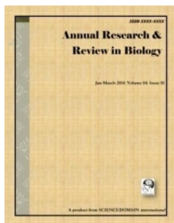

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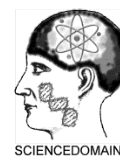

Effect of Cold Atmospheric Pressure Plasma and Gold Nanoparticles on Cell Viability

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SMA and SI designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors SI, ZS and SM managed the analyses of the study. Authors MDJ, MG and SS managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: Cell culture can be used to study of cell proliferation, viability and apoptosis. Purposeful treatment by cold atmospheric plasma is a new approach in cell culture system. Plasma is an ionized gas that has reactive and energetic particles and can be produced by different methods in the laboratory. Nanoparticles have many applications in biology and medicine. In this study we investigated about plasma jet and gold nanoparticle synergy on L929 fibroblast cells viability.
Study Design: The plasma jet consists of a pyrex nozzle tube as an insulating shield (ID=2 mm and OD=4mm). The power supply produces 10 KV high voltage pulses with a

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frequency of 6 kHz and 30 microsecond pulse width.
Place and Duration of Study: Biotechnology engineering Center**,** Department of biology, Science and Research Branch, Islamic Azad University between June 2012 and May 2013.
Methodology: In this study, the plasma jet was employed. Gold nanoparticles produced through Strohoff methods; size and charges were measured by zetasizer. Cell cytotoxicity and cell viability was shown by MTT assay.
Results: The results showed that the cell proliferation has been increased by using of cold atmospheric plasma and gold nanoparticles. Gold nanoparticles and cold plasma alone did not have effect on L929 cells proliferation.
Conclusion: It seems that the cell viability has been enhanced by synergistic combination of nanotechnology and plasma technology in cell culture systems.

Keywords: Nano particles; non-thermal plasma; ICP discharge.

ABBREVIATIONS

GNP: Gold nanoparticles; CAP: Cold atmospheric plasma; RNS: reactive nitrogen species; ROS: reactive oxygen species.

1. INTRODUCTION

Cell culture can be used to analyze cell cycle, cells viability, proliferation and apoptosis [1]. Mammalian cells culture can be used in tissue engineering to cell therapies, gene therapies and cytotoxicity tests. Fate of cultured cells depends not only on medium and chemical but also on some external factors [2]. Cell behavior could have influenced by chemical and physical factors. Physical factors include electromagnetic, light, temperature and plasma [2]. It is possible that a new technology would have made a change in the permeability of cells and the activation of the intracellular secondary messenger signaling. Plasma technology is one of the new technologies that appear to be a promising approach for cell behavior. The first study about plasma was done by Langmuir and Colabrite in 1920 when they were working on vacuum tubes. Langmuir had been used “plasma” for ionized gas [3]. Plasma is a quasi-neutral gas that does not behavior look like the solid, liquid or gas. Therefore plasma is the fourth state of matter and it makes up 99% of the material world [4].

Plasma is an ionized gas that has chemically reactive species such as positive and negative ions, electrons and free radicals, atoms and molecules of the gas [4]. Plasma can form in different temperatures and pressures that feature of created plasma are varied. Discharged gas is changed when high energy is received by it; because the electrical discharge is the most common method of plasma producing. In this method the electron temperature is higher than ion temperature. The ion temperature is close to room temperature; consequently the plasma temperature remains at room temperature [5]. This plasma is called non-thermal plasma (cold) and it is produced in the low temperatures. It can be very important for biology and medicine application; it is used for sterilization animate and inanimate surface [6], genes transfection, cell detachment [7,8], growth factor release, induced cell proliferation, wound healing [9], blood coagulation [10] and induction of apoptosis in cancer cells. The cold plasma has a selective effect on cell proliferation at low dose, but increases apoptosis at high dose [11,12].

Cold atmospheric plasma can act locally so that it can become a very powerful treatment for some of disease. The cold atmospheric plasma does not have heat so it does not lead to tissue damage. Reactive oxygen and nitrogen species is generated by cold plasma and it leaves a therapeutic effect on the body and the tissues. The effect of cold atmospheric plasma could have increased by the gold nanoparticles.

Nanotechnology has capability in penetrating into tissues. Nano particles are characterized from 1 to 100 nanometers that they have many applications due to their unique characteristics.

