Original Article

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A Study of Mechanism and Rate of PC12 Cancer Cell Destruction Induced by Lysine-Coated Gold Nanoparticle

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ABSTRACT

BACKGROUND AND OBJECTIVE: Using gold nanoparticles for cancer treatment has attracted the attention of many researchers in recent years. Entry of these nanoparticles into cells is one the problems that influences the effectiveness of the treatment. The purpose of this study is to identify the destructive effects of amino acid lysine-coated gold nanoparticles on PC12 cancer cells.

METHODS: In this empirical study, gold nanoparticles were prepared using low temperature hydrothermal technique. After obtaining PC12 cells from Pasteur Institute of Iran and culturing them, we divided them into 7 treatment groups with concentrations of 0.5, 2.5 and 5 µM gold nanoparticles, with and without lysine coating; plus one control group. The cytotoxic effect of nanoparticles was assessed using MTT assay and LDH release from cells. In addition, cell morphology and amount of ROS (reactive oxygen species) within cells were examined.

FINDINGS: According to the results of this study, a 40% increase in cell death occurred as a result of exposure to 25 µM amino acid lysine-coated gold nanoparticles, which was significantly higher than the group treated by uncoated gold nanoparticles (18% cell death) ($p \leq 0.001$). In addition, incubating cells with coated and uncoated nanoparticles increased the amount of ROS and mechanism of cell death through apoptosis.

CONCLUSION: Results of the study revealed that lysine-coated gold nanoparticles have promising effects on destruction of PC12 cancer cells and they direct cell death towards apoptosis.

KEY WORDS: *Cancer, Nanoparticle, Lysine, Apoptosis*.

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Introduction

Cancer is the second leading cause of death in developed countries, right behind cardiovascular diseases (1, 2). Various factors are involved in invasion of cancer cells, all of which induce cellular genome damage. Therefore, DNA (the building block of genome) damage is the major cause of cancer. As a result, damaged genes can create many differences between cancer cells and normal cells (3-7). One can make use of these differences to identify cancer cells and destroy them. PC12 cell is a type of cancer cell separated from a rat pheochromocytoma; it grows in culture medium and demonstrates the features of neurons (8, 9).

Surgery, chemotherapy and radiotherapy are the main techniques used to treat cancer. In radiotherapy, ionizing radiation is used to destroy cancer cells, which damages healthy cells inside radiation field (10, 11). Hence, increasing radiation dose is practically impossible in radiotherapy (12). However, to enhance the effectiveness of treatment, certain compounds can be used to increase the radiation resistance of healthy tissue or radiation sensitivity of tumoral tissue (13). Mechanism of action associated with sensitizer compounds is accompanied by generation of ROS and free radical in many cases.

Regarding sensitizers, several studies have been carried out on different physical and chemical compounds (14,15), among which nano compounds, particularly gold nanoparticles (16-18), have a special status. Nanomaterials can damage DNA and membrane and kill cancer cells by causing oxidative stress and lipid peroxidation, which may ultimately provide us with effective treatment methods (6, 14, 15). Studies have shown that gold nanoparticles coated with anticancer antibodies can effectively bind to cancer cells. Most cancer cells contain a protein called “epidermal growth factor receptor” (EFGR) on their surface, which is usually absent in healthy cells. Researchers have managed to bind gold nanoparticles to cancer cells by attaching them to EGFR antibody (anti-EGFR) and, as a result, destroy cancer cells using gold nanoparticles (19, 20).

Nevertheless, the attachment and entry of these nanoparticles into cancer cells are the major problems that need to be dealt with to improve the effectiveness of nanoparticles. Lysine is an amino acid, normally found on the exterior surface of the cell membrane and is small in size. When nanoparticles are coated with amino acid lysine, it is likely that the level of attachment and entry of nanoparticles into cancer cells will improve and the effectiveness of nanoparticles will increase. The present study was carried out to identify the mechanism and rate of PC12 cancer cell death as a result of exposure to lysine-coated and uncoated gold nanoparticles.

