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Silver Nanoparticles (AgNPs) exhibit size-dependent differential toxicity and induce expression of syncytin-1 in FA-AML1 and MOLT-4 Leukaemia cell lines.

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Abstract

Human endogenous retrovirus (HERV) sequences make up approximately 8% of the human genome and increased expression of some HERV proteins has been observed in various pathologies including leukaemia and multiple sclerosis (MS). However, little is known about the function of these HERV proteins or environmental factors which regulate their expression. Silver nanoparticles (AgNPs) are used very extensively as antimicrobials and antivirals in numerous consumer products although their effect on the expression of HERV gene products is unknown. Cell proliferation and cell toxicity assays were carried out on human acute T lymphoblastic leukaemia (MOLT-4) and Fanconi anaemia associated acute myeloid leukaemia (FA-AML1) cells treated with two different sizes of AgNPs (7 nm and 50 nm diameter). RT-PCR and Western blotting were then used to assess expression of HERV-W syncytin-1 mRNA and protein in these cells. FA-AML1 cells were more sensitive overall than MOLT-4 to treatment with the smaller 7 nm sized AgNPs being the most toxic in these cells. MOLT-4 cells were more resistant and showed no evidence of differential toxicity to the different sized particles. Syncytin-1 mRNA and protein were induced by both 7 and 50 nm AgNPs in both cell types yet with different kinetics. In summary, the observation that AgNPs induce expression of syncytin-1 in FA-AML1 and MOLT-4 cells at doses as little as 5 µg/ml is grounds for concern since this protein is up-regulated in both malignant and neurodegenerative diseases. Considering the widespread use of AgNPs in the environment it is clear that their ability to induce syncytin-1 should be investigated further in other cell types.

Introduction

Silver is a toxic heavy metal and is often found associated with environmental contamination with other toxic heavy metals such as lead and mercury. However, driven by the emergence antibiotic resistance, the use of silver as an antimicrobial has increased dramatically over the last few years (1). Moreover, recent advances in nanotechnology have led to the creation of nano-sized silver particles which are now used in a diverse array of both medical and domestic products including catheters, sutures, disinfectants, furniture, paints, textiles, clothes and even telephones (2). Indeed, in 2010 it was estimated that around 320 tons of AgNPs were manufactured globally (3).

Nanoparticles are usually between 10 and 100 nm in diameter and, when compared with the parent material, they often exhibit new properties. Advantages include increased bioavailability due to improved solubility, longer biological half-life and increased catalytic activities as a result of the higher surface to volume ratio. Thus the main benefit is that a reduced concentration is needed to produce a desired response with less overall toxicity to normal cells (4). The potential adverse effects of AgNp's on mammalian cells have been extensively studied both in *vivo* and in *vitro* although the data are conflicting and it is clear that more work is needed (5,6). This lack of clarity is due in part to a number of variables such as choice of methods for synthesis and purification, size difference, coatings and cell types tested.

Cytotoxicity of AgNPs has been tested in many cell types including those derived from cancers of the haemopoietic system (7). The effects are varied but include inhibition of the normal respiratory functions of the cells by targeting mitochondria and endoplasmic reticulum inducing oxidative stress and DNA damage (8).

Although HERVs are expressed in both normal and diseased human tissues (9-12) they are often deregulated in cancers (reviewed (13)) and have been implicated in inflammatory disorders such as multiple sclerosis (MS) (14). Syncytin-1 is unusual for a HERV protein in that it performs an essential role in the morphogenesis of normal placenta by facilitating trophoblast cell fusion to form syncytiotrophoblasts and yet it has also been shown to promote the fusion of cancer cells (15,16). The increasingly wide range of products incorporating nanosilver particles has raised questions about their safety. Indeed their mode of action it is still poorly understood and the current study assesses the effects of AgNPs on leukaemic cell lines and explores the ability of these compounds induce expression of the HERV product syncytin-1.

