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Short Communication

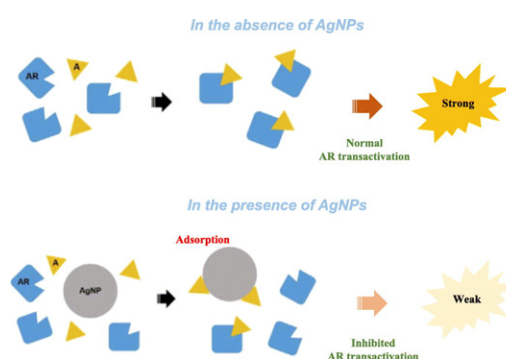
Insight on cytotoxic effects of silver nanoparticles: Alternative androgenic transactivation by adsorption with DHT

Jae Soon Kang^a, June-Woo Park^{a,b,*}^a Gyeongnam Department of Environmental Toxicology and Chemistry, Korea Institute of Toxicology, Jinju, Gyeongnam, Republic of Korea^b Human and Environmental Toxicology Program, Korea University of Science and Technology (UST), Daejeon, Republic of Korea

HIGHLIGHTS

- AgNPs are mostly maintained as a particle form in 22Rv1 cells via endocytosis in a size-dependent manner.
- AgNPs are mostly maintained as a particle form in 22Rv1 cells.
- The AR transactivation was inhibited through DHT adsorption to AgNPs in 22Rv1 cells.

GRAPHICAL ABSTRACT



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ABSTRACT

Silver nanoparticles (AgNPs) are accumulated in the male reproductive organs for a long time and cause several adverse effects in there. Up to now, there is little of information for the cytotoxic effects in male reproductive cells. In this study, the stable AgNPs with a minimal silver ion (Ag^+) dissolution below concentration inducing cytotoxicity in the cell medium were exposed to the human prostate carcinoma cell line 22Rv1. Moreover particle uptake and androgen receptor (AR) transactivation were evaluated. In cell medium, AgNPs exhibited stability in an aqueous environment and minimal Ag^+ release. Transmission electron microscopy (TEM) and energy dispersive spectrometer (EDS) analysis demonstrated uptake of AgNPs into cells via endocytosis, and a quantitative Ag assay showed that uptake of AgNPs was size-dependent with the majority of Ag retained in the particle form. To evaluate if the presence of AgNPs can change androgenic potentials of dihydrotestosterone (DHT, strong human androgen), we conducted an AR transactivation assay using the transgenic prostate cell line 22Rv1-MMTV-Hyg and found that AgNPs lowered androgenic transactivation of DHT, which is due to decreased bioavailability of DHT.

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1. Introduction

Silver nanoparticles (AgNPs) are a useful material, but many studies have warned that they cause adverse effects such as cytotoxicity, genotoxicity and immune responses in mammalian models and human cells (Hussain et al., 2006; Kim et al., 2014; Kim et al., 2008; Koedrich et al., 2014; Park et al., 2010; Yen et al., 2009). The underlying

* Corresponding author at: Gyeongnam Department of Environmental Toxicology and Chemistry, Korea Institute of Toxicology, 52834 Jinju, Republic of Korea.

E-mail address: jwpark@kitox.re.kr (J.-W. Park).

mechanisms of AgNPs toxicity have been suggested to be induced by two prevailing theories: the dissolution of silver ions (Ag^+) from AgNPs and the nanoparticles themselves. Nowadays, it is known that Ag^+ is primarily but not solely responsible for the toxicity of AgNP (Beer et al., 2012; Gomes et al., 2013; Kim et al., 2009; Xu et al., 2011). The oxidative damage by reactive oxidative species (ROS) generated from the nanoparticles themselves and the interaction between AgNPs and biomolecules are the major sources of the adverse effects caused by AgNPs.

Nanoparticles, including AgNPs, have a remarkably surface-to-volume ratio, resulting in strong reactivity with other chemicals and biomolecules. AgNPs can internalize into cells and able to form a protein corona therein. This may disrupt the biological function or activity of intracellular proteins (Duran et al., 2015; Shannahan et al., 2015). In fact, the interaction between AgNPs and biomolecules is not expected to result in severe cytotoxicity, such as cell death. However, depending on the type of cells exposed to AgNPs, the influence induced by AgNPs may vary. For example, in the reproductive cells, the reaction between AgNPs and hormone seems to interfere with the function of the cells or organs. Since some studies have shown that nanoparticles could interact with biomolecules such as natural or synthetic hormones, e.g., 17β -estradiol and 17α -ethynylestradiol (Park et al., 2011; Sun et al., 2015), it wouldn't be an overstatement to say that internalized AgNPs in cells may associate with natural hormones and interrupt normal hormone-hormone receptor interaction.

AgNPs exposure in animal models results in the accumulation of particles in the reproductive organs over an extended period of time (Kim et al., 2008; Kim et al., 2010; van der Zande et al., 2012), and some studies have shown that testicular toxicity can be induced by AgNPs. In the testes of rats and mice, AgNPs causes adverse effects such as a thickened capsule, congested blood vessels, disorganization of the seminiferous tubules (Sts), detachment or reduction of the germinal epithelium, shrinking of the Sts, the absence of sperm, an increase in the abnormal sperm count and a reduction in sperm concentration (Ahmed et al., 2016; Zhang et al., 2015b). Besides, AgNPs were also reported to cause several cytotoxic effects such as DNA damage, apoptosis and necrosis in several types of testicular cells (i.e., Leydig cells, Sertoli cells and testicular embryonic carcinoma cells) and spermatogonial stem cells (Asare et al., 2012; Zhang et al., 2015a). Ong et al. (2016) showed that AgNPs might disrupt germline stem cell maintenance in the *Drosophila* testis.

