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Effect of Gold Nanoparticles on Proliferation and Apoptosis during Spermatogenesis in Rats

Yu. I. Velikorodnaya[a], A. Ya. Pocheptsov[a], O. I. Sokolov[b], V. A. Bogatyrev[b], and L. A. Dykman[b]

[a] *Research Institute of Hygiene, Toxicology, and Occupational Pathology, Federal Medical-Biological Agency, ul. Zemlaychki 12, Volgograd, 400048 Russia*

[b] *Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, pr. Entuziastov 13, Saratov, 410049 Russia*

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Abstract—In the present study we investigate the effect of 5-nm gold nanoparticles on the proliferation and apoptosis of spermatogenesis in rats. It is found that the per os administration of gold nanoparticles to white rats for 8 weeks at a dose of 0.57 mg/kg/day did not change the expression of proliferation proteins (KI-67 and D1 cyclin) and did not alter the activity of apoptosis (TUNEL method) in the spermatogenic epithelium. At the same time we revealed an appreciable accumulation of gold nanoparticles in the connective tissue sheath of the testes and an insignificant presence in the lumen of the convoluted seminiferous tubules.

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INTRODUCTION

The unique physical and chemical properties of nanomaterials (high penetration ability, large specific surface area, and chemical activity) have led to their wide implementation in practical human activity, from food processing to biomedical technology.

Today there are many publications dedicated to the use of nanoparticles (NPs), primarily gold nanoparticles (GNPs), in various fields of biomedicine. Reviews dedicated to the biodistribution and toxicity of GNPs contain detailed lists of numerous branches of medicine and nanobiotechnology where GNPs are used, the prospects for their further use are given, and biosafety issues are discussed [1, 2].

However, despite the abundance of experimental data about GNP cytotoxicity in vitro, there is not enough information about their potential toxicity or biological effect on the organism of mammals in general and on spermatogenesis in particular.

Spermatogenesisis a complex process of male germinal cell development including phenomena such as self-renewal and commitment, proliferation and apoptosis, differentiation and meiosis, reparation, and regeneration. The functioning of the reproductive system is controlled at the genetic and hormonal levels, but environmental conditions can also directly affect the functional state of the testes. Many chemicals have both a direct negative effect on the development of the spermatogenic epithelium and an indirect effect, affecting the somatic cells of the testis (Sertoli cells) [3, 4].

Experimental studies of the effects of NPs on spermatogenesis are mainly performed on the cell culture, which causes difficulties in the extrapolation of the data for the whole body as a multicomponent self-regulating system. The following cell lines are used most often:

(i) NT2—human embryonal carcinoma cell lines derived from testicular cells [5];

(ii) Wild-type primary mice testicular cells C57BL6 [5];

(iii) Primary testicular cells from 8-oxoguanine DNA glycosylase knock mice (this cell culture mimics the sensitivity of generative epithelium cells to oxidative stress and thus, according to the authors, is a good model for the study of the male reproductive function) [5];

(iv) TM3 cell line derived from mouse Leydig cells [6];

(v) Primary mouse spermatogonial stem cells [7];

(vi) Pig cumulus–oocyte complexes [8];

(vii) Mature human [9, 10], stag [11], or a bull [12] sperm.

In studies [5, 7], the effect of silver NPs on spermatogenesis was investigated; in studies [10–12], the effect of GNPs was investigated; in studies [8, 9] the effect of GNPs, silver NPs, and gold–silver NPs was investigated, and in study [6] the effect of titanium dioxide NPs was investigated.

Studies that focus on the effects of NPs on spermatogenic epithelium in vivo, are mainly related to the assessment of structural and functional changes, using indicators such as the weight of the testes and the

number and quality of sperm [80–11]. Histopathological parameters are more sensitive, but they are mainly used for estimating the spatial location of cells and NPs in the structure of spermatogenic epithelium [12–14]. Thus, in the study [14] based on pathological and biochemical studies, the authors demonstrated the absence of changes in male and female mice reproductive systems after the intraperitoneal injection of pegylated GNPs ranging in size from 4 to 36 nm at a dose of 4 mg/kg. Meanwhile, the effect of metal NPs on the molecular mechanisms of spermatogenesis in vivo remains poorly understood. This is probably due to the fact that the processes of spermatogenesis like ovariogenesis are cyclical and at different stages of the cycle they are affected by a variety of regulatory mechanisms, which complicates the interpretation of the obtained results.

