Acta Biochim Biophys Sin 2011, 43: 316–323 | © The Author 2011. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmr015.
Advance Access Publication 3 March 2011

Original Article

Silver nanocrystals sensitize magnetic-nanoparticle-mediated thermo-induced killing of cancer cells

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Magnetic nanoparticles (MNPs) can heat up tumor tissues and induce killing of cancer cells under external AC magnetic field. However, magnetic nanoparticles hyperthermia (MNPH) requires high concentration of MNPs that are injected into the tumor in order to obtain clinically needed thermal dose because of the complicated heat transfer *in vivo* and the limited heat quality of MNPs. To cut down the dose of MNPs and enhance the effect of this Nanotherapy, we prepared silver nanoparticles (AgNPs) with different sizes and investigated the effects of these AgNPs on cancer cells in MNPH treatment. It was found that AgNPs could enhance thermo-sensitivity of glioma cells and this effect was size dependent. AgNPs could induce cell cycles arrested in G_2/M phase and enhanced the apoptosis rate of cancer cells after hyperthermia. In glioma bearing rats model, MNPH combined with AgNPs could enhance Bax expression in cancer cells. Our results suggested that AgNPs could be a potential thermo-sensitizer and could be further developed for the design of Ag nanostructure-based thermal seeds for MNPH therapy.

Keywords glioma cells; magnetic nanoparticles; silver nanoparticles

Received: December 5, 2010 Accepted: December 17, 2010

Introduction

Magnetic nanoparticles (MNPs) can heat up tumor tissues *in vivo* under external AC magnetic field and induce thermo-killing of cancer cells. Although this therapeutic method is considered effective and has been advanced in clinical test phases [1,2], detailed studies have shown that the tumor should be injected with high concentration of MNPs to obtain clinically needed thermal dose because of complicated heat transfer *in vivo* and limited heat quality of MNPs [3,4]. In order to decrease the MNPs dose and enhance the effect of cancer cell killing, magnetic nanoparticles hyperthermia (MNPH) requires thermo-sensitive agents to enhance its thermo-sensitivity on cancer cells. Recent study of nanoparticles on radiosensitivity of cancer cells has shown that silver nanoparticles (AgNPs) could enhance the effect of cancer cell killing on radiation treatment [5].

AgNPs have been studied for their potential use in biological field owing to the development of nanotechnology. By virtue of the nanocores that could release bactericidal Ag^+ ions, AgNPs are taken as attractive anti-microbial agents [6–9]. Besides, AgNPs have unique plasmon-resonant optical scattering properties that are used in optical labels such as signal enhancers, optical sensors, and biomarkers [9–12]. Moreover, the study in nanomedicine has shown that AgNPs could inhibit virus replication [13–15]. Recently, the toxicity of starch-coated AgNPs have been studied in normal human lung fibroblast cells (IMR-90) and human glioblastoma cells (U251) [16]. The results have showed that AgNPs could reduce ATP content of the cell, causing the damage of mitochondria and increasing the production of reactive oxygen species (ROS) in a dose-dependent manner. Nanoparticle treatment on cancer cells has shown no massive apoptosis or necrosis. The experimental analysis has indicated that AgNPs were present inside the mitochondria and nucleus, implicating their direct involvement in the mitochondrial toxicity and DNA damage. A possible mechanism of toxicity has been proposed that AgNPs may be involved in the disruption of the mitochondrial respiratory chain, leading to production of ROS, and interruption of ATP synthesis, which in turn

causes DNA damage. It is anticipated that DNA damage is augmented by deposition, followed by interaction of AgNPs with the DNA, leading to cell cycle arrest in the G_2/M phase [16].

Glioblastoma multiforme (GBM, WHO Grade IV) has a very poor prognosis due to its treatment resistance and ability to infiltrate into the surrounding brain tissue, as well as the impossibility to make a complete resection. Treatment of GBM has therefore been among the most challenging fields in oncology for >20 years and has led investigators to develop new innovative therapies to augment local control [2,17–20]. In this study, we investigated the effect of AgNPs combined with MNPH on the human glioma U251 cells. Because Ag nanostructures have different ability to release Ag^+ due to their sizes [5–9], we prepared three types of AgNPs with different sizes to explore and evaluate the potential use of the particular system containing AgNPs with MNPs in anti-cancer thermotherapy.

Materials and Methods

AgNPs preparation

Three different sizes of AgNPs were prepared by the following three protocols, respectively. All chemicals were purchased from Sigma (St Louis, USA).

