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OPEN PEER REVIEW

Apoptotic Impact of Heliox Cold Plasma on a Cervical Cell Line Using Gold Nanoparticle-Doped Graphene Oxide Nanosheets

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1 Original submission

2 comments · 18 Jul 2024 – 2 Aug 2024

Referee 1 Reviewer

MINOR REVISION 18 Jul 2024

The manuscript entitled “Apoptosis impact of heliox cold plasma on a cervical cell line by gold nanoparticle doped on graphene oxide nanosheets” is well-written and presents a carefully designed and well-reasoned analysis. The literature review was thorough, the methods appear to be appropriate for the research question and objectives, and the paper presents a range of data to support its claims. However, addressing several unanswered questions and expanding on certain areas could strengthen the research:

1. Comparison with Other Treatments: The study does not seem to include a comparison with other treatment modalities for cervical cancer, which could help to contextualize the efficacy of the proposed approach.
2. Long-Term Effects and Toxicity: The paper may not address the long-term effects or potential toxicity of the treatment, which is important for clinical translation.
3. In Vivo Studies: The research appears to be limited to in vitro studies. In vivo studies would be necessary to further support the claims and assess the potential of the treatment in a more physiologically relevant setting.
4. Statistical Significance: The paper should include statistical analysis to confirm the significance of the results, which is not clear from the provided excerpts.

Associate Editor

MAJOR REVISION 2 Aug 2024

Review for manuscript ID: 150385
Reviewer 1:
Dear Editor
- In the Abstract: the method section should be revised. Increase the number of keywords.
- The Background section is very short and not acceptable, and the number of references is very small.
- All parts of the Methods are written very briefly, and explanations should be added for each test. A valid reference must be added to each section in the Methods.
- Authors should describe the plasma treatment scheme in more detail.
- How could plasma be applied directly to the cells?
- What is the basis for choosing the times (30 and 60 s) and concentrations used?
- In the figure legends: the significance (p-value) should be mentioned.
- Why was the apoptotic phenotype not investigated?
- The plasma dose/concentration was not stated, and only the time was mentioned.
- The article needs to be revised in all sections.
- In Figure 6B-D, which groups were compared for statistical significance? All 4 groups have significance markers in all 3 graphs.
- How many times was each experiment repeated?
- Figure 8 is not understandable to the readers. Each group has several significance markers, which complicates the understanding of the figure.
- A statistical analysis section should be added.
Reviewer 2:
The manuscript entitled “Apoptosis impact of heliox cold plasma on a cervical cell line by gold nanoparticle doped on graphene oxide nanosheets” is well-written and presents a carefully designed and well-reasoned

analysis. The literature review was thorough, the methods appear to be appropriate for the research question and objectives, and the paper presents a range of data to support its claims. However, several questions remain unanswered, and some areas could be expanded upon to strengthen the research:

1. Comparison with Other Treatments: The study does not seem to include a comparison with other treatment modalities for cervical cancer, which could help to contextualize the efficacy of the proposed approach.
2. Long-Term Effects and Toxicity: The paper may not address the long-term effects or potential toxicity of the treatment, which is important for clinical translation.
3. In Vivo Studies: The research appears to be limited to in vitro studies. In vivo studies would be necessary to further support the claims and assess the potential of the treatment in a more physiologically relevant setting.
4. Statistical Significance: The paper should include statistical analysis to confirm the significance of the results, which is not clear from the provided excerpts.

2

Revision 1

AUTHORS' RESPONSE TO REVIEWERS

Authors' reply to reviewers

The next pages contain the authors' point-by-point reply to the comments of this revision round, as submitted with the revised manuscript. It is published as the authors wrote it.

Dear Editor and reviewers

Thanks for your consideration to our article. All points were applied in the article carefully and highlighted in the text. We had limitation about word count to 3500. Therefore, we had to increase the number of words to apply the revision.

Best regards,

Reviewer 1:
The manuscript entitled "Apoptosis impact of heliox cold plasma on a cervical cell line by gold nanoparticle doped on graphene oxide nanosheets" is well-written and presents a carefully designed and well-reasoned analysis. The literature review was thorough, the methods appear to be appropriate for the research question and objectives, and the paper presents a range of data to support its claims. However, several unanswered questions and areas could be expanded upon to strengthen the research:

1. Comparison with Other Treatments: The study does not seem to include a comparison with other treatment modalities for cervical cancer, which could help to contextualize the efficacy of the proposed approach.

The discussion was re-written thoroughly and the results were compared with other reports. Some references were collected in the following paragraphs:

In contrast, the cell groups treated by the doped nanoparticle type, considerably indicated the round cellular morphology further than the regular polygonal shape, confirming the possibility of apoptosis in accordance agreement with previous studies (1).

At the higher plasma duration, the photo-activity of gold nanoparticle was negligible because of the extreme effect of the plasma. The observation contradicts a related study that reported the percentage of cell death around 34% after the plasma irradiation for 150 s (2).

A similar study confirmed that cold plasma with closer jet and longer exposure, can make further cancer cell death. This result depends on greater radical species which are developed with the treatment condition (3).

A related investigation reported the distance and treatment time as 1 cm and 5 min to get IC50 point (4).

The result agrees with an evaluation that indicated the critical parameter for the bactericidal capability of plasma is the distance between the plasma tip and bacteria. In this manner, the sterilizing power of CAP is weakened by increasing the gap between the nozzle and cells (5).

A related investigation was conducted using iron nanoparticles and CAP, and the results confirmed a lower expression of Bcl2 in contrast to the non-considerable relationship compared to other groups (6).

