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An Evaluation of the Effect of Graphene Oxide-Gold Nanocomposite on HepG2 Cell Line

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ABSTRACT

Background and Objective: One of the most important effects of nanomaterials on cells is the induction of programmed cell death (apoptosis). The aim of this study is to investigate the toxicity of graphene oxide-gold nanoparticles and to evaluate the expression of caspase 3 gene in HepG2 liver cancer cell line.

Methods: In this experimental study, hepatocellular carcinoma cell line (HepG2) was prepared from Pasteur Institute of Iran. Cells were treated with concentrations of 10 to 500 µg/ml Gold-rGO and untreated cells were used as control. The level of caspase 3 gene expression and cell viability were investigated and compared within 24 and 48 hours in three groups of A, B and control (C). The effect of concentration was investigated by XTT method and acridine orange ethidium bromide staining. Caspase 3 gene expression was measured by relative quantification using real time PCR test.

Findings: Based on XTT results, EC20, EC50, EC80 values were calculated as 386.420, 90.680, 24.151 µg/ml in 24 hours and 358.146, 89.536, 22.384 µg/ml in 48 hours. Examining the level of caspase 3 gene expression change in these concentrations compared to untreated cells showed mean fold changes (mean±SEM) of 3.436±1.022 in 24 hours and 4.054±0.02 in 48 hours, according to which the level of gene expression changes significantly. The viability of control cells (C) compared to cells treated with concentrations higher than 50 µg/ml (B) has a relative increase, which was statistically significant (p<0.0001). Examination with AO/EB staining to determine the level of apoptosis showed a higher level of apoptosis with increasing dose.

Conclusion: The results of the study showed that shorter times and lower concentrations should be used in the application of this nanoparticle.

Keywords: *Cytotoxicity, Biocompatibility, Graphene, Gold-Graphene, Caspase 3.*

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Introduction

Nanoparticles are particles that are smaller than 100 nanometers and have unique compositions, size, shape and surface chemical properties. The importance of nanotechnology has increased due to its small dimensions. Development and progress in the area of making highly sensitive nano-biosensors for the detection of bacteria, fungal pathogens, viruses and cancer cells is necessary in biological, and clinical medicine and food. Gold nanoparticles have had a great impact in the field of medical applications such as drug and gene delivery, non-invasive imaging of the body, and cancer treatment (1). Having characteristics such as ultra-small size, excellent luminescence properties, high chemical stability and excellent biocompatibility have drawn much attention to this nanoparticle (2).

Graphene is one of the allotropes of carbon nanomaterials, which has unique physicochemical properties such as high surface-to-volume ratio, excellent electrical and thermal conductivity, and strong mechanical strength. Composite structures made of graphene and metal nanoparticles can have new properties such as reducing metal oxidation, reducing plate resistance and positive synergistic effects by combining the properties of graphene and metal nanoparticles (3).

Today, with the development of nano-biotechnology, nano-materials have entered the environment of human life and ecosystem. Considering the increase in the production of nanomaterials and their presence in the human environment, paying attention to their effects is an important issue. Research has shown that nanomaterials can have negative effects on cell growth and survival and cause a wide range of acute and chronic effects. Therefore, it is important to evaluate the toxicity of these substances in order to prevent human damage caused by these substances.

Despite the potential benefits of nanoparticles, the toxicity of nanoparticles has raised concerns about their impact on health. Graphene derivatives dramatically reduce the expression of various genes that are responsible for the structure and function of the cell membrane (4). These derivatives are transferred to the cell through various methods and lead to the production of ROS, increase in LDH and calcium release, and then they cause a variety of cell damage such as cell membrane damage, inflammation, DNA damage, mitochondrial disorders, apoptosis and necrosis (5).

Cancer is one of the main causes of death in the world. The liver is one of the internal organs of the body, which plays an important role in detoxification and disposal of body waste. Thus, investigating the effect of nanomaterials on the liver is very important. Liver cancer is one of the major diseases with which the medical community is involved and the fifth most common cancer. The treatment of this condition and drug delivery to these cells have limitations. One of the ways of drug delivery is the use of nanomaterials. Studies have determined that some nanomaterials can have adverse effects such as liver inflammation (6).