The preparation of sample 1 (AgNPs of 20.6 ± 2.7 nm): 0.5 ml of 0.1 M $AgNO_3$ was added into 40 ml deionized water, and then 1 ml of freshly prepared 0.02 M $NaBH_4$ aqueous solution was added at once with vigorous stirring. A solution of 1% sodium citrate (10 ml) was added during the reduction. The solution was stirred for an additional 30 s [21] and then centrifuged at 14,000 *g* for 30 min. The pellet was collected and resuspended in 2 ml of fetal bovine serum (FBS, KeyGEN, Nanjing, China), then transferred into Dulbecco's modified eagle's medium (DMEM, KeyGEN) (FBS: DMEM = 1:9).

The preparation of sample 2 (AgNPs of 53.8 ± 7.6 nm): 900 mg $AgNO_3$ was dissolved in 500 ml of H_2O and brought to boiling. Then, 10 ml of 1% sodium citrate was added. The solution was kept on boiling for ~1 h [21] and then centrifuged at 7400 *g* for 10 min. The pellet was collected and resuspended in 0.3 ml of FBS, then transferred into DMEM (FBS: DMEM = 1:9).

The preparation of sample 3 (AgNPs of 137.3 ± 43.0 nm): the reactions were carried out in a two-necked round-bottom flask of 250 ml with a high-speed electric stirrer. First, 150 ml of solution A containing 1.465 g ascorbic acid and 75 mg gum arabic was put into the reaction flask. Then, 100 ml of solution B containing 0.353 g silver nitrate and 100 mg gum arabic was quickly added to the vigorously stirred solution A. The solutions were stirred up to 24 h at room temperature [22] and then centrifuged at 7400 *g* for 10 min. The pellet was collected and resuspended in 0.175 ml of FBS, then transferred into DMEM (FBS: DMEM = 1:9).

All the prepared samples received 20 Gy dose of X-rays irritation for sterilization and were characterized by scanning electron microscope (SEM, Hitachi S-4800, Tokyo, Japan) and transparent electron microscope (JEM-2010, JEOL Ltd., Tokyo, Japan). Ag contents of all samples were quantitated by element analysis using a graphite furnace atomic absorption spectrophotometer (Z2000, Hitachi).

Cell culture

The human glioma U251 cells were supplied by Shanghai Institutes for Biological Sciences (Shanghai, China) and cells were cultured in DMEM, supplemented with 10% FBS. Cell cultures were incubated at 37°C and equilibrated in 5% CO_2 and air.

AgNPs uptake by cells

U251 cells (1×10^5 cells) were cultured with the same amount (100 μg) of three types of AgNPs for 24 h. At the end of the culture, the medium was removed. Cells were washed three times with phosphate-buffered saline (PBS). Then 200 μl of 0.25% trypsin/ethylene diamine tetraacetic acid (EDTA) solution was added to detach the cells and cells were counted immediately in a hemocytometer. Then cells were transferred to a certain volume of water in which cell number was kept at 2×10^4 cells/ml. Ag content was determined in the solution by element analysis using a graphite furnace atomic absorption spectrophotometer (Z2000, Hitachi).

MTT assay

U251 cells (1×10^4 cells) were seeded in 24-well plates and cultured with 25 μg/ml AgNPs (20.6 ± 2.7 nm) and 50 μg/ml AgNPs (53.8 ± 7.6 nm), respectively, for 24 h. Then cells were heated to 40, 42, and 44°C for 15 min (for details see cell hyperthermia treatment). After that, the medium was removed. Cells were washed with PBS, and 200 μl of 0.25% trypsin/EDTA solution was added to detach the cells, and cells were counted immediately in a hemocytometer. Then cells were transferred into 96-well micro-plates at a density of 1×10^3 cells/well. At the end of culture (1, 2, 3, and 4 days), 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT, Sigma) (5 mg/l) was added to each well, and placed at 37°C for 4 h. Then, 150 μl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the dark blue crystal products. Absorbance was measured at a wavelength of 570 nm using a multi-well spectrophotometer (Bio-Rad, Hercules, USA).

