Interference of Steroidogenesis by Gold Nanorod Core/Silver Shell Nanostructures: Implications for Reproductive Toxicity of Silver Nanomaterials

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As a widely used nanomaterial in daily life, silver nanomaterials may cause great concern to female reproductive system as they are found to penetrate the blood–placental barrier and gain access to the ovary. However, it is largely unknown about how silver nanomaterials influence ovarian physiology and functions such as hormone production. This study performs in vitro toxicology study of silver nanomaterials, focusing especially on cytotoxicity and steroidogenesis and explores their underlying mechanisms. This study exposes primary rat granulosa cells to gold nanorod core/silver shell nanostructures (Au@Ag NRs), and compares outcomes with cells exposed to gold nanorods. The Au@Ag NRs generate more reactive oxygen species and reduce mitochondrial membrane potential and less production of adenosine triphosphate. Au@Ag NRs promote steroidogenesis, including progesterone and estradiol, in a time- and dose-dependent manner. Chemical reactivity and transformation of Au@Ag NRs are then studied by electron spin resonance spectroscopy and X-ray absorption near edge structure, which analyze the generation of free radical and intracellular silver species. Results suggest that both particle-specific activity and intracellular silver ion release of Au@Ag NR contribute to the toxic response of granulosa cells.

1. Introduction

Silver nanomaterials have been noted for their antimicrobial activity and used in many medical devices (wound dressing, catheters) and consumer products (cosmetics and textiles). Some manufacturers have been using silver nanomaterials in gynecological supplies (feminine hygiene products) for...
contraception and anti-infections purpose. These applications of silver nanomaterials raised concerns regarding their effects on female reproductive system. Several recent studies reported that silver nanomaterials could penetrate the blood–placental barrier and gain access to the ovary.[12–15] The transcellular (cellular uptake) and paracellular (NanoEL effect) transport of nanomaterials through endothelial barrier could be the possible ways that involved in Ag nanoparticles (NPs) entering into ovary and granulosa cells.[6–7] Hazardous effects of silver nanomaterials have been studied for many years. It was found that silver nanomaterials might induce cytotoxicity and genotoxicity in various kinds of mammalian cell lines because of their degradation and induction of free radicals within cells.[8–12] To date, evaluations of reproductive toxicity of nanomaterials are rare and have been primarily carried out in vivo.[3–5,13] Potential effects of nanomaterials on physiological functions of reproductive system such as steroidogenesis and their underlying mechanisms are largely unknown. We hypothesized that an in vitro study combining novel analytical techniques can address the challenge and correlate physicochemical information of nanomaterials to their biologic response to uncover the underlying mechanisms.[12,14]

The action of Ag NPs induced toxicity has been described as a Trojan-horse effect, where Ag NPs are taken up by cells as a particle and subsequently release silver ion intracellularly, leading to cytotoxicity and cell death.[15,16] Our previous study also showed fast intracellular dissolution and a persistent cellular uptake of Ag NPs, causing time-dependent increase of cytotoxicity.[14] Unlike silver nanomaterials, gold nanomaterials especially gold nanorods (Au NRs) are chemically inert and mainly exist as elemental Au during their presence in tissues.[17,18] The chemical stability and good biocompatibility of cationic electrolyte poly(diallyldimethylammonium chloride) (PDDAC)-coated Au NRs make it a very good candidate to load cargo into biological systems.[19,20] Here, we synthesized gold nanorod core/silver shell nanostructure (Au@Ag NR) to compare with Au NR and studied the effects of silver nanomaterials induced in female reproductive systems.

In the female reproductive system, the ovary is not only as the site of egg production but also as an endocrine gland producing progesterone, estrogen, and androgen. Within the ovary, fundamental unites are called follicles, which consist of a central oocyte, surrounded by inner granulose cell layers and outer layers of thecal cells. Among them, granulosa cells are the largest cell population and play a central role in steroidogenesis. In granulosa cells, follicle-stimulating hormone (FSH) binds to FSH receptor (FSHR) on the cell membrane and induces the expression of steroidogenic acute regulatory (StAR) protein and cholesterol side-chain cleavage enzyme (P450scc), which are key factors in steroidogenesis.[21] Cholesterol is transferred by StAR from the outer mitochondrial membrane to the cholesterol side-chain cleavage enzyme (P450scc) located on the inner membrane matrix side, where the conversion of cholesterol into progesterone happens. Afterward, thecal cells convert progesterone to androgens by enzyme CYP17 and these androgens subsequently become estradiol in granulosa cells catalyzed by aromatase (P450arom).[22]

In reproductive cycle, both the number and the physiological functions of follicles are crucial because only a few follicles take part in ovulation and most of them will degenerate in atresia.[23] Several studies have shown that follicular atresia is governed by granulosa cell apoptosis, as apoptosis of granulosa cell will deprive oocyte from growth factors, nutrients, and survival factors, resulting in apoptosis of oocytes and follicular atresia.[24–26] Therefore, toxicants that affect viability and function of granulosa cells may disrupt follicular development and eventually cause infertility. Due to this sensitivity to toxicant, granulosa cells can serve as an in vitro model for effectively evaluating reproductive toxicity of silver nanomaterials.