Gold nanoparticles are very important among these nanoparticles because they are very different from gold bulk in synthesis and property [13-15]. They have many applications in biology and medicine [16,17]; for example diagnostic [18], therapeutic, drug delivery and targeting [19]. Also the effect of these substances should be examined on living organisms. Biocompatibility of gold nanoparticles is very important for medical purposes. Although the nanoparticle materials may not be biocompatible, but the nanoparticles do not have a non-desirable effect on cell function. However, the toxic effect of nanoparticles has been reported in some cases; the impact of nanoparticles on the human and the environment is not clear yet. The size of the gold nanoparticles is very small, below 20nm. For this reason GNPs enter to cytoplasm and nucleus and react with DNA. The effect of GNPs is very dependent on size, shape, concentration and charge. Gold nanoparticles can increase the effect of cold atmospheric plasma too.

In this study we analyzed the plasma-nanoparticle synergies on L929 (mouse fibroblast) cell proliferation.

2. MATERIALS AND METHODS

2.1 Plasma Source Specification

The plasma jet consists of a pyrex nozzle tube as an insulating shield (ID=2 mm and OD=4mm). The working gas is fed from the tip of the tube. The inlet gas for this study is helium (99.99%) with 5% oxygen as additional gas to main feeding gas. The helium and oxygen injected in to the tube with a flow rate of 2 Lit/min. A copper electrode in the width of 6 mm wrapped around the tube and connected to the power supply. The applied voltage to the electrode ionized the inlet gas fed into the tube and the generated plasma emitted out from end of the tube and propagates about a few centimeters into the ambient air. The power supply produces 10 KV high voltage pulses with a frequency of 6 kHz and 30 microsecond pulse width Figs. 1 and 2.

2.2 Gold Nanoparticle Production

Materials $HAuCl4.3H_2O$ and trisodium citrate used in this study are analytical grad and without further purification. All glassware was cleaned in aqua regia (4part HCl, 0.5 part HNO_3) rinsed with sonication followed by nano pure H_2O then oven dried prior to use.

GNPs preparation gold nanoparticles (GNPs) were prepared by the citrate reduction of $HAuCl4.3H_2O$ following the methods of Strohoff et al. [20]. 50 ml of a 38.3 mM trisodium citrate solution was added quickly at one time to magnetically stirred boiling aqueous solution of HAuCl4 (1mM, 500μl), resulting in a change in color solution from pale yellow to

deep red. After the color change, the solution was allowed to cool and subsequently filtered through a micron separation Inc.0.45 µm nylon filter. Analytical solution of 55 nm diameter GNPs exhibited a characteristic surface plasmon band (Kontron uvkon 922) centered at 520 nm. The dynamic size and zeta potential were measured using a Malvern (Worcestershire, UK).

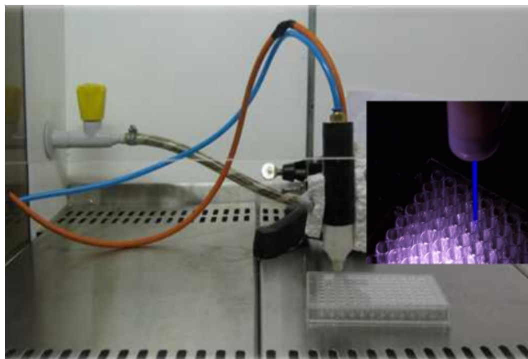


Fig. 1. Experimental of plasma jet

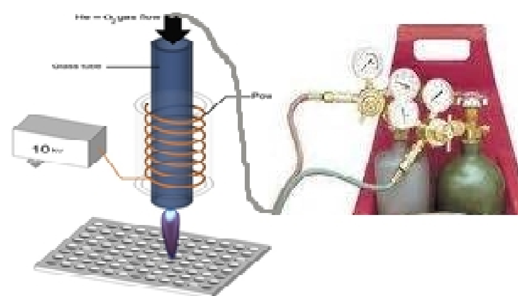


Fig. 2. Schematic of the experimental set up

2.3 Cell Culture

L929 mouse fibroblast cell line was obtained from the Cell Bank department of Pasteur institute of Iran. Cell culture was started from a frozen stock. After thawing rapidly in a 37°C water bath, the cells were cultured in a standard medium consisting of RPMI-1640 with 10% (vol/vol) fetal bovine serum (FBS) (RPMI-1640-10% FBS) and 1% (vol/vol) antibiotic-antimycotic in a 50-cm^3 flask and incubated for 48 h. The medium was then replaced with fresh RPMI 1640-10% FBS. The cells were maintained at sub- by culturing them after arriving at an acceptable confluence. In order to ensure the accuracy of the test conditions we did lived/dead assay before each experiment, using trypan blue staining and Neubauer lam to determine the cell counts.