Clogenic assay (colony-forming assay)

The cells (~200 cells in control, 40 and 42°C heat groups, 2000 cells in 44°C heat groups) were cultured in 60 mm

Petri dishes or six-well plates at 37°C and 5% CO_2 in a humidified incubator (three replicates). Twenty-four hours later, AgNPs were added into the medium and the final concentration of AgNPs was kept at 25, 50, and 100 μg/ml for samples 1, 2, and 3, respectively. Cells were cultured with AgNPs for another 24 h. Then cells were heated to 40, 42, and 44°C for 15 min (for details see Cell hyperthermia treatment). After heating, cells were washed twice with PBS and cultured in the medium containing 10% FBS for another 12 days. Colonies were fixed with methanol, treated with Giemsa stain, and counted using microscopy.

Annexin V-FITC and Propidium Iodide staining

An *in situ* apoptosis detection kit was used for Annexin V-FITC binding and PI staining (KeyGEN) [23]. After incubation with AgNPs, heat, and AgNPs+ heat for 24 h, 2×10^5 cells from each group were harvested and washed with PBS, and then resuspended in binding buffer (10 mM HEPES, 140 mM sodium chloride, 1.8 mM calcium chloride, pH 7.4). Fluorescein-conjugated Annexin V-FITC (1 μg/ml) and PI reagent (5 μg/ml) were added into cell suspensions. Then cells were incubated in the dark for 15 min at room temperature and analyzed immediately by flow cytometry (BD Sciences, San Diego, USA).

Cell cycle test

After cultured with AgNPs for 24 h, cells were heated to 42°C for 15 min. They were harvested after treatment and were fixed in 70% ethanol at 4°C for 24 h. Before analysis, cells were washed once in PBS, digested with 500 U/ml RNase for at least 30 min at 37°C and then stained with PBS containing 50 μg/ml PI for 30 min. Analyses were performed with a flow cytometer (BD Sciences). Cell cycle distributions were calculated on DNA plots by BD FACSDiva sofeware (Verity Software House, Topsham, USA).

Cell hyperthermia treatment

Cells were assigned to four groups: group 1, MNPH combined with AgNPs; group 2, MNPH; group 3, AgNPs; and group 4, control. Cells in groups 1 and 2 were incubated with MNPs of 15 nm [2 mg/ml of the medium (90% DMEM with 10%FBS)] and exposed under an external AC magnetic field by the device (0–100 kA/m, 40 kHz, designed by Southeast University, Nanjing, China). The temperature was regulated by magnetic field intensity and monitored by a fiber optic temperature sensor (FOTS, FISO, Canada). The temperature was controlled at 40, 42, and 44°C, respectively, for 15 min.

Tumor-bearing rats hyperthermia treatment

A total of 40 glioma-bearing rats (Sprague Dawley, purchased from animal experiment center of Southeast University, injected with 10^6 C6 cells in the brain) were randomly assigned to four groups (10 rats per group): group 1, MNPH combined with AgNPs; group 2, MNPH; group 3, AgNPs; and group 4, MNPs. Magnetic resonance imaging scans were used to measure the tumor size and to evaluate the tumor volume. In groups 1, 2, and 4, the dose of MNPs of 15 nm injected was 3 mg/cm^3 of the tumor volume. In groups 1 and 2, the dose of AgNPs injected was 10 μg/cm^3 of the tumor volume. The temperature during hyperthermia was monitored by a FOTS and controlled at 42°C for 30 min. The tumor-bearing rats received hyperthermia once a day and continued three times.

Immunohistochemistry examination

Bouin-fixed, paraffin-embedded testicular sections were deparaffinized, hydrated by successive series of ethanol, rinsed in distilled water, and then incubated in 0.01 mol/l citrate buffer (pH = 6.0) for microwave repairing for 15 min, and subsequently incubated with rat anti-Bax polyclonal antibody (1:50 dilution; Beijing Zhongshan Company, Beijing, China) and biotinylated goat anti-rat IgG secondary antibody (1:500 dilution) sequentially. Immunoreactivity was detected by avidin-conjugated horse radish peroxidase complex, visualized with diaminobenzidine tetrahydrochloride following the manufacturer's instructions (Zhongshan Company).

Statistical analysis

Statistical differences between control and experimental groups were analyzed using Student's *t*-test. The data represent the mean ± SD of three independent experiments. $P < 0.05$ was considered statistically significant.