Methods

In this empirical study, the effect of lysine-coated gold nanoparticles on type and death rate of PC12 cancer cells was investigated. This study includes five steps:

Cell culture: In this study, PC12 cells obtained from Pasteur Institute of Iran were used for in vitro culture. Cells were cultured in RPMI 1640 (Gibco**)** medium along with 0.2% (w/v) bovine serum albumin (BSA), 1% (v/v) L-glutamine and 1% (v/v) nonessential amino acids (NEAA) inside T-25 (NUNC) cell culture flasks, in an incubator containing 5% CO2 and adequate humidity at 37°C. After 48 hours, old culture medium was replaced with new culture medium. When cells occupied 70 to 80 percent of the flask, they were passaged using trypsin–EDTA solution (200 unit/ml) (21, 22).

Producing uncoated gold nanoparticles: To produce uncoated gold nanoparticles, HAuCl4 (254169) was dissolved in 0.01 M water and its ionic strength and pH were set at 0.005 M and 7.8, respectively by phosphate buffer system. A non-aqueous phase (toluene, C6H5CH3) containing 0.02 M sodium tetrahydridoborate was prepared separately. Then, both phases were mixed and shaken severely. After isolating the organic phase under low pressure at 50°C, solvent was removed using a rotary device. Finally, gold nanoparticles left at the bottom of the container were dispersed in phosphate buffer solution with 0.005 M ionic strength and pH 7.8, producing a homogenized solution (23).

Producing lysine-coated gold nanoparticles: To produce lysine-coated gold nanoparticles, 300 µl amino acid solutions with a concentration of 25 mM were added to a beaker containing 15 mL deionized water. Then, the beaker was placed on the heater so that the solution inside would reach boiling point. At this moment, 1 mL solution and 1 mM gold salt (HAuCl4) were added to the boiling solution. When the reaction was finished and the color changed, the container was immediately placed in an ice bath so that the colloidal solution would cool down completely.

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Finally, the colloidal solution was filtered using a 0.2 µm filter (23, 24). In order to prepare samples, 5 µl gold nanoparticle was placed on a carbon-coated copper grid and was incubated for 1 minute. After drying the grid, the sample was rinsed twice with double distilled water (ddH2O) and was stained with 2% uranyl acetate for 1 minute (25). The sample was analyzed using Hu-12A electron microscope (Hitachi, Japan).

Studying the effects of different gold nanoparticle samples on cancer cells: In order to study the possible effect of amino acid coating on toxicity of gold nanoparticle, PC12 cancer cells were exposed to various concentrations of gold nanoparticles (0.5, 2.5 and 5 µM) for 48h. Cell death rate was assessed according to level of LDH (lactate dehydrogenase) released from cells. Activity rate of LDH enzyme in supernatant and lysed cellular sediment (in control group) were measured by spectrophotometry (LDH Cytotoxicity Assay Kit). Percentage of total LDH released into the culture medium was calculated using the following equation:

$$\text{Percentage of total LDH released} = \frac{\text{LDH in culture medium}}{\text{LDH in culture medium} + \text{LDH in lysed cellular solution}} \times 100$$

PerSimultaneously, MTT assay was done to determine cell viability. In addition, to examine cell morphology, control and treatment cells with different gold nanoparticle samples were studied by phase contrast microscopy. In order to study the form of cell death induced by lysine-coated and uncoated nanoparticles, Acridine Orange/Ethidium Bromide (AO/EB) staining was used.

This kind of staining specifies type and rate of cell death. Moreover, DNA in cells that undergo apoptotic death is dense and sliced, whereas necrotic cells reveal dense and unsliced chromatin (26, 27). Finally, the amount of ROS within cell was measured using fluorescence probes 2', 7'-dichlorofluorescin diacetate (DCFH-DA) (28).

Statistical data analysis: The collected data were analyzed using SPSS 16 software, one-way analysis of variance (ANOVA) and Tukey's test and $p<0.05$ was considered significant.

Results

Electron microscope was used to study the structure of lysine-coated gold nanoparticle. The images show the presence of amino acid coating around nanoparticle (Fig 1). The mean diameter of lysine-coated and uncoated gold nanoparticles was measured at 10±0.2 and 29±0.3, respectively. Incubating PC12 cancer cells with lysine-coated gold nanoparticles demonstrated that this nanoparticle had negligible effects on cell viability in low concentrations (0.5 to 5 µM).

Still, it had considerable effects on metabolic activity in high concentrations in a dose-dependent manner, so that cell viability in highest concentration (25 µM) decreased by 62% compared with control cells. However, when exposed to uncoated gold nanoparticles, cell viability decreased by 22% compared with control group (Fig 2&3).

