Graphene oxide-silver nanoparticle nanocomposites induces apoptosis in caprine fetal fibroblast cells via induction of oxidative stress

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Abstract

**Background:** Graphene oxide (GO) has drawn much attention as excellent platform to which silver nanoparticles (AgNPs) can be anchored for the production of biomedical nanocomposites. Yet, the potential toxicity of GO-AgNPs nanocomposites to animal and human is complex to evaluate and remains largely unknown.

**Results:** Our data indicated that GO-AgNPs caused cytotoxicity in dose-dependent manner. GO-AgNPs induced significant cytotoxicity by the loss of cell viability, production of reactive oxygen species (ROS), cell cycle arrest, increasing leakage of lactate dehydrogenase (LDH) and level of Malondialdehyde (MDA), increasing expression of pro-apoptotic genes and decreasing expression of anti-apoptotic genes.

**Conclusions:** Taken together, our study demonstrated that GO-AgNPs potentially induce oxidative damage to DNA, which result in toxicity and cell apoptosis in caprine fetal fibroblast cell due to an increased generation of ROS. It strongly suggests that applications of GO-AgNPs nanocomposite in animal must be further evaluated.

**Background**

Nanotechnology is related to the controlled design, characterization, production and application of nano-sized materials, which are defined as small particles ranging in size between 1 and 100 nm. The increasing application of nanoparticles in microelectronics, cosmetics, ceramics, catalysts and food has made them widely present in our lives and attracts increasing investment from governments and industry around the world [1–4]. Currently, because of their unique physiochemical properties, a growing number of nanomaterials have been applied in the area of biomedicine, which emphasis on the diagnostic and therapeutic purposes, such as cancer diagnosis and therapy, nanocarriers for targeted delivery of drugs and genes, design of novel candidate nanoscale constructs for drug development [5–10]. Consequently, the bio-application and biosafety of the nanomaterials need to be further explored.

In recent decades, graphene and graphene related nanocomposite have attracted much attention both in the industry and scientific community due to their unique physiochemical and biological
properties [11-13]. Silver nanoparticles (AgNPs) are one of the most frequently used nanoparticles in a variety of biomedical applications [14]. Recent advances in nanotechnology have widened the potential combination of AgNPs with graphene-based nanocomposite, which offers a novel graphene-silver hybrid nanomaterials with unique functions in biomedical nanotechnology, and nanomedicine [15, 16]. For example, AgNPs attaching on the surface of Graphene oxide (GO) sheets can prevent AgNPs from aggregating, allow a more controlled release of AgNPs$^+$ ions and lead to the increase antibacterial and anticancer activity [17, 18]. Despite their wide applications in several areas, many evidences suggested that some nanomaterials accumulates in human and animal tissue, such as heart, kidney and other organs [19-22], which modifies the transcription of genes related to transport pathways, nuclear signaling, endocytosis, reproductive behavior and general defenses [23, 24]. Thus, any potential nanotoxicity should be thoroughly and carefully evaluated for promoting its safe application as a clinical agent.

Nanotoxicity involves the understanding of adverse biological effects of nanoparticles using both in vitro and in vivo model systems such as cell, tissue, organ and organism [25]. Several studies have been dedicated to examine the effects of graphene and graphene-related nanomaterials in various cell culture systems, including HeLa, MCF-7, SKBR3, NIH3T3, epithelial lung carcinoma, primary mouse embryonic fibroblast, human breast cancer and ovarian cancer [15, 26-28]. Graphene and graphene related nanomaterials have been proved to lead to inflammatory response in the liver and kidney [29-31], genotoxicity and DNA damage [30, 32], and adverse functional effects in the lungs, heart, intestine, and spleen [33-35].

