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Toxicogenomic analysis for livers from sprague-daley rats following 12-week inhalation exposure to silver nanoparticles

Abstract

Silver nanoparticles (AgNPs) have been extensively applied to many industrial and biomedical fields due to their antibacterial effect. However, a large number of applications is also lead to health and environmental safety concerns. Up to date, it was well-known that AgNPs induced reactive oxygen species (ROS) production, cytotoxicity, pro-inflammatory effect, DNA damage, cell cycle disturb, necrosis and apoptosis by many researches. Also, several studies have been performed to investigate the microarray test for AgNPs in many cell types. However, no work reports the AgNPs toxicogenomic study in liver cell line and tissue until now. For this reason, we performed to *in vivo* toxicogenomic study for AgNPs inhalation exposed liver tissue. After 12 weeks inhalation exposure to AgNPs for the Sprague-Daley rats, we carried out silver concentration measurement for liver tissues and toxicogenomic analysis. As a result, we found that silver concentrations in livers were dose-dependently increased in male and female rats. However, a gender-different accumulation of silver in the livers did not observe. In toxicogenomic study, we observed that 109 and 150 genes significantly up- and down regulated by AgNPs inhalation exposure in male and female rats, respectively. The significantly altered male rat genes were involved in 54 biological pathways which were typically related with diabetes and metabolism. In female rat, the significantly expression changed genes were involved in 89 biological pathways which were mainly connected with metabolism and cell signaling. Plus, the gender-dependent gene expression changes of more than 2 fold were linked to 240 genes, with 114 genes in the male livers and 126 genes in the female livers. These were related to steroids and xenobiotics metabolism pathway.

Keywords: Silver nanoparticles, *in vivo* toxicogenomic study, Liver tissue, Inhalation, Gene expression

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Kim JS,[1] Sung JH,[1] Song KS,[1] Dong MS,[2] Lee JH,[3] Song NW,[4] Ji JH,[5] Ahn K,[6] Kim TG,[3] Kim E,[3] Yu IJ[3]

[1]KCL, Incheon, Korea
[2]Korea University, Seoul, Korea
[3]Institute of Nanoproduct Safety Research, Hoseo University, Asan, Korea
[4]Korea Research Institute of Standards and Science, Daejeon, Korea
[5]Ecopictures, Seoul, Korea
[6]Hanyang University, Ansan, Korea

Correspondence: Il Je Yu, Institute of Nanoproduct Safety Research, Hoseo University, Asan, Korea, Tel 82-41-540-9630, Fax 82-41-540-9846, Email u1670916@chollian.net

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Abbreviations: AgNPs, Silver Nanoparticles; ROS, Reactive Oxygen Species; SPF, Specific-Pathogen-Free; OEL, Occupational Exposure Limit; DMAS, Differential Mobility Analyzing System; SMPS, Scanning Mobility Particle Sizer; GMD, Geometric Mean Diameter; GSD, Geometric Standard Deviation

Introduction

Nanoparticles increasingly used in consumer and industrial products, due to distinctive physicochemical properties as high reactivity, color change, lower melting temperature and greater solar radiation absorption.[1] One of the most commonly used nanoparticles is silver nanoparticles (AgNPs) which have been applied to clothing, food industry, paints, and electronics.[2-6] Also, AgNPs used in medical applications as the constituent elements of catheters, implant surfaces, dental alloys and for treating wounds and burns-related infections as well as in drug delivery in cancer therapies.[7-10] Such an increasingly widespread usage of AgNPs leads to the exposure of humans, animals and plants through industrial or domestic waste which could produce harmful biological response.[11] The target organs for silver nanoparticles have been shown to be the liver in a 28-day oral toxicity study[12-13] and 90-day oral subchronic study,[14] and the liver and lungs in 90-day inhalation studies.[15-16] It was reported that AgNPs induced reactive oxygen species (ROS) production, cytotoxicity, pro-inflammatory effect, DNA damage, cell cycle disturb, necrosis and apoptosis in various *in vitro* test.[17-20] However, *in vitro* studies could not reflect cell-cell and cell-matrix interaction and hormonal effect.[21] For this reason, the importance of the *in vivo* studies has already been highlighted in the nanotoxicology fields.[22] Additionally, the exact mode of action for silver nanoparticle was still poorly known. Accordingly, this study conducted a toxicogenomic examination of rat livers following 12 weeks of inhalation exposure to AgNPs. Fresh air exposed controls were compared with low and high concentration AgNPs exposed groups of male and female rats.

Materials and methods

Generation of silver nanoparticles

The silver nanoparticles were generated as described in previous reports.[23] The rats were exposed in a whole-body-type exposure chamber (1.3m^3, Dusturbo, Seoul), which consisted of a small ceramic heater (50 × 5 × 1.5 mm^3) that was housed within a quartz tube case (70mm diameter, 140mm length). Connected to an AC power supply, the heater achieved a surface temperature of about 1500^{0}C within a local heating area of 5 × 10 mm^2 within about 10s. For the long-term testing, the source material (about 160 mg) was positioned at the highest temperature point. Clean (dry and filtered) air was used as the carrier gas, and the gas flow was maintained at 30 L/min (Re=572, laminar flow regime) using a mass flow controller (MFC, AERA, FC-7810CD-4V, Japan).[23] In this study, the system produced different concentrations of nanoparticles (high, medium, and low) in three separate chambers. The nanoparticle generator was operated for 45.99±0.02 L/min (mean±SE) using the MFC and mixed with 200 L/min at the main flow rate through the high-concentration chamber. Using the MFC for the first dilutor (27.19±0.05 L/min, mean±SE), a portion of the high-nanoparticle-concentration was then diverted to the medium-concentration chamber. Similarly, a portion of the

medium-nanoparticle-concentration was then diverted to the low-concentration chamber using a second MFC (2.33 ± 0.01 L/min).[16]