Apoptosis mechanism is one of the main ways of removing unwanted cells in which internal and external stimuli cause cellular suicide. Apoptotic cells have certain morphological characteristics, the most explicit of which are apoptotic bodies. Three major pathways are known to initiate apoptosis. These pathways are controlled by death receptors (extracellular pathway), mitochondria (intracellular pathway) or endoplasmic reticulum.

At the center of the apoptosis process are a family of proteases called caspase. These proteases are involved in the initiation and execution of apoptosis and can be activated in both intrinsic and extrinsic ways. During stress signals, DNA damage or defects in cell signaling pathways, the internal pathway is activated and causes the development and processing of the immune system of the external pathway (7).

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Today, about 14 types of caspases are known, eleven of which are of human origin. By categorizing based on function, these proteases are divided into three distinct subfamilies: 1-initiator caspases, which include caspases 2, 8, 9, and 10. 2- Executioner or effector caspases, which include caspases 3, 6 and 7. 3- Inflammatory caspases, which include caspases 1, 4, 5, 11, 12, and 14, and are involved in inflammatory reactions and the processing and maturation of cytokines. Caspase 3, which consists of two units of 17 and 12 kDa, is activated by caspase 8, caspase 9 and caspase 10 and in turn activates caspase 6 and caspase 7.

In a study conducted by Xiang et al. on primary corneal epithelial cells and conjunctival epithelial cells in 2016, they were able to discover the potential toxicity and mechanism of graphene oxide (8). Regarding gold nanoparticles alone, Lopez-Chaves et al. have recently investigated the 29-HT and HepG2 cell lines in vitro and on a rat model in vivo and reported a particle size-dependent effect (9). In 2021, a study conducted by Yuan et al. on graphene oxide-silver nanoparticles (GO-AgNPs) nanocomposites investigated the cytotoxicity and epigenetic modification status mediated by GO-AgNPs in goat fetal fibroblast cells (CFFCs) and the data showed that GO-AgNPs cause cytotoxicity in a dose-dependent manner (10).

There are no sufficient records and studies regarding the composite nanomaterial of modified graphene oxide with gold. Therefore, this study was designed to obtain information about the effect of this substance on the induction of apoptosis on the HepG2 cell line. The aim of this study is to investigate the apoptotic and cytotoxic effects of graphene-gold nanoparticles using human HepG2 liver cells. Cytotoxicity mechanisms were investigated using vital parameters such as cell viability and apoptosis in HepG2 cells. In addition, the expression level of the apoptotic gene caspase 3 was evaluated by real time method.

Methods

In this experimental study with code of ethics IR.SBMU.RIGLD.REC.1395.98, the synthesis of Gold-rGO nanoparticles was carried out by precipitation method with the reduction of gold ions according to the method of previous studies (1). Gold nanoparticles were placed on graphene oxide nanoparticles through direct chemical reduction of chloroauric acid (NAUCL4) by sodium borohydride NaBH4. Briefly, 200 mL of HAUCL4 (200 mg) was gradually added to the GO solution (1 mg/mL), and the mixture was stirred at room temperature for 2 h. Then, 0.1 ml of NaBH4 was slowly poured into the mixture and stirring continued for another 1 hour at room temperature, and at the end, the sample was washed many times with distilled water. After drying, its image was prepared with the help of transmission electron microscope (TEM).

HepG2 cell line was obtained from Pasteur Institute, Tehran. The culture medium used was RPMI (Gibco Co.) enriched with 10% Fetal bovine serum (FBS) (Sigma-Aldrich), 1% non-essential amino acid (Gibco) NEAA, 1% penicillin-streptomycin antibiotic and 1% L-glutamine. The cells were passaged several times in special T25 flasks with 0.25% trypsin-EDTA and kept at 37°C and 5% CO2.