Recently, due to rapid growing of nanotechnology, investigation of nanomaterials toxicity and their potential risks are attracting interests [24]. Although a wide spectrum of studies have been performed to determine the potential toxicity of graphene and graphene related nanomaterials in different cancer cell lines and small animal, such as rat and mouse, so far the effect of graphene oxide-silver nanoparticles (GO-AgNPs) nanocomposites on caprine fetal fibroblast cells remained to be illuminated. Therefore, the present study was conducted to investigate the effect of GO-AgNPs nanocomposites on caprine fetal fibroblast cells in vitro and to determine the underlying molecular mechanisms of their
cytotoxicity. To our knowledge, this is the first report in caprine fetal fibroblast cells to demonstrate cellular responses and functional aspects of GO-AgNPs.

Results
Characterization of GO-AgNPs
TEM analysis was conducted to confirm the structural and surface morphology of the GO-AgNPs composites. The size distribution of the GO-AgNPs was about 20 nm as shown in the image of TEM (Fig. 1). AgNPs were uniformly distributed on the GO sheets. GO-AgNPs images clearly showed transparent, single-layer sheets containing flake-like wrinkles (Fig. 1). The wrinkled silk waves were presented on the GO sheets.

Effect Of Go-agnps On Caprine Fetal Fibroblast Cells Viability
The viability of caprine fetal fibroblast cells was determined by CCK-8 assay. As shown in Fig. 2, there were no significant differences in cell viability between control cells and those exposed to 1 µg/mL GO-AgNPs for 24 h, however, the viability of cells was significantly reduced when the concentration increased (4, 8, 12 and 16 µg/mL), suggesting that GO-AgNPs induced toxicity in caprine fetal fibroblast cells in a dose-dependent manner.

Effect Of Go-agnps On Cell Morphology
The morphologies of caprine fetal fibroblast cells after exposure to GO-AgNPs for 24 h were shown in Fig. 3. Caprine fetal fibroblast cells that had been exposed to 4 µg/mL and 8 µg/mL GO-AgNPs exhibited marked morphological changes, with obvious reduction in number of cells in the group of 8 µg/mL GO-AgNPs.

Effect Of Go-agnps On Reactive Oxygen Species (ros) Production
To study whether GO-AgNPs induced oxidative impact involving in the apoptosis, the intracellular ROS level in caprine fetal fibroblast cells was analyzed. As shown in Fig. 4, the level of intracellular ROS in caprine fetal fibroblast cells was significantly increased (P < 0.05) when the cells were treated with 4 and 8 µg/mL of GO-AgNPs for 24 h compared to the control group.

Effects Of Go-agnps On Apoptosis
An Annexin V/PI apoptosis kit was used to quantify the percentage of caprine fetal fibroblast cells undergoing apoptosis and dying by flow cytometry. The results suggested that the GO-AgNPs induced significant apoptosis and dead in caprine fetal fibroblast cells (Fig. 5).
Effects Of Go-agnps On Superoxide Dismutase (sod) Production
Effects of GO-AgNPs on the production of SOD in caprine fetal fibroblast cells were determined with SOD assay kit. As shown in Fig. 6, SOD activity decreased significantly (P < 0.05) in caprine fetal fibroblast cells treated with 4 µg/mL GO-AgNPs for 24 h when compared to the control group. Furthermore, treatment of caprine fetal fibroblast cells with 8 µg/mLGO-AgNPs decreased significantly (P < 0.01) in SOD activity compared to non-treated caprine fetal fibroblast cells.

Effects Of Go-agnps On Malondialdehyde (mda) Production
The production of MDA in caprine fetal fibroblast cells was determined with the MDA assay kit after treated with different concentrations of GO-AgNPs (0, 4 and 8 µg/mL) for 24 h. The results showed that levels of MDA increased significantly (P < 0.05) in the 4 and 8 µg/mL treated groups, when compared to the control group (Fig. 7).

Effects Of Go-agnps On Lactate Dehydrogenase (ldh)
Caprine fetal fibroblast cells were treated with different concentrations of GO-AgNPs (0, 4 and 8 µg/mL) for 24 h, and the level of leakage of LDH was measured. The results indicated that GO-AgNPs significantly increased the leakage level of LDH in caprine fetal fibroblast cells compared to the control group (Fig. 8; P < 0.05).

