

## RESEARCH ARTICLE

# Dual Function of Gold Nanoparticles in Synergism with Mitoxantrone and Microwave Hyperthermia Against Melanoma Cells

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### Abstract

**Background:** This study was performed to evaluate any synergetic effects of mitoxantrone (MX) and gold nanoparticles (GNPs) as dual therapeutic approach, along with microwave (MW) hyperthermia for melanoma cancer. **Methods:** Various tests were performed on the DFW melanoma cell line in the presence of MX and different concentrations of GNPs, with and without MW irradiation. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyltetrazolium bromide] assays were conducted to evaluate the effectiveness of the used therapeutic methods in terms of cell survival. Relative lethal synergism (RLS) was calculated as the ratio of cell death following hyperthermia in the presence of a treatment agent to that after applying hyperthermia in the absence of the same treatment agent. **Results:** Results showed MX and GNPs under MW irradiation to provide maximum cell death ( $P < 0.001$  compared to the other groups). The mean RLS for MW hyperthermia along with the MX-GNP combination was 4.14, whereas in the absence of GNP the value for MX chemotherapy was 0.94. **Conclusion:** MX chemotherapy in the presence of different concentrations of GNP did not alter cell survival as compared to its absence.

**Keywords:** Microwave hyperthermia- chemotherapy- mitoxantrone- gold nanoparticles- melanoma

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### Introduction

Melanoma is one of the lethal diseases that cause millions of deaths worldwide and therefore made an important public health concern in the world (Ries et al., 2008; Siegel et al., 2014). The incidence of melanoma continues to rise dramatically faster than that of any other malignancy, with an approximate increase rate of 3 to 7% per year (Johnson et al., 1998).

Chemotherapy is a selected systemic treatment for non-operable melanoma, however, many patients deemed unsuitable for this kind of treatment mainly due to their underlying conditions such as drug resistance (Michaud et al., 2014; Carvajal et al., 2015; Lee et al., 2015). Over the last several years, many therapeutic methods have been developed or in progress in order to overcome drug resistance by combining with other therapies, i.e., hyperthermia, and other chemotherapy drugs such as mitoxantrone (MX), to improve the survival rate of melanoma patients (Lindsay and Barras, 2015; Macesic et al., 2015; Shields and Shields, 2015; Werthmoller et al., 2015). Hyperthermia is a rapidly developing method

of cancer therapy, which can selectively exploit greater sensitivity of tumor cells to heating at 42 °C to 46 °C without affecting normal cells (Ichinoseki-Sekine et al., 2008; Ghahremani et al., 2011; Jian-Yuan Huang et al., 2015).

On the other hand, magnetic gold-nanoparticles (GNPs) have been demonstrated effective in the treatment of disease (Gannon et al., 2008; Huang et al., 2008; Ghahremani et al., 2011; Sazgarnia et al., 2011; Sazgarnia et al., 2013). The high extinction coefficient of radiation by the GNPs, “which is due to the coherent oscillations of electrons in gold metal”, is intensified by inducing microwave (MW) radiation (Gautier et al., 2015; Kaneko et al., 2015; Rodriguez et al., 2015). This phenomenon can be used in various applications such as hyperthermia of melanoma (Sazgarnia et al., 2011; Sazgarnia et al., 2013). It has been reported that hyperthermia using microwave radiation in the presence of GNPs may be proposed as a new approach to treat leishmaniasis in future studies (Sazgarnia et al., 2013). According to the more recent publications, the excessive heat by GNPs hyperthermia cannot only damage cancer cell, but also increase the

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penetration of the drug into tumor cells (Jian-Yuan et al., 2015).

This study aimed to evaluate the synergetic effects of MX and GNPs as a dual therapeutic agent to induce chemotherapy and MW hyperthermia for melanoma cancer therapy. To the best of our knowledge, no published articles have presented a study on this issue with the methodology and analysis described here.