By considering the p53 gene, the result of the present report contradicts a study that shows a time-dependent increase in p53 in CAP (7).

The result agrees with a study investigating the photothermal therapy of CAP in the presence of gold nanoparticles (8).

Similar to another paper, the caspase 8 expression remained intact after plasma treatment (9) in the present report.

2. Long-Term Effects and Toxicity: The paper may not address the long-term effects or potential toxicity of the treatment, which is important for clinical translation.

Thanks for your careful point. The long term toxicity needs to evaluate the strategy in in vivo models which we are planning to. Accordance with this, below paragraphs were added to the background and discussion, respectively:

A study approved the higher toxic effect of gold nanoparticles as 25-fold when this method was coupled by cold plasma on the glioblastoma multiform cell line. The involved mechanism was stronger endocytosis due to the synergistic impact of the nanoparticles and the plasma. These nanoparticles accumulated better in the lysosomes of the cells treated with both nanoparticles and plasma. This phenomenon increased cell apoptosis as a result of gold nanoparticle toxicity, and interestingly, the plasma and nanoparticles could not alter the transient physical integrity of cellular membranes (10).

Regarding to toxicity of these nanoparticles, it should be noted that in spite of the high biocompatibility of graphene nanoparticles, these materials are physiologically stable in different human cells (11) and bacteria (12). In accordance with other studies, GO labeled by 188Re indicated high stability in pure water and cell-cultured media. Also, in mice, almost 67% of these nanosheets were kept in blood after 24 h of injection. There were no toxic results when these nanoparticles were 1 mg/ 1 kg of body weight for 14 days (13). Another report emphasized the stability of these nanoparticles in the body or in the cells that had uptaken these nanoparticles (14). These nanoparticles were distributed in PBS, and their agglomerates were confirmed by dynamic light scattering (DLS). In the same manner, in *in vivo* conditions, these particles made protein coronas, facilitating their distribution in a media such as blood and based on the observations of atomic force microscopy (AFM), the morphology of GO aggregates was changed after creating the coronas (15). However, the modification of these particles with hydrophilic compounds, such as manganese or dextran, may increase their stability in biological media or blood (16). Similar to carbon nanotube degradation by neutrophils via a myeloperoxidase (MPO)-dependent mechanism, GO nanoparticles can be degraded via this mechanism. In spite of their degradation, the bronchial epithelial cell line had no toxicological observations and no DNA damage

(17). Another literature recommended employing dispersants, and as an optimum condition, the GO functionalized with polyethylene glycol, had the best biocompatibility at the concentration range of 3 and 0.025 mg/ml on fibroblast cells (18). Also, gold nanoparticles have considerable stability against oxidation, and because of that, these nanoparticles have been suggested for clinical diagnosis and even for therapeutic approaches. After intravenous injection of gold nanoparticles loaded in the gum-arabic matrix, these nanoparticles were detected after 30 min in the heart, jejunum, brain, liver, spleen, kidney and lung in juvenile swine. The lowest and highest amounts of the nanoparticles were in the brain and liver. After 24 hr, the concentrations were significantly the same as the results of after 30 min. The observations confirmed the strong affinity of gold nanoparticles with tissue receptors (19). Additionally, by decreasing gold nanoparticle diameter, their stability is enhanced in animal models of mice. The gold nanoparticles were detected in blood and liver after 72 h post-injection. However, the concentration of these nanoparticles was huge in the liver compared to the blood (20). On the other hand, it was approved that smaller nanoparticles indicated wider distribution in the tissues, including rat skin and intestine (21). Similar to GO nanoparticles, the stabilization of these nanoparticles reduced their toxicity from mild to severe, as approved in animal models (20). As a whole, for the examination of GO-gold nanoparticles in clinical studies, their hydrophilic modification will be necessary to remove their toxicity. Also, their degradation will be feasible due to the lower degree of aggregations as a result of their surface modification.

3. In Vivo Studies: The research appears to be limited to in vitro studies. In vivo studies would be necessary to further support the claims and assess the potential of the treatment in a more physiologically relevant setting.

Thanks for your professional point. The study is related to the thesis of a student and it is evident, the *in vivo* studies even on small animals is necessary to proceed this field. Therefore, in the next step, we are planning to examine the results on mice. Also, we had similar studies on laboratory animals such as the below reference and we have enough experience to do the animal phase. Not only, we will investigate the physiological state of tumor after applying cold plasma and nanoparticles, but the other tissues are our target to evaluate the toxic effects of this strategy:

Hatamie, Shadie, Zahra Malaie Balasi, Mohammad Mahdi Ahadian, Tohid Mortezazadeh, Forough Shams, and Simzar Hosseinzadeh. "Hyperthermia of breast cancer tumor using graphene oxide-cobalt ferrite magnetic nanoparticles in mice." Journal of Drug Delivery Science and Technology 65 (2021): 102680.

4. Statistical Significance: The paper should include statistical analysis to confirm the significance of the results, which is not clear from the provided excerpts.

The point was added to the main text and highlighted:

Statistical part

All experiments were repeated at least in triplicate except XRD. Sigma-plot software was used for all experiments to calculate significant relations

between the groups. Student's t-test and one-way ANOVA statistical tests were used to compare 2 and more than 2 groups, respectively. Values were expressed as mean ± standard deviation (SD), and p-values ≤ 0.05 were considered significant differences.

Reviewer 2:
Dear Editor
- In Abstract: the method section should be revised.