Results

Size-dependent effect of cells uptake of AgNPs

We prepared three types of AgNPs and characterized them by SEM (**Fig. 1**). The SEM images showed the sizes of AgNPs were 20.6 ± 2.7, 53.8 ± 7.6, and 137.3 ± 43.0 nm, respectively. We cultured U251 cells with AgNPs for 24 h. The micrograph showed that when external darkness field was focused on them, the light intensity of cells was enhanced [**Fig. 2(B)**], while cells without AgNPs had no effect [**Fig. 2(A)**]. It indicated that AgNPs could be abundantly absorbed by cell membranes, which could further induce particles straddle across the membrane into cells [16]. To further study the amount of AgNPs by cell uptake among three sizes of nanoparticles, we cultured U251 cells with three types of AgNPs for 24 h, respectively, and used element analysis to evaluate the mass of silver element in four groups of cells (control, cells cultured with AgNPs Samples 1, 2, and 3). The results showed that the content of silver element increased

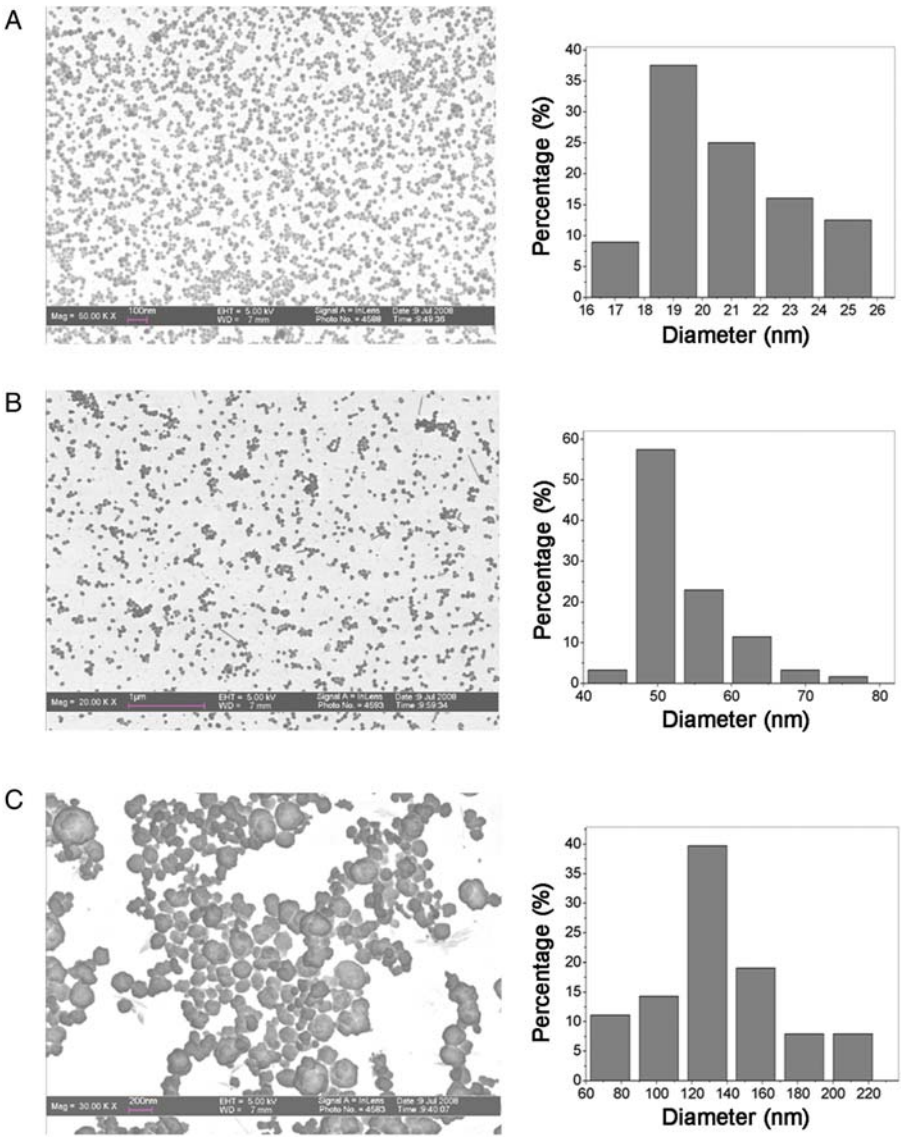

Figure 1 SEM images of three types of sizes of silver nanoparticles (A) 20.6 ± 2.7 nm; (B) 53.8 ± 7.6 nm; and (C) 137.3 ± 43.0 nm.