The main mechanisms to maintain a balance in spermatogenesis are the proliferation of spermatogonia (mitotic division) and apoptosis. The common recognized biomarkers of cell proliferation are proteins Ki-67 and cyclins from D group. Ki-67 is a nuclear nonhistone protein which is present in low concentrations in resting cells. Its quantity increased in dividing cells, especially G2, M, and S phases of the second half of mitosis [15]. Cyclins of D family, including D1, control the transition of the cell cycle from G1 to S phase. Furthermore, group D cyclins may be involved in differentiation and apoptosis. According to the literature, cyclin D1 in the process of spermatogenesis is expressed in nondividing Sertoli cells and participates in the regulation of proliferation of spermatogonia [16]. Apoptosis that occurs in the testes is the main physiological mechanism limiting the number of germinal cells in the epithelium of the testes. Thus, according to some authors, up to 75% of the potentially mature population of germinal cells in the testes lose their activity and are utilized through the mechanism of apoptosis [17].

The present work is devoted to a study of the in vivo effects of GNPs on the processes of proliferation and apoptosis, which play an important role in maintaining the homeostasis of spermatogenesis and, consequently, in maintaining the stability of the functional state of the reproductive system.

MATERIALS AND METHODS

GNPS 5 nm in diameter were prepared by the reduction of hydrochloro-auric acid ($HAuCl_4$; Sigma-Aldrich, United States) by sodium borohydride (Serva, Germany) in the presence of sodium salt of ethylenediaminetetraacetic acid (EDTA-Na_2; Serva, Germany) at room temperature and upon vigorous stirring [18]. Briefly, 49 mL of cold (4°C) aqueous solution of EDTA-Na_2 with a concentration of 0.3 mM was poured in an Erlenmeyer flask, and stirring was started without heating, followed by the addition of 0.2 mL of 0.2 M potassium carbonate solution (the reaction takes place under alkaline conditions) and 0.5 mL of 1% $HAuCl_4$; the stirrer speed was increased and 125 μL of 0.5% cold sodium borohydride solution was added very quickly. As a result, the formation of an orange-red sol was observed.

GNP size was determined using particle size, a Zetasizer Nano-ZS zeta potential analyzer (Malvern, United Kingdom), and a Libra-120 electron transmission microscope (Carl Zeiss, Germany). The calculated concentration of 5-nm gold particles was 7×10^{13} in 1 mL (57 μg Au/mL) [18].

Experiments were carried out on mongrel white male rats weighing 220–270 g. The study was conducted in accordance with Order of the Ministry of Health Care and Social Development of the Russian Federation no. 708n of 23.08.2010 "Approval of the Rules of Good Laboratory Practice." The rats were housed in rooms with artificial lighting (from 8:00 a.m. to 8:00 p.m. in light and from 8:00 p.m. to 8:00 a.m. in darkness) at 20–22°C under conditions of free access to food and water. GNPs were administered per os at a dose solution of 0.57 mg/kg/day to the animals of the experimental group (ten animals). The dose was selected based on the previously determined toxic dose of GNPs in vivo [19]. Control animals (ten animals) were injected with deionized water in a similar amount. The duration of the experiment was 8 weeks, which corresponds to the duration of the maturation of germinal cells of rats [20]. After the experiment, the rats were decapitated and the testes were extracted.

For the detection of GNPs in testis tissue, we used the automatic metallographic method [21]. The principle of this method is the photocatalytic deposition of silver on gold particles. For reaction, the testis was frozen at –30°C and cryostat sections with thicknesses of ~10 μm were obtained. Sections were mounted on glass with poly-L-lysine (Menzel, Germany) and dried and coated with gelatin to prevent nonspecific staining. Thereafter, sections were placed in a solution containing 250 mg of hydroquinone (Sigma-Aldrich, United States) and 100 mg of silver acetate (Sigma-Aldrich, United States) in 100 mL of 1 M citrate buffer (pH = 3.8). Reduction time was dependent on the thickness of sections and varies from 10 to 30 min.

To abort the recovery process, the window was immersed in a 5% solution of sodium thiosulfate (Sigma-Aldrich, United States) for 10 min and washed with warm running water, contrasted with 0.1% toluidine blue, dehydrated, and embedded in antireflective immersion medium DEPEX (Sigma-Aldrich, United States).