## Materials and Methods

### Cell culture

Experiments were carried out on DFW melanoma cancer cell line, provided from Iran Cell Bank of Pasteur Institute (Pasteur, Tehran, Iran). The cells were cultured in RPMI 1640, purchased from Gibco (Gibco, Germany), supplemented with 10% fetal bovine serum (FBS, Gibco Laboratories, Cergy Pontoise, France), 2 mM glutamine, 100 U per ml penicillin, and 100 mg per ml streptomycin (Gibco Laboratories, Cergy Pontoise, France). Cells were grown in a humidified cell incubator at 37 °C under 5% CO<sub>2</sub> atmosphere and 95% air. To perform the experiments, 96 well plates were used. Cells were seeded and 5000 cell/well was put in each plate. Before applying treatment protocols, they were allowed to adhere and grown overnight in 200 µl medium.

### Synthesis of the GNPs

GNPs were synthesized according to standard wet chemical methods, using sodium borohydride (Bhattacharya et al., 2004). Fifty mL aqueous solution was prepared by dissolving HAuCl<sub>4</sub> (Sigma-Fluka, St. Louis) with a concentration of 0.01 M in water. Using the phosphate buffer system, the ionic strength of the prepared solution was adjusted to 0.005 M and the pH was set to 7.8. Then, 50 mL non-aqueous phase (toluene) containing sodium tetraborohydrate (NaBH<sub>4</sub>) (Sigma-Aldrich, Lot no. 247677) and sodium borohydride in 0.02 M concentration were prepared separately. Both phases were mixed together and rapidly stirred. Next, the organic phase was separated and the solvent was removed by a rotary device at low pressure and at 50 °C. GNPs gathered at the bottom of the container were dispersed in a phosphate buffer solution with 0.005 M ion strength and a pH of 7.6. The solution was kept under vigorous stirring overnight (Bhattacharya et al., 2004). According to our basic protocol, stability of the solution was studied by recording its absorption spectrum by a spectrophotometer (Sazgarnia et al., 2013). Then, the GNPs were filtered through 0.22 µm paper filter. The size of nanoparticle was investigated and calculated by the use of a particle size analyzer (Zetasizer, Malvern, Massachusetts).

Before any therapeutic application, the toxicity of GNPs must be determined for healthy tissues in terms of size, shape, surface charge, and surface coating (Huang et al., 2008). In other words, there is a need to investigate the results of exposure to nanoparticles before any potential therapeutic applications, however, the overall toxicity dose of GNPs has been reported to be at an acceptable level (Fisher and Patterson, 1992). In order to evaluate the toxic effects of the synthesized GNPs, the melanoma

cells were incubated with different concentrations of GNPs including; 40, 80, 120, 134, 160, and 200 µg/mL for 40 h.

During cells incubation with GNPs the cell culture was performed in the presence of 3% FBS. It should be noted that, complete deletion of FBS may affect the cells' survival.

After synthesis, the GNP solution was kept as a stock solution. Before using solutions containing GNPs, 5 min sonication was performed.

### MTT assay

In order to investigate the effects of the used treatments methods on the cells' survival, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-iphenyltetrazolium bromide] assay was performed after 40 h post-incubation.

The MTT assay involves the conversion of the water-soluble MTT to an insoluble formazan. The MTTs was dissolved in sterile phosphate-buffered saline (PBS) at 5 mg/ml. The solution was filtered through a 0.22 µm filter and stored in dark condition at 4 °C for a period lasting less than 3 weeks.

Five wells of a 96-well plate were used for every experimental condition. The culture medium was renewed every two days to avoid possible medium product inaccuracy. Since the cell line were adhesive, their media could simply be renewed without making any damage to the cells. To perform the MTT assay, the MTT solution at appropriate concentrations (10 µl MTT solutions in each 100 µl media) was added to each well and the plates were then incubated at 37 °C for 4 h. The incubation was followed by formation of purple formazan salts crystals, from metabolically active cells. Following the incubation, the remaining MTT solution was removed and 100 µl of Dimethyl sulphoxide (DMSO) was added to each well to dissolve the formazan crystals. The plates were shaken for 5 min on a plate shaker to ensure adequate solubility. Absorbance readings of each well was performed at 570 nm (single wavelength) using an ELISA reader (Stat Fax 2100, Awareness Technology, Palm City). The number of viable cells was directly correlated to the amount of formazan crystals formed. To reduce the variability inherent to the assay used, all tests were performed for three independent experiments.