Monitoring of inhalation chamber and analysis of silver nanoparticles

In the individual chambers, the nanoparticle distribution with respect to size was measured directly in real time using a differential mobility analyzer (Short type DMA, 4220, HCT Co., Ltd. Korea, range 5–150 nm) and condensation particle counter (CPC, 4312, HCT Co., Ltd. Korea, 0–10^8/cm^3 detection range), in combination referred to as a DMAS (differential mobility analyzing system) or SMPS (scanning mobility particle sizer). Nanoparticles from 4.23 to 46.95 nm were measured using sheath air at 5 L/min and polydispersed aerosol air at 1 L/min, in order to meet the operational conditions for the DMA and CPC, respectively. The particle concentration in the fresh-air control chamber was measured using a particle sensor (4123, HCT Co., Ltd. Korea) that consisted of 2 channels: 300–1000 nm and over 1000 nm.

Animals and conditions

The animal and exposure conditions were as described in the previous report by Song et al.[13] We purchased five-week-old specific-pathogen-free (SPF) Sprague Dawley male and female rats from KOATECH Co. (Korea) and acclimated for 1 week before starting the experiments. During the acclimation and experimental periods, the rats were housed in polycarbonate cages (3 rats per cage) in a room with controlled temperature (21.6 ± 1.2^{0}C), humidity (43.7 ± 6.8%), and a 12 hr light/dark cycle. The rats were fed a rodent diet (Harlan Teklab, Plaster International Co., Seoul) and filtered water *ad libitum*. The 6-week-old rats, weighing about 171 g for the males and 135g for the females, were then divided into 4 groups (each group consisted of 17 male rats: 5 rats for 12-week exposure, 4 rats for 4-week recovery, 4 rats for 12-week recovery, and 4 rats for micronucleus test after 12-week exposure; plus 12 female rats: 4 rats for 12-week exposure, 4 rats for 4-week recovery, and 4 rats for 12-week recovery): fresh-air control, low-dose group (target dose, 0.6×10^6 particles/cm^3, 1.0×10^9 nm^2/cm^2, 48.76 μg/m^3), medium-dose group (target dose, 1.4 × 10^6 particles/cm^3, 2.5 × 10^9 nm^2/cm^2, 117.14 381.43 μg/m^3) and high-dose group (target dose, 3.0 × 10^6 particles/cm^3, 5.0 × 10^9 nm^2/cm^2, 381.43 μg/m^3), and exposed to silver nanoparticles for 6 hr/day, 5 days/week, for 12 weeks. The doses were selected based on the current silver dust occupational exposure limit (OEL) (100 μg/m^3), the medium dose was similar to the OEL, and the high and low-doses were higher and lower than the OEL, respectively. Eight animals (4 male and 4 female) from the control, low-dose, and high-dose groups were used for the gene expression profile study after 12 weeks of exposure. The animals were examined daily on weekdays for any evidence of exposure-related effects, including respiratory, dermal, behavioral, nasal or genitourinary changes suggestive of irritancy. The body weights were measured at the time of purchase, at the time of grouping, one day after exposure, once a week during the inhalation exposure and recovery, and before necropsy.

RNA preparation and Oligonucleotide chip microarray

After 12 weeks of silver nanoparticle inhalation exposure, the total RNA was isolated from the liver using the TRIZOL reagent (Life Technologies, USA) according to the manufacturer's instructions. The RNA was then analyzed for quantity and purity based on the absorbance at 260 and 280 nm using NanoDrop ND-1000 (NanoDropTechnologies, Wilmington, DE). The gene expression profiling was performed using the Illumina Rat Ref-12 Expression BeadChip platform that contains 22,226 probes (Illumina Inc., San Diego, CA, USA). The quality control was repeated with labeled cRNA, and the hybridization on the Illumina® Rat Ref-12 Expression BeadChips was only performed with intact samples according to the manufacturers' recommendations (Illumina® protocol, www.illumina.com). Briefly, 500 ng of total RNA was labeled using an Illumina Total PrepRNA Amplification kit (Illumina Inc., USA) based on cDNA synthesis with an oligo-dT primer containing a T7 RNA polymerase promoter. The double-stranded cDNA was then used in an *in vitro* transcription reaction, and the generated single-stranded RNA (cRNA) was labeled by incorporating biotin-16-UTP (Roche Diagnosics GmbH, Mannheim, Germany). 750 ng of the biotin-labeled cRNA was hybridized (16 hrs) to a RatRef-12 Expression BeadChip (Illumina, San Diego, CA). The hybridized biotinylated cRNA was then detected with streptavidin-Cy3 (Amersham Biosciences, USA) and quantitated using an Illumina Bead Station 500GX Genetic Analysis System scanner.[24,25]