In the present study, we used rat ovary primary granulosa cells as an in vitro model to evaluate the cytotoxicity and steroidalogenic effects of Au@Ag NRs. By combining inductively coupled plasma mass spectrometry (ICP-MS) and transmission electron microscope (TEM), we quantified the amounts of intracellular accumulation and observed the localization of Au NRs and Au@Ag NRs. Damage in mitochondria, including mitochondrial membrane potential (MMP) and adenosine triphosphate (ATP) production, and cytoskeleton disruption were measured by flow cytometry and confocal laser scanning microscope (CLSM). Cell apoptosis and steroidalogenesis were measured and the accompanied signaling pathways were analyzed by western blotting. To understand the chemical mechanism by which Au@Ag NR induced the biological response of rat granulosa cells, electron spin resonance (ESR) spectroscopy and X-ray absorption near-edge structure (XANES) were applied to study the surface chemistry of both nanomaterials and characterize the chemical transformation of silver nanomaterials in granulosa cells. Based on these findings, we propose a mechanism by which nanosilver mediates toxic effects in ovary granulosa cells.

2. Results and Discussion

2.1. Physicochemical Characterization of Au NRs and Au@Ag NRs

The structure diagrams of Au NRs and Au@Ag NRs are shown in Figure 1A,B. As representative TEM images in Figure 1 C,D illustrate, these two types of nanostructures are rod-like and are uniform in size and shape. The longitudinal surface plasmon resonance (LSPR) peak of Au NRs solution is 850 nm while that of Au@Ag NRs is blue-shifted to 682 nm (Figure 1E). These LSPR peaks are consistent with simulated values for single rods (Figure S1, Supporting Information). As shown in Table 1, the Au NRs were 63.9 ± 0.9 nm in length and 13.5 ± 2.5 nm in diameter, with an aspect ratio of 4.8 ± 1.0. The Au@Ag NRs were 65.3 ± 9.5 nm in length and 23.9 ± 3.6 nm in diameter, with an aspect ratio of 2.8 ± 0.5 (based on statistics from 100 NRs in TEM images). The weight ratio of gold to silver of Au@Ag NRs is 2.03, as measured by ICP-MS. Both Au NRs and Au@Ag NRs were coated first with cetyltrimethylammonium bromide (CTAB) and then polystyrene sulfonate (PSS) and finally with PDDAC, resulting in CTAB–PSS–PDDAC layers on the metal nanorods, which will be referred
as PDDAC–Au NRs and PDDAC–Au@Ag NRs, respectively. The zeta potentials of PDDAC–Au NRs and PDDAC–Au@Ag NRs in H2O were +44.4 mV and +43.1 mV, respectively. The elemental components in both nanorods are characterized by energy-dispersive X-ray spectroscopy (EDX), which is shown in Figure S2 (Supporting Information). Both Au NRs and Au@Ag NRs dispersed well in water and Dulbecco’s Modified Eagle Medium (DMEM)/F12 medium due to the layer by layer surface coating, which is consistent with our previous work.[19]

2.2. Isolation and Identification of Primary Ovary Granulosa Cells

Ovary granulosa cells were obtained from 3 weeks old female Sprague–Dawley rats after gonadotropin stimulation. In brief, rats were intraperitoneally injected with 40 IU per rat pregnant mare serum gonadotropin (PMSG) to stimulate the development of ovarian follicles. After 48 h, rats were sacrificed and granulosa cells were isolated from the ovary then seeded in plates and cultured in complete DMEM/F12 medium (see schematics in Figure S3, Supporting Information). Granulosa cells that adhere to the culture dishes exhibit a fibroblast-like shape and grow into a single layer (Figure S3, Supporting Information). Importantly, the FSHR is highly expressed on the surface of granulosa cell[27] which allows us to verify our results by using immunostaining. Cultured cells showed high levels of FSHR expression confirming that isolated cells were in fact granulosa cells (Figure S3, Supporting Information).

2.3. Cellular Uptake and Intracellular Localization of Au NRs and Au@Ag NRs

The cellular uptake and intracellular localization of Au NRs and Au@Ag NRs were characterized with ICP-MS and TEM. Uptake of nanomaterials is governed by their surface chemistry and chemical composition.[28–30] We have demonstrated that PDDAC-coated Au NRs have the highest endocytosis and the lowest cytotoxicity compared with PSS- and CTAB-coated Au NRs,[19] due to which this coating was used for the Au NRs and Au@Ag NRs in this work. As shown in Figure 2A, we observed a time-dependent increase in the cellular uptake of Au NRs and Au@Ag NRs. With cellular uptake of Au NRs from 1080.1 ± 295.3 ng Au/10^6 cells after 3 h to 2079.1 ± 519.1 ng Au/10^6 cells after 24 h exposure to Au NRs, while uptake of Au@Ag NRs increase from (1278.4 ± 187.1 ng Au and 312.5 ± 260.8 ng Ag, Au/Ag = 4.1)/10^6 cells after 3 h to (3281.2 ± 272 ng Au and 1177.6 ± 153.4 ng Ag, Au/Ag = 2.8)/10^6 cells after 24 h exposure to Au@Ag NRs. Compared with as-synthesized Au@Ag NRs, the intracellular Au/Ag ratio was higher initially and then decreased, indicating the solubilization of silver either due to silver ion release or re-reduction to nanosilver affected the intracellular accumulation of silver.

We then used TEM to observe the intracellular localizations of these nanomaterials. As shown in Figure 2B, both Au NRs and Au@Ag NRs were taken up by cells and translocated into lysosome/endosome structures, without entering cytoplasm and nucleus.[8,14]
2.4. Cytotoxicity of Au NRs and Au@Ag NRs in Granulosa Cells

To study the potential cytotoxicity of Au@Ag NRs, we used MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to measure the cell viability after exposure to Au NRs or Au@Ag NRs. As shown in Figure 3A, after 24 h exposure, Au NRs had negligible effect on granulosa cell viability, while Au@Ag NRs induced a dose-dependent decrease in granulosa cell viability. Our previous study suggested that the toxicity of Ag NPs is mostly due to the intracellular release of Ag ions. Thus, we speculate that the toxicity of Au@Ag NRs might also be induced by the release of silver ions and the resulting enhanced levels of intracellular reactive oxygen species (ROS). To test this hypothesis, we eliminated Ag⁺ and ROS using N-acetyl-cysteine (NAC), an effective antioxidant and silver ion chelator, to test whether the addition of NAC could prevent cytotoxicity.