Conventional methods were used for the production of immunohistochemical reactions [22]. Immediately after removal, the testes were placed in 4% paraformaldehyde in a 0.01 M phosphate-buffer saline (pH = 7.4). The samples were then embedded in par-

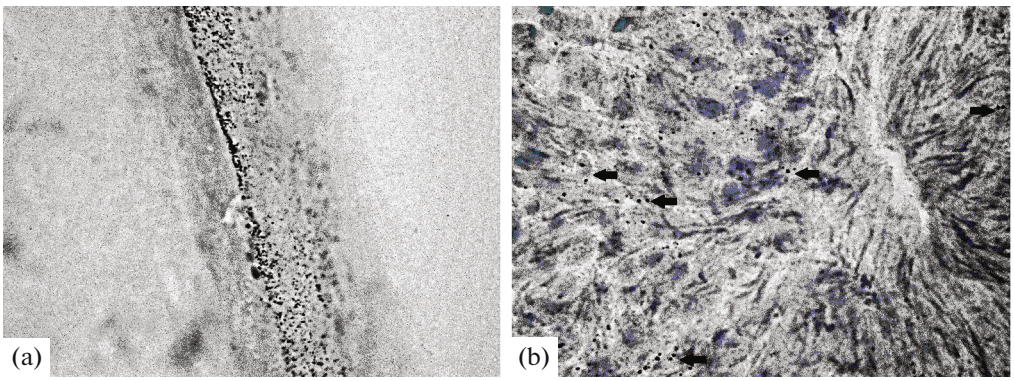

Fig. 1. Accumulation of 5-nm gold particles: (a) in the connective tissue of the testis and (b) in the lumen of the seminiferous tubule (indicated by an arrow). Cryosections; automatic metallography; and additional staining with toluidine blue. Magnification ×400.

affin Histomix (BioVitrum, Russia). The paraffin sections with a thickness of 4 μm were placed on glass treated with poly-L-lysine. For immunohistochemical studies, rabbit Anti-KI-67 polyclonal antibody (Abcam, United Kingdom) and Anti-cyclin D1polyclonal antibody (NeoMarkers, United States) were used. Sections were incubated for 20 min in 3% hydrogen peroxide solution in order to block endogenous peroxidase. The immunohistochemical reaction was carried out using the Dako detection system (Denmark). 3-3'-diaminobenzidine was used as a peroxidase substrate. At the final stage, sections were stained with Mayers hematoxylin. Preparations without incubation with primary antibodies were used as a negative control with the full compliance of the remaining phases of the protocol.

Apoptotic cells were detected using the TUNEL method for the detection of the final stages of apoptosis: DNA fragmentation [23]. The principle of the method is making the end breaks of DNA strand with terminal deoxynucleotidyl transferase (TdT) derivative and deoxyuridine triphosphate (dUTP), which can then be detected by chromogenic or fluorescent labels. In our study, an incubation medium containing TdT (Fermentas, United States) and biotinylated derivatives of deoxyuridine triphosphate—biotin-16-dUTP (Biotium, United States)—were used for the identification of apoptotic cells. As a secondary label we used streptavidin conjugated with peroxidase. The resulting complex was treated with diaminobenzidine and, as a result, cells containing fragmented DNA were stained brown.

The samples were studied, photographed, and morphometric measurements were made using an AxioScopeA1 microscope (Carl Zeiss, Germany) equipped with AxioCam MRc5 digital camera and processed using ZEN 2012 software. For the quantitative determination of the proliferative potential of seminiferous epithelium, Ki-67- and cyclin D1-immunopositive cells were counted in 50 radial seminiferous tubules of each animal of the group. We noted that in approximately 30% of studied tubule, cells of generative epithelium had a negative reaction to both Ki-67 and cyclin D1. Also, the number of apoptotic cells, including Sertoli cells, was counted in 50 rounded tubules. We calculated average number of Ki-67^+ and cyclin $D1^+$ cells and apoptotic cells per one semen tubule in the group. Statistical processing of the data was performed using the Statistica 7.0 program with a nonparametric Mann–Whitney U test.

RESULTS AND DISCUSSION

The study of germinal epithelium in experimental animals after automatic metallography revealed a significant accumulation of gold nanoparticles in the connective tissue of the testes (Fig. 1a). In addition, a few GNPs were registered in the lumen of the convoluted seminiferous tubules (Fig. 1b). This observation does not contradict the literature data, according to which the individual NPs can penetrate through blood–testis barrier (BTB) formed by tight junctions between Sertoli cells [24, 25]. Thus, in study [24], the penetration of 40-nm silver NPs through the BTB was shown for the oral administration of the particles to mice at a dose of 1 mg/kg for 14 days. In study [26], the accumulation of GNPs not only in target organs (liver, spleen, and kidneys) but also in the testis of rats after a single intravenous injection at a dose of 10 mg/kg was revealed. The authors of study [27] established using mass spectroscopy that silver NPs accumulated and permanently remained in the testes after 28 days of exposure.

