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ORIGINAL ARTICLE

Effects of Subacute Exposure to Gold Nanoparticles on Germ Cells of Zebrafish (*Danio rerio*): An *in vivo* Study

[1]Navami Dayal, [2]Mansee Thakur, [3]Poonam Patil, [4]Niharika Swain, [5]Dattatraya Shankar Joshi

ABSTRACT

Although *in vivo* studies have been modeled using higher mammal systems, the lower vertebrate zebrafish *(Danio rerio)* has gained tremendous attention as a model system. Gold nanoparticles (GNPs) attract the interest of scientists due to their promising potential applications in medicine and targeted drug delivery. The purpose to use GNPs *in vivo* is that gold in bulk form is nontoxic and apply the positive potentials of nanoparticles. Bulk gold is century-long accepted as a safe-to-use metal. Gold in its nanoform has distinct chemical and physical properties and the large amount of surface atoms make GNPs reactive. Moreover, GNPs can potentially access many cellular or subcellular structures, which are unreachable by the larger compound and may induce toxic effects. This paper addresses effects of spherical GNPs of average size 15 nm on reproductive organs after subacute exposure in adult male and female zebrafish. Gold nanoparticles were chemically synthesized and characterized by transmission electron microscope.

The primary objective of this study was to determine if exposure to GNPs altered cellular morphology of gonads. The adult fish of both sexes were administered orally with these GNPs at a dose of 20 µg/gm. At the end of the study, quantification of gold content was estimated using two different tools: inductive coupled plasmon-atomic emission spectroscopy (ICP-AES) and inductive coupled plasmon-mass spectroscopy (ICP-MS). No gold metal accumulation was detected in treated group of male and female zebrafish at subacute exposures on estimation through ICP-AES. On analysis using ICP-MS, 0.44 ± 0.18 µg/gm organ weight was detected in ovaries and 4.6 ± 3.20 µg/gm organ weight was detected in testes of treated groups. However, the pattern of accumulation was found to be nonsignificant when compared with the control group at a p-value >0.05. Histopathological analysis of reproductive organs showed no significant changes in cellular morphology of testes and ovaries.

[1]Tutor, [2,4]Associate Professor, [3]Laboratory Technician [5]Professor

[1]Department of Genetics, MGM Institute of Health Sciences Navi Mumbai-410209, Maharashtra, India

[2,3,5]Department of Biotechnology, Central Research Laboratory MGM Institute of Health Sciences, Navi Mumbai-410209 Maharashtra, India

[4]Department of Oral Pathology, MGM Dental College and Hospital, Navi Mumbai-410209, Maharashtra, India

Corresponding Author: Dattatraya Shankar Joshi, Professor Department of Biotechnology, MGM Institute of Health Sciences, Navi Mumbai-410209, Maharashtra, India Phone: +919702536192, e-mail: josharvind@gmail.com

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INTRODUCTION

Gold nanoparticles (GNPs) have traditionally been considered inert and biocompatible. Its high surface area and volume ratio has resulted in its wide applications in biomedical research.[1] Gold nanoparticles of various size and shape have attracted considerable interest for medical applications, e.g., as carrier for drugs, such as paclitaxel, tumor-detector, photothermal agent or radiotherapy dose enhancer.[2,3] Despite their huge potential benefits in the biomedical applications, very little is known about the short and long-term health effects in organisms.[4,5] Bulk gold was used *in vivo* in the 1950s and was considered nontoxic, but functionalized gold particles showed obvious cytotoxicity.[6,7] An increasing number of scientific reports have appeared in the last decade that highlight the issue of understanding the interactions between different types of nanoparticles and cells as functions of size, shape, and surface chemistry of the nanomaterial.[8] The unexpected accumulation of nanoparticles in organs has a potential risk to induce organ dysfunction and diseases.[9,10] Unfortunately, no simple conclusions have emerged from the available studies due to the variability of parameters, such as the physical and chemical properties of the particle, cell type, dosing parameters, and the biochemical assays used. Moreover, the majority of the scientific reports that investigate the cellular impact of nanomaterials are *in vitro*, with far less effort to understand the real situation *in vivo*.[11] Some of the crucial issues that need to be addressed for toxicity assessments of nanomaterials are effect of shape and size, dosimetry, route of delivery and tracking, development, and validation of test models, *in vitro vs in vivo* extrapolation, etc.

