 1a). The GNPs synthesized showed a defined plasmon resonance peak at 520 nm. This gives brilliant red colour to gold nanoparticles (GNPs), varies according to their size distribution. The colloidal gold synthesized in experiment exhibited absorption max at 520 nm (Fig. 1a). The extremely low absorbance at wavelengths greater than 600 nm indicated their well dispersed state in solution\textsuperscript{20}. GNPs were further analyzed by TEM analysis of the colloidal gold solution. The size of gold nanoparticles has been determined by measuring the diameter of whole particles on TEM images (Fig. 1b). The average diameter of colloidal gold was average size 15 nm. Moreover the TEM images show that most of the gold nano-spheres are round or spherical in shape at different scale bar (Fig. 1c & d) showed particles of small sizes (15.0 ± 5 nm) with regular shapes and narrow size distribution. The size distribution of the GNPs was determined.

The major objective of these studies was to investigate if GNPs treatment produces toxicity due to chronic exposure to GNPs in the rats. For this purpose, it was necessary to determine if GNPs cross blood testes barrier if so, their biodistribution and its possible consequences. Our result indicates indicate that despite the prolonged exposure to nanoparticles, no mortality, morbidity or gross behavioural changes were observed in rats receiving GNPs at the doses studied. Our finding is similar to previous short exposure studies where no mortality and gross behavioral changes were observed\textsuperscript{21}. There was no effect observed on the body weight. In tissue size no evidence of atrophy, congestion, or inflammation could be observed in the treated animals. These observations indicate that extensive inflammation might not be induced in the rat after administration of gold.
nanoparticles, which is confirmed by the macroscopic morphological examination.

**Testes Histology**

The rats from control group displayed normal testicular architecture indicating seminiferous tubules of various shapes and sizes. Stratified epithelium (Fig. 2a) was visible with an orderly arrangement of germinal cells (spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids in addition to spermatozoa) and Sertoli cells. These tissues were separated from one another by a delicate connective tissue stroma (interstitial tissue) containing interstitial Leydig cells.

In contrast, sections in testes of treated rat displayed seminiferous tubules in various shapes and sizes with mild sloughing of germ cells and detachment from the basement membrane. The degenerative tubules were lined by very few spermatogenic cells (Fig. 2b).
Transmission Electron Microscopy: Effects on the Ultrastructure of Testes

Testicular Ultrastructure of Control Group

Electron micrographs of control group of animals showed normal germ cells development starting from spermatogonia to spermatids. Sertoli cells had irregular large nucleus, prominent nucleolus and cytoplasmic extension from the basal lamina of the seminiferous tubule to the lumen supporting the germ cells (Fig. 3A). Cytoplasm of the Sertoli cells contained prosecretory granules, rosettes of glycogen granules and free ribosome (Fig. 3A). Basal lamina was surrounded by myoid cells having elongated nucleus (Fig. 3B). Spermatogonia with a round or oval nucleus and patchy chromatin materials were observed near to the basal lamina (Fig. 3C). In control group animals, a number of spermatocytes and round spermatids were present towards luminal part of seminiferous tubules (Fig. 3D). The spermatocytes showed round prominent nucleus with distinct chromatin networks and well-defined nuclear membranes. Round spermatids had smaller nuclei and their cytoplasm had mitochondria (Fig. 3E). The testes of control group showed different stages of elongating and elongated spermatids with normal ultrastructure (Fig. 3F). Elongated spermatozoa also presented with normal ultrastructure with the characteristic shape of head, intact cell membranes, acrosomes, and homogenous nuclei. The cytoplasm organelles of different cells had no evident morphological abnormalities.

Testicular Ultrastructure of Treated Group

Considerable bioaccumulation of GNPs was found in the testes of treated group of animals. This indicates that the GNPs can cross the blood testes barrier and accumulate in the testes tissue. However, no preferential site of accumulation was seen. Electron micrographs of testes tissues showed abundant gold nanoparticles (GNPs) aggregated in interstitial spaces of the testes (Fig. 4A, B & C). GNPs could be clearly seen in large aggregates near the Leydig cells. GNPs were detected crossing the outer membrane of the Leydig cells (Fig. 4D). Interestingly enough, GNPs did not appear to induce any apparent toxicity to the Leydig cell as they appeared structurally intact. Leydig cell of treated group showed normal cytoplasm and nucleus containing prominent rim of heterochromatin attached to the nuclear membrane.

In experimental group, majority of the developing germ cell were found to be structurally intact. However, some spermatocytes and spermatids presented with vacuolated cytoplasm (Fig. 4E) and disrupted nuclear membrane. Membrane bound GNPs were found to be present close to the developing spermatids. Ectoplasmic specializations were disrupted in those germ cells (Fig. 4F). Damage such as chromatin clumping and fragmentation were also seen in these germ cells (Fig. 4G). Aggregates of GNPs were largely entrapped in vesicular structures and distributed all over the Sertoli cell cytoplasm (Fig. 4H). Some of the spermatogenic cells showed degenerative changes having deformed nucleus (Fig. 4I).