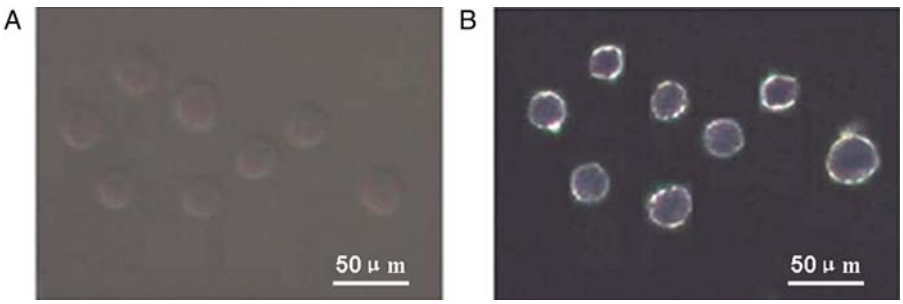

Figure 2 The micrographs of U251 cells uptake of silver nanoparticles on dark field When dark field focused on cells cultured without AgNPs, the light intensity of cells had no effect (A), but after cells cultured with AgNPs for hours, the light intensity was enhanced (B) owing to AgNPs property.

in all three groups cultivated with AgNPs than the control. The mass of silver was the highest in AgNPs (20.6 ± 2.7 nm) group and the lowest in AgNPs (137.3 ± 43.0 nm) group (**Fig. 3**). It indicted that the content of AgNPs by cells uptake was size dependent.

Colony-forming assay of AgNps on U251 cells

To further study the effect of AgNPs on cells upon thermotherapy, we used colony-forming assay to test cell repopulation after MNPs hyperthermia with or without AgNPs. Colony-forming assay is a quantitative method which is

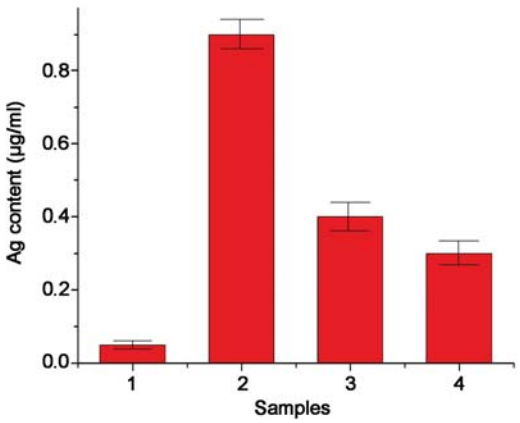

Figure 3 Ag element analysis showed cells uptake AgNPs (20.6 ± 2.7 nm) more than the other two sizes (1) control; (2) cells cultured with AgNPs (20.6 ± 2.7 nm); (3) cells cultured with AgNPs (53.8 ± 7.6 nm); and (4) cells cultured with AgNPs (137.3 ± 43.0 nm).

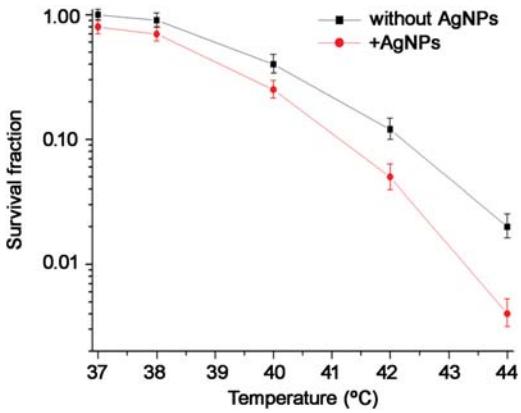

Figure 4 The survival curve of U251 cells showing influence of AgNPs (20.6 ± 2.7 nm, 15 μg/ml) on MNPs thermotherapy.

introduced comprehensively in chemotherapy, radiotherapy, and hyperthermia [5,17]. From the curves of survival fraction of cells, AgNPs (20.6 ± 2.7 nm) showed significant effect on thermo-sensitivity upon U251 cells (**Fig. 4**). Further study showed that the effect of AgNPs was also size dependent. Cells showed highest thermo-sensitivity with AgNPs (20.6 ± 2.7 nm) on hyperthermia treatment, while the effect of AgNPs (53.8 ± 7.6 nm) was weaker, and AgNPs (137.3 ± 43.0 nm) had the least effect (data not shown).