Currently, small animal models are the "gold standards" for nanomaterial toxicity testing. Recently,

zebrafish is becoming a useful vertebrate model system for increasing number of studies on many of the human diseases, drug discovery, drug delivery systems, toxicity assessments, etc.[12,13] Apart from reports on general systemic toxicity of GNPs, there is a paucity of data available on reproductive toxicity associated with GNPs and lack of safety and regulatory guidelines concerning application of GNPs in consumer products. This highlights the need to consider not only the usefulness of NP but also the potentially unpredictable and adverse consequences of human exposure thereto. This applies especially to their potential reproductive toxicology (nanoreprotoxicity), because any shortcomings in this regard would be reflected into the next generation. Moreover, the 1 to 100 nm scale is of interest for biological interfaces; e.g., objects less than 12 nm in diameter may cross the blood-brain barrier[14-16] and objects up to 45 nm can be endocytosed by cells while those more than 70 nm tend to remain on the surface of the cells.[17] Therefore, the purpose of this research is to utilize zebrafish as an *in vivo* vertebrate model in a subacute (14 days) study to assess bioavailability and toxic effects of GNPs with an average size of 15 nm since it is known to have more potential to cross the physiological barriers.

MATERIALS AND METHODS

Gold Nanoparticles Synthesis and Characterization

To produce small nanoparticles, the procedure of the Turkevich et al was used.[18,19] Briefly, 10 mL of 1 mM tetrachloroaurate is heated to near boiling (96°C) followed by addition of 1 mL of 41 mM trisodium citrate. Solution was stirred vigorously on a magnetic stirrer with heating mantle till about 8 to 10 minutes, and then allowed to cool at room temperature.

The formation of GNPs was monitored using double beam UV/visible spectrophotometer (Thermo Scientific, Evolution 201 series). The size and shape of the nanoparticles were confirmed using transmission electron microscopy (TEM) (Philip, Model No. CM200, operating voltages: 20 to 200 kV resolution 24 Å). This solution was stored at 4°C for further use. Stability of the suspension was monitored every week using UV-visible spectrophotometer and was found to be stable for 2 months.

Dose Determination: GNPs to be Administered to Adult Zebrafish

Prior to initiating studies on adult zebrafish, preliminary experiment was performed on zebrafish embryos to determine LC_{50} value for GNP's. For this purpose, five different test concentrations were selected with distilled water as a control. Three replicates of 20 zebrafish embryos were exposed per concentration at 4–6 hours post fertilization (HPF) as per the organization for economic cooperation and development (OECD) guidelines[20] and monitored for their viability at every 24 hours till 96 HPF.

Experimental Design

Animal experiments were performed in the zebrafish facility at the Central Research Laboratory, fulfilling the criteria of good laboratory practices. Experiments were designed according to OECD guidelines for fish (Test no. 204; 1984).[21] Indigenous wild type male and female adult zebrafish strains (3–4 months old) were used for this study. Fish was stocked in static systems with continuous supply of aeration under 14:10 hours light and dark cycle. They were fed twice a day by local fish feed and once with GNPs at an interval of 4 hours daily. During this period, the water temperature was maintained at 28 ± 1°C and no fish died throughout the test period. Studies were divided into two groups: Control group (males and females) and treated group (males and females).

Route of Administration of GNPs to Adult Zebrafish

For treated group, GNPs were administered orally according to the protocol explained previously[22] at a repeated dosing for duration of 14 days. At the same time, control groups were administered with equal volume of distilled water. Experiments for each group (control and treated) was conducted in triplicates with seven healthy zebrafish per sex.