Materials and methods

AgNP preparation

AgNP's of 7 nm and 50 nm sizes were prepared according to previously published work using AgNO₃, sodium citrate and with NaBH₄ as the reducing agent (17-19). Particle size and morphology were determined by TEM (CM30, Philips, Philips Analytical, Holland) at an accelerating voltage of 300 kV. The diameter of AgNP's (mean \pm S.D.) was determined by measurement of more than 200 particles in random fields of view. The TEM size of the two AgNP preparations was 7.6 ± 1.2 nm ("7nm" AgNPs) and 52.5 ± 17.9 nm ("50nm" AgNPs). Stock solutions (200 μ g/ml) were prepared in sterile distilled water and stored at 4°C until required for cell culture experiments. Immediately prior to cell treatments they were diluted to the required concentrations using cell culture medium.

Cell culture

The human T Lymphoblastic leukemia (MOLT-4) cell line was obtained from the American Type Culture Collection (ATCC CRL-1582; Manassas, VA, USA) and the Fanconi Anaemia (FA) Acute Myeloid Leukaemia (FA-AML1) cell line was provided by Dr Stefan Meyer (Consultant Paediatric Haematologist, Christie Hospital, UK). Cells were grown in RPMI 1640 medium (Invitrogen, Paisley, UK) supplemented with 2 mM L-glutamine (Sigma-Aldrich, Dorset, UK) and 10% heat-inactivated Foetal Calf Serum (FCS) (Sigma-Aldrich, Dorset, UK). The cells were cultured in a humidified 5% CO₂ incubator at 37°C. Exponentially growing cells were seeded at 5×10^5 cells per well in 6-well culture plates and treated with varying concentrations (5 µg, 10 µg, 25 µg and 50 µg) of either 7 nm or 50 nm AgNPs for 24 h. Following this they were harvested for further study.

MTS proliferation assay

Cell viability was measured using CellTiter AQ96 reagent (Promega, Southhampton, UK) according to the manufacturer's protocol. Briefly, cells were seeded in a 96-well plate at a seeding density of 5×10^3 cells per well and incubated at 37°C with 5% CO₂ for 24 and 48 h. Following exposure to various concentrations of both sizes of AgNPs (5, 10, 25 and 50 µg), 20 µL of AQ reagent was added and the cells further incubated for 4 h at 37°C in 5% CO₂. The absorbance was measured at 490 nm using a Dynex MRX plate reader (Dynex Technologies Ltd, West Sussex, UK) and results were presented as the percentage of viable cells in AgNPs-treated cells as compared to untreated cells (control).

Trypan blue exclusion test

FA-AML1 and MOLT-4 cells were seeded into 6 well plates (0.5×10^6 cells/well) and incubated for 24 h in the presence or absence of varying concentrations of both sizes of AgNPs (5, 10, 25 and 50 $\mu\text{g/ml}$). Cells were stained using Trypan blue (Sigma, Aldrich, Dorset, UK) and counted using a haemocytometer. Non-viable cells were expressed as a percentage of the total number of cells counted.

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was prepared from treated and untreated cells using TRIzol reagent (Life Technologies, Paisley, UK) and cDNA synthesised using the 'Cells-to-cDNA™ II Kit' (Ambion, Warrington, UK) both according to the manufacturer's instructions. The PCR reactions were performed in a total volume of 50 μl , containing 5 μL of $10 \times \text{CL}$ buffer (Qiagen Inc.), 2.5 μl of MgCl_2 , 1 μl each of syncytin-1 forward and reverse primers (5' TGCCCCATCGTATAGGAGTCT-3') and (5'-CATGTACCCGGGTGAGTTGG-3'), 0.4 μL of dNTPs, 0.5 μl of Taq polymerase and 2 μl of each cDNA sample. PCR conditions were: $94^\circ\text{C} \times 5$ minutes followed by 30 cycles of $94^\circ\text{C} \times 30$ seconds, $56^\circ\text{C} \times 30$ seconds and $72^\circ\text{C} \times 30$ seconds. A final extension of 72°C for 7 minutes was then applied. GAPDH was amplified using the forward primer (5'-CATTGACCTCAACTACATGGT-3') and reverse primer (5'-TCGCTCCTGGAAGATGGTGAT-3'). PCR was carried out using a VerityH 96 well Fast Thermocycler (Applied Biosystems, Paisley, UK). Products were separated through a 1.5% agarose gel and visualised using ethidium bromide under UV light. The relative intensity of each DNA band was semi-quantified using image J software.