We preincubated granulosa cells with 10 × 10⁻³ m NAC in fetal bovine serum (FBS)-free DMEM/F12 medium for 2 h before exposing to Au NRs or Au@Ag NRs for 24 h. In Figure 3B, MTT results show that preincubation with NAC significantly decreases the cytotoxicity of Au@Ag NRs. Live–dead cell staining results are also in consistent with the MTT data, with more live cells in the NAC preincubated cell culture at high Au@Ag NRs concentrations (Figure S4, Supporting Information). The NAC concentration and exposure condition used in this study did not interfere the cell viability of granulosa cells (Figure S4, Supporting Information). Taken together, these results suggest that the cytotoxicity of Au@Ag NRs is related to silver ion release and subsequent intracellular ROS generation.

Reactive oxygen species are important for cell growth and cell signaling. However, excessive intracellular ROS can result in oxidative stress, which damage biomolecules such as proteins, lipids, and nucleotides, and even cause cell death. Studies have shown that exposure to silver nanomaterials could disturb the intracellular redox balance and cause oxidative stress. We measured the intracellular ROS levels of granulosa cells after exposing them to Au NRs or Au@Ag NRs and found that the intracellular ROS increased from 3 to 6 h after treatment then gradually decreased to normal level after 12 h (Figure S5, Supporting Information). This may be due to the activation of intracellular antioxidant system, including low-molecular-weight scavengers glutathione (GSH), reduced nicotinamide adenine dinucleotide (NADH) and antioxidant enzymes superoxide dismutase (SOD) and catalase, which restores the intracellular redox balance. Leakage of electrons from the mitochondria during ATP synthesis is the major source of intracellular ROS. Therefore, damage to the mitochondrial membrane integrity could further increase cytoplasmic ROS levels, causing oxidative stress. MMP is a very sensitive indicator of the integrity of mitochondrial membrane and changes in MMP can be monitored by a cell-permeant, cationic fluorescent dye rhodamine 123. Therefore, we used rhodamine 123 to measure MMP with CLSM and flow cytometry after treating the cells with Au NRs or Au@Ag NRs. As shown in Figure 3C,D, when the Au concentration was higher than 0.5 µg mL⁻¹, a dose-dependent decrease of MMP was observed in both Au NRs and Au@Ag NRs exposed granulosa cells. Au@Ag NRs induced a more significant decrease of MMP than Au NRs at the same Au concentration. These results suggest that...
exposure to Au NRs and Au@Ag NRs decreased the mitochondrial membrane potential and increased cellular ROS levels.

Mitochondria are organelles that perform many complex biological processes, including the production of ATP, which serves as the primary energy source of cells. Mitochondrial dysfunction or damage to mitochondrial integrity therefore leads to severe impairments of energy metabolism. It has been suggested that exposure to silver nanomaterials weakens ATP synthesis and alters the energy metabolism from oxidative phosphorylation-based aerobic metabolism to anaerobic glycolysis.[36] In our study, we found that after 24 h exposure, Au NRs had negligible effect on ATP production in granulosa cell while Au@Ag NRs increased ATP synthesis at Au concentrations of 0.5 and 1 µg mL⁻¹, but decreased ATP synthesis when Au concentration was higher than 1 µg mL⁻¹. The similar phenomenon has been reported that low concentration of silver nanoparticles could increase the cellular viability,[37] probably due to slightly increased intracellular ROS production and stimulated cell metabolism. In combination with reduced MMP levels, these results suggest that in high concentration Au@Ag NRs damage mitochondrial function and interferes with ATP synthesis in granulosa cells.

Figure 3. Structural and functional damage of mitochondria upon Au NRs and Au@Ag NRs exposure to granulosa cells. MTT results of cell viability after exposure to Au NRs and Au@Ag NRs for 24 h A) without or B) with NAC preincubation. C) Qualitative MMP assay after Au NRs or Au@Ag NRs exposure for 24 h (measured by CLSM). The scale bars in these images represent 50 µm. D) Relative MMP after Au NRs or Au@Ag NRs exposure for 24 h (measured with flow cytometry). E) Quantification of intracellular ATP content after Au NRs or Au@Ag NRs exposure for 24 h. The data are presented as mean ± SD, n = 3. The significant statistical differences of nanomaterial-exposed group compared with control and between Au NRs and Au@Ag NRs-exposed groups at the same Au concentration are expressed as * (P < 0.05) and # (P < 0.05), respectively.
2.5. Au@Ag NRs Exposure Induced Mitochondria-Mediated Cell Apoptosis