Thanks for your point and the method was corrected as below:

Methods: GO nanosheets was synthesized by hammer method and then, doped by gold nanoparticles. The nanoparticles were characterized by transmission electron microscopy (TEM) and the diffraction peaks of GO and gold nanoparticles were confirmed by X-ray diffraction (XRD) analysis. Also, the optical absorbance of the nanoparticles was measured in the range of 200-900 nm by UV-Visible spectroscopy. After the fabrication of a plasma generator, cold plasma was developed by using He and O2 gases at the ratio of 99:1. The produced radicals of this cold plasma were studied by optical emission spectroscopy (OES). The cell treatment was carried out by applying a serial concentration of GO and GO/Au nanoparticles. The cellular phenotype was monitored by optical microscopy and biocompatible concentrations of both nanoparticles were obtained by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT). After that, the cold plasma at various distances and durations was applied to the cells which had been treated by the nanoparticle. Also, the developed radicals of the treated cells and the expression of apoptotic genes were respectively investigated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and Real-Time PCR.

Increase the number of keywords.

Thanks for your point and the keywords was corrected as below:

Keywords: Cervical carcinoma, cold atmospheric plasma, cancer, gold nanoparticles, radical species, graphene oxide

- The Background section is very short and not acceptable and the number of references is very few.

Thanks for your point and the background was corrected as below:

Regarding mortality, cervical cancer is ranked fourth among cancers worldwide, and almost 90% of the prevalence of this disease occurs in developing countries (22). A new strategy to evolve reactive oxygen species (ROS) and reactive nitrogen species (RNS) is cold atmospheric plasma (CAP). These active chemical compounds correspond to microbial destruction by macrophages (23), vasodilatation (24), maintenance of homeostasis (25), immune system response (26) and control of cell proliferation (27) and differentiation (28). CAP can selectively affect cancer cells compared to traditional cancer therapies by just killing them

(29). A higher level of ROS can damage cancer cells, while normal cells can tolerate this high concentration of radicals. Therefore, radicals can selectively destroy cells depending on their physiological conditions (30). In this way, cold plasma was introduced as a factor with chemotherapeutic properties, especially in cancers with high resistance to anticancer drugs, such as chondrosarcoma (31). Previous studies approved that CAP induces apoptosis in 46% of multidrug-resistant malignant cells (32). The CAP-depended cell death pathway is related to the resulting oxidative pressure of CAP, and this oxidative stress causes dysfunction of the mitochondria and endoplasmic reticulum system and, eventually, cell death (33).

On the other hand, traditional methods of cancer therapy, including chemotherapy, radiation and surgery, have side effects and can damage surrounding tissues (34). In addition, cancer drugs are expensive, and their administration is particularly accompanied by pain and side effects. In contrast, CAP as a non-invasive option causes the least amount of damage to healthy tissues, along with its economic cost system (35). Therefore, this near-room temperature ionization method may function better compared to UV and cancer drugs. A study confirmed that UV photons have no effect on melanoma cancer cells (B16/F10) (36). Fridman et al. reported in 2007 on the apoptotic properties of cold plasma against cancer cells for the first time (37).

Regarding tumor cell transformation, gold nanoparticles activated the PI3K/AKT signaling axis strongly in the presence of cold plasma. Also, this treatment strategy reversed epithelial-mesenchymal transition (EMT) via higher expression of epithelial markers (8). Gold nanoparticles bound to p-FAK made cell death selectively in oral squamous cell cancer (OSCC) in the synergistic model with cold plasma, and the apoptotic role of these nanoparticles and the plasma was removed when there was no combination between them (38). In the same manner, a group employed magnetic nanoparticles loaded by paclitaxel and cold plasma on A549 cells. The observations confirmed lower drug resistance of the cells (39). In other investigations, magnetic nanoparticles and non-thermal plasma led to lower expression of epidermal growth factor receptor (EGFR) (40). Moreover, a group examined the synergistic effect of some nanoparticles, including silica, silver, iron oxide, cerium oxide, titanium oxide, and iron-doped titanium oxide, in the presence of cold plasma on melanoma cell lines. Their data confirmed the higher anticancer effect of these nanoparticles. However, their function is strictly dependent on the nanoparticle and cell line types (41). Iron magnetic nanoparticles were also used to apply the expression of the Bax/Bcl-2 ratio in favor of apoptosis, and the combination of these nanoparticles with cold plasma indicated higher cytotoxicity (42). Silver is another metal that has been

used to examine its combination with cold plasma, and the outcomes approved the cell toxicity against glioblastoma as 100-fold further. This toxicity was ROS-dependent and quenched when N-Acetyl Cysteine was added (43). In a report, Ag/TiO2-reduced graphene oxide (rGO) was used to increase water purification, and the results confirmed there was no successful destruction of bacteria in contrast to viruses (44). Besides noted materials, cerium oxide nanoparticles were used in the presence of cold plasma to examine the protective role of these nanoparticles on primary embryonic mouse fibroblasts (45). In a similar study, platinum nanoparticles reduced the production of radical species in U-251 MG cells when cold atmospheric plasma was applied (46). Also, the silica nanoparticles loaded by doxorubicin and cold plasma were simultaneously applied to the MCF-7 cell line, and the results confirmed a higher apoptosis level with the combination treatment (47). Silicon dioxide nanoparticles are another derivative of silica that was used to induce growth in Astragalus fridae in the presence of cold plasma (48). Among the materials, gold nanoparticles have been introduced as a US Food and Drug Administration-approved particle for photothermal applications due to their surface plasmon resonance (SPR), strong scattering and biocompatibility (49). In one study, gold nanoparticles killed oral squamous cell carcinoma (OSCC) faster in the presence of CAP than the cells treated with CAP alone (38). However, the corresponding property is strictly size dependent, but in the absence of CAP, the photothermal property fails considerably (50). If biological molecules such as proteins, enzymes, biological markers and even drugs and antibodies were doped with these nanoparticles, they would become efficient markers to detect bacteria and cancerous cells and collect conjugated drugs at cancer site (51). Owing to the enhanced permeability and retention (EPR) effect, nanoparticles accumulate in tumor depending on leaky vascular systems of solid tumors (52). Transcytosis is another pathway that has been activated to transport nanoparticles from the blood through tight junctions of endothelial cells into tumor tissue (53). Meanwhile, due to renal and hepatic clearance of these nanoparticles, it is better to load them into nanoparticles with longer circulation time such as graphene derivatives. On the other hand, graphene sheets show prominent photothermal effect due to its large number of π-π conjugations between its sheets (54). Furthermore, graphene platelets have a great ability to conduct the developed heat to the loaded nanoparticles such as MnO_2 (55). In this manner, the low quantum yield of graphene particles could be promoted, and the conversion of radiation into heat could be improved accordingly. As a whole, after plasma irradiation, the free electrons of the graphene nanoparticles start to oscillate and the generated heat travels to their surface where the gold nanoparticles have been doped. CAP not only