Cells were treated with concentrations of 10, 50, 75, 100, 150, 200, 400, 500 µg/ml of Gold-rGO and untreated cells were used as control. The level of caspase 3 gene expression and cell viability (after treatment with gold-rGO) was evaluated and compared in three cell groups of control (C), group A (which showed no significant difference with the control) and group B (which showed a significant difference with the control group) within 24 and 48 hours.

To evaluate the viability of cells after the impact of nanoparticles, the Cell Proliferation Kit II (XTT) (Roche company) was used. This test is based on the breakdown of the yellow salt of tetrazolium XTT and the formation of the orange color of formazan through the mitochondrial succinate dehydrogenase of living

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cells. Formazan is soluble in water and its value is calculated directly in the Elisa reader and read at a wavelength of 450 nm. The incubation period of XTT Mixture to complete the test is 18 to 24 hours. In this test, the cells were incubated for 24 hours with XTT solution at 37°C and 5% CO2.

Eight groups of cells were considered for treatment with nanoparticles at concentrations of 10, 50, 75, 100, 150, 200, 400 and 500 micrograms per ml and one untreated control group and one group treated with DMSO and all the tests were repeated twice. In order to perform the XTT test, cells were transferred to 96-well plates after full growth in cell culture flasks. For each sample, two wells were considered. 2×10^4 cells were seeded in each well and the amount of 200 microliters of the final volume was calculated in each well. Before cell treatment, the cells were first washed with PBS and then the new medium containing nanoparticles was added to it.

The results of the XTT test were used to find EC20, EC50 and EC80 concentration values. The amounts of EC50 expressing the concentration of nanoparticle that inhibits 50% of the growth of cells compared to the control, as well as the amount of EC20 in which 80% of the cells survived and EC80 in which 20% of the cells survived were calculated and these values were used to evaluate gene expression. To evaluate the expression of caspase 3 gene, the following steps were performed:

RNA was extracted using the RNasy Plus mini kit (Qiagen Co.) according to the protocol.

Before the synthesis of cDNA by RT-PCR method, the concentration of purified RNA was determined by reading their OD by Nano Drop device, and similar concentrations of RNA from different samples were used in all reactions, and RNA adjusting was done.

cDNA was synthesized using Revert Aid Reverse Transcriptase kit (Thermo Fisher Scientific) with amounts of mix 1 including random hexamer 1 µl and deionized water 6.5 µl and RNA and mix 2 including buffer 4 µl, deionized water 6.5 µl, enzyme 1 µl, Mix dNTP 2 µl and RiboLock RNase Inhibitor 0.5 µl and the reaction conditions were 65˚C for 5 minutes, 25˚C for 10 minutes, 45˚C for 45 minutes and 70˚C for 70 minutes.

The gene involved in the induction of apoptosis (caspase 3) at the transcript level and the GAPDH gene as a reference gene were evaluated using the Real-time PCR method by the 7500ABI machine and by the SYBR GREEN method with Takara Master Mix. The sequence of primers is given in Table 1 (11). The reaction was carried out with a final volume of 20 µl, which included Cyber Master Mix 10 µl and each of the primers 0.4 µl, deionized water 7.12 µl and ROX 0.08 µl. The reaction temperature conditions included 95˚C for 10 minutes, 95˚C for 15 seconds and 60˚C for 1 minute.

The data were analyzed using ANOVA and Student's T-test statistical tests, and $p<0.05$ was considered significant.

Table 1. Sequence of primers related to Caspase 3 and GAPDH

Primer title	Primer sequence	Product size
Caspase3-F	5- CCA GAG TCCATTGATTCGCT -3	280 bp
Caspase3-R	5- GCTATTGTGAGGCGGTTGTA -3	280 bp
GAPDH-F	5- GCTCTCTGCTCCTCCTGTTC -3	220 bp
GAPDH-R	5- ACGACCAAATCCGTTGACTC -3	220 bp

Results

In order to study the structure of reduced graphene with gold coating, transmission electron microscope was used. The prepared images show the presence of gold coatings in the form of black dots on the nanoparticle (Figure 1).