Western blotting

5×10^5 control or AgNPs treated cells were pelleted by centrifugation at $295 \times g$ and protein samples were prepared using Laemmli sample buffer. Proteins were separated by 12% SDS

PAGE at 20 mA for 2 hours (Biometra, UK), membrane blotted (Amersham Biosciences, Buckinghamshire, UK) and blocked with 5% skimmed milk in PBS-Tween for 2 hours. The membrane was incubated with primary antibodies to syncytin-1 at 1:100 in PBS, v/v or GAPDH 1:1500 in PBS (Both from Abcam, Cambridge, UK). After washing the membrane was incubated with HRP conjugated secondary antibodies at 1:2000 in 5% skimmed milk in PBS (Dako, Cambridge, UK). The target proteins were visualised using an enhanced chemilluminescence kit and exposure to Hyperfilm® (Amersham Biosciences, Bucks, UK) according to the manufacturer's instructions.

Statistical analysis

AQ96 assays and Trypan blue exclusion tests were all carried out in triplicate and the results were analysed using one way (ANOVA) with SPSS software. The data were presented as the mean value \pm standard deviation (SD). To assess the level between treatments and respective controls, *P* values < 0.05 were considered statistically significant. All EC50 values were predicted using R version 3.2.2 drc package and Microsoft Excel 2010.

Results

Effect of AgNPs on cell growth and viability

The sensitivity of the two leukaemic cell lines MOLT-4 and FA-AML1 to AgNPs was determined by MTS proliferation and Trypan blue live/dead cell assays. Both 7 and 50 nm AgNPs reduced the growth of both cell lines in a dose-dependent manner after incubation for 24 and 48 h (Figure 1). Similar growth rate reductions were observed with increasing concentrations of 50 nm AgNPs for FA-AML1 and MOLT-4 cells and 7 nm AgNPs for MOLT-4 cells (Figure 1 B, C and D). In contrast the 7 nm nanoparticles were much more

growth inhibitory against FA-AML1 cells (Figure 1A) which were also judged to be significant by Dunnet's T3 post hoc test.

Assessment of cell death by Trypan blue exclusion confirmed the trends seen with the proliferation assays (Figure 2). As can be seen there was a marked increase in FA-AML1 cell death after exposure to 5, 10, 25 and 50 $\mu\text{g/ml}$ of the smaller 7 nm sized AgNPs for 24 h (15%, 23%, 54% and 69% dead cells respectively) whereas these cells were much less sensitive to the larger sized 50 nm AgNPs with only 30% death after 24 h of treatment at 50 $\mu\text{g/ml}$ (Figure 2B). Treatment of MOLT-4 cells with 50 $\mu\text{g/ml}$ of the 50 nm AgNPs produced 20% cell death at 24hrs whereas 25 and 50 $\mu\text{g/ml}$ of the 7 nm particles produced 27% and 37% death respectively (Figures 2 C and D) confirming these cells were less sensitive than FA-AML1 cells to the smaller particles.

From the growth assays (Figure 1) EC50s of 7nm AgNps on FA-AML1 cells were predicted to be $19.6 \pm 4.9 \mu\text{g/ml}$ at 24h and $23 \pm 2.7 \mu\text{g/ml}$ 48h. EC50's of 50nm AgNp's on FA-AML-1 cells were predicted to be >65 and $55 \mu\text{g/ml}$ at 24h and 48h respectively. For MOLT-4 cells EC50's for both 7 nm and 50nm AgNPs were predicted to be $> 50 \mu\text{g/ml}$.

From the live/dead assays (Figure 2) EC50s of 7nm AgNp's on FA-AML1 cells were predicted to be $17.9 \pm 2.9 \mu\text{g/ml}$ and $>50 \mu\text{g/ml}$ for 50nm particles. EC50s for MOLT-4 cell death were $>50 \mu\text{g/ml}$ for both sizes of particles.