The results of Annexin-V-FITC binding and PI staining

The colony-forming assay showed hyperthermia combined with AgNPs (20.6 ± 2.7 nm) could decrease the cell survival rate. Then we used Annexin-V-FITC binding and PI staining to investigate the apoptosis rate of cells in four groups. The results showed that after MNPs hyperthermia, the percentages of ($FITC^+/PI^-$) and ($FITC^+/PI^+$) in cells in the MNPH combined with AgNPs (20.6 ± 2.7 nm) group was significantly enhanced ($P < 0.05$) compared with the merely MNPH group (**Fig. 5**).The ratios of $FITC^+/PI^-$ in different group were as followed: 3.5% ± 0.9% (control group), 4.2% ± 0.9% (heat group), 4.5% ± 1.1% (AgNPs group), and 7.2% ± 1.6% (AgNPs+heat group). The ratios of $FITC^+/PI^+$ in different group were: 1.0% ± 0.5% (control group), 1.2% ± 0.7% (heat group), 1.5% ± 0.7% (AgNPs group), and 8.1% ± 2.0% (AgNPs + heat group). It indicated that MNPH combined with AgNPs could induce more cancer cells into apoptosis phase.

Cell cycle test

The cell cycle test showed that AgNPs (20.6 ± 2.7 nm) could induce G_2/M arrest (15.0% ± 0.4% in the control and 30.8% ± 0.5% in the cells cultured with AgNPs group), which was similar to the effect of starch-coated AgNPs on cancer cells [16]. Although after hyperthermia, G_2/M ratio decreased (**Fig. 6**). The G_2 ratios in different groups were: 15.0% ± 0.4% (control group), 16.3% ± 0.3% (heat group), 30.8% ± 0.5% (AgNPs group), and 22.3% ± 0.4% (AgNPs + heat group). The result showed MNPH could clear this G_2/M arrest in the MNPH combined with AgNPs group. It indicated that after magnetic heating, more cells with the uptake of AgNPs (20.6 ± 2.7 nm) could enter the apoptosis phase.

Effect of AgNPs on thermo-sensitivity

Based on the results of the *in vitro* experiments, we further studied the effect of AgNPs on thermo-sensitivity of cancer cells in the glioma-bearing rat model. We chose 20.6 ± 2.7 nm AgNPs (10 μg/cm^3 of the tumor volume) and directly injected AgNPs into the volume of brain tumor of rats, along with 15 nm MNPs (3 mg/cm^3 of the tumor volume) for hyperthermia (**Fig. 7**). The dose of NPs guaranteed the safe and therapy requirements for rats.

The *in vivo* expression of Bax protein

After thermotherapy, the expression of Bax was tests by immunohistochemistry in pathological sections from tumor-bearing rats. The results showed that AgNPs (20.6 ± 2.7 nm) along with MNPH could enhance glioma cells bax expression *in vivo* (40.1% ± 6.31%) compared with merely MNPH group (35.20% ± 5.78%) (**Fig. 8**). Bax plays an important role in cell apoptosis [24,25], and our results indicated that MNPH combined with AgNPs could induce more cancer cell apoptosis *in vivo*.

Discussion

AgNPs show cytotoxicity and genotoxicity on cancer cells and would have applications in the anti-cancer drug field [16]. The cell experiments also showed that AgNPs could

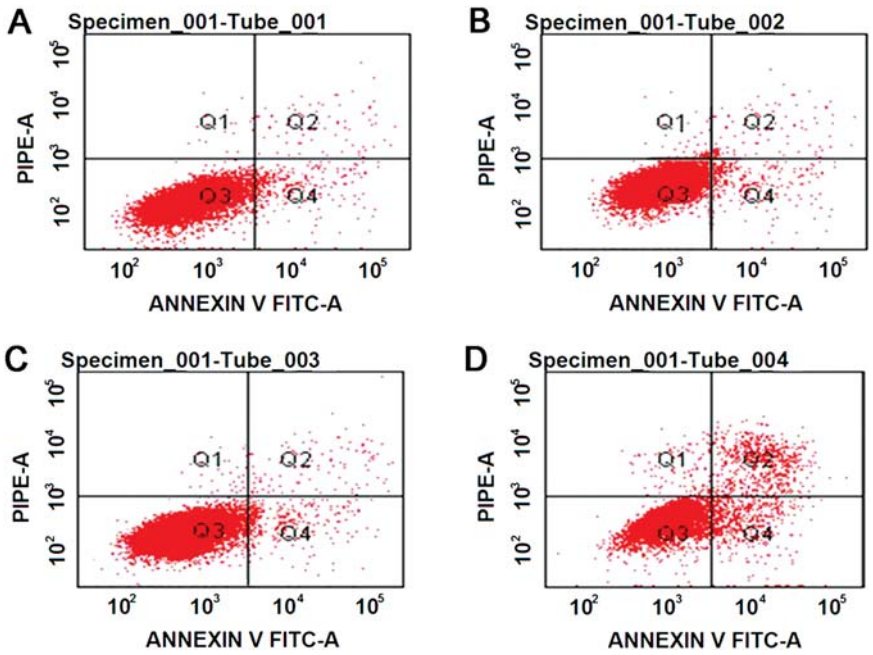

Figure 5 Cell apoptosis of U251 cells treated with AgNPs (20.6 ± 2.7 nm) and heat (A) control; (B) heat; (C) AgNPs; and (D) AgNPs + heat.