2.4 Cell Viability Analysis

MTT assay was employed to determine the viable L929 cell numbers, based on the mitochondrial conversion of the tetrazolium salt, [3-4,5] (dimethylthiazol-2-yl) [2,5] diphenyltetrazolium bromide (MTT). The fibroblast cells were trypsinized, resuspended in RPMI-10% FBS, counted, and carefully seeded at a concentration 5×10^3 cells per 96 well and incubated for 24 h.Then the cells were treated by helium-oxygen plasma and GNPs. Exposure time of plasma treatment was 120 seconds and also GNPs with 375 ppm concentration micro plates were incubated for 48h then 100ml of MTT solution was added to each the wells were mixed well and incubated for 4h in 37 degree centigrade in the dark. Then 100 ml of DMSO was added to each well and was mixed and OD was measured by ELISA Reader in 570 nm (back ground wave length was 630nm).

2.5 Statistical Analysis

Data were evaluated by a Dunnet one way analysis of variance (ANOVA) using software SPSS version 16.0. The a priori alpha value was set at 0.05 with the level of significance for all statistical analyses $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 The Result of Size and the Zeta Potential Determination

DLS measurements of GNPs show the nanoparticles have an average hydrodynamic diameter of 56 nm that is close to value reported by Strohoff. Malvern (Worcestershire, UK) was used to measure the zeta potential of the gold nanoparticles. From zeta-potential measurements, it has been observed that gold nanoparticles have negative surface charge (-3.20 mV) and can be used for biomedical applications Diagrams 1 and 2.

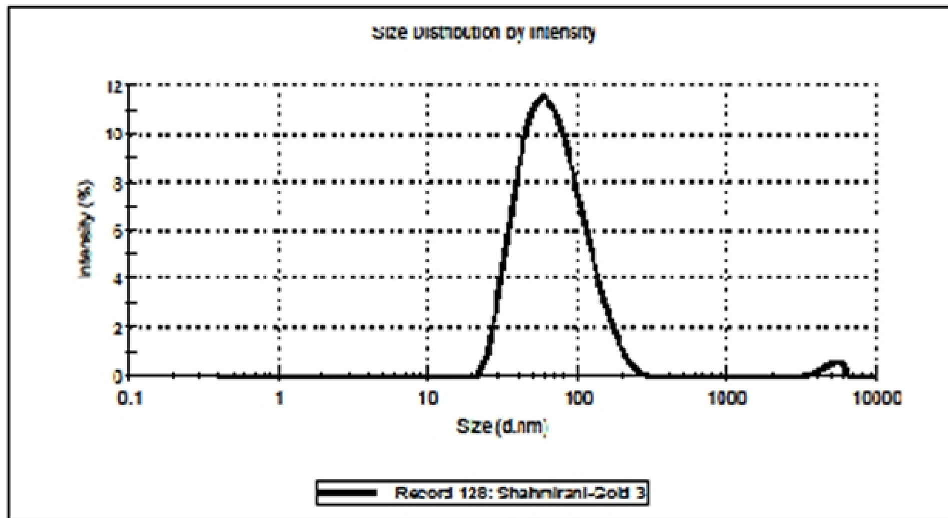


Diagram 1. The result of size determination of gold nanoparticles by zeta sizer system (Malnern model). The mean size of GNPs was 56nm

