Biomolecules & Therapeutics, **17(1)**, 92-97 (2009)
www.biomolther.org

ISSN: 1976-9148(print)/2005-4483(online)
DOI: 10.4062/biomolther.2009.17.1.92

Induction of DNA Damage in L5178Y Cells Treated with Gold Nanoparticle

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(Received December 10, 2008; Revised December 29, 2008; Accepted January 7, 2009)

Abstract − As nanomaterials might enter into cells and have high reactivity with intracellular structures, it is necessary to assay possible genotoxic risk of them. One of these approaches, we investigated possible genotoxic potential of gold nanoparticle (AuNP) using L5178Y cells. Four different sizes of AuNP (4, 15, 100 or 200 nm) were synthesized and the sizes and structures of AuNP were analyzed using transmission electron microscopy (TEM), scanning electron microscopy (SEM) and stability was analyzed by a UV/Vis. Spectrophotometer. Cytotoxicity was assessed by direct cell counting, and cellular location was detected by dark field microscope at 6, 24 and 48 h after treatment of AuNP. Comet assay was conducted to examine DNA damage and tumor necrosis factor (TNF)-α mRNA level was assay by real-time reverse transcription polymerase chain reaction. Synthetic AuNP (4, 50, 100 and 200 nm size) had constant characteristics and stability confirmed by TEM, SEM and spectrophotometer for 10 days, respectively. Dark field microscope revealed the location of AuNP in the cytoplasm at 6, 24 and 48 h. Treatment of 4 nm AuNP induced dose and time dependent cytotoxicity, while other sizes of AuNP did not. However, Comet assay represented that treatment of 100 nm and 200 nm AuNP significantly increased DNA damage compared to vehicle control ($p < 0.01$). Treatment of 100 nm and 200 nm AuNP significantly increased TNF-α mRNA expression compared to vehicle control ($p < 0.05$, $p < 0.01$, respectively). Taken together, AuNP induced DNA damage in L5178Y cell, associated with induction of oxidative stress.

Keywords: Genetic toxicity, Gold nanoparticle, L5178Y cell, DNA damage

INTRODUCTION

Natural occurring nanomaterials include bacteria, virus, ultrafine dust and synthetic materials include metal-based ones, carbon-based ones, dendrimers and so on. Nanomaterials can be applied to bioimaging, cell isolation, therapeutics and diagnostics by using nanobiotechnology. Due to their size, nanomaterials often exhibit unique physical/chemical properties and they are often much more reactive due to their larger surface area (Thomas and Sayre, 2005; Nel *et al.,* 2006). Using these characteristics, many scientists have big interest and enthusiasm in many fields using nanomaterials.

While nanotechnology seems to have commercial promise and potential benefit, an equally large issue is the evaluation of potential effects on human and environmental health (Holsapple and Lehman-McKeeman, 2005). It is urgently required and achievable to ensure safe manufacture and use of nanomaterials in the marketplace (Nel *et al.,* 2006). International efforts to develop risk-based safety evaluations for nanomaterials have been started for safety evaluations (Thomas *et al.,* 2006a). Furthermore, there is very little information available regarding associated risks from these exposures (Thomas *et al.,* 2006b).

And it seems that the development of improved strategies for assessing risk and improving public health are needed (Balshaw *et al.,* 2005), and it should be set safety evaluation for nanomaterials to develop nanotechnology (Maynard *et al.,* 2006).

It has been reported that nanomaterials might facilitates their uptake into cells and transcytosis across epithelial

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and endothelial cells into body and lymph circulation to reach potentially sensitive target organs (Oberdorster *et al.,* 2005) and they have increased reactivity in cells (Thomas and Sayre, 2005). And even more, recent reports suggest that they can bind to DNA or amino acid (Nel *et al.,* 2006) and carbon nanotubes were seen to enter the cytoplasm and localize within the cell nucleus, causing cell mortality in a dose-dependent manner (Porter *et al.,* 2007). From these results, it is possible that nanomaterials may induce genotoxicity in certain circumstances.