produces active chemical species but also increases cell penetration by creating a large electric field, as in electroporation methods, allowing large molecules to be easily transferred to the cell membrane (56). Graphene/gold nanohybrid was produced in a study and modified with folate and paclitaxel. A high rate of cancer cell death was observed as a result of hyperthermia and laser beam (57). Another investigation assessed the efficiency of NIR light excitation and graphene/gold nanoparticles in human glioblastoma astrocytoma cells and the observations confirmed tumor growth suppression (58). Moreover, this nanocomposite restricted cell viability according to its concentration in human cervical cancer (HeLa) cells and made apoptotic and autophagic gene expression and a higher amount of reactive oxygen molecules (59). A study approved the higher toxic effect of gold nanoparticles as 25-fold when this method was coupled by cold plasma on the glioblastoma multiform cell line. The involved mechanism was stronger endocytosis due to the synergistic impact of the nanoparticles and the plasma. These nanoparticles accumulated better in the lysosomes of the cells treated with both nanoparticles and plasma. This phenomenon increased cell apoptosis as a result of gold nanoparticle toxicity, and interestingly, the plasma and nanoparticles could not alter the transient physical integrity of cellular membranes (10). Another investigation showed the combination of gold nanoparticles and cold plasma on mice carrying breast cancer, and the results approved rapid tumor inhibition in its early treatment stages. However, the in vitro study on the L929 cell line was not satisfied (60). In an experiment, gold nanoparticles was bound to antibodies and its effect in the presence of air-cold plasma was studied on melanoma. The results indicated the potential positive effect of this technique on cell death rate. The proposed mechanism of this study was related to collecting the nanoparticle inside of cells, and this route stimulates higher toxicity even at a low dose of cold plasma (61). Again, regarding glioblastoma, the combination of gold nanoparticles and cold plasma made higher ROS intensity, leading to oxidative shock, and this stress alters intercellular signaling pathways, damaging proteins, lipids and DNA (62). Against colorectal cancer, a group employed gold nanoparticles in combination with cold plasma, and the results approved the significant impact of the plasma on cell death. In this study, helium was utilized to develop plasma, although oxygen was added to increase the percentage of ROS (63).

Additionally, in some investigations, these nanoparticles were modified with some growth factors, such as the epidermal one (EGF) that is overexpressed on cancer cells, such as lung carcinoma cells. The observations indicated higher cell apoptosis after applying the plasma method (64). Therefore, there are no reports regarding the combination of

gold nanoparticles doped on graphene oxide sheets and cold plasma against cancer. Also, cervical cancer has not been intentioned to examine the impact of this strategy as a therapeutic method. In this study, GO nanosheets were coupled with gold nanoparticles and a helium/oxygen plasma jet was developed. The HeLa cell line was cultured in the presence of the nanoparticles and CAP parameters including time and distance were optimized. Next, cell viability, gene expression and also, radical generated by the treated cells were investigated.

- All the parts of the Methods are written in briefly form and explanations should be added for each test. A valid reference must be added to each section in the method.

Thanks for your point and the methods was corrected and referenced as below:

1.1. Fabrication of cold plasma generator

An atmospheric plasma jet (High Tech Company, 0045, Tehran, Iran) filled with helium and oxygen gases at the ratio of 99:1, was developed for this study. The mixture of helium and oxygen is commonly known as “Heliox.” The corresponding apparatus was equipped with 2 parallel cylindrical copper electrodes. These electrodes consist of a conical-shaped copper one (cathode) and another cylindrical outer electrode (anode) with 8 copper rods, and the height and diameter of the corresponding electrodes were 45 and 0.12 mm, respectively. A stable plasma jet was developed under a high AC power supply with a voltage, frequency and power of 6 kV, 35 kHz and 12 W, respectively. To obtain these voltage and frequency values, a transformer (Phenix Technologies, MD, USA) was used, and the initial plasma was developed with assumed values and then, the values were adjusted to get stable irradiations, as shown in Figure 2a (65).

1.2. Measurement of radical species

Optical emission spectroscopy (TIDA Spectrometer 350-1100, Teksan, Tehran, Iran, OES) was employed to examine the spectra of UV–visible emission related to the generated radicals from the cold plasma. This apparatus was operated at a resolution of 0.02 nm and a power of 200 mA at +5 VDC. The range of wavelengths was between 300-900 nm. The light emitted by the radicals originated from the plasma, was read by a spectrometer (66).