Microarray data analysis

Beadstudio v3.0 was used to evaluate the expression signals generated by the Illumina RatRef-12 Expression BeadChip arrays. The gene expression data were input into GenPlex™ v3.0 software (ISTECH Inc., Korea), normalized using the Quantile method, and the normalized data log-transformed using base 2. Thereafter, the fold change and Welch t-test were applied to select the differentially expressed genes (DEGs) using a volcano plot with a fold change threshold of 1.5 fold and $p < 0.05$ to indicate significance. The 1.5-fold DEGs were clustered based on hierarchical clustering using the Pearson correlation as the similarity measure and complete linkage as the linkage method. In addition, the gene ontology classification was provided by the KEGG database. A three-dimensional principal component analysis (PCA) was used to analyze and visualize the distribution of the 6 different biological sample types (NC, MLK, MHK, FLK, and FHK) within the three orthogonal components, which were linear combinations of the expression profiles of the selected genes. The number of genes used for the PCA was chosen by a Kruskal Wallis H-test, which classified the six different sample types with the maximum accuracy in the error estimation procedure. The weighted K-nearest neighbor (K-NN) classifier and leave-one-out-cross-validation (LOOCV) were used for the unsupervised classification and error estimation, respectively.

Results

The generated silver nanoparticles (AgNPs) properties and concentrations were measured regularly in exposure chamber.[16] In summary, In the high-concentration chamber, the geometric mean diameter (GMD), geometric standard deviation (GSD), total number concentration, and mass concentration and surface area of the silver nanoparticles were 15.00 ±1.75 nm, 3.24 ± 10^6 particles/cm^3, 381.43 mg/m^3 and 4.85 ± 10^9 nm^2/cm^3, respectively, in the medium-concentration chamber, these measurements were 14.38 ± 1.64 nm, 1.41 ± 10^6 particles/cm^3, 117.14 mg/m^3 and 1.70 ± 109nm^2/cm^3, respectively, and in the low-concentration chamber they were 14.54 ± 1.62 nm, 0.66 ± 10^6 particles/cm^3, 48.76 mg/m^3, and 0.76 ± 109 nm^2/cm^3, respectively. The concentrations of silver nanoparticles in each chamber were well maintained during the 12-week exposure period (Supplement 1). These data were summarized in Supplement 1. In each exposure chamber, the AgNPs were well maintained during the 12 weeks exposure period. The AgNPs observed by FE-TEM were spherical in shape and non-aggregated/non-agglomerated forms with diameters under 47 nm.[16] The diameters were log normally distributed from 4 to 47 nm and the Count media diameter (CMD) and geometric standard deviation (GSD) were 14.09 nm and 1.71, respectively, with a good correspondence to the mobility diameters.[16] To investigate tissue

distribution of AgNPs, we measured silver concentration in livers. As a result, we observed that the silver concentration in the livers dose-dependently increased in both male and female rats after 12 weeks of inhalation exposure to AgNPs (supplement 2). However, we could not observe the significant gender difference of silver concentration in livers. Then, we analyzed *in vivo* gene expression profiles with obtained liver tissue after 12 weeks AgNPs inhalation exposure. Using the DNA microarray, the gene expression changes were evaluated for the livers which obtained from male and female rats exposed to the low and high concentration of AgNPs. The over 1.3-fold up- or down-regulated genes ($p<0.05$) were regarded as significant and used for the data mining categories. Among 403 genes, 109 and 150 genes were observed expression changes by AgNPs inhalation exposure in male and female rats, respectively. (Figure 1A) (Table 1&2).

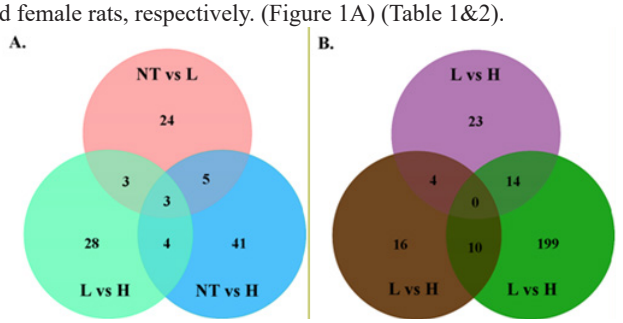

Figure 1 Venn diagram for the numbers of genes expressed significantly different to each control group (Fold change > 1.3, $p<0.05$).

NT: No Treatment; L: Low; H: High

Table 1 Gene expression associated with silver nanoparticles (AgNPs) inhalation exposure in male livers

NCBI Accession No.	Gene Symbol	Average Fold Change		
		NT vs L	NT vs H	L vs H
NM_053299.1	Ubd	0.183	0.47	3.612
XR_009519.1	RGD1562652_predicted	0.259	0.661	2.585
NM_001008857.1	RT1-S2	0.379	0.664	1.754
XM_574280.1	LOC498989	0.479		
NM_001009651.1	Clic2	0.511		1.777
XM_001063078.1	Pex11b	0.564		
NM_024385.1	Hhex	0.588		
XM_575533.1	LOC500181	0.638		1.717
XM_001061818.1	RGD1565561_predicted	0.652	0.676	
XM_228301.4	RGD1311375_predicted	0.654		
XM_001063114.1	Prcp_predicted	0.664		
NM_207603.1	Fcgr3a	0.685	0.74	
XM_001068101.1	isg12(b)	0.699	0.691	
NM_019316.1	Mafb	0.705		
XM_578320.1	LOC502820	0.706		0.743
NM_198740.1	Hla-dmb	0.725		
XM_001066853.1	RGD1562052_predicted	0.733		
NM_012896.1	Adora3	0.738		
XM_232259.3	LOC312688	0.752	0.654	
XM_001056311.1	Fhl3_predicted	0.754		
XM_001061010.1	RGD1564163_predicted	0.755		
NM_001025750.1	Plek	0.762		1.408
NM_001024289.1	LOC499300	0.765		
NM_053535.1	Enpp1	0.768		
XM_231833.4	Ppm1k_predicted	1.302		
XM_001053727.1	Bmp7	1.374		
NM_053371.1	Fxc1	1.382		
NM_001012072.1	Ppp1r3c	1.393		
XM_575738.1	LOC500380	1.394		

Table Continued...