During mammalian follicular growth and development, more than 99% of follicles undergo degeneration, a phenomenon known as follicular atresia. Inappropriate follicular atresia can result in certain reproductive disorders, like polycystic ovarian syndrome and premature ovarian failure, leading to infertility in women. Granulosa cells have been demonstrated to play a major role in the fate of follicles and apoptosis of granulosa cells results in follicular atresia. Damage to mitochondrial structure and function may further trigger cell apoptosis. TEM images of Au@Ag NR-exposed cells shows cytoplasm vacuolation, and disrupted cytoskeleton as observed with CLSM (Figure S6, Supporting Information), a sign of apoptosis induced by Au@Ag NRs exposure was indicated. We then studied whether Au@Ag NRs exposure induced apoptosis of granulosa cells. Bcl-2 protein families play significant roles in follicular growth/atresia by regulating apoptosis of germ cells as well as somatic cells. Increased ratio of apoptotic promoter Bax to suppressor Bcl-2 within a given cell is an indicator of mitochondrion-mediated cell apoptosis. We measured the expression level of Bax and Bcl-2 genes after Au NRs or Au@Ag NRs exposure. As shown in Figure 4A,B, exposure to Au NRs did not interfere significantly with the expression of apoptosis genes, while Au@Ag NRs exposure upregulated the expression of Bax and down regulated Bcl-2. Cell apoptosis also involves pro-apoptotic gene including apoptotic protease-activating factor-1 (Apaf-1) was also upregulated in Au@Ag NR-exposed granulosa cells, indicating gene-regulated apoptosis. Caspases are a family of cysteine-dependent aspartate-directed proteases that cleave intracellular polypeptides and disrupt cellular architecture, resulting in morphological changes characteristic of cell apoptosis. The involvement of caspase-3 activation during oocyte apoptosis has been reported in mouse and rat oocytes. Next, we investigated potential alterations to caspase activities after Au NRs or Au@Ag NRs exposure. We used western blotting to analyze apoptotic protein expression of caspase 3, caspase 9, PARP , and Bcl-2. As shown in Figure 4C, exposure to Au@Ag NRs increased the protein expression of p53 and Bax in a dose-dependent manner, while caspase 3, caspase 9, and PARP were decreased after Au@Ag NRs exposure, indicating the activation of mitochondria-mediated cell apoptosis. To further investigate whether and to what extent apoptosis

Figure 4. Real-time PCR and western blotting analysis of apoptosis genes and proteins and apoptosis quantification for granulosa cells. Relative expression levels of Apaf, Bax, Bcl-2, and p53 genes after 24 h exposure to A) Au NRs or B) Au@Ag NRs. 18s rRNA is used as an internal reference gene for PCR analysis and unexposed cells serve as control. C) Protein levels of p53, Bax, Caspase-3, Caspase-9, PARP, and Bcl-2 after 24 h exposure to Au NRs or Au@Ag NRs. β-Actin is used as the loading control for western blotting. D) Percentage of live, apoptotic, and necrotic cells after 24 h exposure to Au NRs or Au@Ag NRs. The data are expressed as mean ± SD. n = 3.
of granulosa cells occur upon exposure to Au NRs and Au@Ag NRs, we stained granulosa cells with fluorescein isothiocyanate (FITC)–Annexin V and PI after exposure to Au NRs or Au@Ag NRs and analyzed the relative percentages of live, apoptotic, and necrotic cells. As shown in Figure 4D, exposure to Au@Ag NRs led to a dose-dependent increase of apoptotic and necrotic granulosa cells, while exposure to Au NRs did not result in apoptosis. These results suggest that it is the silver shell and not the gold nanorod core that causes granulosa cell apoptosis. Similar results were reported with silver nanomaterial-exposed mammalian cells, in which a dose-dependent increase in cell apoptosis was induced.[9,48–50]

2.6. Au@Ag NRs Promoted Steroidogenesis in Granulosa Cells

Granulosa cells comprise the largest cell population in ovarian follicles and play crucial roles in follicular development and steroidogenesis, including the production of sex steroids, progesterone, and estradiol, as well as important growth factors that interact with the oocyte during its development. In order to ensure the normal secretion of estradiol and progesterone by isolated primary granulosa cells, we added FSH and androstenedione to the granulosa cell culture medium as the promoter and precursors for estradiol and progesterone synthesis. We investigated the effects of exposure to Au NRs and Au@Ag NRs on steroidogenesis of granulosa cells by monitoring hormone secretion in granulosa cells. As shown in Figure 5, the progesterone level of unexposed granulosa cells remained unchanged from 6 to 48 h, while the estradiol level increased over time. This was expected, as progesterone is primarily secreted by luteinized ovary cells, and estradiol is produced continuously. After exposure, only cells exposed to high concentrations of Au@Ag NRs exhibited increased progesterone production, while granulosa cells exposed to either Au NRs or Au@Ag NRs increased their production of estradiol in a time- and dose-dependent manner. Notably, exposure to Au@Ag NRs increased more progesterone production than Au NRs at the same Au concentration.

To understand the mechanism that is responsible for the elevated levels of estradiol and progesterone secretion induced by Au NRs and Au@Ag NRs, we examined the expression levels of several proteins and enzymes, which play essential roles in steroidogenesis, including FSHR, StAR, P450scc, and P450arom. As shown in Figure 6A–D, the protein levels of FSHR, StAR, P450scc, and P450arom were significantly increased in a time and dose-dependent manner upon Au@Ag NRs exposure. During the first 12 h of exposure, Au NRs increased the protein levels of FSHR but did not alter the expression of StAR. However, after 24 h, decreased levels of FSHR and increased levels of StAR were observed in Au NRs exposed cells. The protein levels of P450scc and P450arom remained unchanged in Au NRs exposed granulosa cells. Taken together, these results suggest that exposure to Au@Ag NRs could interfere with the steroidogenesis of granulosa cells in a time- and dose-dependent manner, and the effects of Au@Ag NRs is higher than that of Au NRs at the same gold concentration and incubation time.