Histological Examination

Histological examination was performed following 14 days of subacute exposure. For this purpose, the fish was anesthetized in ice water and dissected to obtain testes and ovaries. The organs were fixed in 10% formalin for 24 hours at room temperature. Fixed tissue was dehydrated and embedded in the paraffin wax. Serial cross sections of 5 µm were cut by microtome (Leica RM255) and stained with hematoxylin and eosin. The samples were examined under the light microscope (Olympus Magnus, Model no. 11F589). Staging of germ cells was observed as described by Menke et al 2003.[23]

Pattern of Bioaccumulation in Gonad

At the end of the test period, fish tissues (testes and ovaries) were sampled for estimation of gold content in respective organs. Prior to digestion for gold content

measurements, the samples were thoroughly rinsed using distilled water and dried for 48 hours at 55°C. After cooling, they were weighed followed by digestion of tissues in 3 mL HNO_3 (15.3 M) by heating at 90°C (180 min) on a sand bath.[24] After complete digestion, samples were then evaporated to incipient dryness (100°C). The digestion process was completed by the addition of 2 mL of H_2O_2 (1M) and evaporation to incipient dryness (60 min, 100°C). Prior to measurements by inductive coupled plasmon-atomic emission spectroscopy (ICP-AES) (ARCOS from M/s Spectro, Germany) and inductive coupled plasmon-mass spectroscopy (ICP-MS) (Thermo Fischer Scientific, Germany), acidified ultrapure water (2% v/v, HNO_3, 15.3 M) was added. Gold content in respective samples was measured to determine the accumulation pattern of the GNPs in testes and ovaries.

RESULTS

Synthesis and Characterization of Gold Nanoparticles

A simple one-step synthesis method for the preparation of uniform and stable GNPs was employed (Fig. 1A). The UV/visible spectrum of synthesized GNPs showed maximum absorbance at 520 nm (Fig. 1B). The TEM image and size distribution plot of GNPs indicated spherical shaped particles with an average diameter of 15 ± 5 nm (Figs 1C and D).

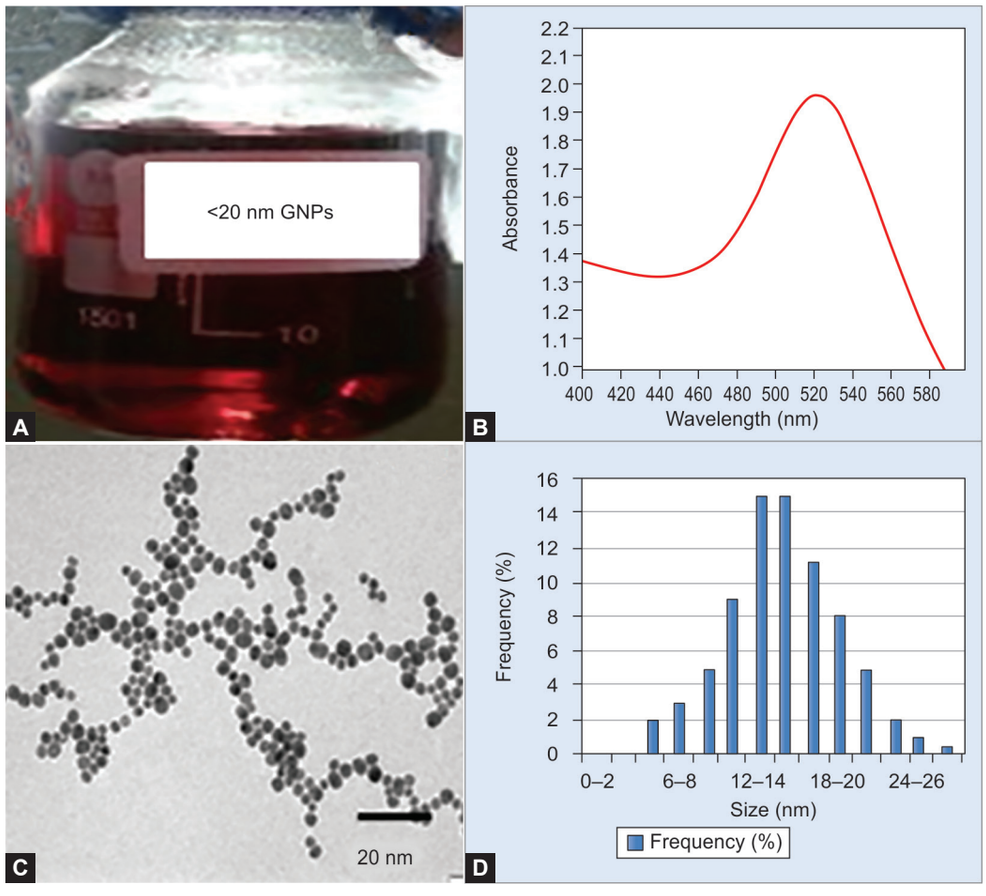
Dose Determination: GNPs to be Administered to Adult Zebrafish