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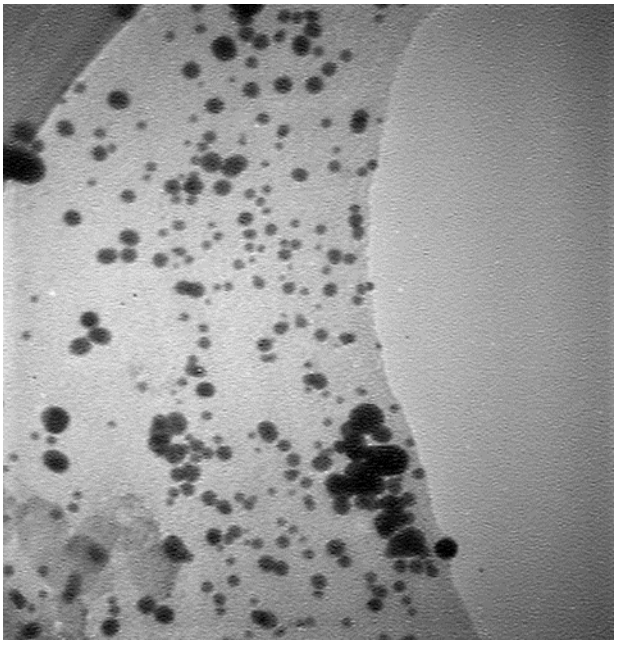

Figure 1. Electron microscope image of Gold-rGo nanoparticle. **Black dots represent gold particles.**

The cytotoxicity of Gold-rGO on HepG2 cell line was evaluated using the XTT test in 8 concentrations prepared from Gold-rGO with two repetitions in the wells containing the differentiated cells during 24 and 48 hours (Diagrams 1 and 2).

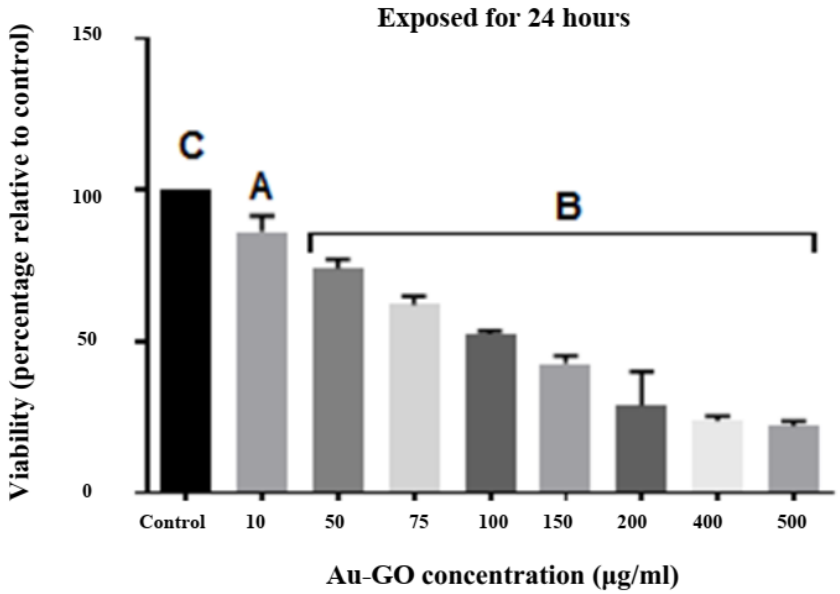

Diagram 1. Cytotoxicity of Gold-rGO on HepG2 cells in 24 hours ($p<0.0001$). **At the concentration of 10 µg/ml (A), there is a difference compared to the control (C), but this difference is not significant. A significant difference can be seen in the concentration of 50 µg/ml (B) compared to the control group (C).**