1.3. GO fabrication

The famous hammer method was used to produce GOe nanoparticles. For this method, graphite (Sigma-Aldrich) was added in an amount of 0.5 g of

sulfuric acid (%98, Merck). The suspension was stirred at a temperature below 0∘ C for 10 min. Then, sodium nitrite (Sigma, 0.5 g) was mixed with the above suspension, and after adding potassium permanganate (3 g), the temperature was increased to 30∘ C. The reaction was continued for 4 h, and then 100 ml of water was added. Again, the temperature was raised to 90∘ C, and extra water (50 ml) was poured in with the addition of 3 ml of hydrogen peroxide (Merck). An ultra-sonication step was performed, and finally, the solution was washed several times to change the pH to 7. Finally, the solution was dried at 50∘ C for 24 h (67).

1.4. Gold doping

First, an aqueous solution was prepared by mixing GO and gold nanoparticles (US-Nano, USA) in water at the respective concentrations of 0.3 mg/ml and 0.05 mg/ml in a ratio of 3:10. The mixture was then heated at 80∘ C for 30 min and sodium citrate was added as a chemical reducing agent. The sample was shaken for 5 h, and the resulting nanoparticles were collected by the centrifuge method at 6000 rpm. The container was stored in an oven at 40∘ C to remove any liquid. The method was taken from other reports with some modifications (68).

1.5. TEM, XRD and UV–Vis spectroscopy

The doped and non-doped nanoparticles were analyzed for their size and homogeneity using transmission electron microscopy (TEM). For this assay, the nanoparticles were fixed on carbon grids and examined with a TEM instrument (Philips CM-30) with an acceleration voltage of 250 kV. Furthermore, the addition of gold nanoparticles into GO nanosheets was verified by an X-ray diffraction (XRD, AXS Bruker diffractometer) device equipped with a CuKα source to generate a wavelength of 1.5418 Å. The doped and non-doped GO nanoparticles were examined by UV-Vis spectroscopy (V-670, JASCO, Japan) at the wavelength range of 200-900 nm (69, 70). The quartz cuvette had an optical path length of 1 cm.

1.6. MTT analysis

Human epithelial carcinoma (HeLa) cell line was purchased from the Pasteur Institute of Iran and cultured using high glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco) and 2 mM 1-glutamine (Gibco). For cell culture approaches, the cells were stored at 37∘ C in a 5% CO_2 and 20% O_2

incubator and passaged with trypsin (Gibco) when their population increased beyond 80%. Cell viability was examined at a gradient concentration of both nanoparticle types. For this part, the cell viability was evaluated by MTT method. Briefly, HeLa cells were seeded in a 96-well plate at 5×10^3 cells per well and incubated for 24 h. Then, the composite and non-composite nanoparticles were treated at a serial concentration of 5, 10, 15, 20, 25 and 30 µg/100 µl and the untreated cells were used as the control group. After 24, 48 and 72 h, the MTT solution at a concentration of 0.1% was added to the wells and after 3.5 h, the reduced formazan crystals were collected using dimethyl sulfoxide (DMSO, Merck) as a solvent. Their absorbance values at 570 nm were read with a microplate reader (BioTek Instruments, USA) (71). The cell viabilities were calculated according to the following formula:

Cell viability % = OD of treated cells / OD of TCPS × 100

Where OD stands for optical density at 570 nm, and TCPS corresponds to the untreated cells.

The concentration with a cell viability value of > 90% was reported as the biocompatible value and used for the following cellular investigations in the presence of the cold plasma. This assessment was repeated after the combination of the nanoparticles and the plasma. This step was necessary to detect the plasma conditions that was able to kill at least 50% of the cells in a synergistic strategy with the nanoparticles. The time and distance of the irritation conditions were 0.5, 1 and 3 min at a distance of 3 cm and an additional group with the respective time and distance of 1 min and 5 cm.

1.7. DPPH assessment

The measurement of the radicals formed the treated cells by the nanoparticles and plasma, was carried out using a DPPH assay. Here, the plasma was applied to all groups for 30 and 60 s at a distance of 3 cm, and DPPH solution (Sigma, 0.1 mM) was added as 0.5 ml per well in a 24-well plate, and methanol (Merck) was used as the dissolution media. The scavenging activity needs at least 30 min at room temperature, and then, the absorbance values of the samples were reported at 520 nm. The following formula was used to calculate the radical inhibition percentages:

Radical inhibition percentage = $(OD_{blank}-OD_{sample})/OD_{blank} \times 100$

Here, the OD value of the blank group shows the absorbance of the DPPH solution without any treatments and the OD of sample is the absorbance of the treated groups (67).

1.8. Real-Time PCR

For genetic analysis, HeLa cells were cultured at a density of 2 × 10^4 cells/ well of a 24-well plate. The groups were listed as TCPS and the nanoparticle-treated groups. All groups were exposed to the plasma for 30 and 60 s at a distance of 3 cm. After 72 h, the cells were processed for RNA, and here, TCPS was used as the control group to normalize the gene expression values. First, TRIzol reagent (Gibco) was recruited to extract total RNA. The quality of the developed RNA was checked by running 5 µl of the extracted RNA on an agarose gel (Gibco, 1.5%) and electrophoresis process. In order to ensure the purity of the RNA, its absorbance was examined at 260/ 280 after dilution using RNase-DNase-free water (Gibco). Then, a cDNA synthesis kit (Yekta Tajhiz Azma kit, Teheran, Iran) was recruited, and for this step, Random Hexamer primer (SinaClon, Iran, 1 µl) was mixed with 1 µg of the RNA in 9 µl of RNase-DNase free water. The cDNA samples (0.5 µl) were analyzed by Corbett Rotor-Gene 6000 (QIAGEN Rotor-Gene Q, Germany) to indicate the expression of the genes listed in Table 1. The GAPDH gene was used as a reference gene to normalize the expression of the marker genes. The PCR master mix was purchased from SYBR Green, and the primers were made by Cinagen, Iran (72).