### MX chemotherapy

MX was purchased from EBEWE Pharma (Ges.m.b.H, Austria). MX chemotherapy drug utilized in this project had a molecular weight of 444.48 g/M and a chemical formula of C<sub>22</sub>H<sub>28</sub>N<sub>4</sub>O<sub>6</sub>. MX chemotherapy was performed at different concentrations, including 1, 3, 6, 12 and 50 µg/mL. The incubation time of MX with melanoma cancer cells was 40 h. The percentage of dead cells was calculated and compared with untreated cells.

### MX-incubated GNPs chemotherapy

It has been reported that GNPs can facilitate the macromolecules' entry into host cells (Jen et al., 2004; Gautier et al., 2015; Kaneko et al., 2015). Therefore, GNPs are promising vehicles for drug and gene delivery because these particles are readily conjugated with biomolecules at a high packing density (Rojas-Chapana et al., 2005). This

can be used in various applications such as MX delivery drugs into melanoma cells. In this study, MX with GNPs chemotherapy was also performed to investigate the effects of this treatment modality on melanoma cancer cells.

#### *MX chemotherapy and MW hyperthermia*

The hyperthermia protocol was in accordance to that in our previous publication (Ghahremani et al., 2011). Flasks containing 10 mL cell suspension in the culture medium were exposed to 2450 MHz MW source (Panasonic MW generator, model NN-ST565W, Japan). Since the efficiency of MW hyperthermia depends on the heating time and temperature, in separate triplet measurements, the changes in temperatures were recorded at 5 s intervals by a digital thermometer (APPA51, Shenzhen Laesent Technology, Shenzhen, China) equipped with K-type thermocouple and 0.1 °C sensitivity. The curve of temperature variations versus exposure time was then drawn. The exposure time required for the samples to reach 39.5 °C was determined as 50 s. It must be noted that 42 °C is in the temperature range which normal tissue cells can tolerate (Sukiyama et al., 1994). The MW hyperthermia was done on the plates containing different concentrations of MX including 1, 3, 6, 12 and 50 µg/mL. The hyperthermia temperature achieved in the presence of GNPs was 2.5 °C higher in comparison with that in the absence of GNPs. Considering the temperatures curve of the hyperthermia procedure and our general protocol (Ghahremani et al., 2011), the hyperthermia irradiation was adjusted on 42 °C for 10, 15 and 20 min.

#### *Microwave hyperthermia mediated GNPs*

Taking into account that thermal power dissipation of MW exposure is mainly dependent on the GNP concentrations (Ghahremani et al., 2011), in this work changes in temperatures was separately followed at all the above mentioned GNPs concentrations.

#### *MX-incubated GNPs chemotherapy and MW hyperthermia*

MX-incubated GNPs chemotherapy was performed at 10, 15 and 20 min MW hyperthermia. At first, to determine the optimum GNPs and MX concentrations the cells were incubated with fresh medium containing serial concentrations of GNPs (40, 80, 120, 134, 160, and 200 µg/mL) and MX (1, 3, 6, 12 and 50 µg/mL) for 40 h. Then, the survival of the cells was investigated and compared with the control groups.

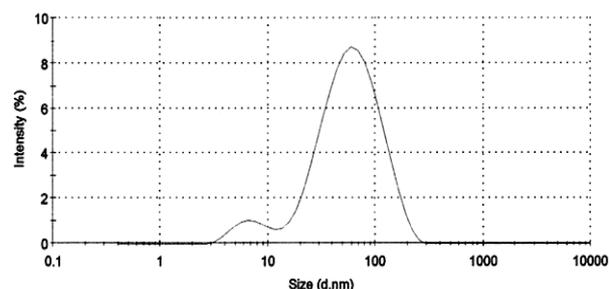


Figure 1. Distribution Curve of GNPs, Based on Their Particle Size (Average Diameter, 49 nm)

#### *Statistical analysis*

Mean values and standard deviations were calculated and statistical significance of the differences between the studied groups was evaluated. All data were analyzed using one-way ANOVA and Tukey test on SPSS software (version 16.0, Chicago, IL) after performing normality test. A significant level of 0.05 was considered to compare the data.