Effects Of Go-agnps On Caspase-3 Activity
To confirm whether caspase-3 is involved in apoptosis of caprine fetal fibroblast cells induced by GO-AgNPs (4 and 8 µg/mL), caspase-3 activity was measured by the caspase-3 kit. The activity of caspase-3 in the 4 and 8 µg/mL group was significantly (P < 0.05) higher after treatment than that in the control group (Fig. 9).

Effects Of Go-agnps On Gene Expression
The expression level of apoptotic genes was determined in the caprine fetal fibroblast cells treated with GO-AgNPs (4 and 8 µg/mL) for 24 h. The results showed that the level of caspase-3, Cyt-C, BAX, Smac and p53 were significantly (P < 0.05) upregulated in the GO-AgNPs treated groups, compared to the control group (Fig. 10). However, the level of BCL2 in the GO-AgNPs treated group was significantly (P < 0.05) downregulated, compared with the control group (Fig. 10).

Discussion
Nanotechnology has become an indispensable field tool to develop various kinds of nanoparticles with
unique properties [36]. As an efficient support material, graphene sheets can disperse and stabilize silver nanoparticles preventing their agglomeration, which open up a way for the nanomaterial development. Therefore, the combination of graphene and AgNPs based nanocomposites has been widely produced to enhanced antibacterial and anticancer activity [37]. This kind of nanomaterial can easily enter cells, thus affect the physiology of organisms, which may have the potential toxicity both on human and animal health or ecosystems. Therefore, the adverse effects of GO-AgNPs composites have been considered as a major limitation for its broad applications. Numerous studies have proved the toxicological effects of GO-AgNPs nanocomposite on animal and human normal cells [21, 37, 38].

In the present study, a GO-AgNPs nanocomposite was synthesized using quercetin and was confirmed its surface and structural morphology, and the uniform distribution of AgNPs on the GO sheets with TEM, and investigated its toxic effect on caprine fetal fibroblast cells.

In order to evaluate the effect of GO-AgNPs on caprine fetal fibroblast cell viability, cells were treated with different concentrations of GO-AgNPs for 24 h. The data showed that 4 or 8 µg/mL GO-AgNPs reduced cell growth, viability and induced morphological changes in a concentration-dependent manner. In our previous study, GO-AgNPs significantly decreased the human ovarian cancer cell viability with an IC50 of 5 µg/mL [39], which is lower than that in the present study, suggesting that caprine fetal fibroblast cells are less sensitive to GO-AgNPs than human cancer or mouse cells. Similar results were reported that 5 µg/mL rGO-Ag nanocomposite did not induce cytotoxicity in human normal cells (CHANG cell) but could slightly induce toxic effect on HepG2 cells, however, when the concentration of rGO-Ag increased up to 25 µg/mL, cell viability significantly decreased [38]. The loss of cell viability by graphene materials may depend on many factors, such as the surface chemistry charge, size and physicochemical properties of the materials.

To investigate whether GO-AgNPs inducing the cytotoxicity was the cause of cell apoptosis, caprine fetal fibroblast cells were analyzed with Annexin V/PI double labeling assay. The data by flow cytometry showed that GO-AgNPs induced more apoptosis and inhibited cell proliferation compared to that of control group. Similarly, the level of caspase-3 was higher in GO-AgNPs treated groups, which confirms the results of apoptosis. Oxidative stress inducing ROS is one of the proposed toxicological
mechanisms of various nanomaterials such as Ag or Ag-graphene nanocomposites, can cause mitochondrial damage, and initiation of lipid peroxidation [39–41]. In current study, the production of ROS in caprine fetal fibroblast cells was evaluated. After treatment with 4 and 8 µg/mL GO-AgNPs for 24 h, the level of ROS increased significantly at a 1.4- and 1.8-fold in caprine fetal fibroblast cells. The present data suggests that the production of ROS is a common toxicity mechanism of GO-AgNPs for animal cells.

