Male- and female-derived somatic and germ cell specific toxicity of silver nanoparticles in mouse

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Running title: silver nanoparticles toxicity in mouse germ cells

Supplementary methods

Synthesis and characterization of AgNPs

Synthesis and characterization of AgNPs were performed according to the method described previously (Gurunathan et al., 2009). The Dynamic Light Scattering (DLS), UV-visible spectra and X-ray diffraction (XRD) of AgNPs were measured by using Zetasizer Nano ZS90 (Malvern Instruments; Limited; UK), Optizen POP (Mecasys, South Korea), and Bruker D8 Discover (Bruker AXS GmbH, Karlsruhe, Germany) instruments respectively in National Center for Inter-University Research Facilities (Seoul National University, South Korea). The high resolution XRD patterns were measured at 3 kW with Cu target using a scintillation counter ($\lambda = 1.5406\text{\AA}$) at 40 kV and 40 mA; recorded range value is $2\theta = 5^\circ\text{--}80^\circ$. The EDX spectra were performed to analyze the purity of the material and the complete chemical composition of synthesized silver nanoparticles. Transmission electron microscopy (TEM), using a JEM-1200EX microscope was performed to determine the size and morphology of AgNPs. TEM images of AgNPs were obtained at an accelerating voltage of 300 kV.

Transmission electron microscopy (TEM) analysis

After fixation of arterial specimens in 2.5% glutaraldehyde (Redding, CA, USA) in PBS (pH, 7.2), samples were postfixed in 1% osmium tetroxide (Heraeus, Boksburg, South Africa), dehydrated in a graded series of ethanol baths and propylene oxide (Acros Organics, New Jersey, USA), and then embedded in epoxy resin [EMbed 812 containing nadic methyl anhydride (NMA), dodenyl succinic anhydride (DDSA), and DMP-30] as described previously (Kuo, 2007). Serial ultrathin sections were cut on an LKB-III ultratome (LEICA, Wetzlar, Germany). Sections were stained with uranyl acetate and lead citrate (Redding, CA, USA). Sections were examined under a Hitachi H7600 electron microscope (Hitachi, Tokyo,

Japan) at an accelerating voltage of 100 kV.

Real-time quantitative reverse transcription polymerase chain reaction

Polymerase chain reaction (PCR) was performed according to the instructions of the real-time PCR machine manufacturer (ViiATM7, Applied Biosystems, Foster City, CA). For the analysis of cytokines, we determined that a 400- μ L aliquot of blood collected after two different doses (0.5 mg/kg and 1.0 mg/kg) of AgNPs provided sufficient mRNA for gene expression studies. We successfully quantified the expression levels of tumor necrosis factor α (TNF- α), INF- γ , interleukins-6 (IL-6) and - β (IL- β), and monocyte chemoattractant protein-1 (MCP-1) with this approach. The RT-PCR primer sets are shown in **Supplementary Table 1**. Target gene expression levels were normalized to GAPDH gene expression.

Hematological analysis

Hematological and clinical chemistry analyses were performed on blood samples obtained at autopsy. Blood was collected in EDTA-coated tubes. Hematological parameters included white blood cell (WBC), lymphocyte (LY), monocyte (MO), neutrophil (NE), eosinophil (EO), and red blood cell (RBC) counts; mean corpuscular volume (MCV); hematocrit (HCT); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC); hemoglobin (HGB); red blood cell distribution width (RDW); platelet (PLT) count; and mean platelet volume (MPV). All hematological parameters were determined using an Advia 120 Hematology Analyzer (Siemens, Germany). Blood smears were also prepared for visual evaluation.

Histopathology and TUNEL staining

The kidney, liver, lung, spleen, testes, and ovaries were removed and examined microscopically for gross lesions. All specimens were fixed in 4 % paraformaldehyde and embedded in paraffin. Cross-sections were obtained, stained with hematoxylin and eosin, and mounted with Vectashield medium. Cross-sections were examined under a light microscope.

Supplementary Table 1.