Viability percentage of the embryos was determined at the end of 96 HPF to estimate the LC_{50} value for spherical GNP's with average diameter of 15 nm. LC_{50} for 15 ± 5 nm GNPs was obtained at 10 µg/mL (Fig. 2).

Thus, a concentration of 10 µg/mL was used for further studies on adult zebrafish. The average weight obtained for male and female zebrafish used in the present study was 0.5 gm, thus, the dose calculated is 10 µg/0.5 gm body weight of the fish, i.e., 20 µg/gm body weight of fish.

Gold Content Estimation in Gonads of Zebrafish

In order to validate the success of the protocol of oral administration whether GNPs have reached its target organ, i.e., gonads, the reproductive organs were dissected posttreatment followed by acid digestion and analyzed for accumulation of gold content using ICP-AES and ICP-MS. No gold metal accumulation was detected



Figs 1A to D: Synthesis and characterization of GNPs: (A) Chemically synthesized GNPs, (B) UV-visible spectrum with absorption maxima at 520 nm, (C) TEM image at a scale bar of 20 nm indicating GNPs with spherical shape, and (D) size distribution plot

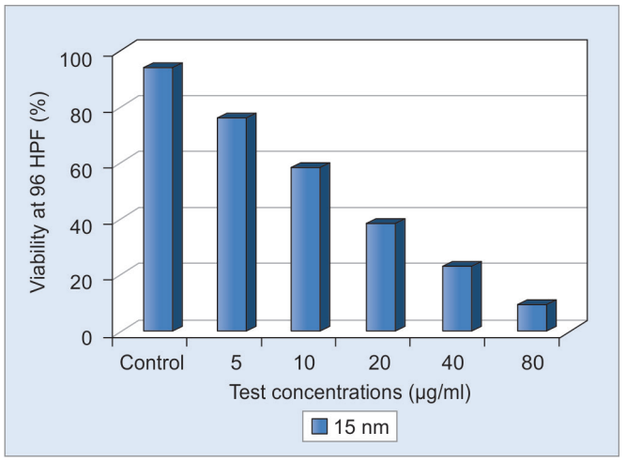


Fig. 2: Histogram showing percentage viability at 96 HPF for embryos exposed to spherical GNPs of average diameter of 15 nm

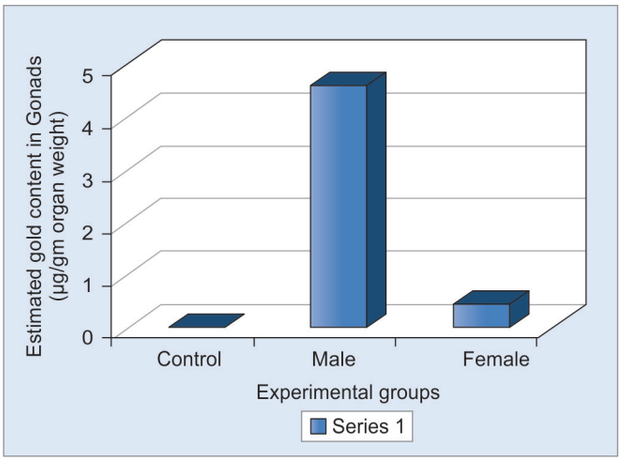


Fig. 3: Histogram plot for estimation of gold content (µg/gm organ weight) in Gonads using ICP-MS