- Authors should describe plasma treatment scheme in more details.

Thanks for your point and the caption was edited as below:

Figure 1. The schematic graphical abstract summarizes the employment of Helium (He) and O2 gases at the ratio of 99:1 to develop cold plasma beside doped graphene oxide nanoparticles by gold in the present study. The nanoparticles were prepared and characterized by XRD, OES and TEM. After confimation of the nanoparticles properties, the cell treatments were started to reach biocompatible values. The values were resulted as 30 and 20 µg/100 µl for non-doped and doped GO, respectively. For investigation of the combination effect of the nanoparticles and the plasma, the cells were cultured and their plasma treatments were done. The stable cold plasma jet was obtained with voltage of 6 kV, frequency of 35 kHz and power of 12 W. The values were kept same for all assessments. The cellular phenotype was studied by optical miccroscopy and the expression of apoptotic genes by Real-Time PCR. Accordance with the cell morphology, the round shape against normal polygonal phenotype, clearly approved apoptosis. Moreover, the molecular technique, indicated highest expression of apoptotic genes when the .duration of the treatment was 60 s at the distancec of 3 cm

- How could you apply plasma to cell directly?

Thanks for your careful consideration. Under the laminar flow cell culture hood, the lid of the cell plates was removed and then, the probe was fixed at a predetermined distance by a ruler before starting the

cold plasma. The transformer of the plasma was set at specific voltage, frequency and power and cold plasma was developed. After ending the irradiation time, the probe was replaced to another cell plate. This method was taken from other similar studies such as below. These references were cited in the present study:

1- Shiva, I., S. Zhohreh, A. Seyed Mohammad, and M. Shahriar. "Induction of growth arrest in colorectal cancer cells by cold plasma and gold nanoparticles. Arch." *Med. Sci* 11, no. 6 (2015): 1286-1295.
2- Wang, Mian, Xiaoqian Cheng, Wei Zhu, Benjamin Holmes, Michael Keidar, and Lijie Grace Zhang. "Design of biomimetic and bioactive cold plasma-modified nanostructured scaffolds for enhanced osteogenic differentiation of bone marrow-derived mesenchymal stem cells." *Tissue Engineering Part A* 20, no. 5-6 (2014): 1060-1071.
3- Kim, Sun Ja, and T. H. Chung. "Cold atmospheric plasma jet-generated RONS and their selective effects on normal and carcinoma cells." *Scientific reports* 6, no. 1 (2016): 20332.

- What is the basis for choosing the time (30 and 60 s) and concentrations used?

Thanks for your point. The effect of cold plasma on cells strictly depends on the voltage, frequency and power of the plasma apparatus. In this regard, when these values are high, the incubation time, inevitably was low and vice versa. By considering this fact, each research group has to set the parameters independently. Although, the initial values to optimize the cold plasma in the present study were extracted from other studies. These values were examined to develop radical species and the appearance of these radicals was major criteria to get the treatment times. Some of the references which were recruited here, were listed at below:

1- Shiva, I., S. Zhohreh, A. Seyed Mohammad, and M. Shahriar. "Induction of growth arrest in colorectal cancer cells by cold plasma and gold nanoparticles. Arch." *Med. Sci* 11, no. 6 (2015): 1286-1295.

The times were 60, 90, 120 and 180 s.

2- Bekeschus, Sander, Anke Schmidt, Lydia Bethge, Kai Masur, Thomas von Woedtke, Sybille Hasse, and Kristian Wende. "Redox stimulation of human THP-1 monocytes in response to cold physical plasma." *Oxidative medicine and cellular longevity* 2016, no. 1 (2016): 5910695.

The times were 1, 3 and 6 min.

3- Masur, Kai, Magdalena von Behr, Sander Bekeschus, Klaus-Dieter Weltmann, Christine Hackbarth, Claus-Dieter Heidecke, Wolfram von Bernstorff, Thomas von Woedtke, and Lars Ivo Partecke. "Synergistic inhibition of tumor cell proliferation by cold plasma and gemcitabine." *Plasma Processes and Polymers* 12, no. 12 (2015): 1377-1382.

The times were 4, 9, 18, 36, 75, 150 and 300 s.

4- Baniya, Hom Bahadur, Pabitra Khadka, Sudip Panday, Anusuya Nepal, Rajesh Prakash Guragain, Tika Ram Lamichhane, Santosh Dhungana, Bhupal Govinda Shrestha, and Deepak Prasad Subedi. "Characterization of cold atmospheric pressure plasma technology and its anticancer properties." *Plasma Medicine* 11, no. 4 (2021).

The times were 0.5 to 3 min.

5- Fridman, Gregory, Alexey Shereshevsky, Monika M. Jost, Ari D. Brooks, Alexander Fridman, Alexander Gutsol, Victor Vasilets, and Gary Friedman. "Floating electrode dielectric barrier discharge plasma in air promoting apoptotic behavior in melanoma skin cancer cell lines." *Plasma Chemistry and Plasma Processing* 27 (2007): 163-176.

The times were 5, 10, 15, 20, or 30 s.

- In the Figures legends: the significance (p-value) should be mentioned.