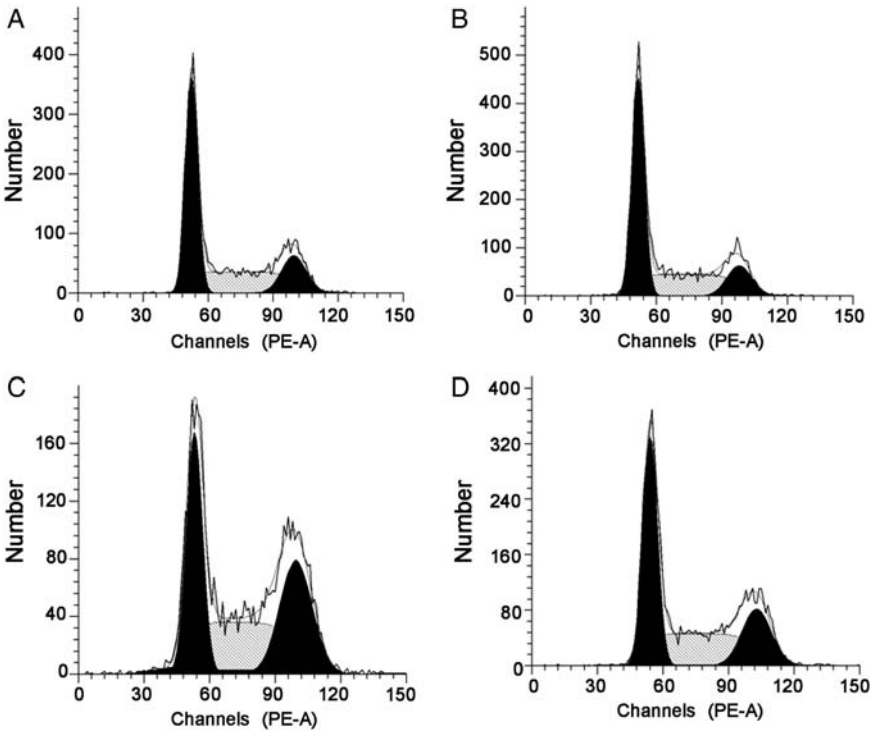

Figure 6 Effective of AgNPs (20.6 ± 2.7 nm) on cell cycle distribution before and after heat in U251 cells (A) control; (B) heat; (C) AgNPs; and (D) AgNPs + heat.

be potential radio-sensitizers for radiotherapy [5]. Our results demonstrated that AgNPs had the effect on thermo-sensitivity of cancer cells and this effect was size dependent. Among three sizes of AgNPs, 20.6 ± 2.7 nm AgNPs had significant effect on enhancing thermo-induced killing of glioma cells *in vitro*. In the glioma-bearing rats model, AgNPs combined MNPH could enhance Bax expression in cancer cells, which was correlated with cell apoptosis and could be worth getting further studies on possible mechanism. We choose glioma cells as a model system because most of GBM is insensitive or even resistant to clinical therapy, which is also reflected in the established glioma