Despite the importance of the prostate as a male reproductive organ—specifically, its secretion of an alkaline fluid to prolong the lifespan of sperm within the vaginal tract, there is little information regarding the prostatic toxicities associated with AgNPs. A study reported that AgNPs caused adverse effects in the prostate, i.e., disruption of the epithelial lining of prostatic alveoli, enlarged prostatic alveoli and excessive oozing of prostatic alveoli secretions in male rats (Mohammed et al., 2016). Besides, since dihydrotestosterone (DHT) which is a metabolite of testosterone is abundant in prostate and regulates prostatic functions (Carson and Rittmaster, 2003), the adverse effects by interaction between AgNPs and DHT have to be assessed in prostate or prostate cell.

Herein, we exposed three AgNPs which are stable in cell medium (with very low dissolution of Ag^+) to 22Rv1 cells, a carcinoma cell line isolated from the human prostate and observed the intracellular uptake of them. To find the novel cytotoxic effects of AgNPs on male reproductive cells having abundant androgenic hormone DHT, we estimated the effects on androgenic transactivation using transgenic cell line 22Rv1-MMTV-Hyg which is genetically modified for androgen receptor (AR) transactivation assay.

2. Materials and methods

2.1. Cell lines

The prostate carcinoma cell line 22Rv1 was purchased from the American Type Culture Collection (Manassas, VA, USA). The 22Rv1-

MMTV-Hyg cell line was kindly supplied by Dr. Hee Seok Lee at the National Institute of Food and Drug Safety Evaluation (NIFDS, Cheoun-ju, South Korea). This cell line was produced by stably transfecting 22Rv1 cells with a pGL4/hyg luciferase vector containing the androgen response element and was established to evaluate AR transactivation activity (Sun et al., 2016). The two cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Invitrogen, Waltham, MA, USA), $1 \times \text{GlutaMAX}^{\text{TM}}$ (Invitrogen) and $1 \times \text{Antibiotic-Antimycotic}$ solution (finally 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 0.25 $\mu\text{g}/\text{ml}$ amphotericin B, Invitrogen) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 ; the cells were passaged once per week. All cell culture reagents in this study were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Prior to exposing to AgNPs, the cells were seeded in plates (Peri dishes or 96-well plates) suitable for the corresponding experiments for 24 h at 37°C in a humidified atmosphere containing 5% CO_2 .

2.2. Aqueous characterization of the AgNPs in cell medium

Three well-controlled PVP-coated AgNPs of different sizes (NanoComposix, Inc., San Diego, CA, USA) in the form of stock dispersions (1000 mg/l) were used in this study: 10 nm OECD PVP BioPure Silver (AgNPs-10), 20 nm PVP BioPure Silver (AgNPs-20) and 50 nm PVP BioPure Silver (AgNPs-50). The aqueous properties of these AgNPs were provided by the manufacturer and are summarized in Supplementary Fig. S1. The ζ -potential and hydrodynamic diameter of each 10 mg/l AgNPs suspension in RPMI 1640 medium were measured using a Zetasizer Nano (Nano ZS 90, Malvern Instrument, Worcestershire, UK) every 24 h for a total time period of 72 h. The dissolved Ag^+ from each 10 mg/l AgNPs suspension in RPMI 1640 medium was exclusively obtained by using an Amicon Ultra-4 (Ultracel-3 K, Merck Millipore, Billerica, MA, USA), and the concentration was evaluated by inductively coupled plasma-optical emission spectroscopy (ICP-OES, PerkinElmer, Waltham, MA, USA).

2.3. Observation of AgNPs internalization

To verify the internalization of AgNPs into 22Rv1 cells, transmission electron microscopy (TEM) and energy-dispersive X-ray spectroscopy (EDS) analysis were conducted. Briefly, cells were exposed to 2 mg/l AgNPs for 24 h, rinsed with phosphate-buffered saline (PBS) to remove any AgNPs remaining in the medium and harvested via trypsin treatment and centrifugation. The harvested cells were fixed using a 2.5% glutaraldehyde solution (Sigma-Aldrich), and the preparation of these samples for TEM and EDS analysis was performed at the Korea Advanced Institute of Science and Technology (Daejeon, South Korea). To quantify total Ag internalized into cells, 5×10^6 cells in 100-mm petri dishes were exposed to 2 mg/l AgNPs for 24 h at 37°C in a humidified incubator containing 5% CO_2 . To remove any remaining AgNPs and dissolved Ag^+ from the medium, cells were rinsed five times with PBS. After the cells were harvested by trypsin treatment and centrifugation, the resulting pellet was resuspended in fresh cell medium, and the cells were lysed using a TissueLyser (Qiagen, Hilden, Germany). Approximately half of the cell lysate was dissolved with nitric acid (12.5%, v/v), and total Ag was quantified by ICP-OES. The remaining cell lysate was filtered using an Amicon Ultra-4 (Ultracel-3 K, Merck Millipore), and the Ag^+ released from the AgNPs was quantified by ICP-OES.

2.4. AR transactivation assay

To find the exposure range of AgNPs, 22Rv1-MMTV-hyg cells (3×10^4 per well) in clear 96-well plates were exposed to 2 to 100 mg/l AgNPs for 24, 48 and 72 h. Untreated cells were used as a control. To measure cell viability, 20 μl of CellTiter 96 Aqueous One Solution reagent (Promega, Fitchburg, WI, USA) was added to each well. After the cells were

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