NCBI Accession No.	Gene Symbol	Average Fold Change		
		NT vs L	NT vs H	L vs H
XM_576498.1	LOC501086	1.399		
XM_574174.2	RGD1562987_predicted	1.403		
NM_022701.1	Flot1	1.405		
NM_057133.1	Nr0b2	1.481		
NM_013098.1	G6pc	1.52		
XM_579222.1	RT1-149	1.522		
NM_001024334.2	LOC500300	1.79	2.056	
NM_013144.1	Igfbp1	2.137		
NM_012703.2	Thrsp		0.526	
NM_173096.2	Mx1		0.529	0.658
NM_031742.1	Kcnh1		0.621	
XM_001078678.1	RGD1560242_predicted		0.628	
NM_001011922.1	Nedd9		0.64	
XM_238366.4	RGD1563633_predicted		0.669	
NM_012621.3	Pfkfb1		0.681	
NM_001007235.1	Itpr1		0.698	0.746
NM_022604.2	Esm1		0.699	
XR_008204.1	RGD1565690_predicted		0.705	
XM_001072449.1	RT1-S3		0.713	
XM_224824.3	LOC306428		0.732	
XM_001075137.1	Tyki_predicted		0.734	
XM_579055.1	LOC497712		0.738	
XM_573861.1	LOC364514		0.745	
XM_225947.4	Trim36_predicted		0.745	
NM_032082.1	Hao2		0.748	
NM_001004277.2	Lypla3		0.75	
XM_001075502.1	Ms4a11_predicted		0.751	
XM_001054526.1	Zbp1		0.752	
XM_001065014.1	RGD1307882_predicted		0.756	
NM_133593.2	Ap3m1		0.757	
XM_001056345.1	Rtp4_predicted		0.761	
XM_001059523.1	Rarb		0.762	
NM_032612.2	Stat1		0.765	
NM_017159.1	Hal		1.302	1.542
NM_001014193.1	RGD1359529		1.325	
XM_219785.4	Gldc_predicted		1.327	
NM_031345.1	Tsc22d3		1.345	
NM_001035255.1	LOC502603		1.352	
XM_223945.4	Samd4_predicted		1.356	
NM_152936.1	Spink3		1.366	
XM_214172.4	Pabpn1		1.419	
NM_001012111.1	Lpin1		1.432	1.616
NM_031108.1	Rps9		1.437	
XR_009081.1	RGD1564649_predicted		1.446	
NM_017127.1	Chka		1.459	
XM_577408.1	LOC501979		1.505	
NM_021762.1	Tsn		1.59	
NM_032074.1	Irs3		1.613	
NM_022238.1	Abcb9		1.628	
NM_031776.1	Gda		1.661	
NM_172335.2	Gm2a		1.706	
XM_574013.2	LOC498736		1.916	
NM_019216.1	Gdf15		2.002	
NM_212505.1	Ier3			0.65
NM_031986.1	Sdcbp			0.695
NM_133543.2	Rdh3			0.706
XM_226988.4	Fndc3b_predicted			0.717
XM_576619.1	LOC501191			0.725
NM_001004261.1	RGD1303232			0.73

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Table Continued...

NCBI Accession No.	Gene Symbol	Average Fold Change		
		NT vs L	NT vs H	L vs H
XM_573464.1	LOC498241			0.743
NM_001008859.1	Mrps10			0.745
XM_001056150.1	LOC362068			0.754
NM_031510.1	Idh1			0.754
NM_001012007.1	Irgm			0.755
XM_001073260.1	RGD1306565_predicted			0.758
NM_053995.3	Bdh1			0.766
XM_213574.4	Polr2h_predicted			0.767
NM_001014076.1	Nol10			0.768
NM_134415.1	Cdk105			1.313
NM_017171.1	Prkce			1.316
NM_153308.1	Grina			1.374
XM_344454.2	LOC364468			1.381
XM_345140.2	Arsb			1.392
NM_001003706.1	LOC360228			1.402
XM_235156.4	Ptprb_predicted			1.409
NM_001012206.1	Phlda3			1.435
NM_012603.2	Myc			1.521
XM_579988.1	LOC499620			1.529
NM_019278.1	Resp18			2.524
XM_001054526.1	Zbp1		0.752	

Table 2 Gene expression associated with silver nanoparticles (AgNPs) inhalation exposure in female livers