Thanks for your point. The value was added generally to all figures as (p-value ≤ 0.05). Such as below:

Figure 6. MTT results of a) a gradient of particles in the absence of plasma: the comparisons are between 2 cell groups treated by GO (control group) and GO/Au (test group) nanoparticles, b) after 24 h treated with 30 and 20 µg/100 µl of non-doped GO and doped GO nanoparticles, respectively: the comparisions are between the cell groups which were treated by only the cold plasma (control) and the groups by the cold plasma and the nanopartilces (test groups), c) after 48 h treated with 30 and 20 µg/100 µl of non-doped GO and doped GO nanoparticles, respectively: the comparisions are between the cell groups which were treated by only the cold plasma (control) and the groups by the cold plasma and the nanopartilces (test groups), and d) after 72 h treated by 30 and 20 µg/100 µl of non-doped GO and doped GO nanoparticles, respectively: the comparisions are between the cell groups which were treated by only the cold plasma (control) and the groups by the cold plasma and the nanopartilces (test groups). The star (*) assigns the statistical differences when p-value≤0.05.

Figure 7. The curve of remaining DPPH (%) by measuring the absorbance values at 520 nm after the treatment of Hela cells by 30 and 20 µg/100 µl of non-doped GO and doped GO nanoparticles, respectively. The star (*) symbol assigns the statistical differences between the doped groups and TCPS, and the number sign (#) symbol assigns the statistical differences between the doped and the non-doped group when p-value≤0.05.

- Apoptotic phenotype not investigated?

Thanks for your valuable point. This part was completed in results and also, discussion parts as the below:

Results:

Cell phenotype

The morphology of the plasma-treated cells was examined under a light microscope after 24 and 72 h and summarized in Figures 4 and 5, respectively. Among the groups without any nanoparticles, the one treated with the plasma at a distance of 5 cm for 1 min showed a higher cell number than the group at a distance of 3 cm. In this manner, the group with 3 min and 3 cm contained only a few single cells in contrast to the other two groups with cell colonies. Considering other studies, the distance between the plasma nozzle and a treated surface has a significant impact on the formation of radical species, and a higher value of radicals can be obtained at short distances (73). On the other hand, the groups containing the non-doped GO nanoparticles showed no difference in their cell morphology. Regarding this, it is evident that the effect of these nanoparticles on the number of cells is undetectable by using an optical microscope and needs more careful methods, such as MTT analysis. The round cell shape, compared to the regular polygonal one, confirms the

establishment of apoptosis (1). The considerable difference relates to the groups treated with the coupled nanoparticles. Herein, the cell morphology has obviously changed to a collapsed one, and the cells lost their adhesion. Moreover, when the plasma jet distance was changed to 3 cm, the number of the attached cells was increased, although the treatment time was 3 min.

Discussion:

Regarding biological investigations, the non-doped GO nanoparticles were not able to alter cell morphology, which could be related to the limited photoactivity of these nanoparticles. However, depending on its π-π bonds, GO might have the photocatalytic activity to generate free radicals (74). In contrast, the cell groups treated by the doped nanoparticle type, considerably indicated the round cellular morphology further than the regular polygonal shape, confirming the possibility of apoptosis in accordance agreement with previous studies (1). The observations of cellular morphology clearly confirmed the distance of cells from the plasma tip can have a stronger effect on cells as opposed to the exposure time. A similar study confirmed that cold plasma with closer jet and longer exposure, can make further cancer cell death. This result depends on greater radical species which are developed with the treatment condition (3). Except for the groups with the coupled nanoparticles, the cells of the other group were able to compensate for their cell death over time.

- The plasma dose/concentration was not stated and only the time was mentioned.

Thanks for your point. The anticancer effect of the plasma depends on the presence of radicals. Therefore, we produced the plasma with various voltage, frequency and power and then, examined the radicals by OES.

- The radicals which are needed to establish cell apoptosis, could be listed such as N2 and oxygen radicals. Therefore, accordance with the results of OES method, the plasma jet was fixed at the voltage of 6 kV, frequency of 35 kHz and power of 12 W. These values of the plasma parameters were minimum to develop the radicals.
- Beside, another important criterion to set the parameters to specific values, is electron temperature which could be calculated by the evaluation of emission lines under NIST formula. This is another application of OES method. The value (Te) was 0.7843 eV confirming development of cold plasma (75).

- The article needs to be revised in all sections.

The whole text was revised and highlighted.

- In Figure 6B-D, significance between which groups have been investigated? All 4 groups have significant signs in all 3 graphs.

The legend was corrected as the below:

Figure 6. MTT results of a) a gradient of particles in the absence of plasma: the comparisons are between 2 cell groups treated by GO (control group) and GO/Au (test group) nanoparticles, b) after 24 h treated with 30 and 20 µg/100 µl of non-doped GO and doped GO nanoparticles, respectively: the comparisions are between the cell groups which were treated by only the cold plasma (control) and the groups by the cold plasma and the nanopartilces (test groups), c) after 48 h treated with 30 and 20 µg/100 µl of non-doped GO and doped GO nanoparticles, respectively: the comparisions are between the cell groups which were treated by only the cold plasma (control) and the groups by the cold plasma and the nanopartilces (test groups), and d) after 72 h treated by 30 and 20 µg/100 µl of non-doped GO and doped GO nanoparticles, respectively: the comparisions are between the cell groups which were treated by only the cold plasma (control) and the groups by the cold plasma and the nanopartilces (test groups). The star (*) assigns the statistical differences when p-value≤0.05.

- How many times was each experiment repeated?