Effect of AgNPs on syncytin-1 mRNA expression

RT-PCR was used to assess syncytin-1 expression in both FA-AML1 and MOLT-4 cells. The resulting gel images were quantitated and normalised to GAPDH using Image J software (Figure 3). Syncytin-1 was induced in both cell types in response to treatment with both sizes of AgNPs albeit with different kinetics. In FA-AML1 cells the 7 nm particles induced syncytin-1 mRNA in a dose dependent manner up to $10 \mu\text{g/ml}$ after which the levels dropped

markedly (Figure 3A). Interestingly, although the mode of action is unknown, the 50 nm particle size showed a biphasic response with maximum induction of syncytin-1 transcript occurring at 5 $\mu\text{g/ml}$ followed by a reduction in signal at 10 and 25 $\mu\text{g/ml}$ with this increasing again at 50 $\mu\text{g/ml}$ (Figure 3B). MOLT-4 cells showed an induction of syncytin-1 transcript at 5 $\mu\text{g/ml}$ of the 7 nm particles which remained constant for all higher concentrations of AgNPs (Figure 3C). When these cells were treated with the 50 nm sized particles, similar to FA-AML cells, a biphasic response was observed with maximum induction of syncytin-1 occurring at 10 $\mu\text{g/ml}$ followed by a reduction at 25 and an increase at 50 $\mu\text{g/ml}$ (Figure 3D).

Induction of syncytin-1 protein by AgNPs

The induction of syncytin-1 protein by AgNPs was evaluated by western immunoblotting of protein lysates prepared from the same cells used in the previous RT-PCR analysis (Figure 4). In both cell lines syncytin-1 levels increased in response to treatment with both sizes of AgNP, albeit with different kinetics. For FA-AML1 cells, 7 nm AgNPs induced syncytin-1 protein upto 10 $\mu\text{g/ml}$ after which the level declined (Figure 4A). Treatment of these cells with 50 nm AgNP showed a more consistent induction of syncytin-1 with all concentrations used (Figure 4B). Treatment of MOLT-4 cells with both 7 nm and 50 nm AgNPs caused a pronounced induction of syncytin-1 protein from 5 $\mu\text{g/ml}$ which persisted with all the higher concentrations of AgNPs used (Figure 4C and D).

Discussion

By virtue of their unique physicochemical properties, nanoparticles cause effects that are related to their shape, size and chemical composition (20-24). Silver nanoparticles are widely used in common consumer products including cosmetics, pesticides, detergents, face creams, toothpastes, medical products and even dietary supplements (25). Hence it is important to

understand their full range of biological activities in human cells. Since previous studies have indicated that the response to different size of AgNPs was variable in different cancer cell lines (26) we opted to evaluate the effects of two sizes of AgNPs on the myeloid FA-AML1 and lymphoid MOLT-4 leukaemic cell lines. Results presented herein are consistent with these earlier observations as the FA-AML1 cells were much more sensitive to growth inhibition and cell death induced by the smaller 7 nm AgNp particles than MOLT-4 cells.

What factors might explain this differential toxicity? One possibility is that this could be related to differences in the phagocytic activity of these cell types. Myeloid precursor cells can develop into macrophages which have high rates of phagocytosis that may simply result in higher uptake of AgNPs although how this relates to different particle size is unclear. Another possibility is differential sensitivity of cells to oxidative stress (27,28) since it has been shown that the production of reactive oxygen species (ROS) is the primary cause of cytotoxicity produced by different sizes of AgNPs particles (26,29). Moreover it has been shown that ROS production is more pronounced with smaller AgNPs which is also consistent with our results.

Irrespective of the differences in cytotoxicity displayed by the two sizes of AgNPs in FA-AML1 cells, of more concern was the ability of both particle sizes to induce expression of syncytin-1 mRNA and protein in both cell types tested. The reason for this is that syncytin-1 is known to be up-regulated in multiple sclerosis (30-32) and a variety of cancers (33,34), including lymphoma and leukaemia (35). It is also known to promote the fusion of malignant cells, both to themselves and normal endothelial cells which contributes to enhanced genetic instability (15,16,34) and recent work has shown that mutations in the HERV-W genomic 3'-long terminal repeat induce up-regulation of syncytin-1 expression in urothelial cell carcinoma (36).

In normal cells expression of syncytin-1 is mostly confined to the placenta (37) where it functions to promote both proliferation (38) and fusion of trophoblasts (39,40) in order to form syncytio-trophoblasts. It is also known to suppress apoptosis in choriocarcinoma cells (41) and low levels of syncytin-1 have been linked to placental dysfunction in pre-eclampsia (42-44). Most significantly expression of syncytin-1 has also been shown to suppress staurosporin-induced apoptosis in Chinese hamster ovary (CHO) cells (45) demonstrating the ability of this protein to produce these effects in cells other than those of placental origin and we have shown that siRNA silencing of syncytin-1 in Jurkat T cell lymphoma cells induces a marked drop in the growth rate of these cells (manuscript in preparation).