Gene assession no.	Gene symbol	Average fold change		
		Nt vs l	Nt vs h	L vs h
NM_017272.15	Aldh1a7	0.026		
NM_019314.1	Kcnn2	0.544		
XM_342602.3	RGD1306721	0.576		
XM_574196.1	LOC498907	0.605	0.627	
XM_214618.4	Abhd3_predicted	0.613	0.675	
NM_001005530.1	Slc16a13	0.675		
XM_574750.1	LOC365566	0.719	0.767	
XM_223397.3	LOC305332	0.728	0.742	
NM_031073.2	Ntf3	0.736		
NM_024135.2	Limk2	0.738		
NM_152790.2	Carhsp1	0.744	0.719	
XM_220690.2	LOC287518	0.747		
NM_133543.2	Rdh3	0.751		
NM_020976.1	Tmem27	0.754		
NM_057114.1	Prdx1	0.756		
XM_579805.1	LOC498268	0.759	0.761	
NM_053883.2	Dusp6	0.764		
XM_217254.4	Ifrd2_predicted	1.303		
NM_178096.2	Nrep	1.307		0.701
XM_231599.4	RGD1306697_predicted	1.335	1.368	
XM_001058675.1	LOC368158	1.336		
NM_001037787.1	MGC112727	1.338	1.480	
XM_222245.4	Hps4_predicted	1.343		
XR_007378.1	RGD1563521_predicted	1.348		
NM_001007671.1	Cyb5d2	1.353		
XM_578335.1	LOC502835	1.358		
NM_017016.1	Hdc	1.374		
NM_001037355.1	Mettl7a	1.416	1.377	
XM_233551.2	LOC313615	1.421		0.768
NM_001017496.1	LOC498335	1.426	1.439	
NM_173322.1	Pnrc1	1.426	1.449	

Table Continued...

Gene assession no.	Gene symbol	Average fold change		
		Nt vs l	Nt vs h	L vs h
XM_579556.1	LOC497689	1.438		
XM_001054760.1	Rnf125_predicted	1.452		
NM_013215.1	Akr7a3	1.465		0.708
XM_213769.4	Vps37b_predicted	1.470		
NM_012603.2	Myc	1.490	1.914	
NM_199113.1	Popdc2	1.491	1.400	
NM_001009681.1	Oasl1	1.507		
XM_576044.2	RGD1564008_predicted	1.685		
NM_012571.1	Got1	1.800		
NM_053380.1	Slc34a2	1.887		
NM_206950.1	Mig12		0.447	
XM_342965.3	Arhgef19_predicted		0.449	
XM_213329.4	Srebf1		0.495	
NM_012703.2	Thrsp		0.534	
XM_216452.4	Dhcr24		0.544	
NM_017136.1	Sqle		0.580	
NM_013134.2	Hmgcr		0.597	
XM_343823.2	Serpina7		0.605	
NM_138863.2	Ltb4dh		0.610	0.601
XM_001059113.1	Slc35d2_predicted		0.615	
NM_130408.1	Cyp26a1		0.631	
NM_022389.2	Dhcr7		0.644	
NM_013105.1	Cyp3a3		0.644	0.619
NM_171992.2	Ccnd1		0.645	
NM_001014216.1	RGD1306658		0.665	
NM_144748.1	LOC246263		0.667	
XM_576589.1	Ihh		0.668	
NM_019339.1	Rgs12		0.673	
XM_238366.4	RGD1563633_predicted		0.675	
NM_017080.2	Hsd11b1		0.681	
NM_173144.1	Cyp3a1		0.681	
NM_175762.2	Ldlr		0.695	
XM_216756.4	RGD1310769_predicted	RGD131	0.698	
NM_173293.1	Olr59		0.706	
NM_199101.1	Plekha4		0.719	
NM_031649.1	Klrg1		0.719	
NM_001004214.1	Nqo2		0.730	
XM_342979.2	Pgd		0.733	
NM_138836.1	Prss8		0.734	
NM_001033694.1	Srebf2		0.735	
XM_001078178.1	Klb_predicted		0.739	
XM_221047.4	Polg2_predicted		0.740	
XR_008878.1	RGD1560462_predicted		0.742	
XM_577565.1	Gcnt2		0.742	
NM_001009399.1	Nsdhl		0.745	
XM_342986.2	Tas1r1		0.746	
NM_001007672.1	Tmem98		0.751	
NM_203335.2	Vkorc1		0.752	
XM_575223.1	LOC499880		0.752	
NM_138710.1	Dab2ip		0.753	
XM_215423.4	Sesn1_predicted		0.754	
XM_575430.1	LOC500080		0.757	
XM_001078486.1	RGD1308481_predicted		0.757	
NM_053019.2	Avpr1a		0.758	

Table Continued...