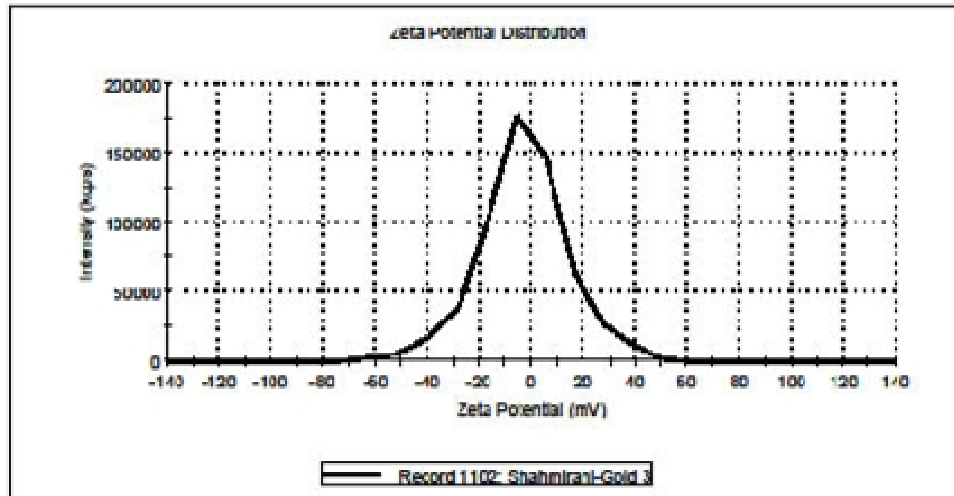


Diagram 2. The result of charge determination of gold nanoparticles by zeta sizer system (Malnern model). GNPs have negative surface charge (-3.20 mV)

3.2 Cell Culture

A gas only treatment was used to analyze the gas effects of cold atmospheric plasma on cell proliferation. The results of optical microscopy showed that the cells were treated by cold plasma at 10 KV for 120 seconds; plasma had not any significant effect on L929 cells Fig. 1. After that the cells treated by gold nanoparticles only, similar results were observed. Indeed the results of optical microscopy showed that nanoparticle-only treatment to L929 cells with 375ppm concentration GNPs showed no significant effect on cell proliferation Fig. 3.

3.3 Cell Viability

L929 cells that were treated with gold nanoparticles and cold plasma, incubated for 48h. The supernatant was removed and MTT solution was added and incubated for 4h, and dark-colored crystals were formed, by dissolving these crystals in DMSO solution, light absorption was measured by ELISA reader (bio tech). The result of MTT assay shows no effect on the L929 cells growth or death rate during 120 seconds of plasma radiation exposure Thus, plasma does not have any toxic effect on the L929 cells. The result of MTT assay also shows that cell growth or death rate are not affected by 375 ppm concentration gold nanoparticles, wich represents gold nanoparticles non-toxicity to the L929 cells. But, the simultaneous presence of cold plasma and gold nanoparticles results in growth of the cells Diagram 3.

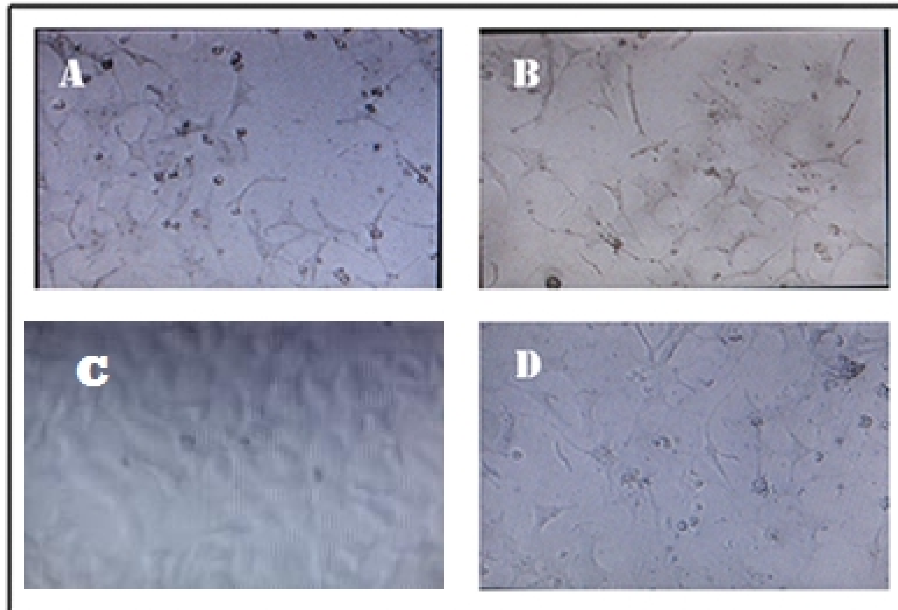
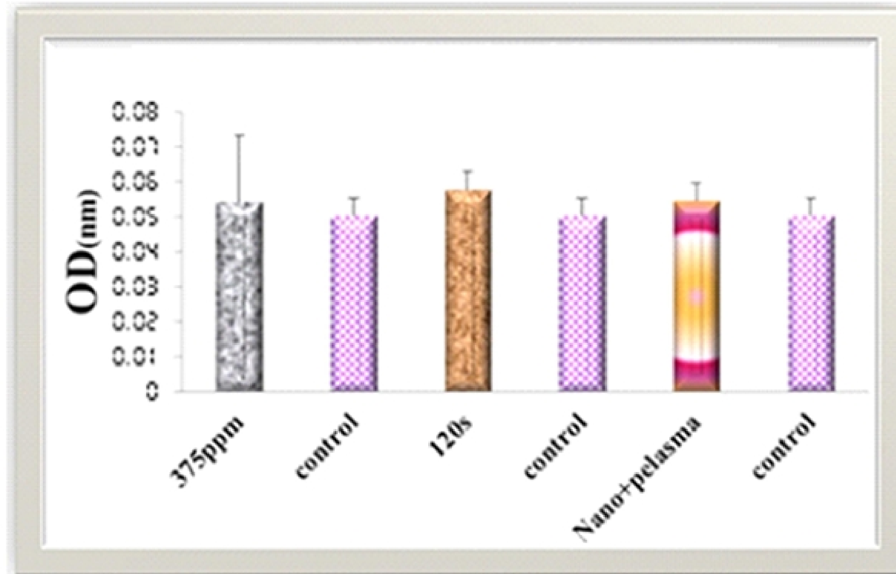