How do these observations assimilate with our finding that AgNPs can induce syncytin-1 expression in FA-AML1 and MOLT-4 cells? In light of the documented growth-promoting anti-apoptotic activity of syncytin-1, it is most likely that its induction by AgNPs represents a survival response to ROS-induced cell damage rather than the protein playing any causative role in subsequent cytotoxicity. Indeed the very marked induction of syncytin-1 expression in FA-AML1 cells treated with as little as 5 µg/ml AgNP accompanied by only low levels of corresponding cell death at this concentration (Figures 2B and 3B) supports this hypothesis. Thus, considering their widespread use, the ability of AgNPs to promote the expression of HERV-W-derived syncytin-1 in cultured leukaemia cells is significant since syncytin-1 clearly promotes the survival of malignant cells. Furthermore, it is also known to promote cell-cell fusion of malignant cells which implies that it could also contribute to neoplastic progression by promoting polyploidy and aneuploidy with resulting genetic instability (46). Moreover it has recently been shown that chronic treatment of HepG2 cells with AgNPs induces persistent growth stimulation and anti-apoptotic changes in gene expression in these cells (47) which is consistent with AgNP-induced syncytin-1 playing a role in this process.

Although there is significant evidence for syncytin-1 conferring a growth/survival advantage in malignant cells, there is also evidence that its expression induces demyelination and cell death in oligodendrocytes which is consistent with the protein playing a causative role in neurodegenerative disease (48). Indeed, as previously mentioned, syncytin-1 is thought to play an instrumental role in MS (30,32,49). Thus it is significant that AgNPs are also known to cause neuronal damage (50,51) and we speculate this could be augmented by induction of syncytin-1.

In conclusion, the widespread use of AgNPs in many products means that humans will invariably be exposed to these agents and, although speculative, our results support the conclusions of others that this may not be without risk (for review see (52)). AgNPs are known to promote ROS production and DNA damage (26,53,54) which is consistent with their potential use as cancer therapeutics (29,55,56). However, as with most cytotoxic drugs, there are invariably off-target effects which restrict their use and our data indicates that AgNPs induce syncytin-1 expression in leukaemic cell lines. Given that expression of syncytin-1 is known to enhance genetic instability and to play a role in neurodegenerative disease, more studies on this indication are clearly warranted.

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Figure Legends

Figure 1. Proliferation assays to assess the effects of AgNPs on leukemic cell lines.

Cells were treated with 5, 10, 25 and 50 µg/ml of AgNPs for 24 and 48 h. MTS assay was used to assess cell growth comparing 7 nm and 50 nm sizes on FA-AML1 (A and B) and MOLT-4 (C and D) cells respectively. Each value was expressed as the mean ± SD of three independent experiments, with **P* <0.05 compared to the control. The normal cell line population doubling times was 38 h for FA-AML1 cells and 42 h. for MOLT-4 cells.

Figure 2. Cytotoxic Effects of AgNPs on leukemic cell lines.

Cells were treated with 5, 10, 25 and 50 µg/ml of AgNPs for 24 h. Trypan blue exclusion assay was used to assess cell death of the cells comparing 7 nm and 50 nm sizes on FA-AML1 (A and B) and MOLT-4 (C and D) cells respectively. Each value was expressed as the mean ± SD of three independent experiments, with **P* <0.05 compared to the control.

Figure 3. RT-PCR analysis of syncytin-1 mRNA in leukemic cell lines.

FA-AML1 and MOLT-4 cells were treated with 7 and 50 nm AgNPs at 5, 10, 25 and 50 µg/ml for 24 h. GAPDH was used as an internal control. Densitometric analysis was performed using Image J software and the signals normalised to GAPDH (A) FA-AML1 cells treated with 7 nm AgNPs. (B) FA-AML1 cells treated with 50 nm AgNPs (C) MOLT-4 cells treated with 7 nm AgNPs (D) MOLT-4 cells treated with 50 nm AgNPs. The results are the mean ± SD of three independent experiments, with * indicating *P* < 0.05 compared to the control.