Gene assession no.	Gene symbol	Average fold change		
		Nt vs l	Nt vs h	L vs h
NM_012770.1	Gucy1b2		0.765	
NM_001025277.1	RGD1308076		1.303	
NM_013086.1	Crem		1.304	
NM_001013193.1	Tial1		1.306	
XM_001063880.1	Dusp8_predicted		1.311	
NM_001007691.1	Prss23		1.315	1.326
NM_199086.1	Nob1p		1.323	
XM_001076614.1	Chic2_predicted		1.326	
XM_001078898.1	LOC691575		1.328	
XM_575373.2	RGD1564980_ predicted		1.329	
XM_230961.4	RGD1306056_ predicted		1.333	1.304
XM_221724.2	Nrip1_predicted		1.338	
XM_341874.3	Isg20l1_predicted		1.346	
NM_031512.1	Il1b		1.347	
NM_022186.1	Nrbf2		1.351	
XM_573296.1	LOC498090		1.352	1.320
NM_022289.2	Snx16		1.353	
XM_236656.3	Tmem7_predicted		1.357	
NM_031732.1	Sult1c1		1.379	1.654
NM_001015004.1	Vgll4		1.396	
XM_574989.2	RGD1560263_ predicted		1.421	
XM_218704.4	Smox_predicted		1.423	
NM_031335.2	Polr2f		1.436	
NM_012743.1	Foxa2		1.453	
NM_017340.1	Acox1		1.463	
XM_001078182.1	Snrp1c_predicted		1.472	
NM_017192.1	Edg5		1.475	
NM_181090.2	Slc38a2		1.476	
NM_212505.1	Ier3		1.477	
NM_031507.1	Egfr		1.495	
XM_222107.4	RGD1559988_ predicted		1.539	
XM_001066533.1	Nnmt_predicted		1.572	
NM_019195.2	Cd47		1.587	
XM_343472.2	Cish		1.688	
NM_053727.2	Nfil3		1.753	
NM_199115.2	Angptl4		1.753	
XM_001063345.1	Zfp364_predicted		2.039	
NM_053551.1	Pdk4		2.058	
NM_001013187.1	Slc25a30		2.119	
XM_001053888.1	Gadd45g		2.163	2.112
XM_001076548.1	RGD1566118_ predicted		2.209	
XM_342803.3	Plekhf2_predicted		2.234	
NM_198750.2	Cry1		2.264	
NM_058208.1	Socs2		2.558	
XM_341791.2	LOC361510		21.003	
XM_232684.3	RGD1309085_ predicted			0.674
NM_030850.1	Bhmt			0.701
XM_001077068.1	Cfd			1.305
XM_341578.3	Riok3_predicted			1.312
XM_001058249.1	LOC680665			1.323
NM_021762.1	Tsn			1.339
XM_001061682.1	Hist1h2bp_predicted			1.361
XM_574280.1	LOC498989			1.362

Table Continued...

Gene assession no.	Gene symbol	Average fold change		
		Nt vs l	Nt vs h	L vs h
NM_017196.2	Aif1			1.363
NM_001013048.1	Igfbp7			1.370
NM_020103.1	Ly6c			1.373
XM_001079607.1	RGD1560542_ predicted			1.425
NM_012823.1	Anxa3			1.450
XM_577145.1	LOC501744			1.496
NM_207603.1	Fcgr3a			1.529
XM_342602.3	RGD1306721			1.742
NM_053372.1	Slpi			1.787
XM_573284.2	Ssg1			2.433
XM_213106.4	RGD1566136_ predicted			5.336
NM_001008829.1	RT1-A2			19.684

In male rats, 35 genes expressions were significantly changed by low concentration exposure, where 12 genes up-regulated and 23 genes down-regulated (Figure 1 & Table 1). Also, the high concentration AgNPs exposure changed the expression of 53 genes which consisted of 21 up-regulated and 32 down-regulated genes (Figure 1A & Table 1). The 80 genes expression changed by both the low and high concentration exposure, and 8 genes were commonly changed where the only one gene (LOC500300) was down-regulated and 7 genes that increased their expression were Ubd, RGD1562652_ predicted, RT1-S2, RGD1565561_ predicted, Fcgr3a, isg12(b) and LOC312688. Among the 109 changed genes by AgNPs exposure, 29 genes were involved in the KEGG pathway and related to 54 biological pathways. We selected 10 representative pathway such as Type I diabetes mellitus, antigen processing and presentation, insulin signaling pathway, cell adhesion molecules, purine metabolism, starch and sucrose metabolism, Type II diabetes mellitus, adipocytokine signaling pathway, TGF-beta signaling pathway and Gap junction (Table 3).

Table 3 Genes showing expression changes following silver nanoparticles (AgNPs) exposure and their relevant KEGG pathways in male rat livers

KEGG pathway	Probe ID	P-Value
Type I diabetes mellitus	RT1-149, RT1-S2, RT1-S3, Hla-dmb	1.17E-06
Antigen processing and presentation	RT1-149, RT1-S2, RT1-S3, Hla-dmb	2.81E-06
Insulin signaling pathway	Flot1, Irs3, Ppp1r3c, G6pc	1.21E-05
Cell adhesion molecules (CAMs)	RT1-149, RT1-S2, RT1-S3, Hla-dmb	2.32E-05
Purine metabolism	Gda, Enpp1, Polr2h_predicted	2.61E-04
Starch and sucrose metabolism	Enpp1, G6pc	0.001037
Type II diabetes mellitus	Irs3, Prkce	0.001272
Adipocytokine signaling pathway	Irs3, G6pc	0.003071
TGF-beta signaling	Bmp7, Myc	0.00336
Gap junction	LOC498736, Itpr1	0.005107

In female rats, the low concentration of AgNPs exposure changed the expression of 41 genes, where 24 genes up-regulated and 17 genes down-regulated their expression (Figure 1B & Table 2). Plus, the high concentration of AgNPs exposure changed the expression of 103 genes, where 52 genes up-regulated and 51 genes down-regulated their expression (Figure 1B & Table 2). 131 genes were significantly changed by low and high concentration of AgNP inhalation exposure. Among these genes, 13 genes were commonly up- (7 genes: LOC498335, Mettl7a, Myc, Pnrc1, Popdc2, RGD1306697_predicted, MGC112727) and down-regulated (6 genes: LOC498907, Abhd3_ predicted, LOC365566, LOC305332, Carhsp1, LOC498268). Among

the 150 changed genes by AgNPs exposure, 43 genes were involved in the KEGG pathway and related to 89 biological pathways. Ten representative pathways are described in Table 4.