In order to determine the relative lethal synergism (RLS) effect of MW hyperthermia in the presence or absence of the MX-incubated GNPs, the RLS fraction was calculated and compared between the studied groups (Ghahremani et al., 2011). RLS was calculated by the ratio of cell death following hyperthermia in the presence of a treatment agent over cell death after applying the hyperthermia in the absence of the same treatment agent. A RLS > 1 confirms the synergic effect between hyperthermia and the agent (Ghahremani et al., 2011).

## Results

#### *GNPs Characterization*

A size histogram curve of the GNPs, recorded by particle size analyzer using dynamic light scattering (DLS) method, showed that the mean diameter of the GNPs was 49 nm. Figure 1, illustrates distribution curve of GNPs, based on their particle size. Other main characteristics of the synthesized GNPs are as follow: 531 nm absorption peak, -4.53 mV Zeta potential (ZP), and Polydispersity index (PDI) of 0.438.

The toxicity of the GNPs at different concentration is shown in Figure 2. According to this figure, the optimum GNP concentration was found to be 134 µg/mL and this concentration was used for the next study experiments.

#### *MX chemotherapy and MX-incubated GNPs chemotherapy*

Figure 3, gives the results of MTT assay on incubated cells with different MX concentrations of 1, 3, 6, 12 and 50

Table 1. The Mean of Calculated RLS for the Groups Treated by MW Hyperthermia in the Presence and Absence of MX Chemotherapy and GNP. The RLS presents the ratio of cell death occurred for several concurrent therapeutic agents divided by the summation of the cell death after applying each one of therapeutic agents. Since cell survival was not entirely independent of GNPs and MX concentrations and MW irradiation time, the Mean Relative Lethal Synergism was used for the groups.

| Therapeutic agents | GNPs concentration (µg/mL) | MX concentration (µg/mL) | Exposure time of MW (min) | Mean Relative Lethal Synergism (RLS ± SD) |
|--------------------|----------------------------|--------------------------|---------------------------|---|
| MX with MW         | 0                          | Jan-50                   | 10-20                     | 0.94 ± 0.10                               |
| GNP with MW        | 134                        | 0                        | 10-20                     | 0.78 ± 0.4                                |
| GNP with MX        | 134                        | Jan-50                   | 0                         | 0.79 ± 0.04                               |
| MW with MX and GNP | 134                        | Jan-50                   | 10-20                     | 4.14 ± 1.67                               |

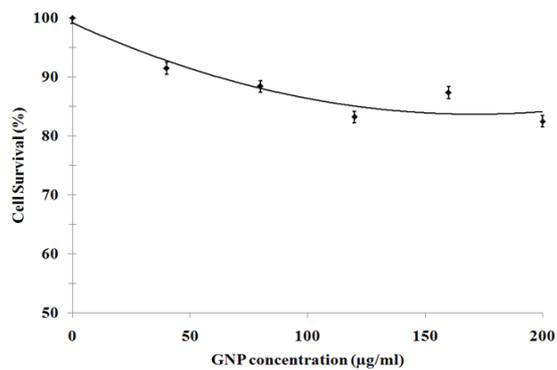


Figure 2. Melanoma Cell Survival Following Incubation at the Different Concentrations of GNPs.

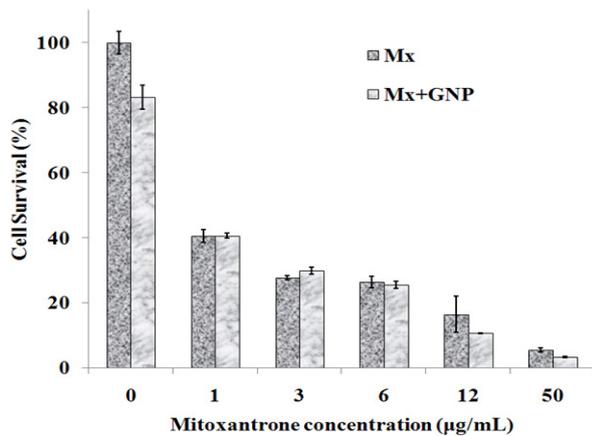


Figure 3. The Obtained Data from MTT Assay in Term of Cell Survival at Different MX Concentrations in the Absence and Presence of 134 µg/mL GNP.