Table 1: Estimation of gold content (µg/gm organ weight) in Gonads using ICP-AES and ICP-MS

Test	*Control*	*Male*	*Female*
ICP-AES	< 0.01	< 0.01	< 0.01
ICP-MS	< 0.01	4.6 ± 3.20	0.44 ± 0.1

Note: < 0.01 means not detected

in treated group of male and female zebrafish at subacute exposures on estimation through ICP-AES (Table 1). On analysis using ICP-MS, 4.6 ± 3.20 µg/gm organ weight was detected in testes and 0.44 ± 0.18 µg/gm organ weight was detected in ovaries of treated groups (Table 1, Fig. 3). However, the pattern of accumulation was found to be nonsignificant when compared with the control group at a p-value > 0.05 on statistical analysis by analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) software. Inductive coupled plasmon-mass spectroscopy is considered to be more sensitive instrument than ICP-AES as the detection limit for ICP-AES is 10 ppb while that for ICP-MS is 0.1 ppb.

Histopathological Analysis of the Gonads

Due to lack of reports available on histology of testes and ovaries in zebrafish, efforts were made to study the normal histological structure of testes and ovaries from the control group of male and female zebrafish respectively followed by histopathological analysis of the treated group of zebrafish males and females after subacute exposure to GNPs with an average size of 15 nm at a dose of 20 µg/gm. Histopathological observations for zebrafish testes were made under 100× objective while that for ovaries were made under 10× objective.

Germ Cells of Male Zebrafish

Testes are lateral, paired organs comprising of tubules or blind sacs, which are lined with spermatogenic epithelium. The seminiferous tubules are separated by thin strands of interstitial connective tissue containing Leydig cells, spermatogonia, spermatocytes, and spermatids.[23, 25] Testes of treated male zebrafish revealed the presence of testicular cells with typical architecture and normal spermatogenesis as evaluated by histology. Thus, no significant changes were observed when compared to control in testes (Figs 4A and B) on subacute exposure to GNPs for the given size at a given dose.