Figure 4. Syncytin -1 protein expression in leukaemic cells lines.

FA-AML1 and MOLT-4 cells were treated with 7 and 50 nm AgNPs at 5, 10, 25 and 50 µg/ml for 24 h. GAPDH was used as an internal control. Densitometric analysis was performed using Image J software and the signals normalised to GAPDH (A) FA-AML1 cells

treated with 7 nm AgNPs. (B) FA-AML1 cells treated with 50 nm AgNPs (C) MOLT-4 cells treated with 7 nm AgNPs (D) MOLT-4 cells treated with 50 nm AgNPs. The results are the mean \pm SD of three independent experiments, with * indicating $P < 0.05$ compared to the control.

Figure 1

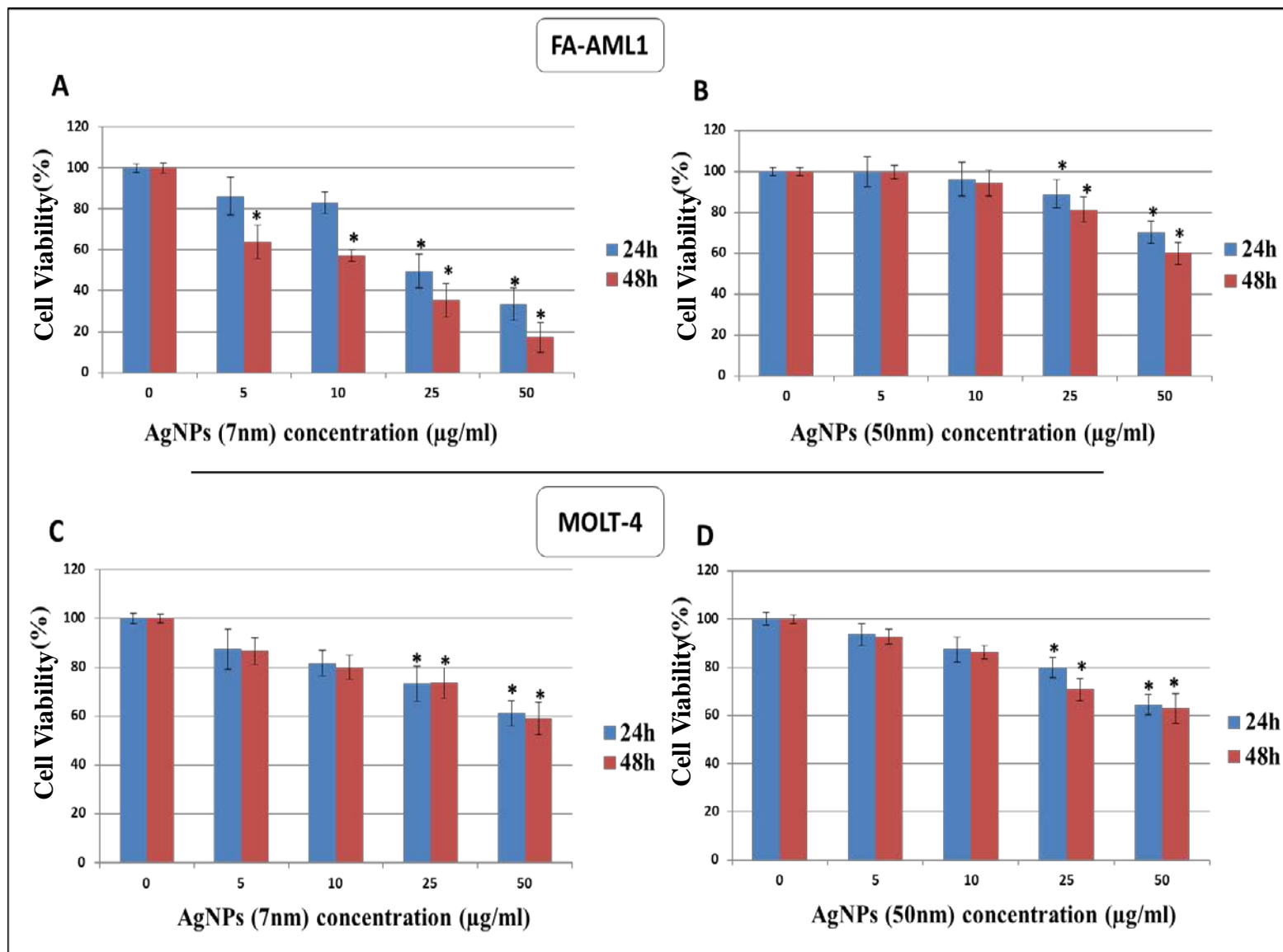


Figure 2

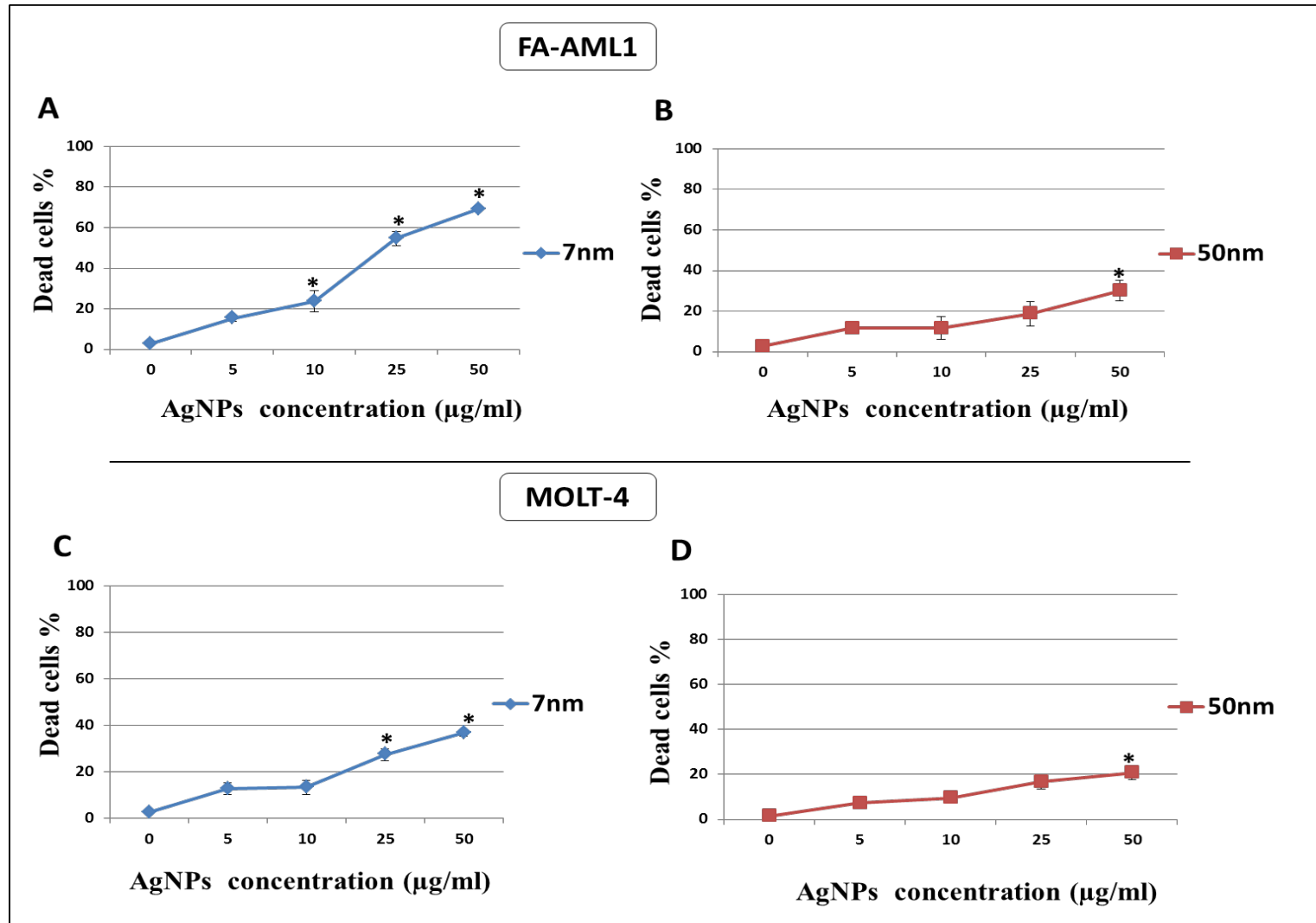


Figure 3

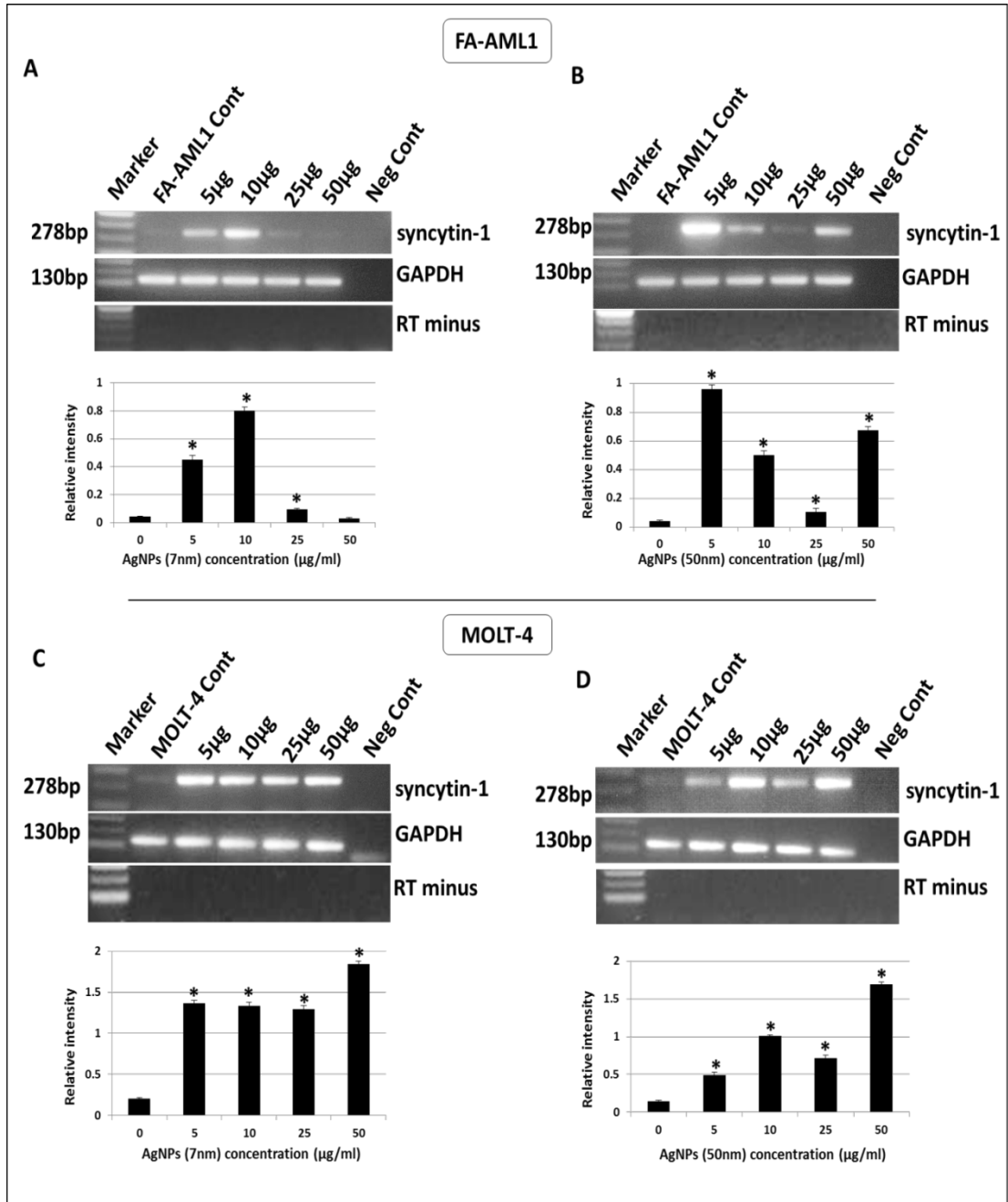


Figure 4