µg/mL. A statistically significant difference ( $p < 0.001$ ) was observed by comparing the mean cell's survival at all the used concentrations of MX with the control group (group that received no MX). The cell survival was significantly ( $P < 0.001$ ) lower for the MX concentration of 50 µg/mL, compared to the other treated groups, whereas, there was no significant difference ( $P = 0.992$ ) between the groups receiving 3 and 6 µg/mL MX.

Data revealed that, MX chemotherapy at the presence of GNP with different concentrations did not alter the

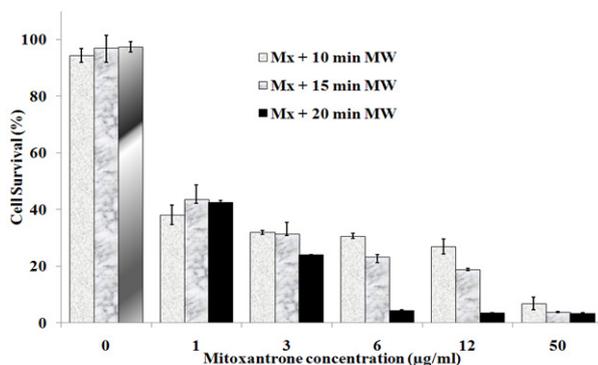


Figure 4. Cell Survival Incubated with Different Concentrations of MX Chemotherapy and Also at 10, 15 and 20 min MW Hyperthermia.

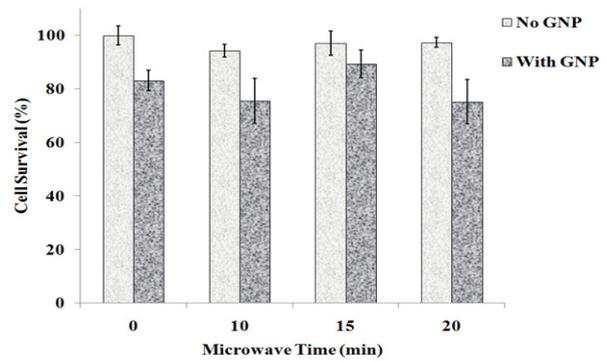


Figure 5. Survival of the Cells Under MW Irradiation for 10, 15 and 20 min in the Presence and Absence of 134 µg/mL Concentrations of GNP.

melanoma cell's survival compared to those in the absence of GNP ( $P > 0.050$ ), except for 0 µg/mL ( $P = 0.012$ ), 3 µg/mL ( $P = 0.038$ ), and 50 µg/mL ( $P = 0.024$ ) concentrations. *MX chemotherapy and MW hyperthermia*

Figure 4, demonstrates the results of MX chemotherapy and MW hyperthermia on the cell survival at different irradiation times of 10, 15 and 20 min. This figure illustrates that, for the MX chemotherapy treated groups at MX concentrations of  $> 1$  µg/mL, the cell survival was significantly ( $p < 0.001$ ) lower for the irradiation times of 20 min, compared to 10 and 15 min. Whereas, there was no significant difference between 10 and 15 min MW irradiated groups at 1 and 3 µg/mL MX concentrations ( $P = 0.512$ ). As expected, the maximum decrease in cell survival was observed for the cells irradiated for 20 min at 12 and 50 µg/mL MX. Differences between these two groups were not significant ( $P = 0.998$ ).

*Microwave hyperthermia mediated GNPs*

Figure 5, shows the survival of the cells incubated with 134 µg/mL concentrations of GNP in the presence of MW irradiation for 10, 15 and 20 min. It was found that MW hyperthermia for 20 min significantly decreased the cell survival ( $P < 0.001$ ) compared to other treated groups for 10 and 15 min. Differences were also significant for the 10 min irradiated group compared to those of 15 min irradiation ( $P < 0.001$ ).