The increased level of MDA is generally considered to imply the cell injury. Human cells treated with AgNPs and GO showed significantly increased levels of MDA [42, 43]. Assessing the release of intracellular LDH in cell, which is the result from the breakdown and alteration in the permeability of the plasma membrane, is one of marker for estimating cytotoxicity [16, 41]. Previous studies reported that the combination of GO with AgNPs could significantly increase the intracellular production of MDA [15, 44]. The present data indicated that the mechanism of increased level of MDA in GO-AgNPs-treated caprine fetal fibroblast cells may be due to the strong hydrophobic interactions with the cell membrane and ROS formation, which influenced the viability and proliferation of cells.

The apoptosis in cell is a highly conserved mechanism, and ROS is an important factor in the apoptotic process [45]. ROS induced by nanomaterial could result in nuclear DNA damages and leakage of lipids, proteins and carbohydrates in the cell. In the present study, apoptosis was observed in GO-AgNPs-treated caprine fetal fibroblast cells. ROS production and lipid peroxidation induced by GO-AgNPs affected cellular redox homeostasis and decrease antioxidant levels, which were evaluated by the SOD, which act as an active oxygen free radical scavenger that can combat the accumulation of ROS and reduce the oxidative injury. The SOD activity has a relationship with the antioxidative ability and also can be used as indicative of mitochondrial activity [46, 47]. The present data showed that the level of SOD significantly decreased in GO-AgNPs-treated caprine fetal fibroblast cells, which corresponded with the high rates of apoptotic cells.

Anti-apoptotic and apoptotic genes play an important role in cell death and survival. A study reported that GO-AgNPs can cause oxidative damage, leakage of LDH, and enhance expression of apoptotic genes, thus lead to mitochondrial dysfunction and trigger apoptosis [48]. All apoptotic pathways
appear to terminate in the activation of the caspase family of proteases [49]. Moreover, oxidative stress is reported to cause the mitochondrial translocation of the pro-apoptotic protein of Bax, increase the total expression of Bax and also down-regulate the expression of the anti-apoptotic protein of BCL-2 [50]. The present data suggested that GO-AgNPs up-regulated the expression apoptotic genes such as caspase-3, Bax, Smac and c-myc, and down-regulated anti-apoptotic genes such as Bcl-2.

Conclusion
Previously, we described a simple and environmentally friendly method to synthesize GO-AgNPs nanocomposite, which exhibited enhanced cytotoxicity to human cancer cells (SH-SY5Y) compared with that of GO at a very low concentration [39]. In the present study, the cytotoxic potential and molecular pathway of the GO-AgNPs nanocomposite were evaluated in caprine fetal fibroblast cells. The synthesized GO-AgNPs nanocomposite displayed cytotoxicity to caprine fetal fibroblast cells at the concentration of 4 and 8 µg/mL with apoptotic induction by enhancing production of ROS, loss of proliferation and cell viability, decrease levels of anti-apoptotic genes and increase expression of pro-apoptotic genes.

Materials And Methods
Chemicals
All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Synthesis And Characterization Of Go-agnps
GO-AgNPs nanocomposites were synthesized using the biomolecule quercetin as described previously [39]. Briefly, 50 mg GO was dispersed in 30 mL water and sonicated for 60 min. One mM AgNO₃ were dissolved in 15 mL water in a 500 mL round-bottom flask. 30 mL of the GO dispersion was added, followed by addition of 5 mL of aqueous 1 mM quercetin, and then stirred at 60 °C for 12 h. The resultant mixture was washed and centrifuged three times with water. The size and shape were observed under a transmission electron microscope (TEM; HT7800, Hitachi High-Technologies Corporation, Tokyo, Japan).