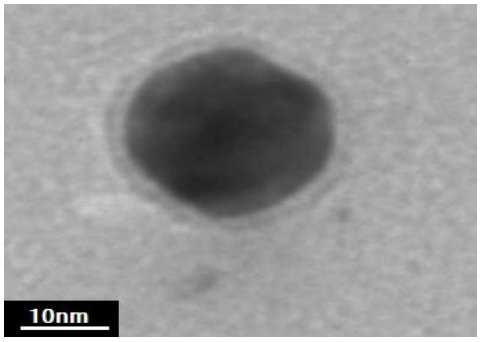


Figure 1. Electron microscope image of lysine-coated nanoparticle

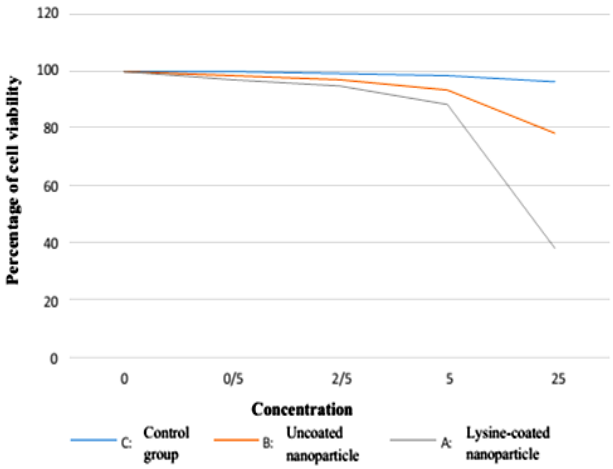


Figure 2. Percentage of cell viability (based on MTT assay) after incubating PC12 cancer cells with various concentrations of lysine-coated gold nanoparticles (A), uncoated gold nanoparticles (B) and control group (C) for 48 hours. There was no significant difference between cell viability in groups A, B and C with concentrations of 0.5, 2.5 and 5 µM ($p>0.05$). However, the difference was significant at concentration of 25 µM ($P_{AC}>0.001$), ($P_{BC}=0.01$) and ($P_{AB}>0.001$).

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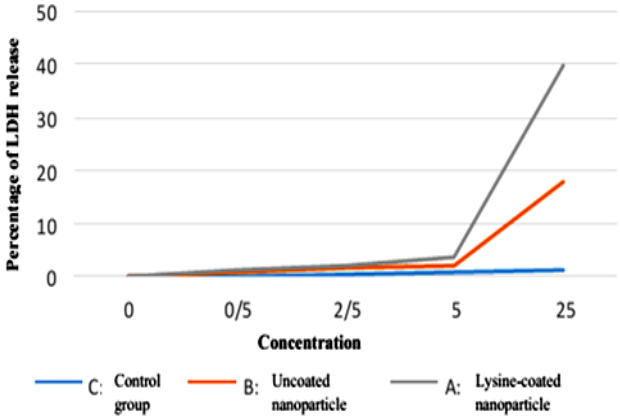


Figure 3. Percentage of LDH (lactate dehydrogenase) release into culture medium after incubating PC12 cancer cells with various concentrations of lysine-coated gold nanoparticles (A), uncoated gold nanoparticles (B) and control group (C) for 48 hours. There was no significant difference between LDH release and cell wall destruction in groups A, B and C with concentrations of 0.5, 2.5 and 5 µM (p>0.05). However, the difference was significant at concentration of 25 µM (P_{AC}>0.001), (P_{BC}=0.003) and (P_{AB}=0.01).

Level of cell viability in lysine-coated gold nanoparticle group was significantly lower than uncoated gold nanoparticle group (p>0.001). Measuring the level of membrane structural integrity also showed that in highest concentration, this nanoparticle increased the level of LDH released into the culture medium by around 38% compared with control cells. However, cell viability in uncoated gold nanoparticle group decreased by 20% compared with control group.

Level of cell viability in lysine-coated gold nanoparticle group was significantly lower than uncoated gold nanoparticle group (p=0.01). Results showed that normal PC12 cancer cells usually have fusiform morphology. During incubation with lysine-coated and uncoated gold nanoparticles, the morphological shape of cells changes from fusiform to spherical (Fig 4). In addition, the results demonstrated that much of cell death due to incubation with 25 µM lysine-coated and uncoated gold nanoparticles in Acridine Orange/Ethidium Bromide (AO/EB) staining is apoptotic (Fig 5). Results also revealed that proximity of PC12 cancer cells to 25 µM lysine-coated gold nanoparticles for 48 hours increased ROS production rate within cells, compared with control cells (p>0.001). Proximity to 25 µM lysine-coated gold nanoparticles indicated a 60% increase in cell death. However, this change in the group treated with uncoated gold nanoparticles was only 20% and ROS production rate did not change noticeably (p=0.031). Difference in cell death rate between the two groups was statistically significant (p<0.001).