Furthermore, there is no doubt that the low permeability of NPs through tissue barriers and their distribution in organs depends on their size. The study described in [28] showed that big GNPs ($\geqslant$50 nm) administered intravenously was primarily determined in the liver, spleen, and blood, while the smaller NPs can be detected in the testes.

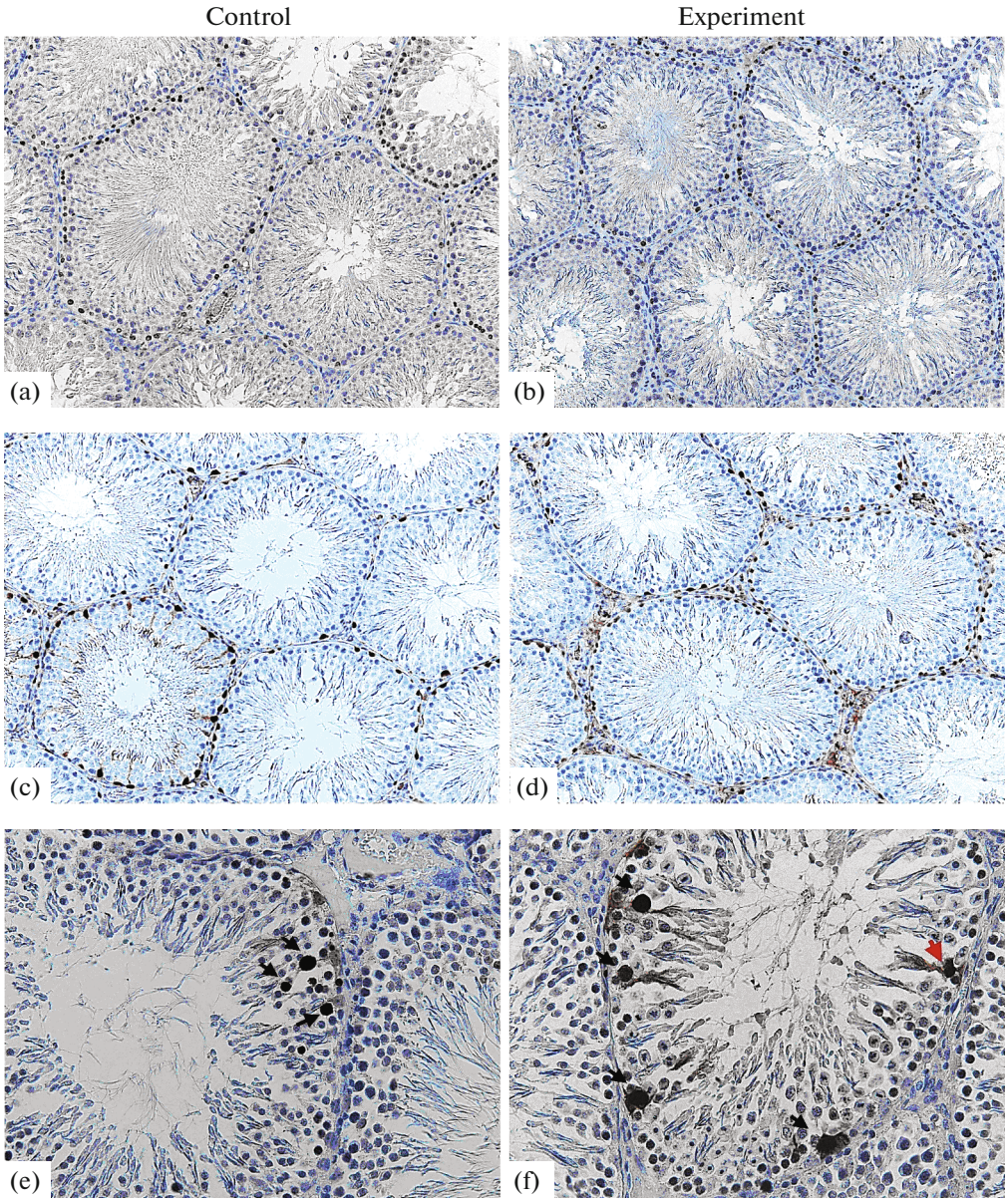

Fig. 2. (Color online) Expression of Ki-67 (a, b), cyclin D1 (c, d) in spermatogonia lying in proximity to the basement membrane of the seminiferous tubules and apoptotic cells of seminiferous epithelium (indicated by the black arrow) and Sertoli (indicated by the red arrow) (e, f). Method peroxydase-antiperoxydase; additional staining with Mayers hematoxylin. TUNEL method. Magnification ×200 (a–d) and ×400 (e, f).