Cell Culture
Caprine fetal fibroblast cells were isolated from 70-day old fetuses that were recovered surgically from a Boer goats as previously described [51]. After removal of the head and internal organs, the remaining tissues were dissociated into small pieces using scissors and digested with 0.25% trypsin (Thermo Fisher Scientific, Waltham, MA, USA). Then cells were washed three times, centrifuged to recover the cells, and cultured in Dulbecco’s Modified Eagle’s Medium/F12 (DMEM/F12; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Hangzhou Sijiqing Hangzhou, China) at 37°C in a humidified atmosphere of 5% CO₂. The cells were used at passage 3–10.

Cell Viability Assay
The cell viability was assessed by using an in vitro cell-counting assay kit (CCK-8; Rockville, MD, USA) as described previously [40]. Caprine fetal fibroblast cells were seeded on 96-well or 6-well plate and cultured for 24 h to allow adherence and stabilization. Then the GO-AgNPs suspension was dispersed in DMEM/F12 for different concentrations (1, 4, 8, 12 and 16 µg/mL) for 24 h at 37°C. After culture, 10 µL CCK-8 was added into each well and were incubated for 30 min at 37°C in the dark. The absorbance at 450 nm was measured using a microplate reader (BioTek Synergy 2, USA).

Cell Morphology
Caprine fetal fibroblast cells were seeded into a 24-well plate for 24 h, and then treated with the different concentrations of GO-AgNPs (0, 4 and 8 µg/mL) for 24 h. The cell morphology was observed using an Olympus BX-UCB microscope (Tokyo, Japan).

Annexin V-ficp/pi Staining Assay
Caprine fetal fibroblast cells were seeded in a 75 mm culture plate and treated with different concentrations of GO-AgNPs (0, 4 and 8 µg/mL) for 24 h. Cell apoptosis of caprine fetal fibroblast cells was detected by Annexin V-FITC and Propidium iodide (PI) staining assay according to manufacturer’s instructions (Bipec Biopharma Corporation, USA). The cells were harvested, centrifuged for 5 min, rinsed with phosphate buffered saline (PBS) twice and resuspended in 500 µL binding buffer containing 5 µL PI and 5 µL V-FITC, and then incubated for 15 min at the room temperature in dark. The cell suspension was determined by flow cytometry to analyze the apoptotic rate.

Measurement Of Ros Production
Dichlorodihydrofluorescein diacetate (DCFH-DA) was used to detect intracellular ROS induced by different concentrations (0, 4 and 8 µg/mL) of GO-AgNPs [40]. Caprine fetal fibroblast cells were incubated in 10 µM DCFH-DA for 30 min at 37°C. The cells were rinsed with PBS twice, and then the intracellular accumulation of ROS was measured by flow cytometry (Beckman-Coulter, USA).

**Measurement Of Total Sod Enzyme Activity**
The SOD activity in caprine fetal fibroblast cells was detected following the the SOD assay kit (Beijing Solarbio Science & Technology, beijin, China) [40]. Cells were treated with 0, 4 and 8 µg/mL of GO-AgNPs for 24 h. Cells were washed with PBS twice, and lysed with lysis buffer on the ice. The lysates were then centrifuged for 15 min. Then, the supernatant was analysed with a U-vis spectrophotometer (Nanodrop, Thermo, USA) at 550 nm.

**Measurement Of Mda Production**
MDA, a convenient index for detecting the extent of lipid peroxidation reactions, was performed using the MDA assay kit (Beijing Solarbio Science & Technology, beijin, China) according to the manufacturer's instructions [52]. Cells were plated into 6-well plates at a density of 1.0 × 10^5 cells per well and cultured for 24 h to allow adherence before exposure to different concentrations (0, 4 and 8 µg/mL) of GO-AgNPs for 24 h. Then the cells were washed with PBS twice and MDA activities were quantitated by reading optical densities using Synergy 2 multi-mode microplate reader (BioTek, USA) at 532 nm.