Table 4 Genes showing expression changes following silver nanoparticles (AgNPs) exposure and their relevant KEGG pathways in female rat livers

KEGG pathway	Probe ID	P-Value
Biosynthesis of steroid	Dhcr7, Hmgcr, Vkorc1, Dhcr24, Nsdhl, Sqle	8.96E-13
Bladder, Colorectal, Endometiral cancer	Ccnd1, Egfr, Myc	3.72E-05
Fatty acid metabolism	Cyp3a3, Acos1, Aldh1a7	4.81E-05
Jak-STAT signaling pathway	Ccnd1, Cish, Socs2, Myc	5.54E-05
MAPK signaling pathway	Il1b, Egfr, Dusp6, Myc, Ntf3	5.92E-05
Cysteine metabolism	Got1, Sult1c1	2.74E-04
Gammar-Hexachlorocyclohexane degradation	Cyp3a3, Sult1c1	3.19E-04
Histidine metabolism	Hdc, Aldh1a7	5.96E-04
Cytokine-cytokine receptor interaction	LOC498335, Il1b, Egfr	0.00237
Thyroid cancer	Ccnd1, Myc	0.001212

The genes with the most significant change in their expression following silver nanoparticle exposure were identified as involved in biosynthesis of steroids, bladder, colorectal, endometrial cancer, fatty acid metabolism, Jak-STAT signaling pathway, MAPK signaling pathway, cysteine metabolism, gamma-Hexachlorocyclohexane degradation, histidine metabolism, cytokine-cytokine receptor interaction and thyroid cancer. Additionally, we analyzed gender difference in liver gene expression levels. According to our result, 240 genes showed a more than 2-fold changes when the male and female rat liver gene expression level compared after AgNPs inhalation exposure. Among these genes, 114 male genes and 126 female genes were increased over 10-fold than their counterparts (Table 5).

Table 5 Significant gene changes (over 10 fold) in liver mRNA levels between male and female rats

Gene association	Gene symbol	Fold Change	P-Value
XM_001062261.1	LOC682605	0.0022	9.84E-10
NM_147212.1	LOC259244	0.0023	1.59E-08
NM_198784.1	Mup4	0.0026	1.47E-08
NM_147214.1	LOC259246	0.0027	2.09E-06
NM_147214.1	LOC259246	0.0032	2.13E-10
NM_153312.2	Cyp3a2	0.0032	8.40E-10
NM_203325.1	Mup5	0.0037	8.22E-09
NM_147212.1	LOC259244	0.0042	3.65E-04
XM_233029.3	LOC298111	0.0048	6.32E-06
XM_001074217.1	Ste2	0.0052	1.07E-06
NM_019184.1	Cyp2c	0.0075	2.50E-04
XM_578458.1	LOC502953	0.0086	0.005275
NM_001003409.1	LOC298116	0.0088	6.06E-08
NM_001013098.1	Dhrs7	0.0094	2.30E-10
XM_575837.1	LOC500473	0.0106	0.001043
NM_147215.2	Obp3	0.012	8.66E-08
NM_012584.1	Hsd3b	0.0153	3.49E-09
XM_001056439.1	Stac3_predicted	0.0282	4.61E-05
NM_031732.1	Sult1c1	0.0361	1.03E-05
NM_134380.1	Ust5r	0.0408	9.79E-08
NM_001014240.2	LOC364773	0.0423	1.41E-05
XM_575821.1	LOC500457	0.0453	7.81E-05
NM_152936.1	Spink3	ss0.0588	1.91E-05
NM_017061.1	Lox	0.0616	9.16E-06
NM_145782.1	Cyp3a18	0.0668	1.15E-04

Table Continued...

Gene association	Gene symbol	Fold Change	P-Value
NM_019292.3	Ca3	0.0871	1.23E-06
NM_053977.1	Cdh17	0.098	0.015137
NM_017070.3	Srd5a1	10.0837	1.16E-04
XM_216107.3	Cpa2_predicted	12.5769	3.25E-04
NM_031572.1	Cyp2c12	24.0966	2.45E-04
NM_012630.1	Prlr	38.9271	3.12E-06
NM_017272.15	Aldh1a7	40.8982	0.005545
NM_016998.2	Cpa1	42.0376	0.002719
NM_022384.1	Ascl1	84.8827	3.28E-04
NM_053781.1	Akr1b7	85.1113	7.73E-08
XM_235351.3	RGD1564515_predicted	169.3112	1.15E-04
NM_022258.2	A1bg	226.3963	2.49E-06

Among the 240 genes which have over 2-fold changes, 68 genes were involved in the KEGG pathway and related to 79 biological pathways. We sorted the major 10 pathway as Biosynthesis of steroid, polysaturated fatty acid biosythesis, Bile acid biosynthesis, PPAR signaling pathway, Terpenoid biosynthesis, Cytokine-cytokine receptor interaction, Linoleic acid metabolism, Jak-STAT signaling pathway and ABC transporters (Table 6).