Thanks for your question. All experiments were repeated at least in triplicate except XRD (this sentence was added to the statistical part). Most of the assays such as TEM, OES, MTT and even Real-Time PCR were repeated more than 3 times. That was because these assays depended on the parameters which were needed to be set. For example, for MTT assessment and cell phenotype, irrespective of the issues related to cell culture such as microbial contamination, the values of the nanoparticle were not divided equally among the plate wells. Therefore, some simple tests such as cell treatments needed more than 6 months to be set.

- Figure 8 is not understandable for the reader. Each group has several significant signs and complicates the understanding of the Figure.

The figure was edited to be easy understandable as below:

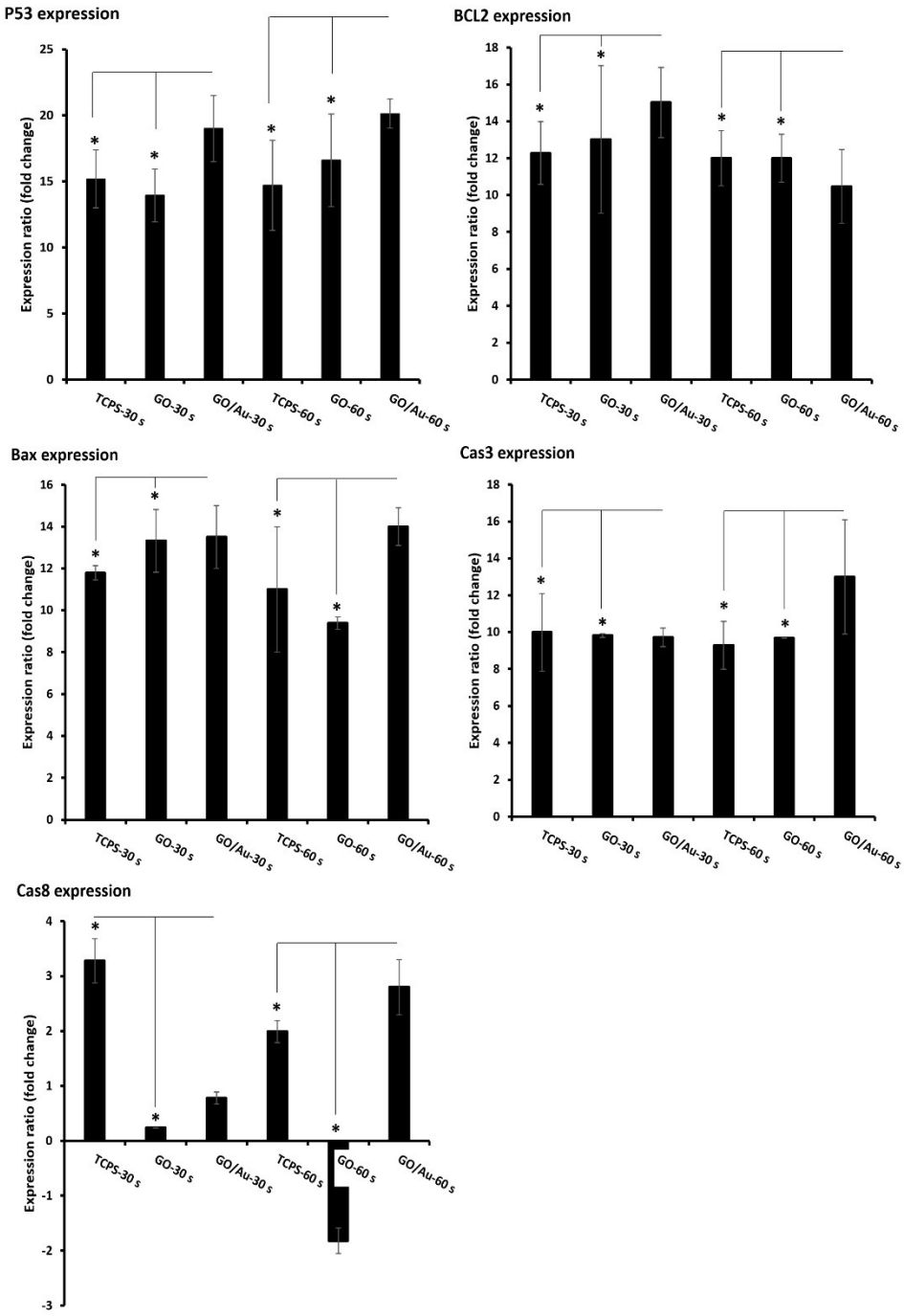


Figure 8. Real-time PCR analysis of HeLa cells after 72 h treated with 30 and 20 µg/100 µl of non-doped GO and doped GO nanoparticles, respectively. The star (*) symbol assigns the statistical differences between the TCPS and nanopartilce-treated groups with a same treatment time of the cold plasma when p-value≤0.05. Also, the statistical differences among all groups were collected in a supplementary file.

- Statistical analysis section should be added.

The point was added to the main text and highlighted:

Statistical part

All experiments were repeated at least in triplicate except XRD. Sigma-plot software was used for all experiments to calculate significant relations between the groups. Student's t-test and one-way ANOVA statistical tests were used to compare 2 and more than 2 groups, respectively. Values were expressed as mean ± standard deviation (SD), and p-values ≤ 0.05 were considered significant differences.

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2 Revision 1

1 comment · 25 Sep 2024

Associate Editor

ACCEPT 25 Sep 2024

The authors improved their manuscript according to reviewers' comments and provided thoughtful answers to the questions. In my opinion, this manuscript is now acceptable for publication in IJ Pharmaceutical Sciences.