More than 99% of mammalian ovarian follicles undergo degeneration by apoptosis. Apoptosis and steroidogenesis are not mutually exclusive. In one investigation, for at least a short time, these two processes could take place simultaneously.[51] Evidence suggests that the production of progesterone by atresia follicles increased when 20%–30% granulosa cell were apoptotic.[52] Another research has shown that caspase 3, which mediates the differentiation and apoptosis of granulosa cells, is indispensable for dexamethasone-enhanced progesterone production.[53] In our study, the highest degree of apoptosis and steroidogenesis occurred simultaneously after 24 h Au@Ag NRs exposure. This implies a correlation between apoptosis induced by silver nanomaterials and elevated steroidogenesis in granulosa cells. The coexistence of promoted apoptosis and steroidogenesis was also reported by other researchers as well, in which they concluded that during the initial steps of granulosa cell apoptosis, steroidogenesis can be increased due to the clustering of steroidogenic organelles in the prenuclear region and their exclusion from apoptotic blebs.[51]

![Figure 5](https://www.small-journal.com)

**Figure 5.** Effects of Au NRs and Au@Ag NRs exposure on steroidogenesis in granulosa cells. The amount of A) progesterone and B) estradiol secreted by granulosa cells after exposure to Au NRs or Au@Ag NRs for 6, 12, 24, and 48 h, as determined by radioimmunoassay. The progesterone and estradiol levels are normalized with total proteins of cells. The data are represented as mean ± SD. n = 3. The significant statistical differences of nanomaterials exposed group compared with control are expressed as * (P < 0.05).
2.7. Free Radical Generation and Intracellular Transformation of Au@Ag NRs

Due to their nanoscale size and high surface to volume ratio, Au NRs and Au@Ag NRs, like other metallic nanomaterials, are known to have catalytic properties.[53–57] High surface reactivity of nanomaterials may also contribute to their cytotoxicity. To verify whether Au NRs and Au@Ag NRs exert any free radical generation reactivity that may contribute to their toxicity, we used ESR spectroscopy to measure the free radical generation of Au NRs and Au@Ag NRs in acidic and neutral environment, as a mimic of intracellular lysosome and cytoplasm environment. ESR is a direct and reliable technique to both qualify and quantify free radical production in chemical and biological environment.[58] Results showed neither Au NRs nor Au@Ag NRs generated any hydroxyl or super oxide radical in the presence of H2O2 in pH 4.6 and pH 7.4 solution as measured with spin trapping agent 5-tert-butoxycarbonyl-5-methyl-1-pyrroline-N-oxide (BMPO) (data not shown). Even though Au NRs and Au@Ag NRs may not generate ROS at biological pH, they may still oxidize redox molecules and disrupt the antioxidant system. To verify this hypothesis, we used sodium ascorbate to measure the oxidase-like activity of Au NRs and Au@Ag NRs. Sodium ascorbate is a common reductant that can be oxidized to form an intermediate ascorbyl radical, which is detectable by ESR instrument.[59] Figure 7A shows the ESR spectra of ascorbyl radical formed with/without Au NRs or Au@Ag NRs. The intensity of the first peak in the ESR spectrum of ascorbyl radical was used to monitor the time dependence. As shown in Figure 7B, the oxidation of sodium ascorbate under ambient conditions is negligible within 9 min, while in the presence of Au@Ag NRs or Au NRs the system showed a dose- and time-dependent increase in the ESR signal intensity in the first 7 min and then decreased over time due to further oxidation of the ascorbyl radical. Au@Ag NRs showed a faster and higher content of ascorbyl radical formation than Au NRs under the same Au concentration, which indicates a stronger oxidase-like activity of Au@Ag NRs than Au NRs.

To further understand the ways in which Au NRs and Au@Ag NRs may disrupt the intracellular redox balance, we applied a cytochrome c (cyt c)/H2O2 electron-transfer model based on the ESR technique. In the cyt c/H2O2 system, H2O2 oxidize Fe3+ cyt c to a peroxidase compound I-type intermediate, $\text{O} = \text{Fe}^{4+} \text{cyt c}$. The $\text{O} = \text{Fe}^{4+} \text{cyt c}$ intermediate is a reactive oxidant and oxidizes reducing compounds, such as 5,5-dimethyl N-oxide pyrroline (DMPO), GSH, NADH, and ascorbic acid, to their corresponding oxidative forms. The oxidation of ESR silent DMPO to ESR detectable 5,5-dimethyl-1-pyrrolidone-N-oxyl (DMPOX) can be monitored.[60] Previous studies have shown that carbon-based nanomaterials could facilitate the electron transfer in cyt c/H2O2 system and increase in vivo oxidative stress. [61] In our study, in the presence of cyt c and H2O2, DMPO was oxidized to DMPOX, which gave a seven-line ESR spectrum as shown in Figure 7C. The intensity of the second peak in the ESR spectrum of DMPOX was used to monitor the time dependence. As shown in Figure 7D, the addition of Au NRs or Au@Ag NRs in the cyt c/H2O2 system significantly enhanced the ESR signal intensity of DMPOX, and Au NRs and Au@Ag NRs show a similar highest DMPOX signal intensity. This indicates that both Au NRs and Au@Ag NRs in the cyt c/H2O2 system promote electron transfer from the reducing agent DMPO to form DMPOX. In cells, the reducing agents could be GSH, NADH, or other reducing agent that were oxidized by the cyt c/H2O2 in the presence of Au NRs or Au@Ag NRs, which may result in disrupting the intracellular antioxidative system. This helps understand how Au NRs and Au@Ag NRs increased the intracellular ROS and decreased the MMP of granulosa cell.
Studies suggest that Ag NPs induced toxicity are through a so-called Trojan-horse mechanism, where Ag NPs are taken up by cells as particles and subsequently release silver ion intracellularly, leading to cytotoxicity and cell death.\cite{15,16} Recent studies have shown that Ag NPs could release silver ions both in cell culture medium and in cells.\cite{12,43,62} The protein corona formed in serum-containing cell culture medium, the acidic, enzyme-rich, lysosome environment facilitates the silver ion release. In previous work, we studied the time-dependent silver ion release of Ag NPs by Triton X-114-based cloud point extraction. We found that silver can be released from Ag NPs both outside and inside the cells, but silver ion release ratio is much higher in cells than in cell culture medium.\cite{14} To study intracellular silver ion release and the transformation of the Au@Ag NRs in granulosa cells, we used Ag K-edge XANES to determine the chemical form of silver after Au@Ag NRs entered the cells. Based on XANES spectra and first derivation of XANES, we can clearly differentiate the information of different compounds and samples. XANES spectra of silver inside cells were distinct from any kind of reference.