**Measurement Of Ldh Production**
Caprine fetal fibroblast cells were seeded in a 24-well culture plate, and treated with 0, 4 and 8 µg/mL of GO-AgNPs for 24 h. LDH level of cells in culture medium were quantified with LDH-cytotoxicity assay Kit (Beijing Solarbio Science & Technology, Beijin, China) [52]. The LDH activities were quantitated by reading optical densities at 490 nm using Synergy 2 multi-mode microplate reader (BioTek, USA).

**Measurement Of Caspase-3 Activity**
Measurement of caspase-3 activity was analyzed with a caspase-3 activity kit (Beijing Solarbio Science & Technology, beijin, China) according to manufacturer's instructions. Briefly, caprine fetal fibroblast cells were seeded in a 24-well culture plate, and treated with 0, 4 and 8 µg/mL GO-AgNPs
for 24 h. Then the cells were washed twice in PBS, lysed using lysis buffer, centrifuged at 16,000 x g at 4°C for 10 min, and the supernatant was incubated with 10 µL of caspase3 substrate for 7 h at 37°C. Substrate cleavage was measured at 405 nm using Synergy 2 multi-mode microplate reader (BioTek, USA).

Quantitative Reverse Transcription PCR (rt-qPCR) Analysis
Total RNA was extracted from caprine fetal fibroblast cells using a RNA Isolation Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. RNA samples were stored at -80°C until used. The mRNA samples were reverse-transcribed into first-strand cDNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Quantitative analysis of the cDNA samples was performed using a CFX98 instrument (Bio-Rad Laboratories) using SYBR Green (Vazyme). Primers were designed based on the mRNA sequences of selected genes available in GenBank (Table 1). The PCR cycle was as follows: initial denaturation at 95 °C for 30 s, followed by 41 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 30 s. RT-qPCR was performed independently four times. The target genes were quantified by the delta-delta Ct method using CFX manager V1.1 software (Bio-Rad Laboratories). Normalization was performed using β-actin as the reference gene.
Table 1

Primers used for quantitative reverse transcription PCR analysis

| Gene  | Primer sequence (5'--3') | Accession no. | Product size (bp) |
|-------|--------------------------|---------------|-------------------|
| caspase-3 | F: CCATGGTGGAAGAGGAATCTTT | AF068837.1 | 78 |
|         | R: TCCCCCTCTGAAGAAACTTGCTAA | | |
| Cyt-C  | F: CAGTGCCATACTTGGGAGAAA | DQ176429.1 | 81 |
|         | R: TGACCTGTCTTCGTCCAAAC | | |
| BAX    | F: GCATCCACCAAGAGCTGAGR: CCGCCACTCGGGAAAAAGAC | XM_002701934.1 | 130 |
| Smac   | F: TGTTCAGTGTGGGCTAACTT | NM_001045882.1 | 171 |
| R: AAGACACAGCCCTCCTCcTATT | | |
| BCL2   | F: ATGTGTGTGGAGACGCTCA | NM_001166486.1 | 182 |
|         | R: AGAGACAGCCAGGAGAATC | | |
| β-actin| F: TCACGGAGCGGTGGCTACAG | U39357.1.1 | 63 |
|         | R: CCTTGATGTCAGCGACGATTT | | |

Abbreviations: F, forward; R, reverse

Statistical analysis

All results were expressed as mean ± S.D. and analyzed by Origin 8.0 and SPSS 18.0 (IBM Corp., Armonk, NY, USA). The statistical significance of the changes between tested groups and control group were analyzed by one-way ANOVA followed Dunnett’s multiple comparison. The level of statistical significance was set at P < 0.05. All experiments were performed at least three times.