Table 6 Different biological pathways between the male and female in the livers of rats

KEGG pathway	Gene counts	P-Value
Biosynthesis of steroids	9	0
Polyunsaturated fatty acid biosynthesis	3	1.72E-06
Bile acid biosynthesis	3	5.36E-06
PPAR signaling pathway	4	5.44E-06
Terpenoid biosynthesis	2	1.75E-05
Cytokine-cytokine receptor interaction	4	9.66E-05
Linoleic acid metabolism	2	7.87E-04
Pyruvate metabolism	2	8.55E-04
Jak-STAT signaling pathway	3	8.88E-04
ABC transporters - General	2	0.001233

Discussion

It was well-known that Silver nanoparticles (AgNPs) have antimicrobial properties and extensively used in various medical and general applications.[2] Thus, consumers and workers may be exposed to AgNPs, which may pose harmful effects on their health.[26] Exposure to silver nanoparticles have been shown to induce liver toxicity including increase of cholesterol and alkaline phosphatase and bile duct hyperplasia in 28-day oral toxicity study[12-14] and 90-day oral subchronic study[14] and a 90-day inhalation studies.[15] The toxicity of AgNPs on cells are well established and it was reported that AgNPs induced cytotoxicity in primary liver cells and increased superoxide dismutase and glutathione.[27-29] In addition, AgNPs produced reactive oxygen species (ROS) which induced oxidative cell damage and mitochondria-involved apoptosis in human liver cell.[2] Moreover, most mechanistic studies were focused on *in vitro* system than *in vivo* model.[30] To find the exact mode of action, this study conducted a toxicogenomic examination with rat livers which were inhalation exposure for 12 weeks to AgNPs. Nanoparticles exposure could occur via inhalation, dermal contact and ingestion during manufacturing and consuming. So, researchers were administered AgNPs with various ways and then they observed tissue distribution for AgNPs to investigate the toxic effect and target organ.

Especially, liver is one of the most importance target organs. Because, the liver is the first line of defense against for exogenous

xenobiotic materials and involved in detoxification of heavy metals, including silver.[10,20] Additionally, Kupffer cells, reticulo-endothelial system in the liver, can efficiently remove impurities from the blood.[20] Also, many studies reported that AgNPs deposition was observed in the liver after oral, inhalation, intraperitoneal and intravenous administration.[20,31] In our study, we also observed AgNPs deposition was observed in liver after 12 weeks inhalation exposure. We found that liver tissue silver contents were dose-dependently increased in both male and female rat after 13 weeks inhalation exposure. However, we could not observe sex difference in silver contents of liver tissues. In case of gold nanoparticles (AuNPs) intravenous injection, AuNPs were rapidly and consistently accumulated in liver and spleen among 25 organs after administration. Also, these liver and spleen tissues were significantly altered to genes related to detoxification, lipid metabolism, cell cycle, defense response and circadian rhythm.[32] To elucidate the exact mode of action of AgNPs, we have conducted a toxicogenomic study to rat livers which have been exposed silver nanoparticles for 12 weeks inhalation. 1.3 fold up or down regulated genes ($p < 0.05$) were regarded as significant and used for data mining. According to Park et al., ICR male mice exposed Ag NPs up to 500 μg/kg by single instillation were conducted for microarrary with the mouse lung tissue at day 1 after exposure.[30] Inflammation and tissue damage relative genes were identified as significant gene alteration. Also, more than 1000 genes were up-regulated by 1.3 μg/ml Ag NPs in the human lung epithelial cell line (A549).

The up-regulated genes included member of metallothionein, heat shock protein and histone family.[33] In present study, we observed that 109 and 150 genes significantly up- and down regulated by AgNPs inhalation exposure in male and female rats, respectively (Table 3&5). The significantly altered male rat genes were involved in 54 biological pathways which were typically related with diabetes and metabolism. In female rat, the significantly expression changed genes were involved in 89 biological pathways which were mainly connected with metabolism and cell signaling. Furthermore, the gender-dependent gene expression changes of more than 2 fold were linked to 240 genes, with 114 genes in the male livers and 126 genes in the female livers. 68 genes of these genes were involved in the KEGG pathway and related to 79 biological pathways which were related to steroids and xenobiotics metabolism. For this result, liver metabolic enzymes activation was different between male and female rat by AgNPs inhalation exposure. Although, we could not observe the significant gene alterations such as the redox system, inflammation, cell cycle, and apoptosis-related genes, metabolism pathway related genes showed a significant alteration.

Conclusion

Silver concentrations in livers were dose-dependently increased in male and female rats. The used-exposure concentration and tissue accumulated concentration of Ag NPs could not induce pivotal toxicogenomic alterations and did not cause serious toxicity in this study. 109 and 150 genes significantly up- and down regulated by AgNPs inhalation exposure in male and female rats, respectively. The significantly altered male rat genes were involved in 54 biological pathways which were typically related with diabetes and metabolism. In female rat, the significantly expression changed genes were involved in 89 biological pathways which were mainly connected with metabolism and cell signaling. Plus, the gender-dependent gene expression changes of more than 2 fold were linked to 240 genes, with 114 genes in the male livers and 126 genes in the female livers. To surely verify the toxicogenomic-mechanism for AgNPs inhalation exposure, we need to find a significant exposure level and carry the long-term study for recovery groups.

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Conflicts of interest

None.

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