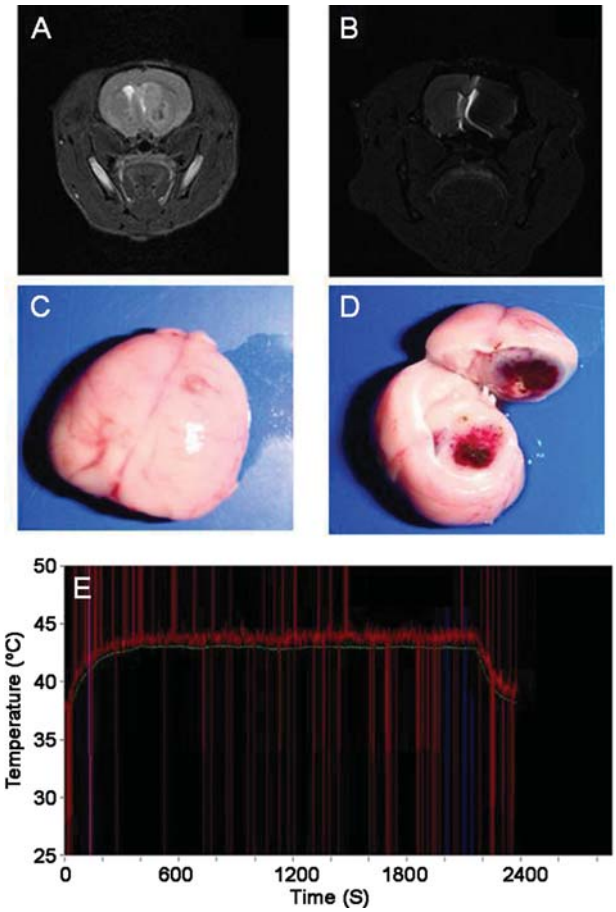

Figure 7 The images of glioma rats experiment on hyperthermia with AgNPs (20.6 ± 2.7 nm) MRI images of glioma rats before (A) and after (B) injecting MNPs for hyperthermia. MNPs can induce MRI images darkness enhanced in nanoparticles injection zones for magnetism. The dissection of mouse brain showed MNPs concentrated on the tumor zone after injection (C, D). The parameters of magnetic field for hyperthermia are 100 KA/m and 40 KHz. The temperature was monitored by a FOTS (E).

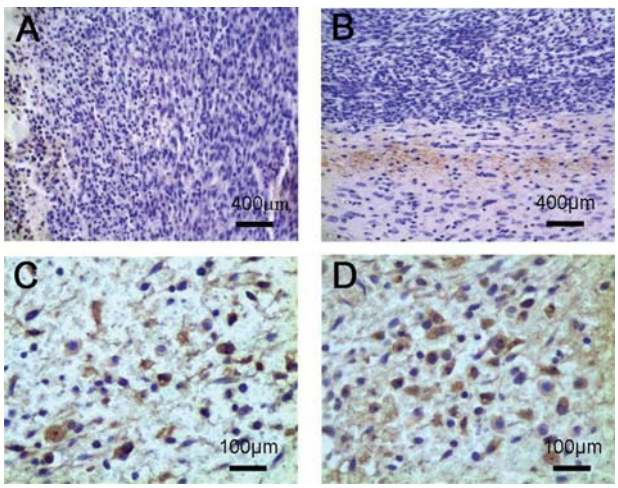

Figure 8 Effect of AgNPs (20.6 ± 2.7 nm) on the expression of Bax protein (A) +MNPs; (B) +AgNPs; (C) +heat; and (D) +AgNPs + heat.

cell lines [17–20]. Thus, enhancing the effect of thermo-induced killing of glioma cells may offer therapeutic benefit for patients with GBM.

We hypothesize that the mechanism of themo-sensitization by AgNPs might be related to the release of Ag^+ cation from the silver nanostructures inside cells. Ag^+ cation has the ability to capture electron and thus functions as an oxidative agent [5,16]. Consistent with our thermo-sensitization results, treating cell with AgNPs (20.6 ± 2.7 nm) led to some cytotoxicity on its own, while it greatly enhanced further cell killing by magnetic heating. **Fig. 3** showed that among three sizes of AgNPs, 20.6 ± 2.7 nm AgNPs had the most quantity of cell uptake. The fact that the thermo-sensitization effect of AgNPs decreases upon the increase in particle size is consistent with the Ag^+ cation release model, as smaller size AgNPs have a better ability to release silver ion.

The cell cycle test showed that after cell uptake of AgNPs (20.6 ± 2.7 nm), more cells stopped in G_2/M phase. Then after MNPH treatment, apoptosis rate was enhanced. In tumor-bearing rats, the Bax tests by immuno-histochemistry method showed that AgNPs (20.6 ± 2.7 nm) with MNPH could enhance cell apoptosis compared with merely magnetic heating group.

Based on our results, we suggest that AgNPs of particular sizes (20.6 ± 2.7 nm) significantly sensitized glioma cells upon hyperthermia. The thermo-sensitization effect of AgNPs suggests that intracellular nanostructures might interact with cell DNA repair system, and has important implications in the design of nanotechnology-based thermo-sensitizers for improving the outcomes of cancer therapy.

Funding

This work was supported by the grants from the National Basic Research Program of China (973 project) (2006CB933206) and the Scientific Research Foundation of Graduate School of Southeast University.

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