Figure 7. Free radical generation of Au NRs and Au@Ag NRs measured by ESR. A) ESR spectra of ascorbyl radical generated by Au NRs or Au@Ag NRs, which are mixed with sodium ascorbate. B) Time-dependent ESR signal of ascorbyl radical generated by Au NRs or Au@Ag NRs mixed with sodium ascorbate. The sample solution contains 25 or 50 µg mL\(^{-1}\) Au NRs or Au@Ag NRs in the presence of 0.5 × 10\(^{-3}\) M sodium ascorbate. C) ESR spectrum of DMPOX formed in the cyt c/H\(_2\)O\(_2\) system. D) Time-dependent ESR signal intensity of DMPOX formed during the oxidation of ESR spin trap DMPO in the cyt c/H\(_2\)O\(_2\) system mixed with Au NRs or Au@Ag NRs. The sample solution contains 25 or 50 µg mL\(^{-1}\) Au NRs or Au@Ag NRs, in addition to 50 × 10\(^{-3}\) M DMPO, 1 × 10\(^{-3}\) M H\(_2\)O\(_2\), and 100 × 10\(^{-6}\) M cyt c.

Figure 8. Silver K-edge XANES of reference materials and testing samples. First derivative of A) Ag K-edge XANES for all reference materials or B) Au@Ag NRs in stock solution and cells which internalize Au@Ag NRs. For first derivative plotting, the peak position represents the characteristic information of a given chemical speciation.
compounds, which suggested a composition of multiple species (Figure 8). Based on least square fitting for XANES data (Figure S7A,B, Supporting Information), we found that the as synthesized Au@Ag NRs in the stock solution are composed of elemental silver (Ag0), Ag−O−, Ag−Cl, and dissolved Ag+ species. After exposure to cells, the chemical forms of silver changed compared to the Au@Ag NRs kept in stock solution. As shown in Table 2, after 12 h exposure, Ag0 form decreased from 72.3% to 63%, while Ag−S− form increased to 34.9%. After 24 h exposure, Ag0 form decreased to 59.9%, Ag−S− form increased to 32.4%. The Au@Ag NRs in this work was synthesized with surface coating agent PSS and PDDAC, which consist of abundant surface groups such as −SO3− and Cl−. These groups may directly interact with dissolved silver ions (Ag+) to form undissolved silver such as Ag−Cl and Ag−S− species that may block the surface of NPs and decrease the dissolution rate of silver. Therefore, these Au@Ag NRs dissolved much slowly when compared to our previous study of bovine serum albumin (BSA)-coated Ag NPs and the ones coated with Tween-20.[12,14] However, the cytotoxicity of Au@Ag NRs measured here was much higher than our previous study with BSA and Tween-20 coated Ag NPs at the same Ag concentration. It is possible that primary granulosa cells are much more sensitive to toxicants than other cell lines, such as CHO-K1 or human monocytes (THP-1).

The underlying mechanisms about the cytotoxic and steroidalogenic effects of Au@Ag NRs are proposed in Figure 9. In
brief, the Au@Ag NRs are internalized by granulosa cells and translocated into lysosome. The internalized Au@Ag NRs and released silver ions induce intracellular oxidative stress, which damages the mitochondrial membrane integrity, resulting in lower MMP and decreased production of ATP. These conditions lead to mitochondria-mediated apoptosis, initiated by the activated cleavage of caspase 3 and caspase 9. Au@Ag NRs also promote the production of progesterone and estradiol through upregulation of steroidogenic proteins and enzymes including FSHR, StAR, P450scc, and P450arom. It is interesting to find that Au NR also upregulate the secretion of estradiol in granulosa cells. Our measurements by MTT assay and Annexin V-PI assay indicated that Au NR did not affect the cell viability (Figure 3A,B) or induce p53-activated cell apoptosis (Figure 4) among granulosa cells after 24 h exposure. But it is noteworthy that Au NRs induced a concentration-dependent decrease of mitochondrial membrane potential (Figure 3D) and an increase in the production of intracellular reactive oxygen species (Figure S5, Supporting Information), which might affect estradiol secretion. Further studies are needed to investigate the pathways about how silver nanomaterials induce upregulation of steroidogenic proteins and enzymes. Bases on ESR results and XANES evidence, we proposed that the toxicity effects are partially due to the surface reactivity of Au@Ag NRs, but mainly come from the silver ion release from the silver shell of Au@Ag NRs. The reason is that Au@Ag NRs have a similar or slightly higher surface reactivity than Au NRs but exert a much higher cytotoxicity and steroidogenic effects.