Gene	Primer	TM(°C)
3β-HSD	F: CTGAATGTTACTGGCAAATTCTC	58
	R: TGTAATAATGGACGCAGCAGGAA	
17β-HSD3	F: GTTCTCGCAGCACCTTTTTC	58
	R: ACAATCTTCACACCGCTTCC	
CYP19	F: CACCCTTCCAAGTGACAGGA	58
	R: AAAAAAGTAAAGTTCTATGGGAA	
CYP17	F: TTTTGGCCCAAGTCAAAGAC	53
	R: CCCTTCTTCACGAGCACTTC	
GATA4	F: CCCTACCCAGAATACATGG	58
	R: ACATATCGAGATTGGGGTGTCT	
STAR	F: GCAGCAGGCAACCTGGTG	58
	R: TGATTGTCTTCGGCAGCC	
CYP11a1	F: CGATACTCTTCTCATGCGAG	58
	R: CTTTCTTCCAGGCATCTGAAC	
Gapdh	F: AGGTCGGTGTGAACGGATTG	58
	R: TGTAGACCATGTAGTTGAGGTCA	
Stra8	F: ATTATAATGGCCACCCCTGG	58
	R: CCAGGCTTTCTTCCTGTTC	
Sohlh1	F: TGACAACGCGCTCTGGCG	60
	R: TGCCTCAGTTTGATGGCC	
SCP1	F: AGGTTGAGAAAGCCAAAGCA	60
	R: GTCTCCAAAGCAATCTTCGC	
SCP3	F: CATTCTGGGAAATCTGGGAA	60
	R: GGAGCCTTTTCATCAGCAAC	
GFRA1	F: GAACTACGCAGACTGCCTCC	58
	R : ATCTTCCAGGTCATTGCCAC	
PLZF	F: AACGGTTCCTGGACAGTTTG	60
	R: ACTCGCACAGCTGGTAGGTT	
Dazl	F: AAGGCAAAATCATGCCAAAC	58
	R: AAAATCAAAGGACGTGGCTG	
Mdc1	F: CACCAGGACACCAACATCTG	58
	R: TCTTCCCCTGATTTCCCTCT	
Dnmt3L	F: CTGCTGACTGAGGATGACCA	58
	R: GCTTGCTCCTGCTTCTGACT	
Miwi2	F: CAACAAGAATGTGGTGGTCG	58
	R: GTCATGCAGACTTTGAGGCA	
Hrb	F: AATTTTGGAGGTTTCCCC	60
	R: TGCTGTCTGATGACTCTGGG	
GOPC	F: GCTGCATGTTGGAGATGCTA	58
	R: ATCTCGCCACTGCTGTCTTT	
PRM1	F: AGCAAAAGCAGGAGCAGATG	58
	R: CCTTATGGTGTATGAGCGGC	
PRM2	F: GTTCGCTACCGAATGAGGAG	58
	R: CCATAGTCCTCTACGCGCTC	
FIGLA	F: ACAGAGCAGGAAGCCCAGTA	60
	R: ACTCGCACAGCTGGTAGGTT	
NOBOX	F: ACAAACGCCATGAGATTTCC	58
	R :AACAGGGCCAGGTTCTAGGT	

LHX8	F: CAGTTCGCTCAGGACAACAA	58
	R: AGCCATTTCTTCCAACATGG	
FOXO3A	F: GATGATGATGGACCCCTGTC	58
	R: TCTTGGCGGTATATGGGAAG	
ABP	F:CACGCAGAATTCAGTCTCCA	60
	R:CCTGTTCCAAGAGCAAGGAG	
GATA1	F:GATGGAATCCAGACGAGGAA	60
	R:CAGCAGAGGTCCAGGAAAAG	
Inhibin B	F:ACAGGCCCTTTGTAGTGGTG	60
	R:AGCCGAAAGTCGATGAAGAA	
Serotonin receptor	F:GGAGAAAAGGCTGCAGTACG	60
	R:ATAACCAGGCAGGACACAGG	
TNF-α	F:CATCTTCTCAAAATTCGAGTGACAA	53
	R: TGGGAGTAGACAAGGTACAACCC	
INF-γ	F: TCAAGTGGCATAGATGTGGAAGAA	58
	R:TGGCTCTGCAGGATTTTCATG	
IL-1β	F: AGGTCGCTCAGGGTCACAAG	58
	R:GTGCTGCCTAATGTCCCCTTGAATC	
IL-6	F: GACAACCTTTGGCATTGTGG	58
	R: ATGCAGGGATGATGTTCTG	
MCP-1	F: AAGTTGACCCGTAAATCT	58
	R: CTAGTTCACTGTCACT	

Supplementary Table 2.