There is a hypothesis of NP penetration into the lumen of the seminiferous tubules which is called "the elevator door." According to this hypothesis, small particles (up to 40 nm) overcome the BTB due to the initiation of the inflammatory response [24], whereby the gap between the Sertoli cells increases and the medium-sized particles penetrate into the seminiferous tubule. Thus, large particles (above 70 nm) cannot pass through the BTB since their size exceeds the size of the resulting gap [25].

Thus the presence of nanosized metal particles in the generative epithelium causes various biological and toxic effects in the reproductive system of the experimental animals. For example, in study [29] it was found that silver NPs 5–20 nm in size had a negative effect on spermatogenesis, causing the depletion of germinal cells and the necrosis of spermatogonia and Leydig cells during prolonged exposure (90 days). The study of the effect of titanium dioxide NPs on the reproductive function of male mice showed that NPs caused the damage to the testes, sperm abnormality, and the dysregulation of spermatogenesis genes [30]. However, in the available sources, we did not find sim-

Number of Ki-67-, cyclin D1-immunopositive, and apoptotic cells per 1 seminiferous tubule in the control and experimental groups (M ± SD)

Cells	Control	GNP
Ki-67$^+$	17.82 ± 10.18	17.75 ± 11.30
Cyclin D1$^+$	23.80 ± 10.80	22.50 ± 10.60
Apoptotic cells	1.94 ± 0.93	1.88 ± 0.86

ilar studies on the effects of GNPs on the reproductive function in vivo.

The expression of Ki-67 (Figs. 2a, 2b) and cyclin D1 (Figs. 2c, 2d), which are essentially proteins of the mitotic cycle, is most clearly manifested in spermatogonia: immature germinal cells, some of which are consistently differentiated into other cell types of the generative epithelium of the seminiferous tubules. In spermatocytes, cyclin D1 was not detected and the Ki-67 protein was detected as fine granules associated with the dividing chromosomes. Since structured chromatin is absent in cells of this type, estimating their proliferative activity was difficult. The different method of calculation using PCNA protein is more suitable for estimating the cell proliferative activity of spermatocytes [31].

Despite the presence of GNPs in seminiferous tubules, the results of an immunohistochemical study of the processes of cell proliferation showed that the subchronic administration of GNPs for 8 weeks had no effect on the basic functions of seminiferous epithelium. There were no negative effects of GNPs on the proliferative function of generative epithelium. The content of Ki-67$^+$ in spermatogonia of the experimental groups with GNPs did not differ from control values. Furthermore, the number of cells of seminiferous epithelium expressing cyclin D1 in the experimental group remained within normal levels (table).

It should be noted that in primary and secondary spermatocytes there is meiosis and, at this stage of spermatogenesis, transformation of nuclear and cytoplasmic materials leading to a substantial simplification of male germ cells and resulting in the loss of many protection and repair mechanisms occurs. Furthermore, as a result of successive meiotic divisions of spermatocytes, chromatin is constantly decondensed and therefore such type of cell is more sensitive to the exogenous factors.

The results of our experiments have shown that spermatogonia retained the ability to divide and reproduce. At the same time, the absence of overexpression of proliferation factors is also a favorable indication that GNP does not trigger uncontrolled cell division, which may lead to the "redundancy" of germinal cells and, as a consequence, the loss of stability of the reproductive system in general.

The counting of TUNEL-positive cells in 50 round tubules of rat testes after the administration of nanosized gold particles showed that the number of apoptotic cells ranged within control values (Table 1). It was noted that the phenomenon of apoptosis in control and experimental groups appeared in seminiferous epithelium cells of various types, and Sertoli cells (Figs. 2d, 2e).

The presence of GNPs in the lumen of seminiferous tubules did not trigger mechanisms of spontaneous "inside apoptosis," which would cause the death of multiple individual cells, or "apoptosis upon command," in which the elimination of the whole layer-syncytium of primary and secondary spermatocytes would occur.

Thus, the results of our experiments have shown that, despite the tight intercellular contacts of the BTB, gold particles 5 nm in size can penetrate into the seminiferous tubules. However, they did not affect the proliferative status of spermatogonia and did not stimulate the apoptosis of mature germinal cells.

However, we must remember that GNPs obtained in another laboratory or by other methods may be different from the described GNPs in terms of physicochemical parameters and have other biological effects. Therefore, any NP for prospective biomedical uses should be carefully studied not only in vitro, but also in vivo.

Under the conditions of our experiment, GNPs presented during prolonged oral administration did not show any negative impact on the spermatogenesis process in experimental animals.

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