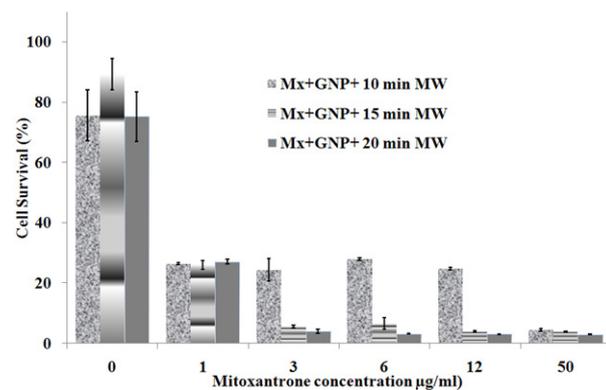


Figure 6. The Cell Survival at Different Concentrations of MX and 134 µg/mL GNP, Irradiated with MW for 10, 15 and 20 min.

*MX-incubated GNPs chemotherapy and MW hyperthermia*

Following MX-incubated GNPs chemotherapy and MW hyperthermia, the cell survival was significantly decreased at 20 irradiation time ( $P < 0.001$ ) compared to the group that received no treatment. A significant decrease of cell survival was also observed for the 10 and 15 min irradiated groups compared to the control one (group that received no MX) ( $P < 0.001$ ). Figure 6, illustrates the cell survival at different concentrations of MX and 134  $\mu\text{g/mL}$  GNP, irradiated with MW for 10, 15 and 20 min.

Moreover, as can be seen in the Table 1, the mean of RLS for MW hyperthermia along with the MX-incubated GNP chemotherapy was obtained 4.14, whereas in the absence of GNP, the RLS for MX chemotherapy was estimated 0.94. Table 1, gives the mean of calculated RLS for the groups treated by MW hyperthermia in the presence and absence of MX chemotherapy and GNP.

## Discussion

The probability of cure of melanoma cancer patients using chemotherapy treatment is mainly related to the stage of cancer (Johnson et al., 1998; Macesic et al., 2015; Shields and Shields, 2015; Werthmoller et al., 2015). In addition, due to drug resistance, a novel therapeutic method is needed in order to overcome this resistance in melanoma patients (Carvajal et al., 2015; Lee et al., 2015; Lindsay and Barras, 2015). Considering the limitations associated with melanoma treatment, in this study, a new combinational approach has been proposed using MX and GNPs to intensify MW hyperthermia and chemotherapy.

MX is a topo II inhibitor that disrupts DNA synthesis and causes double strand breaks (Sazgarnia A et al., 2013). The molecular mechanism of action of this drug is complex, however it includes free radical generation (Fischer and Chan, 2007), the induction of long-term DNA damage (Crooke et al., 1981; Kapuscinski and Darzynkiewicz, 1986) and intercalative binding (Lown JW et al., 1985). Some researchers have investigated that the MX-induced genotoxicity occurred through at least two mechanisms, DNA intercalation and/or redox cycling as well as topo II inhibition (Smart et al., 2008).

In our work, the melanoma cells were incubated with MX at different concentrations and the cell viability was determined by MTT assay. Our findings revealed that, MX concentration played an important role on the cell viability, however for the treatment of melanoma, more MX concentrations ( $>50 \mu\text{g/mL}$ ) may be toxic to the healthy cells (Figure 2).

As stated earlier, GNPs may facilitate the macromolecules' entry into host cells for chemotherapy drug delivery (Huang et al., 2008; Kaneko et al., 2015). In the present study, this phenomenon was used to ease the MX delivery into melanoma cancer cells.

A number of researchers have shown GNPs' uptake by the mammalian cancer cells and its mechanism [Song et al., 2013; Dykman and Khlebtsov, 2014]. Some of them have determined that GNPs endocytosis reaches to maximum at the diameter of 50 nm. In these studies culture medium was contained 10% FBS or FCS (Song et al., 2013; Dykman and Khlebtsov, 2014).

It's necessary to mention that, we utilized the 49 nm nanoparticles. Also, after cells incubation with GNPs the medium was removed and cells washing was performed. Thus GNPs were not remained on the bottom of the dish or in the fluid medium.