Actually, it has been reported that some nanomaterials could induce genotoxicity in some situations. For example, it was reported that fullerenes induced DNA damage, possibly associated with oxidative stress (Dhawan *et al.,* 2006), and genotoxicity of zinc oxide was enhanced in irradiated circumstance (Dufour *et al.,* 2006). And ultrafine titanium dioxide induced micronuclei and apoptosis *in vitro* (Rahman *et al.,* 2002), and it can induce oxidative damage to human bronchial epithelial cells even in the absence of photoactivation (Gurr *et al.,* 2005).

Gold nanoparticle has been developed as an agent for cell imaging, drug delivery and cancer diagnostics (El-Sayed *et al.,* 2005). Recent study reported that gold nanoparticles entered the cells via endocytosis pathway (Chithrani *et al.,* 2006) and they exocytosed out of the cells in a linear relationship to size (Chithrani and Chan, 2007). These studies made us investigate the possible genotoxicity of gold nanoparticles. We tested synthesized gold nanoparticle (AuNP) at four different sizes and at three treatment doses, and investigated cellular toxicity, cellular location, DNA damage and oxidative stress-related genes.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA). Methyl methanesulphonate (MMS) was obtained from Sigma (St. Louis, MO).

Cell line and cell culture

L5178Y was purchased from American Type Culture Collection (ATCC, Manassas, VA), and was cultured in DMEM medium supplemented with 1.5 g/L sodium bicarbonate, 10% FBS and 1% penicillin at 37°C in a 5% CO_2 atmosphere.

Synthesis and chemical characteristics of AuNP

Four different sizes of AuNP (4, 15, 100 or 200 nm) were used in this work. The 4 nm gold nanoparticles were prepared by the method of Jana *et al.* (Jana *et al.,* 2001) following as: two hundred milliliter of aqueous solution containing 0.25 mM $HAuCl_4$ and 0.25 mM citric acid was prepared in conical flask. Then 6 ml of 0.1 M sodium borohydride ($NaBH_4$) were added to the solution with stirring at room temperature. The solution color immediately turned to pink, and the solution kept stirring for 10 min. The 15 nm gold nanoparticles were synthesized by following Fren's method (Frens, 1973). In brief, 250 ml of 1 mM $HAuCl_4$ dissolved in water was heated with stirring, and 50 ml of 1% citric acid was added and further heated for 15 min, then cooled at room temperature. The 100 nm and 200 nm AuNPs were used as-received from BBInternational Co. (UK). All AuNPs were protected by thiol-terminated poly (ethylene)glycol (HS-PEG, MW. 5,000 for 4, 15 nm or MW. 30,000 for 100, 200 nm). Aqueous solution of 0.1% HS-PEG was added into AuNP solution and kept stirring for 4 h. Finally, the AuNP solutions were exchanged with PBS buffer by dialysis (4 nm) or centrifugation (15, 100, 200 nm).

The size and structure of PEG-coated AuNPs were analyzed using a field-emission scanning electron microscopy (FE-SEM, FEI, USA) at an accelerating voltage of 10 kV. The transmission electron microscopy images were obtained using a CM20 (Philips, Eindhoven, Netherlands) at an acceleration voltage of 120 kV. The UV/Vis absorption spectra were recorded on a UV/Vis. Spectrophotometer (DU-800, Beckman Coulter, USA).

Cytotoxicity assay

Cytotoxicity was assessed by direct cell counting. In brief, L5178Y cells (2×10^5 cells/ml) were treated with four sizes of AuNP (4, 15, 100 and 200 nm) and were incubated for 6, 24 or 48 h at the concentration of 0, 6.25, 12.5, 25, 50, 100 and 200 μg/ml, and cell counting was carried out.

Cellular location

L5178Y cells (2×10^5 cells/ml) were treated with four different sizes of AuNP and the cells were fixed with 4% paraformaldehyde in 0.1 M PBS buffer. Cellular location was detected by dark field microscope (Nikon, Japan) at 6, 24 and 48 h after treatment of AuNP.

Comet assay

Cells (1×10^5 cells/ml) were cultured in 12-well plate were treated with four different sizes of AuNP (4, 15, 100 or 200 nm) as low dose (25 μg/ml), middle dose (50 μg/ml) and high dose (100 μg/ml) for 2 h. Cells were mixed with LMAgarose, and these mixture was put into Comet Slide™

(Trevigen, MD) and then into lysis solution for 30-60 min at 4°C, alkaline solution for 30-60 min, and were carried out electrophoresis for 30 min, and were dried out after dipping into 70% alcohol. And then these were stained with ethidium bromide and examined by fluorescene microscope, and were analyzed by Comet assay program (Komet 3.1, Andor Technology, Belfast, UK) to calculate tail moment. MMS (325.75 mg/ml) was used as positive control.