3. Conclusion

To address potential influence of silver nanoparticle-containing products on reproductive health, we systemically evaluated the cytotoxicity and steroidogenic effects of Au NRs and Au@Ag NRs in rat primary granulosa cells and explored the underlying mechanisms. Our results show that both Au NRs and Au@Ag NRs are taken up by granulosa cells and translocate to lysosomes. Au NRs appeared to have minimal effects, while Au@Ag NRs increased ROS generation and decreased viability of granulosa cells. These adverse effects occur as a consequence of the damage to the mitochondria membrane integrity. ATP production was reduced and cytoskeleton was disrupted to the point of mitochondrial-mediated apoptosis. Au@Ag NRs significantly increased the secretion of progesterone and estradiol in a time- and dose-dependent manner. These observations were further validated by measuring the time-dependent upregulation of the steroid regulation proteins and enzymes including FSHR, StAR, P450scc, and P450arom. Exposure to Au@Ag NRs clearly interfered with the physiological functions of granulosa cells. Based on ESR and XANES data indicating free radical generation of both particles and the intracellular release and transformation of silver ions, we propose that both effects contribute to the cytotoxicity and steroidogenesis caused by Au@Ag NRs. The study also revealed relationship between apoptosis and the production of steroids in the ovary mediated by silver, which may pose a threat to follicular development. To conclude, cautious should be taken when considering the use of silver nanomaterials in various biomedical and commercial products.

4. Experimental Section

The Preparation of Au NRs and Au@Ag NRs: Au NRs and Au@Ag NRs coated with PDDAC were synthesized as described in the Supporting Information.

Au NRs and Au@Ag NRs Characterization: UV–Vis–NIR spectra of Au NRs and Au@Ag NRs were recorded with UV5300 (METASH). Morphological and elemental analyses of Au NRs and Au@Ag NRs were performed with a TEM (Tecnai G2 20 S-TWIN) coupled with energy-dispersive analysis of X-rays (Genesis 2000 XMS). The size and aspect ratio of Au NRs and Au@Ag NRs were measured with Image J by manually measuring the length and width in TEM images of Au NRs and Au@Ag NRs. At least, 100 nanorods were counted for each sample. The concentration of Ag and Au in Au NRs and Au@Ag NRs stock solution was determined by ICP-MS. Briefly, 100 µL Au NRs or Au@Ag NRs stock solution was soaked in HNO₃ (4 mL, 69%) overnight and heated to about 140 °C until the solution was colorless and clear, which indicates the complete evaporation of nitrogen oxides. To completely dissolve Au NRs, a strong acidic solution of HCl:HNO₃ volume ratio of 3:1 was added and the mixture was heated to 140 °C until the solution volume reduced to about 0.5 mL. The solution volume was then adjusted to 3 mL by adding mixed acidic solution containing 1% HCl (v/v) and 2% HNO₃ (v/v). Afterward, the Au and Ag contents were analyzed using ICP-MS, with bismuth (20 ng mL⁻¹) and indium (20 ng mL⁻¹) as an internal standard for Au and Ag, respectively.

Animals: Three weeks old female Sprague–Dawley rats were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. and housed in stainless steel cages under standard conditions (temperature: 26–28 °C; relative humidity: 30%–70%). Rats were given free access to distilled water and food. All procedures carried out in this experiment were compliant with the local animal ethics committee.

Rat Granulosa Primary Cell Isolation and Identification: Rat granulosa cells were isolated as described previously with minor modifications.[63] Briefly, female Sprague–Dawley rats (three weeks old) were intraperitoneally injected with 40 IU PMSG. After 48 h, the rats were sacrificed by decapitation and ovaries were isolated and transferred into DMEM/F12 medium supplemented with 15% FBS. Granulosa cells were obtained from follicles by needle puncture. Cells were then centrifuged 1000 rpm, 10 min at room temperature. The cell pellet was resuspended with cell culture medium and seeded in tissue culture plates. Cells were cultured at 37 °C in a 5% CO₂ atmosphere in DMEM/F12 supplemented with FBS (15%, v/v), penicillin (100 IU mL⁻¹), and streptomycin (100 µg mL⁻¹). Immunofluorescent staining with anti-FSHR antibody (Abcam, ab103874) was carried out to identify the isolated granulosa cells. Images were captured with a CLSM and analyzed with Carl Zeiss AIM software.

Cellular Uptake and Intracellular Localization Analysis: The cellular uptake of Au NRs and Au@Ag NRs were measured by ICP-MS. Granulosa cells were seeded in six-well plates and exposed to either Au NRs or Au@Ag NRs for 3, 12, or 24 h, respectively. After exposure, cells were washed three times with PBS to eliminate
the residual nanomaterials on the cell surface. Cells were then trypsinized and collected. Cell number was counted by hemocytometer and calculated with the cell suspension volume. Cell pellets were then added with HNO₃ and heated as described above. The concentration of Au and Ag taken up into cells was calculated by dividing the total internalized Au and Ag by corresponding cell number. The quantity of internalized nanomaterials was expressed as ng Au or Ag/10⁶ cells. The intracellular localization of Au NRs and Au@Ag NRs was characterized by TEM. Cells were seeded in six-well plates and exposed to 1 µg mL⁻¹ Au NRs or Au@Ag NRs for 24 h. Then cells were washed three times with PBS and collected. After centrifugation, cells were washed once with PBS and centrifuged again. Glutaraldehyde (2.5%, 500 µL) was added to the cell pellet and stored at 4 °C overnight. Afterward, cells were dehydrated with ethanol, embed in resin, cut to ultrathin section, stained by osmic acid and imaged with TEM (Hitachi 7700).