Chen et al., (2009) studied the toxicity of a wide size range of injected GNPs with spheres of 3 to 100 nm diameter in mice. They found that, at the dose they were used, the smallest size (less than 5 nm) and the largest size (50 to 100 nm) of GNPs are not toxic. However, they stated that the intermediate size range of 8 to 37 nm had lethal effects on mice. They reported that the systematic toxicity of this size range was linked to major organ damage in the liver, spleen, and lungs. While, in the same study, the same size nanoparticles were not toxic on Hela cell line (Chen et al., 2009). This study has demonstrated a large inconsistency between the in vitro and in vivo results, and highlights the notion that in vitro experiments may not lead to good predictions regarding in vivo results. Nevertheless, in vitro studies are a critical and first step for evaluating treatment outcomes of new modalities.

On the basis of our study, MX-incubated GNP chemotherapy did not affect the melanoma cell's survival compared to MX chemotherapy alone ( $P = 0.231$ ). Chemotherapy was also performed under MW hyperthermia. Hyperthermia, is a rapidly developing method of cancer therapy and is defined as a temperature enhancer of all or a portion of the body to a temperature that exceeds the normal physiologic limit which exploits greater sensitivity of tumor cells to heating at 42–46 °C (Ghahremani et al., 2011). During hyperthermia, cell killing, physiologic changes, and changes in cell signaling and DNA damage repair provide rationale for combining hyperthermia with other cancer therapeutic methods, such as chemotherapy. We considered the experimental groups of MX chemotherapy with MW hyperthermia at different irradiation times. There was no significant difference in the mean survival of treated cells using MX chemotherapy and MW hyperthermia compared to the MX chemotherapy groups. Whereas, the mean survival of the treated cells using MX chemotherapy and MW hyperthermia was significantly decreased compared to the control group (Figure 4). Moreover, the synergetic effects of MW was seen only from MX concentrations of above 3  $\mu\text{g/mL}$ . Whereas, at the highest concentrations and above 15 min MW irradiation the effects were exposure time-independent.

Today's, most conventional hyperthermia systems available in clinical applications are equipped with 2450 MHz generators and non-contact applicators (Ichinoseki-Sekine et al., 2008), similar to the used source in this work. Previously, interaction between oscillating magnetic fields and magnetic nanoparticles in the production of hyperthermia has been studied (Johnsen et al., 2005). Moreover, gastrointestinal cell destruction following radiofrequency exposure in the presence of GNPs has been reported (Gannon et al., 2008). Nonetheless, few studies have evaluated the application of MW hyperthermia and chemotherapy in the presence of GNPs (Gannon et al., 2008; Ghahremani et al., 2011; Gautier et al., 2015). Similar to drug delivery properties, depending on

particle size, composition, structure and physico-chemical properties, nanoparticles have demonstrated a diverse range of useful applications in hyperthermia. Previously, our research team investigated that, the presence of GNPs along with MW irradiation led to a decrease in survival rate of osteosarcoma cell line (Ghahremani et al., 2011).

Survival of the cells under MW irradiation in the presence and absence of 134 µg/mL concentrations of GNP was also studied (Figure 5). The mean cell survival percentage of the groups received MW treatment showed a non-significant difference, in comparison to the groups received no irradiation. This finding reflects that MW treatment had no added effect on GNP toxicity.

Finally, MX-incubated GNPs chemotherapy and MW hyperthermia was performed as a dual treatment for the melanoma cells. It was found that cell's survival was significantly decreased due to MX chemotherapy and MW hyperthermia dual treatment compared to the control group (Figure 6). As can be seen from figure 6, the observed effects were dependent on MW exposure time and MX concentration. The maximum RLS was seen for MX-incubated GNP chemotherapy with MW hyperthermia. But our findings showed that, GNPs cannot be efficiently provided a useful synergism with MX chemotherapy without MW hyperthermia against melanoma cells. Therefore, according to our investigations, in addition to the excessive heat by GNPs hyperthermia another mechanism may be enrolled in cancerous cell toxicity and damage, due to the presence of MX-incubated GNP chemotherapy with MW hyperthermia.

Considering that this is the first study dealing with melanoma treatment using the mentioned methods, further in vivo studies on mammals are of utmost importance.

In conclusion, in this study the synergic effects of MX-incubated GNPs as dual treatment of microwave hyperthermia and chemotherapy for melanoma cancer therapy was evaluated. Results showed that, melanoma cells's survival was significantly decreased due to MX chemotherapy in synergism with GNPs hyperthermia under MW irradiation.

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#### Conflict of Interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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