Tumor necrosis factor (TNF)-α mRNA expression by real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNAs were extracted for gene expression analysis using the RNeasy Mini kit (Qiagene, Valencia, CA). The yield of RNA was determined by Bioanalyzer 2100 (Agilent Technology, CA), and stored at −80°C until use.

For cDNA synthesis TaqMan® Gold RT-PCR Kit (Applied Biosystmes, CA) was used according to manufacturer's guide. In brief, 2 μg of total RNA was mixed with 10 μl of 10X RT Buffer, 22 μl of 25 mM $MgCl_2$, 20 μl of deoxyNTPs mixture, 5 μl of random hexamers, 2 μl of RNase inhibitor, 2.5 μl of MultiScribe Reverse Transcriptase (50 U/μl) and incubate at 25°C for 10 min, at 37°C for 1 h, and at 95°C for 5 min and placed on ice for 10 min and stored at −20°C until use.

cDNAs were amplified using oligonucleotide primers and probe (Applied Biosystems, CA) for TNF-α mRNA. PCR amplification was carried out according to the manufacturer's instruction (Applied Biosystems, CA). The PCR program cycles were set as follows: initial denaturing at 50°C for 20 min, 95°C for 10 min, followed by 40 cycles (95°C for 15 s, 60°C for 1 min).

β-actin mRNA was employed as an internal standard, and each gene expression was determined by real- time RT-PCR and normalized against β-actin mRNA levels. All PCR products were amplified in a linear cycle. Data are the mean ± SD from three samples per group of two independent experiments.

Statistical analysis

Statistical analyses for cytotoxicity, Comet assay and real-time RT-PCR data were performed with the Tukey-Kramer method using JMP program (SAS Institute, Cary, NC). For all comparisons, probability values less than 5% ($p < 0.05$) were considered to be statistically significant.

RESULTS

Chemical characteristics of AuNP

Color of AuNP showed from red to pink depending on size (data not shown). There were no alterations of size and component of AuNP confirmed by TEM (Fig. 1a-d), SEM (Fig. 1e), and spectrophotometer (Fig. 1f).

Cytotoxicity assay

Treatment of 4 nm AuNP induced dose and time dependent cytotoxicity (Fig. 2). At 6 h after treatment of 4 nm AuNP, there was a significant difference at the concen-