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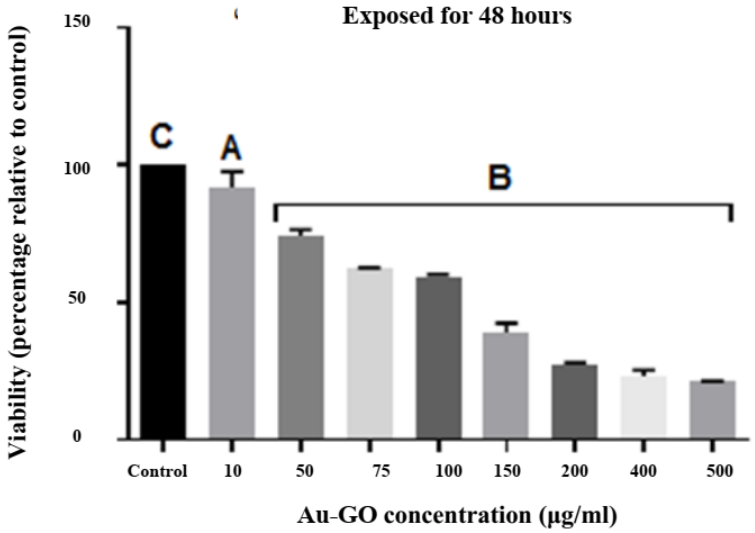

Diagram 2. Cytotoxicity of Gold-rGO on HepG2 cells in 48 hours. In 48 hours, the same concentrations were tested as before and the cell viability compared to untreated control cells is shown in the figure ($p<0.0001$).

The results showed that the viability of control cells (C) compared to cells treated with concentrations higher than 50 µg/ml (B) has a relative increase ($p<0.0001$). The values of EC20 (80% of cells are alive), EC50 and EC80 (20% of cells are alive) were calculated during 24 hours of nanoparticle effect and were 24.151, 90.68 and 386.420 micrograms/ml, respectively. The values of EC20, EC50 and EC80 were also calculated in 48 hours of nanoparticle effect on cells, which were 384.22, 536.89 and 146.358 µg/ml, respectively. EC20, EC50 and EC80 concentrations in 24 and 48 hours were selected as reliable and toxic concentrations for use in subsequent tests. Morphological results showed that normal HepG2 cancer cells mainly have elongated morphology (Figure a2). This morphology changes during the incubation of cells with high amounts of nanoparticles and becomes spherical (Figure b2), so at concentrations higher than 100 µg/ml, all cells are spherical (Figure c2).

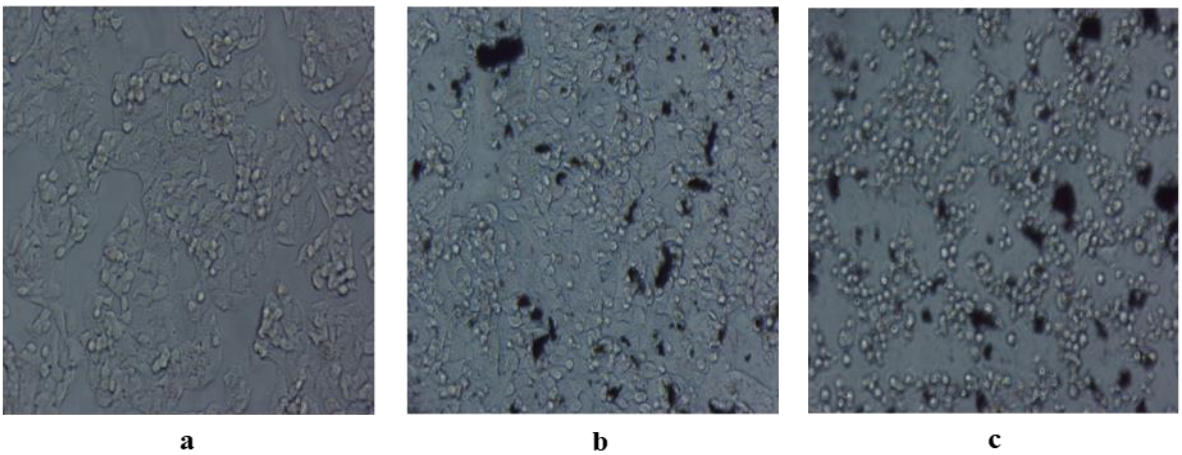

Figure 2. Image of cells treated with different concentrations. a) Untreated control cells: in the normal state, the morphology of the cells is elongated. (b) Cells treated with a concentration of 50 µg/ml: Morphology changes during incubation of cells with 50 µg/ml of nanoparticles and becomes spherical. c) Cells treated with a concentration of 100 µg/ml ml: At concentrations higher than 100 µg/ml, all cells are spherical.