Fig. 3. Images obtained from optical microscopy 48 h after GNPs treatment and plasma treatment on L929 cells. Plasma exposure time: 120 s gold nanoparticles concentration: 375 ppm A) Treated L929cells by gold nanoparticles with 375 ppm concentration. B) Treated L929 cells by cold atmospheric plasma with 120s exposure time. C) Treated L929 cells by gold nanoparticles with 375 ppm concentration and cold atmospheric plasma with 120s exposure time. D) Untreated L929 cells. Cold plasma and GNP treatment together induce cell proliferation.



Diagra 3. MTT test results after 48 h: cells were treated by cold plasma and gold nanoparticles Gold nanoparticles concentration: 375 ppm. Cold plasma exposure times: 120 seconds

P-value<0.05

4. DISCUSSION

Reactive species are produced by hit the surrounding air and liquid inside of cells via the charged particles of plasma. Reactive species are divided into radical species and unradical species. Radical species are included hydroxyl radicals, anion super oxides, peroxides, alkoxyle and nitric oxide. The unradical species are included hydrogen oxide, ozone, singlet oxygen, peroxy nitrit.

The cold plasma can lead to cell proliferation and apoptosis. Sometimes cold plasma has no cytotoxic effect on living cells and tissues. The plasma effect mechanism on living cells and tissues can be caused by heat, UV light and reactive species. Analysis of infrared thermometers and in-vitro studies are showed that these two factors have not had important effect on living cells. Thus, the reactive species have had the greatest effect on living tissue in the plasma. These effects would be dependent on the dose absolutely, that can have beneficial or destructive effect. Plasma can alter the cell membrane permeability transiently, thus ROS and RNS are entered in to the cells. Membrane lipid peroxidation, as a result of high level of ROS, lead to destroy the cell membrane; it is followed by DNA damage that cause to be the cell goes to the apoptosis. However, ROS not only induce cell death but also activate a signaling pathway for the cell proliferation and cell growth. ROS plays an important physiological role in several aspects of the regulation of intracellular signaling; for example, the cells are able to generate ROS for induction and maintenance of signaling pathways are involved in cell growth. Those that produce low amounts of ROS, play an important physiological role as a secondary messenger. It should be noted that low levels of ROS are used in the defense against infection agents, the performance of cell-cell signaling system, increasing in a number of pathological conditions ROS levels and oxidation stress.

Also ROS plays an important role in vascular angiogenesis and angiogenic responses in vascular tissue through ROS messages triggering [21,22] but ROS that produced by cold plasma was generated outside the cells then transported into the cells.