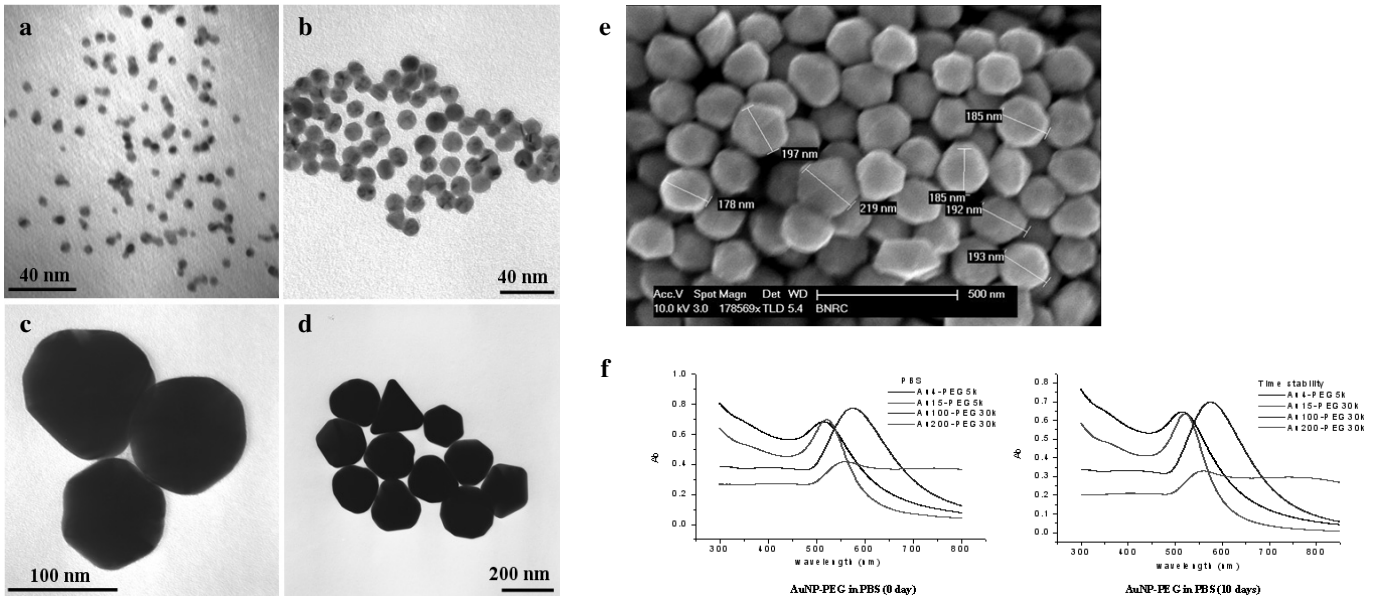


Fig. 1. Characteristics of gold nanoparticle (AuNP). TEM image of 4 (a), 15 (b), 100 (c) and 200 nm AuNP (d); (e) SEM image of 200 nm size AuNP; (f) stability of AuNP measured by spectrophotometry.

tration of 200 μg/ml compared to vehicle control ($p < 0.01$). Moreover, at 24 h after treatment, there were significant differences at the concentration of 25, 50, 100 and 200 μg/ml compared to vehicle control ($p < 0.01$, respectively) and at 48 h after treatment, at the concentration of 50, 100 and 200 μg/ml compared to vehicle control ($p < 0.05$, $p < 0.01$ or $p < 0.01$, respectively). However, 15, 100 and 200 nm AuNP treatment did not induce cytotoxicity at any dose within 48 h (data not shown).

Cellular location

Cells were treated with four different sizes of AuNP (4, 15, 100 or 200 nm) and the cells were fixed with fixatives, and cellular location was detected by dark field microscope. The nanoparticles were existed in cytoplasm at 6, 24 and 48 h after treatment. Representative figure was shown in Fig. 3, illustrating the cellular location of AuNP in cytoplasm, mainly around cell membrane.

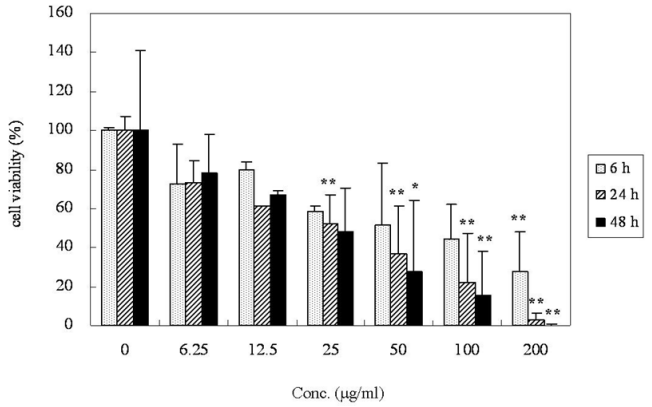


Fig. 2. Cytotoxicity at 4, 24 and 48 h after treatment of 4 nm AuNP. *, **Significantly different from vehicle control ($p < 0.05$, $p < 0.01$, respectively).

Comet assay

Cell were treated with four different sizes of AuNP (4, 15, 100 or 200 nm) as low dose (25 μg/ml), middle dose (50 μg/ml) and high dose (100 μg/ml) for 2 h. Treatment of 100 nm or 200 nm AuNP significantly increased DNA damage ($p < 0.01$), as equivalent level shown in the treatment of MMS as positive control (Fig. 4). However, treatment of 4 nm or 15 nm AuNP did not induce DNA damage.

TNF-α mRNA expression

Real-time RT-PCR analysis showed that treatment of 100 nm or 200 nm AuNP significantly increased TNF-α mRNA expression compared to vehicle control ($p < 0.05$, $p < 0.01$, respectively) (Fig. 5). On the while, treatment of 4 nm or 15 nm AuNP showed increased values of it.