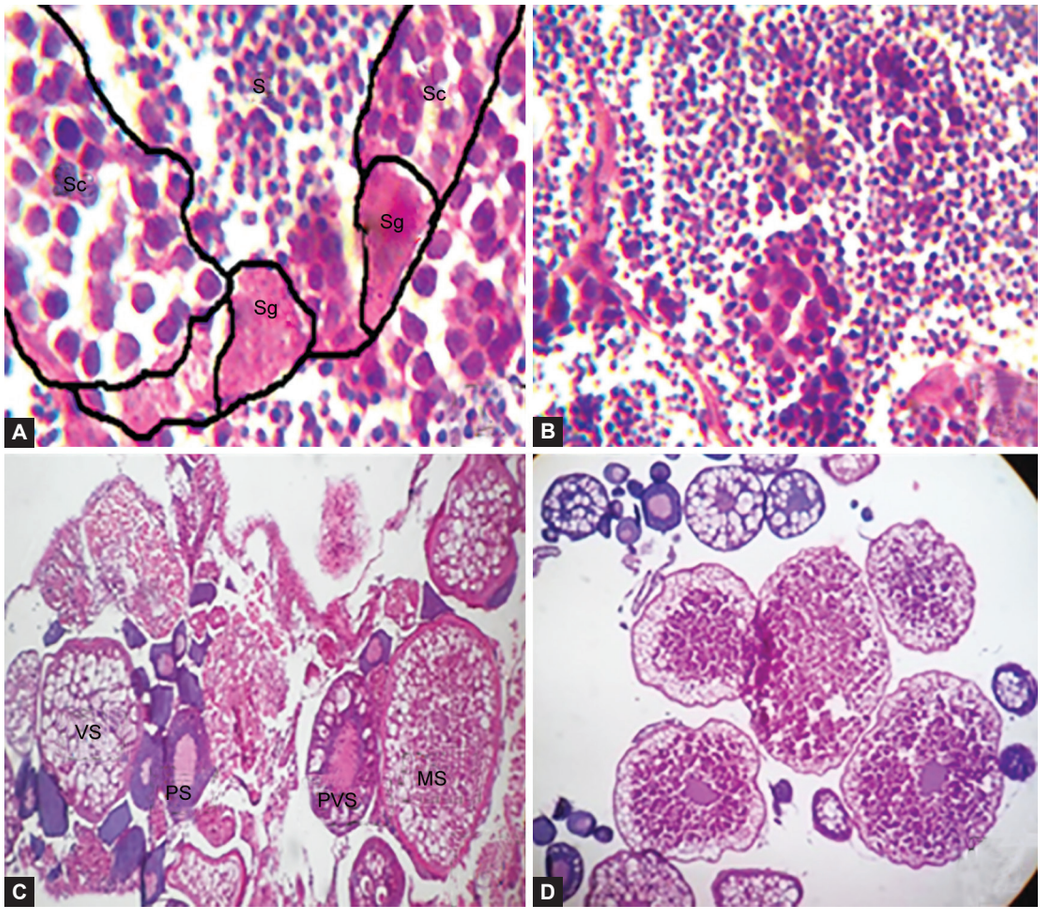
Germ Cells of Female Zebrafish

On examination of whole female gonads, the germinative parenchyma (epithelium) of the ovary showed a membrane-bound structure and constitutively contains oogonia, primary oocytes, pre (previtellogenic, vitellogenic, and mature oocytes) and postfollicular cells.[23,26] All features resembled that of the control and showed no significant changes in ovaries (Figs 4C and D) on subacute exposure to GNPs for the given size at a given dose.

DISCUSSION

This research was designed to explore the *in vivo* effects of GNPs in two types of zebrafish germinal cells; teticular and ovarian cells. So far, there have only been two studies published concerning the impact of GNPs on gametes. However, both trials concentrated on the male side, i.e., the effect of GNPs on spermatozoa. In each study, one uses chemically derived GNPs[27] while the other using laser-generated ligand-free particles.[28] Up to date there are no studies available concerning the impact of GNPs on oocytes.

In the present study, we were interested in exploring the toxic effects of GNPs on testicular and ovarian germ cells, in particular, their possible ability to cross physiological barriers and penetrate inside the germ cells, and in defining their localization. We choose zebrafish as a model mainly because of its biological resemblance to



Figs 4A to D: Histopathological analysis of testes (under 100× objective) and ovaries (under 10× objectives) from control and treated groups: (A) Histology of control male zebrafish showing different spermatogenic populations lined within the seminiferous tubules (Sg: Spermatogonia; Sc: Spermatocytes and S: Sperms), (B) histology of treated male zebrafish showing no changes in cellular morphology, (C) histology of control female zebrafish showing oocytes at different stages of development (PS: Primary oocyte stage; PVS: Previtellogenic stage; VS: Vitellogenic stage; MS: Mature stage), and (D) histology of treated female zebrafish showing no changes in cellular morphology

humans with respect to organ system homology and also due to the difficulty on working with higher vertebrate models.[29,30]

The present article is based on exposing adult male and female zebrafish toward GNPs with an average diameter of 15 nm via oral route and to investigate if gonads are the target for small nanoparticles. Also, study its interaction with both the types of germ cells. The results of this study demonstrate that though gold metal accumulation was detected in testes and ovaries after sub-acute exposure to 15 nm average sized spherical GNPs, it did not alter the cellular morphology of reproductive organs in zebrafish (*Danio rerio*) at a dose of 20 µg/gm. At this point, it cannot be considered as a reproductive toxicant at subacute exposures as there was no evident morphological disruption of germ cells in both males and females. The results of this study will be helpful for further research which can focus on the long-term effects induced by GNPs thus, setting standards for safety evaluation for metallic GNPs.

Although the present study gives a preliminary idea on germ cell response toward spherical GNPs of average size 15 nm, it can be suggested that GNPs of the selected size range used in this study seem to exert no negative effect on zebrafish germ cells, particularly on subacute exposure of 14 days. Further *in vivo* research should focus on prolonged exposure duration and possible genotoxicity of these GNPs on germinal cells to elucidate its effects and mechanism of action in human populations.

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