Abbreviations

GO: Graphene oxide; AgNPs: Silver nanoparticles; ROS: Reactive oxygen species; LDH: Lactate dehydrogenase; MDA: Malondialdehyde; SOD: Superoxide dismutase DCFH-DA: Dichlorodihydrofluorescein diacetate; PBS: Phosphate buffered saline; PI: Propidium iodide

Declarations

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Authors’ contributions

YGY designed the study, analyzed the data, drafted the manuscript and did experiment for nanoparticles and toxicity of cells. MDJ/rand JLW performed cell culture. AM and AMMTR performed
statistical analysis of the data and aided in data analysis. YW and CQ developed and performed qPCR analysis. CQ and IKK aided in reviewing and editing manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

The study was approved by the Animal Ethics Committee of Yangzhou University. Goats were handled in accordance with the Animal Ethics Procedures and Guidelines of the People’s Republic of China. All procedures collecting 70 d caprine fetus were approved by the Animal Ethics Committee of Yangzhou University (no.2018.1109).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare no conflict of interest.

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Figures
Figure 1

Size determination analysis of GO-AgNPs by Transmission electron microscope (TEM). TEM image showed that the size of GO-AgNPs was distributed about 20 nm.
Effects of GO-AgNPs on the proliferation of caparine fetal fibroblast cells. Caparine fetal fibroblast cells were exposed to 0, 1, 4, 8, 12 and 16 µg/mL of GO-AgNPs for 24 h. The percentage of cell viability was then calculated relative to the control group (0 µg/mL). Values are presented as mean±SD of four independent experiments. (* P < 0.05).

Cell morphology following treatment of GO-AgNPs. Caprine fetal fibroblast cells were treated with 0, 4 and 8 µg/mL of GO-AgNPs for 24 h and then visualized under a phase-contrast microscope (magnification, 100×).
Total ROS generation in GO-AgNPs-treated cells. Caprine fetal fibroblast cells were treated with 0, 4 and 8 µg/mL of GO-AgNPs for 24 h and analyzed by FACS (A). The percentage of ROS generation relative to the untreated control group (0 µg/mL) (B). Values are presented as mean±SD of four independent experiments. (*P < 0.05).
Figure 5

Evaluation of GO-AgNPs-induced apoptotic cell death with Annexin V-FITC/PI staining assay. Caprine fetal fibroblast cells were treated with 0 (A), 4 (B) and 8 µg/mL (C) of GO-AgNPs for 24 h and FACS was carried out for detection of fractions of early apoptotic, late apoptotic, and necrotic cell death of caprine fetal fibroblast cells. The corresponding linear diagram of flow cytometry was shown in (D). Values are presented as mean±SD of five independent experiments. (*P < 0.05).
Measurement of SOD production in GO-AgNPs-treated cells. Caprine fetal fibroblast cells were treated with 0, 4 and 8 µg/mL of GO-AgNPs for 24 h. The percentage of SOD relative to the control group (0 µg/mL) was determined. Values are presented as mean±SD of four independent experiments. (*P < 0.05** P < 0.01).
Figure 7

Measurement of MDA production in GO-AgNPs-treated cells. Caprine fetal fibroblast cells were treated with 0, 4 and 8 µg/mL of GO-AgNPs for 24 h. The percentage of MDA relative to the control group (0 µg/mL) was determined. Values are presented as mean±SD of four independent experiments. (*P < 0.05).
Measurement of LDH activity in GO-AgNPs-treated cells. Caprine fetal fibroblast cells were treated with 0, 4 and 8 µg/mL of GO-AgNPs for 24 h. The percentage of LDH activity relative to the control group (0 µg/mL) was determined. Values are presented as mean±SD of five independent experiments. (*P < 0.05).
Figure 9

Measurement of caspase-3 activity in GO-AgNPs-treated cells. Caprine fetal fibroblast cells were treated with 0, 4 and 8 µg/mL of GO-AgNPs for 24 h. The percentage of caspase-3 activity relative to the control group (0 µg/mL) was determined. Values are presented as mean±SD of four independent experiments. (*P < 0.05).
Effects of GO-AgNPs on apoptotic gene expression levels. Caprine fetal fibroblast cells were treated with 0, 4 and 8 µg/mL of GO-AgNPs for 24 h. Relative mRNA levels of genes related to the apoptosis was determined. Values are presented as mean±SD of four independent experiments. (*P < 0.05).