Fig. 3 — Transmission electron microscopy images of control group testicular cells: A. Sertoli cell nucleus (N) with nucleolus (Nu) and cytoplasm (Cy). Scale bar = 2 µm, B. Myoid cells (My). Scale bar = 2 µm, C. Spermatogonia cell. Scale bar = 2 µm, D. Spermatocytes. Scale bar = 2 µm, E. Round spermatid. Scale bar = 1 µm, F. Elongated spermatid. Scale bar= 0.5 µm.
Electron micrographs of the unstained ultrathin sections of testes tissue from GNPs treated group showed some very interesting results. In these sections testes ultrastructure was faintly visible whereas, aggregates of GNPs were clearly visible distributed all over the interstitial spaces and Sertoli cell epithelium. This was due to the fact that, gold being heavy metal; GNPs can scatter electrons and generate better contrast under electron microscope. GNPs could be clearly located inside the testes and exclude the probability of any kind of artefacts. We could clearly see the GNPs inside the Leydig cell nucleus which suggest that GNPs can cross nuclear pore and bind to the chromatin (Fig. 5A). There were abundant GNPs present in the Sertoli as well as in germ cell cytoplasm entrapped in lysosomal bodies (Fig. 5B, C, D, E, and F). The ultrastructure of the testicular cell was not significantly hampered though few abnormalities were noticed such as elongated spermatid having deformed head and tail (Fig. 5G & H). The other remarkable finding was the presence of GNPs in the elongated sperm nucleus (Fig. 5I). In some cases of the developing germ cells, cytoplasm was also noticed vacuolated with presence of GNPs (Fig. 5J).

**Discussion**

The ability to engineer nanoparticles with desired characteristics has allowed nanoparticles to find increased applications in various fields of medicine. Studies have documented the bio distribution and functions of nanoparticles as a function of their size and shape in vitro and in vivo. Among nanoparticles gold based ones are gaining interest due to certain unique properties. Although, gold nanoparticles are considered inert and biocompatible, limited studies in mice have shown dose dependent toxicity including erythrocyte cell death and nephrotoxicity. In addition, histopathological studies of testicular tissues from mice acutely exposed to GNPs have shown them crossing the blood testicle barrier. Our study was aimed at exposing rats to chronic doses of GNPs and to study their ability to cross the blood testicle barrier and to study if there is any reproductive toxicity. This is the first report showing the effects of chronic (90 days) exposure of gold nanoparticles on rat germ cells in the peer-reviewed literature. Similar type of post chronic exposure of GNP in testicular cells of Wistar rat was studied by our team.
The structural and functional integrity of testes is essential for normal reproductive capacity in males. Studies show germinal cells are extremely vulnerable to the interference of external agents. Among them nanoparticles pose a greater concern due to their ability to cross the blood testicle barrier. Previous studies in mice show GNPs crossing the blood testicle barrier after acute exposure with the same. Our TEM analysis of rat testicular tissues post 90 days exposure to GNPs shows accumulation of and distribution of GNPs in majority of the testicular tissues. For this study we used GNPs size range of 15 nm, and our ultrastructural analysis reveals the presence of the same sized nanoparticles in the testicular tissues. This indicates GNPs of the above-mentioned size effectively crosses the blood testicle barrier. Moreover, after crossing the blood testicle barrier the GNPs was found present in the seminiferous tubules and various types of cells in the testes including spermatocytes, Leydig and Sertoli cells. Distribution of GNPs in testicular tissues was shown to be dependent on time and size of exposure. One study, showed significantly higher distribution of GNPs after a sixty-day exposure compared to a week’s exposure. Similarly, the distribution of GNPs sized between 10 and 50 nm are effective compared to large sizes (>50 nm). Our results with GNPs of average size of 15 nm for 90 days in rat testicles corroborate with these previous studies. Prolonged exposure to GNPs could lead to reproductive toxicity through degeneration of testicular tissues. As seen by histological analysis degeneration of testes occurs by sloughing of the germinal epithelium from the basement membrane and reduction in the population of the germ cells. Our results from TEM analysis of treated rat testes sections also show mild degenerative changes. Studies have shown the effect on germ cells and Leydig cell viability post exposure to TiO2, silver and Cb based nanoparticles. Moreover, GNPs were shown to reduce sperm motility. Cormier et al pointed out that particles will affect the human reproductive system, but what kind of nanoparticles (NPs) or (non NPs) affect the human reproductive system were not pointed out. However, it is demonstrated that not all nanoparticles contribute towards reproductive toxicity. Also supplements were shown to have a positive effect on sperms in goats.

Thus it becomes imperative to investigate GNPs induced toxicity depending on the size and time of exposure. Even though we observed mild degeneration of testicular tissues, our histopathological results show presence of all the different stages of testicular cells. They appear healthy and they were highly similar with untreated tissues. Moreover, the testicular tubules remained healthy and normal and also, the relative proportions of spermatogenic cells were not affected. Thus, our results show the ability of the GNPs to cross the blood testicle barrier and prove the distribution of nanoparticles is dependent on their size and time of exposure. The less severity in toxicity inspite of distribution and accumulation of GNPs could possibly be due to the size of the nanoparticles. Further studies would be necessary to conclusively prove GNPs of this size could be safely used for treatment and other purposes at chronic doses. Understanding the effects of nanotoxicology on male reproductive organ will be beneficial in understanding the problem of male infertility and setting up plans to solve it.

References
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