Male				Female		
Parameters	PBS	500ug/kg AgNP	1000ug/kg AgNP	PBS	500ug/kg AgNP	1000ug/kg AgNP
WBC(K/ μ l)	5.635($\pm$ 2)	5.336($\pm$ 1.29)	4.58($\pm$ 0.96)	4.11($\pm$ 1.26)	4.705($\pm$ 0.97)	3.206($\pm$ 0.48)
LY(%)	82.69($\pm$ 6.92)	78.97($\pm$ 3.36)	82.46($\pm$ 3.53)	82.315($\pm$ 4.45)	77.5575($\pm$ 5.39)	88.706($\pm$ 3.72)
MO(%)	3.37($\pm$ 1.29)	4.776($\pm$ 0.55)	3.76($\pm$ 0.82)	4.09($\pm$ 1.06)	4.215($\pm$ 0.5)	2.474($\pm$ 0.4)
NE(%)	10.32($\pm$ 4.85)	13.002($\pm$ 2.46)	12.25($\pm$ 2.78)	13.695($\pm$ 2.86)	8.27($\pm$ 3.57)*	5.93($\pm$ 2.89)*
EO(%)	3.081($\pm$ 3.56)	1.93($\pm$ 1.03)	1.00($\pm$ 0.85)	0.8875($\pm$ 0.3)	10.1475($\pm$ 8)	3.016($\pm$ 3.32)
BA(%)	0.37($\pm$ 0.31)	0.644($\pm$ 0.23)	0.345($\pm$ 0.24)	0.6375($\pm$ 0.33)	0.475($\pm$ 0.19)	0.456($\pm$ 0.13)
RBC(M/ μ l)	6.605($\pm$ 1.08)	7.406($\pm$ 0.34)	6.995($\pm$ 1.42)	6.755($\pm$ 1.09)	6.805($\pm$ 0.91)	6.182($\pm$ 0.62)
MCV(fL)	58.62($\pm$ 3.2)	57.306($\pm$ 1.63)	61.4275($\pm$ 2.87)	58.335($\pm$ 0.6)	58.61($\pm$ 4.32)	57.368($\pm$ 1.39)
HCT(%)	38.97($\pm$ 8.23)	42.706($\pm$ 2.5)	42.9275($\pm$ 8.82)*	45.3975($\pm$ 6.53)	38.0625($\pm$ 4.48)	35.412($\pm$ 2.94)
MCH(pg)	15.81($\pm$ 0.59)	16.582($\pm$ 0.24)	19.69($\pm$ 5.97)	16.3($\pm$ 0.28)	15.955($\pm$ 0.72)	15.686($\pm$ 0.34)*
MCHC(g/dL)	27.03($\pm$ 1.47)	28.116($\pm$ 0.4)	31.96($\pm$ 8.9)	27.9425($\pm$ 0.55)	27.3575($\pm$ 1.18)	27.038($\pm$ 0.91)
Hgb(g/dL)	10.48($\pm$ 1.96)	12.116($\pm$ 0.39)	13.1675($\pm$ 0.99)	12.695($\pm$ 1.91)	12.1025($\pm$ 1.43)	9.534($\pm$ 1.07)
RDW(%)	16.23($\pm$ 0.49)	16.62($\pm$ 0.6)	15.85($\pm$ 0.85)	16.325($\pm$ 0.34)	16.45($\pm$ 0.66)	16.4($\pm$ 0.41)
PLT(K/ μ l)	763.16($\pm$ 228.73)	827.4($\pm$ 343.1)	1145.25($\pm$ 298.6)	877.5($\pm$ 121.05)	826.25($\pm$ 160)	735($\pm$ 234.17)
MPV(fL)	7.37($\pm$ 0.88)	6.404($\pm$ 0.57)	6.6125($\pm$ 0.28)	6.6275($\pm$ 0.12)	6.965($\pm$ 0.13)	7.866($\pm$ 0.6)

Supplementary Table 1.

Primers used for quantitative real-time polymerase chain reaction

Supplementary Table 2.

Effect of silver nanoparticles on blood parameters

Supplementary Fig. 1. UV-visible spectrum (**a**), size distribution analysis by DLS (**b**), XRD

(c) and EDX spectra analysis (d) of AgNPs.

Supplementary Fig. 2.(a) TM4 Cell viability relative to the control (100%) was measured using the Cell Counting *Kit*-8 (*CCK*-8), exposed to different concentrations of AgNO₃ for 24 hr. Experiments were performed in triplicate, and values are expressed as mean ± SEM of three independent experiments. (b) H₂-DCFDA-positive cells measured by flow cytometry in TM4 cells treated with different concentrations of AgNO₃ for 24 h. M2 represents the

population of H₂-DCFDA-positive cells. ** $P < 0.01$ compared with the control.