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Moreover, the results of the present study showed that the majority of cell death resulting from the incubation of cells with Gold-rGO nanoparticles in ethidium bromide/acridine orange (EtBr/Ao) staining is apoptotic and the number of apoptotic cells increases with increasing concentration.

The results of real time PCR test using relative quantification method: evaluation of the expression changes of these genes was done by comparing treated cells with control cells based on relative quantification. The analysis of the results after normalization with the results of the reference gene was done with ΔΔCT method and ABI 7500 software. The change in the expression level of caspase 3 gene in EC20, EC50 and EC80 concentrations compared to untreated cells in 24 hours is shown in diagram 3 (Difference between mean fold change±SEM= 3.436±1.022) and in 48 hours is shown in diagram 4 (Difference between mean fold change±SEM= 4.054±0.02).

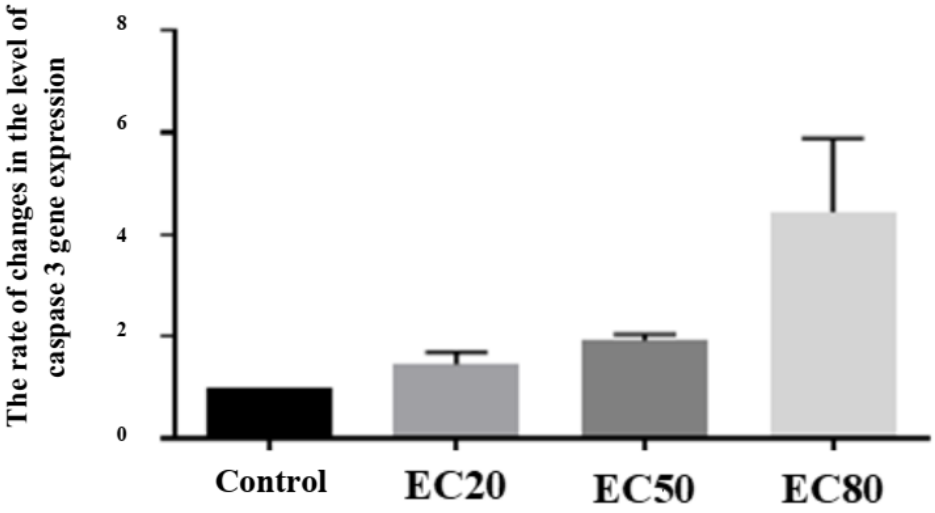

Diagram 3. Changes in the expression level of caspase 3 gene in 24 hours. Changes in the expression level of caspase 3 gene in 24 hours in cells treated with concentrations of EC20, EC50 and EC80. Calibrator: untreated cells (Difference between mean fold change±SEM= 3.436±1.022).