DISCUSSION

In the present study, it was showed that treatment of 4 nm AuNP induced cellular toxicity in L5178Y cells, while 15, 100 or 200 nm AuNP did not show cytotoxicity. However, DNA damage detected by Comet assay was appeared at the treatment of 100 or 200 nm AuNP. And the treatment of 15 nm AuNP did not show cytotoxicity or DNA damage in this study.

It is generally accepted that nanosize materials may be more toxic than micron-sized one, as showing that nano-sized cobalt-chromium alloy induced more DNA damage than micron-sized particles (Papageorgiou *et al.,* 2007) and the ultrafine particles elicited a persistently high inflammatory reaction in the lungs of the animals compared to the larger-sized particles (Oberdorster *et al.,* 1994). In concordance with previous reports, the smallest size of AuNP showed cellular toxicity by dose and time-dependent manner in this study.

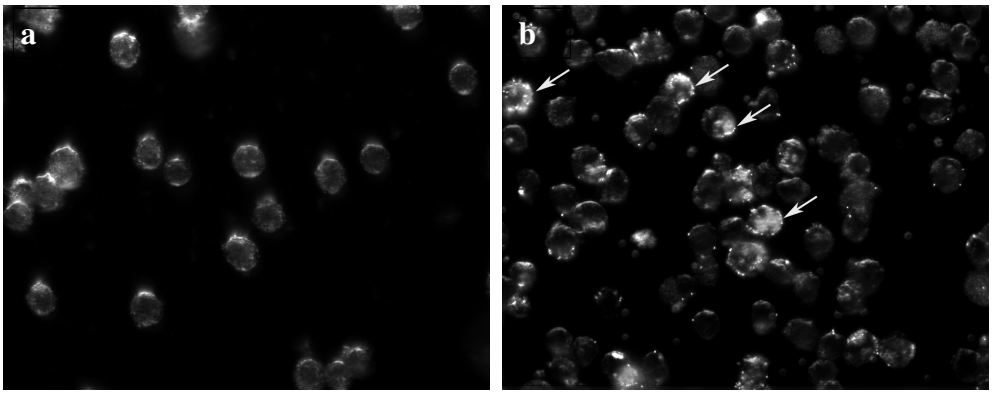
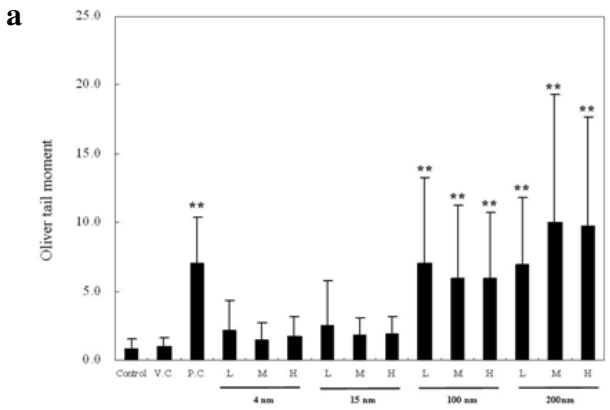


Fig. 3. Cellular location detected by dark field microscope after treatment of AuNP. a) control; b) AuNP 200 nm (200 μg/ml) at 48 h after treatment, ×800 magnification; Note the cellular location of AuNP in cytoplasm, mainly around cell membrane (Arrow).