Supplementary Fig. 3. H&E staining of lung, liver, kidney, and spleen of control and AgNPs-treated mice. A, alveoli; B, bronchiole; PA, pulmonary artery; PT, proximal tubules; IA, interloper artery; CV, central vein; SV, sub lobular vein; GC, germinal center

Supplementary Fig. 4. Abundance of macrophage in spleen. M indicate macrophage.

Supplementary Fig. 5. Effect of AgNPs on the expression of cytokines. Cytokine levels measured from pooled samples of 6 male (A) and female (B) mice after 15 days of treatment with 0.5 mg/kg and 1.0 mg/kg body weight of AgNPs. Experiments were performed in triplicate, and values are expressed as mean $\pm$ SEM of three independent experiments.

**** $P < 0.01$, *** $P < 0.001$** compared with the control. *TNF- α* , tumor necrosis factor α ; *IFN- γ* , interferon- γ ; *IL-6*, interleukin 6; *IL- β* , interleukin β ; *MCP-1*, monocyte chemoattractant protein 1.

Supplementary Fig. 6. Analysis of caput epididymis in control and AgNPs-treated mice.

Dark stained signals in each tubule indicate spermatozoa. Of note, control mouse-derived tubules contained a lot of spermatozoa, whereas in AgNPs-treated shows mouse derived

tubules have rare spermatozoa.

Supplementary Fig. 7. Male somatic cells (Sertoli and Leydig cells)-specific gene expressions and apoptosis analysis. (a) RT-PCR analysis of Sertoli cells-specific gene expression. *Abp*, *Gata 1* *Inhibin b* and *Serotonin receptor*. (b) Relative mRNA expression of Leydig-specific genes (*3 β -HSD*, *17 β -HSD*, *Cyp 17a1* and *Cyp19a1*) in the testes of control and AgNP-treated mice. Each target gene expression levels were normalized to GAPDH gene expression. Experiments were performed in triplicate, and values are expressed as $\pm$ SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the control. (c) Tunnel assay analysis. Green color indicates apoptosis in male germ cells. ST, seminiferous tubule; *Abp*, Androgen binding protein; *Gata1*, GATA binding protein 1.

Supplementary Fig. 8. Ovary analysis after AgNPs treatment. (a) The status of ovaries condition was analyzed in control and AgNPs treated mice. Hard ovary tissues changed to soft tissues after AgNPs treatment. (b) Ovary tissue section analysis. After AgNPs treatment, the numbers of follicles in ovaries are dramatically reduced in a time-dependent manner and atrophy of developing follicles can be found in the ovaries. At 8 weeks, most of developing follicles are disappeared.

Supplementary Fig. 9. Analysis of primordial specific mRNA and protein expression.

(a) RT-qPCR analysis of female germ cell-specific genes. The expression levels of *Figla*, *Nobox*, *Lhx8*, and *Foxo3A* were significantly lower in AgNPs-treated mice than those in the control. Each target gene expression levels were normalized to GAPDH gene expression.

Experiments were performed in triplicate, and values are expressed as $\pm$ SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the control. **(b)** Lhx8 immunohistochemistry analysis. After AgNPs treatment, the number of follicles in ovaries was dramatically reduced in a time-dependent manner. At 8 weeks after AgNPs treatment, Lhx 8 positive signals were not detected in the ovaries. *Nobox*, newborn ovary homeobox; *Lhx8*, LIM homeobox 8.

Control male X control Female

AgNPs-treated male X control Female

Control male X AgNPs-treated Female

AgNPs-treated male X AgNPs-treated Female

Supplementary Fig. 10. Representative images of pregnant uterus, which contains developing or arrested/aborted fetuses after mating with AgNPs-treated female or male mice.

Supplementary Fig. 11. The hypothetical model to explain the mechanism of AgNPs induced cell death in TM4 cells. We propose AgNPs induced ROS mediated apoptosis in TM4 cells involve oxidative stress, organelle damage, induction of autophagy, membrane damage, down regulation of survival protein, Akt and up regulation of p53 and cytochrome c release. The thickness of the arrows indicates the importance of the biological process

regulated by AgNPs in the cell. N, nucleus.

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