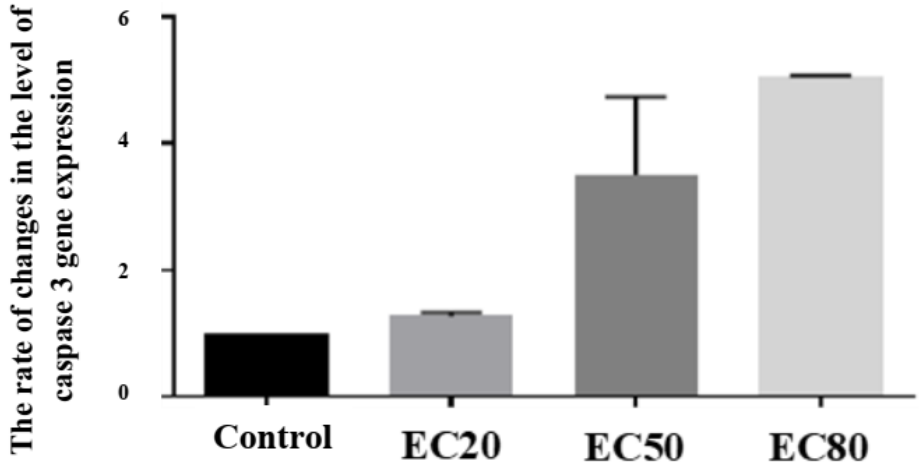

Diagram 4. Changes in the level of caspase 3 gene expression in 48 hours. Changes in the level of caspase 3 gene expression in 48 hours in cells treated with concentrations of EC20, EC50 and EC80. Calibrator: untreated cells (Difference between mean fold change±SEM= 4.054±0.02).

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Discussion

The results of examining cell viability by XTT test mainly indicated dose- and time-dependent cytotoxic effects. The difference in concentration and time have been the effective factors in causing the toxicity of nanoparticles on cancer cells in this study.

Researchers use the HepG2 cell line in many biological studies investigating cytotoxicity. Hepatocellular carcinoma is an important and common cancer worldwide (12). One of the most important effects of nanoparticles on cells is the induction of programmed death (apoptosis) (13).

The results of examining cell viability by XTT test mainly indicate dose- and time-dependent cytotoxic effects. The difference in concentration and time has been the effective factors in causing the toxicity of nanoparticles on cancer cells in this study.

The study of Yang et al. regarding the toxic effect of reduced graphene oxide on the HepG2 cell line shows a dose- and time-dependent effect on cellular DNA, oxidative stress and finally cell lethality (14). These findings were confirmed in the present study regarding Gold-rGO nanoparticles in HepG2 cell line. Based on the results of this study, the lethality of nanoparticles in high concentrations was higher than the control group, and increasing the duration of incubation of nanoparticles with cells also increased the lethality.

Vallabani et al. investigated the toxicity of graphene oxide at different times and concentrations on the cell line (BEAS-2B) and the results of the XTT test in this study showed a significant decrease in the level of cell viability, which increased in a dose-dependent and time-dependent manner (15).

The results obtained in the examination of the cells treated with nanoparticles by invert microscope were in line with the results of the XTT test and the higher the concentration of nanoparticles and the longer the duration, the more vacuoles were seen and the number of living cells decreased.

The acridine elbow ethidium bromide (EtBr/AO) test showed that gold-rGO nanoparticles induced apoptotic death in HepG2 cancer cells at high concentrations. In the study of Siddique et al., the apoptotic effect of graphene and zinc oxide was investigated by (EtBr/AO) test (16). By increasing the expression of caspase 3 gene, cells go towards death through apoptosis. Caspase 3 is the terminal caspase in both the mitochondrial and extrinsic pathways. The comparison of the results of the caspase 3 gene expression in this study showed that with the increase in the dose of nanoparticles and longer duration increased the expression of the caspase 3 gene, which caused an increase in the rate of apoptosis and cell death (17).

Chatterjee et al. treated two substances of graphene oxide and reduced graphene oxide in different concentrations and durations on the cells and evaluated the level of caspase 3 gene expression in them. The results of the investigation showed an increase in the expression of the caspase 3 gene and as a result apoptosis with increasing concentration and duration, which is in line with the results of our study (18).

The evaluation of the results of the present research showed that the gold-rGO nanomaterial can have a toxic effect on HepG2 cells by increasing the expression of caspase 3 gene at a concentration higher than 50 µg/ml (group B). Therefore, in cases of therapeutic and diagnostic applications, this should be considered and shorter times and lower concentrations of this nanoparticle should be used.

Conflict of interest: None of the authors of this study, individuals or the sponsoring center have any conflict of interest to publish this article.

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