ROS and RNS have a short half-life and they have a very low penetration depth in the water. Their penetration depth is about 10 microns; penetration depth is dependent on physiological conditions, for example hydroxyl radicals do not spread over several angstrom in liquid mediums [23,24]. A half–life of singlet oxygen is about a few microseconds in liquid medium while a half- life of hydrogen peroxide is longer. It can spread throughout the cell [23,24] although it is less reactive. Thus ROS\RNS penetration was limited in to the skin and living tissues and this fact causes the plasma to act locally. Thus, the plasma position and plasma treatment are not made harmful to healthy tissue by this property [25,26] so plasma is used for wound healing and skin infection removal. PH regulation can increase the half-life of some of ROS [27,28] for example anion superoxide that produces for 10000 s in an aqueous environment.

Treatment via plasma will be very difficult if skin disease is far from the surface, because in order to have effective plasma, its dose should be raised. However the toxicity levels in the dose should be considered. Therefore additional factors are required for the plasma to increase the life time of ROS [29].

It has been proved that the regulated pH can increase the half- life of ROS. There are channels in the skin that allow small molecules and ROS to release and spread. Lipidic pathways are 5-36 nm in diameter and the trance –follicular route is about 10-210 micrometers [30]. We assume that aqueous channels are about 1mm. Diffusion coefficient of superoxide anion in the liquid is $D=8\times10^{-5}$ m^2s^{-1} [31].

Diffusion length of superoxide anions is about $2(D_t)^{0.5}$=17.9 mm for 10000 s half -life. In fact if the half –life of superoxide anions is about 32 s, they can pass from channels with 1mm in diameter. So it is possible that reactive species pass from aqueous channel and reach the site of the disease that is deeper. In addition the diameter of the lipid pathway is increased by plasma temporarily. It provides a better and more effective path for ROS and RNS to pass through the aqueous channel in living tissues. Another way for ROS production with long –life is to excite. This can cause an imbalance between intracellular ROS and antioxidants [32,33]. Electrons move about 10 km/s that cause ROS and RNS to produce in tissues because this energy is sufficient for these electrons to react with water or chemical bonds in material structures. Thus these electrons produce an imbalance between intracellular ROS /RNS and the antioxidants [34]. The effect of plasma is increased by gold nanoparticles. Materials in nanometer measurement increase the surface to volume so that the particles tend to sedimentation so avoiding aggregation in the colloidal system and prevent electrostatic interaction with the cytoplasmic membrane negatively charged gold nanoparticles were selected. The shape of nanoparticles were spherical that had the lowest level of energy and were the most effective for monolayer cell culture. Using the rod nanoparticles is the most effective for multiple layer cell culture because of their ability to reflect and scatter light. The size of gold nanoparticles was 55.5 nm so that they could pass from membrane pores and accumulate in the cytoplasm around the nucleus, because nanoparticles in this diameter cannot pass from nucleus pore. Clusters nucleus surrounding is formed due to their size. According to Stocks –Einstein relation, referring to spherical particles, their smaller size enters the cells by a low friction factor which makes a greater diffusion coefficient. According to calculations, the best size for spherical gold nanoparticles

is about 30-50 nm. Gold nanoparticles can produce ROS and increase the half- life of intracellular ROs.

The nanoparticles uptake mechanism into cells can be passive or adhesive interaction. Two mentioned mechanisms can be initiated by electrostatic charge , van der vals forces, steric interaction and interfacial tension effects therefore there is not any vesicle formation [35,36]. in non-phagocytic uptake, nanoparticles could be found in various place inside cells which depends on nonoparticles size. In non-phagocytic uptake nanoparticles could not be entered into nucleus and remain in cytoplasm around the nucleus. Nanoparticles produce ROS. Intracellular calcium concentration and reactive transcription factor are modulated, and production of cytokine is promoted by ROS. ROS also can change signaling function [37]. In addition ROS can be produced by nanoparticles surface when oxidants and free radicals exist on it.

5. CONCLUSION

In this study the effect of cold atmospheric plasma and gold nanoparticles on L929 cells was investigated. It has shown that when L929 cells treated by gold nanoparticles only with a concentration of 375 ppm and size of 55 nm and plasma irradiated only for 120 seconds, the number of L929 cells do not change significantly. But if cold plasma and gold nanoparticles work together, the number of L929 cells increased.

It seems plasma and gold nanoparticles increase the production of reactive oxygen species and maybe plasma temporarily increases the permeability of cell membrane. Therefore they increase the intracellular ROS and lead to cell viability. Gold nanoparticles and cold atmospheric plasma had no cytotoxicity effects on L929 cells.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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