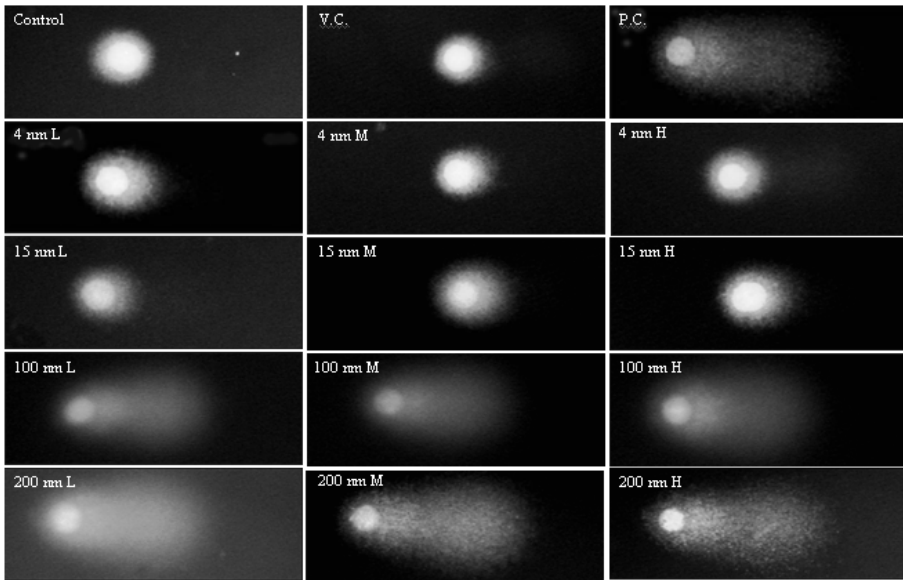


Fig. 4. Comet assay for AuNP. a) DNA damage as represented as oliver tail moment. Note treatment of 100 nm or 200 nm AuNP increased DNA damage, as equivalent level shown in the treatment of methyl methanesulphonate (MMS) as positive control; b) Representative figures from control and 4, 15, 100 or 200 nm AuNP treatment groups; L, M and H mean low (25 μg/ml), middle (50 μg/ml) and high dose (100 μg/ml) treatment of four different size of AuNP, respectively; VC: vehicle control; PC: positive control; **Significantly different from vehicle control ($p < 0.01$).

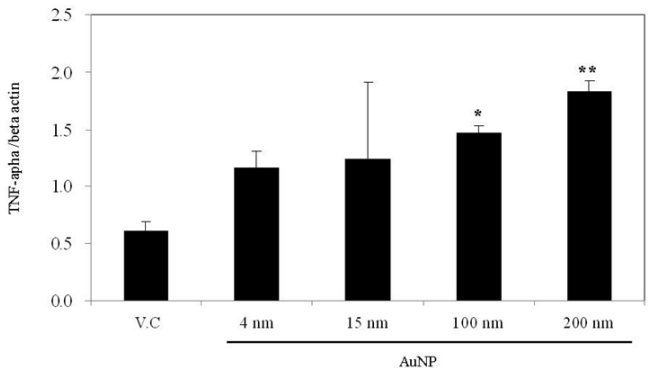


Fig. 5. Real-time RT-PCR analysis of TNF-α mRNA in L5178Y cell treated with AuNP. TNF-α mRNA expression was quantified and normalized with β-actin mRNA expression as described in the Materials and Methods. Note the significant differences of the gene expression among vehicle control and AuNP treatment (100 and 200 nm); *,**Significantly different from vehicle control ($p < 0.05$, $p < 0.01$, respectively).

It was reported that uptake of the gold nanoparticles significantly increased for the first 2 h, but the uptake rate gradually slowed and reached a plateau at 4-7 h, depending on size (Chithrani *et al.,* 2006). We examined cellular location of AuNP occurred at 6, 24 and 48 h after treatment. However, we did not clearly determine the quantity by dark-filed microscopy in this study. On the while, we determined DNA damage at 2 h after treatment of AuNP by Comet assay, which was microgel electorphresis method to find DNA damage directly in cellular level (McNamee *et al.,* 2000). In our study, the treatment of 100 nm and 200 nm AuNP significantly increased DNA damage compared to control. So, it seems that AuNP may enter the cell within 2 h and caused DNA damage only in 100 and 200 nm AuNP. Interestingly, we found that there were little variation in control and positive control value, in contrast with large variation in the groups of 100 or 200 nm of AuNP treatment. It seems that there may be different cellular susceptibility and variable level of cellular damage in treated

cells.

To investigate mechanism of DNA damage, we carried out real-time RT-PCR, representing that AuNP treatment induced TNF-α mRNA. However there was no change of p53 mRNA or Mdm2 mRNA (data not shown). We assume that 4 nm AuNP might induce cellular toxicity by direct pathway(s), however 100 or 200 nm AuNP induce DNA damage by indirect mechanism(s), associated with increase of oxidative stress. Further studies will be required to investigate detail mechanism(s) for DNA damage induced by AuNP.

Taken together, AuNP induced DNA damage in L5178Y cell, associated with induction of oxidative stress.

ACKNOWLEDGMENTS

This research was supported by a research grant (07141KFDA762) from Korea Food and Drug Administration in 2007.

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