**Cell Viability Measurement:** Viability of primary granulosa cells after Au NRs or Au@Ag NRs exposure was measured by MTT assay. Cells were seeded in 96-well plates and cultured until 80% confluency. Au NRs or Au@Ag NRs of different concentrations were added into cells and incubated for 24 h. After incubation, cells were washed once with PBS to eliminate the residual nanomaterials on the cell surface, then 100 µL MTT (0.5 mg mL⁻¹, diluted with culturing medium) was added and cultured in the incubator. After 2 h incubation, MTT solution was removed and 100 µL dimethyl sulfoxide was added. After shaking for 2 min, the absorbance was measured with a microplate reader (Tecan infinite M200) at 570 nm.

**Cell Apoptosis and Necrosis Analysis:** Alexa Fluor 488-Annexin V and propidium iodide (PI) staining were applied to analyze the ratio of apoptosis and necrosis cells after Au NRs or Au@Ag NRs exposure. Briefly, cells were seeded in 24-well plates and exposed to Au NRs or Au@Ag NRs for 24 h. After exposure, cells were collected and stained with Alexa Fluor 488 Annexin V and PI according to the manufacturer’s instruction. Samples were incubated on ice until analysis. For color compensation, Alexa Fluor 488 Annexin V staining and PI staining were applied as single staining. Cells that had not been exposed to either Au NRs or Au@Ag NRs were used as controls.

**Real-Time PCR Gene Expression Analysis:** Total RNA was extracted using TRIzol reagent (Sigma) according to the manufacturer’s instruction. Granulosa cells were seeded in six-well plates and exposed to Au NRs or Au@Ag NRs for 24 h. Cells were collected and total RNA was isolated according to the manufacturer’s instruction. Total RNA (2 µg) was used for reverse transcription using a Promega reverse transcriptase system (Invitrogen, ThermoFisher). Real-time quantitative PCR was performed using a real-time PCR instrument (Realplex4, Eppendorf). Each sample was measured in triplicate. Primers used in PCR reactions were designed by Primer Premier 5.0 and those sequences are listed in Table S1 of the Supporting Information.

**Western Blotting Analysis:** Western blotting was applied to analyze the expression levels of apoptosis-related proteins and steroidogenesis regulation proteins and enzymes. Granulosa cells were seeded in six-well plates and exposed to Au NRs or Au@Ag NRs for 24 h in DMEM/F12 culturing medium or in DMEM/F12 medium containing FSH (30 ng mL⁻¹) and androstendione (9 ng mL⁻¹) for 6, 12, 24, and 48 h, respectively. After incubation, cells were lysed in radio-immunoprecipitation assay buffer (100 µL) containing 1 × 10⁻³ M phenylmethylsulfonyl fluoride (PMSF). The total protein concentration was measured by the bicinchoninic acid (BCA) method (Pierce). For blotting analysis, 30 µg protein was electrophoresed on 10%–12% sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose membranes. The immunoblotting was performed with various primary antibodies and secondary antibodies conjugated to hors eradish peroxidase. Proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, USA).

**Progesterone and Estradiol Quantification:** Progesterone and estradiol secreted by granulosa cells were measured by radioimmunoassay (RIA). Granulosa cells were seeded in 24-well plates and exposed to Au NRs or Au@Ag NRs in DMEM/F12 medium containing FSH (30 ng mL⁻¹) and androstenedione (9 ng mL⁻¹) for 6, 12, 24, and 48 h respectively. After exposure, the supernatant was collected for RIA, and granulosa cells were collected and lysed for protein quantification. The progesterone and estradiol secreted by cells were normalized with corresponding total cellular protein content.

**Electron Spin Resonance Measurements:** ESR spectra were obtained with a Bruker EMX ESR Spectrometer (Billerica, MA, USA). Each sample was mixed with reactants and transferred to a quartz capillary tube that was placed into the microwave cavity of the ESR spectrometer.

**Characterization of Intracellular Silver Chemical Species:** To study the chemical transformation of silver during cellular uptake, we exposed granulosa cells to Au@Ag NRs (0.5 µg mL⁻¹) for 12 and 24 h. Totally, 1 × 10⁷ cells were collected, rinsed three times with PBS, centrifuged to be a cell pellet, lyophilized, and transferred to a sealed tube. Before XANES measurement, dried cell samples were pressed to a flat and uniform pellet adhering to a tape (3M). XANES spectra of silver K-edge were mainly recorded on beamline BL-14W1 at Shanghai Synchrotron Radiation Facility (SSRF) in China. The transmission mode was used to measure XANES spectra for reference materials (Ag foil, Ag₂S, Ag₂O, AgCl, AgNO₃, Ag₂SO₄ from Sigma) and Au@Ag NRs suspension (10 µg mL⁻¹). Equipped with a 32-element germanium solid-state detector, the fluorescence mode was adopted to collect XANES spectra for NP-exposed cells. XANES data were first normalized in order to facilitate comparison of spectra from the varied samples, modes, or facilities. The preprocessed data were then analyzed with least-squares fitting to calculate the ratio of silver species using IFEFFIT Athena software (CARS, the Consortium for Advanced Radiation Sources at University of Chicago).

**Statistical Analysis:** Data are expressed as mean ± standard deviation. Statistical significance was calculated using the Student’s t-test or one-way ANOVA to compare differences between different groups. P value of <0.05 was considered as statistically significant.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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