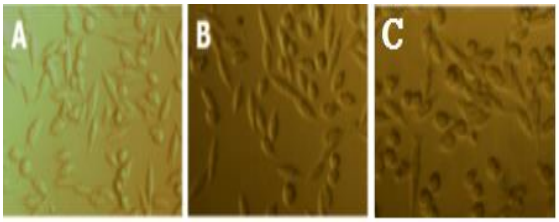


Figure 4. Phase contrast microscope images of PC12 cancer cells treated with various types of gold nanoparticles at concentration of 25 µM. A: Control sample, B: incubated with uncoated nanoparticle and C: Lysine-coated nanoparticle.

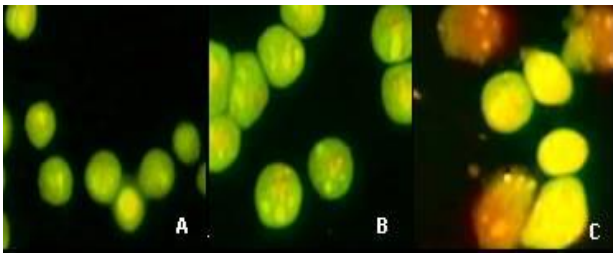


Figure 5. Apoptosis/necrosis induction by 25 µM gold nanoparticles. A: Acridine Orange/Ethidium Bromide (AO/EB) staining in untreated cells, B: treated with uncoated nanoparticles and C: treated with lysine-coated gold nanoparticles

Discussion

This study demonstrated that coating gold nanoparticles with amino acid lysine increases cell death rate among PC12 cancer cells, compared with uncoated gold nanoparticles. For years, researchers have used PC12 cells to study cancer cells and biological issues (22). Results of MTT assay and level of LDH released into the culture medium indicated more destructive effects of lysine-coated gold nanoparticles compared with uncoated gold nanoparticles. In this study, difference in shape, size and surface charge of gold nanoparticles increased toxic effects of nanoparticles on cancer cells (29). Study of Suh et al. regarding membrane integrity has shown that presence of amino acid coating can disrupt cancer cell membrane integrity by increasing the interaction between nanoparticles and cellular levels or creating pores in cell membranes (29).

This result was in accord with the results of the present study regarding PC12 cancer cells. According to the results of this study, cell death rate due to proximity to lysine-coated gold nanoparticles was

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higher than proximity to uncoated gold nanoparticles and control group. In addition, cell death due to proximity of PC12 cancer cells to lysine-coated and uncoated gold nanoparticles was apoptotic. Moreover, results showed that ROS production rate in cells that were in proximity to lysine-coated gold nanoparticles was significantly higher than cells in proximity to uncoated gold nanoparticles.

This indicates apoptotic death in these cells induced by gold nanoparticles through increasing ROS production rate. Shubayev et al. have indicated that nanoparticles increase ROS production rate and damage DNA molecules and therefore induce cell death through apoptosis (15).

Study of Wassan et al. on sensitivity of pancreatic cancer cells in proximity to nanoparticles has demonstrated that ROS production rate in this type of cancer cells increases in proximity to nanoparticles and leads to selective death in these cells (14). However, study of Connor et al. has indicated nontoxicity of nanoparticles after 3 days of proximity to cells. According to this study, some precursors of nanoparticles may be toxic, but nanoparticles do not damage cells on their own (27). Our complementary tests on other amino acids revealed that mechanism and rate of cell death changes according to the type of coating nanoparticles have. Based on our results, lysine is the most effective coating for inducing apoptosis in PC12 cancer cells, suggesting a strong interaction between this type of nanoparticle and death receptors on the surface of cancer cells. These results are in accord with the results obtained by Selvakannan et al. (30). Overall, proximity of PC12 cancer cells to lysine-coated gold nanoparticles and their proximity to uncoated gold nanoparticles revealed that lysine coating increases cell death rate. Furthermore, this type of cell death is accompanied by increase in ROS production rate and apoptotic process.

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