

The Application of ATR Kinase Inhibitor AZD6738 in Combination with Radiotherapy for the Treatment of Melanoma

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ABSTRACT

Background: Melanoma is categorized as one of the most malignant, severe, and lethal cancers of the skin. Regarding the lack of efficiency of conventional therapies for most patients, novel therapeutic strategies are strongly required.

Objective: The current study aimed to assess the impact of AZD6738- an ATR kinase inhibitor- in combination with 6 MV X-ray on the human melanoma cell line (A375).

Material and Methods: In this experimental study, cells were treated with different concentrations of AZD6738 for 24 and 48 h in the presence and absence of radiation (2 Gy, 4 Gy, and 6 Gy). The cell viability and cell proliferation assay were examined in both experimental and control groups by MTT and colony formation techniques, respectively.

Results: The results indicated that by increasing the concentration of AZD6738, the cell viability was markedly diminished in all treatment groups. As expected, the cell viability of the cells treated with AZD6738 and radiation was significantly lower than the group treated with AZD6738 alone. Besides, the combinatory treatment significantly decreased cell proliferation in the melanoma cell line. The combination of AZD6738 with radiation resulted in a significant increase in cytotoxicity by a 50% increase in cell death when used at concentrations of 0.3 μ M, 1 μ M, 1.51 μ M, and 1.61 μ M, respectively.

Conclusion: The combination of AZD6738 with radiation possesses a synergistic effect on the reduction of the cell viability and proliferation of melanoma cells. This present study provides insight into the impact of Ataxia Telangiectasia and Rad3-related kinase (ATR) inhibition on the potential role of this kinase in the suppression of melanoma cell proliferation.

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Keywords

AZD6738; Melanoma; Radiotherapy; Cell Proliferation

Introduction

Melanoma is clinically classified as one of the most aggressive and deadly types of skin cancers. The etiology of melanoma is still unclear; however, different factors, including UV radiation, genetic susceptibility, and environmental factors, are thought to play a role in developing the disease. Melanoma is simply characterized by the uncontrolled proliferation of melanocytes. Melanocytes

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are responsible for the production of pigment in the skin's epidermis, Iris, and hair follicles. Therefore, melanoma tumors usually appear as brown- or dark-colored patches. Nevertheless, in rare conditions, melanoma cells do not synthesize any pigment, and cancerous tissue is manifested as red-, pink-, or purple-colored patches. The incidence of melanoma has sharply increased in recent years that may be due to direct exposure of the skin to ultraviolet radiation, especially in Caucasians [1-4].

Various international strategies might be employed for the treatment of patients with melanoma, such as surgery, chemotherapy, biological treatments, and radiotherapy, or even a combination of them. Surgery is a conventional therapeutic method for the removal of typical tumors. Radiotherapy is another intervention that could be used as a major treatment or complementary therapy, targeting the depth of tumors by means of X or gamma rays. The dose of 1.8-2 (Gy) per fraction is usually used for most cases with melanoma [5-10].

Melanoma cells are pathologically categorized as one the most resistant cells to irradiation [11], and the recurrence and metastasis are considered major problems following surgery [12-16]. On the other hand, chemotherapy generally cause severe cytotoxicity and other complications in patients with melanoma [17, 18].

Hence, to prevent the progression of tumor cells, different techniques have been designed to overcome the resistance of cancerous cells to irradiation. One of these strategies is the simultaneous usage of radio-sensitizer drugs. In the current research, a combination of both AZD6738, as an Ataxia Telangiectasia and Rad3-related kinase (ATR) inhibitor, and radiotherapy was applied to induce cell death in the melanoma cancer cell line.

AZD6738 is a potent inhibitor of ATR and a radio-sensitizer drug, possessing an IC₅₀ (half maximal inhibitory concentration) of 1 nM and considerable pharmacokinetic properties [19, 20]. ATR proteins are essential proteins

widely expressed in mammals and belong to the phosphatidylinositol 3-kinase-related kinase (PIKK) family. These classes of proteins are involved in phosphorylation of thousands of other proteins, such as Chk1, to control the cell cycle process, as well as DNA replication repair. The kinases activity ATR family members are increased when DNA is damaged, and it can repair breaks in double-stranded DNA. Several lines of evidence show that ATRs effectively contribute to cell proliferation.

As a result, specific inhibitors of ATR proteins not only cause a decrease in the expression of Chk1 and the accumulation of cells in the S phase of the cell cycle but also impede proliferative signals and halt cell proliferation. These types of proteins are able to diminish the rate of phosphorylation in those molecules that play a role in DNA Damage Repair (DDR) as well as the expression of the HU protein. In other words, ATR inhibitors are capable of deactivating DDR molecules in the nucleus and prevent the transfer of these molecules into the cytoplasm [20-26].

It has been demonstrated that the kinase activity of ATR proteins is increased in response to hypoxia, and the inhibitors of these proteins could sensitize radioresistant cells in hypoxic conditions. So, ATR inhibitors could have marked therapeutic properties; as they possess a major impact on cancer cells when compared with normal cells [27-29]. AZD6738 is currently used in phase II of clinical trials. This drug is employed alone or in combination with other anticancer agents, such as chemotherapeutic molecules or irradiation. It has been revealed that this drug sensitizes different cell lines to anticancer agents and causes synergistic effects when combined with other genotoxic agents [20-23]. According to previous studies, AZD6738 can induce cell death and senescence in cancer cells [30-32]. In the present study, the cytotoxicity of AZD6738 in combination with 2, 4, and 6 (Gy) of 6 MV X-ray was investigated on the A375 cell-line by means of MTT and colony formation assays.

Material and Methods

Cell Culture

In this experimental research, the A375 cell-line derived from human melanoma cells was procured from the Pasteur Institute, Tehran, Iran. At first, cells were first cultured in high-glutamine Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and penicillin/streptomycin. They were incubated in a humidified atmosphere with 5% CO₂ at 37 °C.

Afterward, cells were recovered from flask cell culture flasks by 0.25% Trypsin-Ethylendiaminetetraacetic Acid (EDTA) solution, according to the manufacturer's recommendations.

Irradiation and the treatment protocol

AZD6738 was obtained from Selleckchem (Texas, US). For the cell culture purpose, AZD6738 was first dissolved in dimethyl sulfoxide (DMSO) at a concentration of 30 mM and then diluted by DMSO to reach chosen concentrations. Next, A375 cells were irradiated with the X-Ray at doses of 2, 4, and 6 Gy using a linear accelerator (Linac 600, GMV; Varian Medical Systems; USA). A dose rate of the apparatus was set at 200 cGy/min and a field size of 35×30 cm². The cell culture flasks were irradiated from the posterior side, and 3 layers of tissue-equivalent materials at a depth of 1 cm were positioned under the cell culture flasks to guarantee the electronic equilibrium.

MTT assay

For the determination of the cytotoxicity of AZD6738 and irradiation, the cell viability was examined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described. In brief, A375 Cells at a density of 1×10⁴ cells per well were seeded onto a 96-well plate and then incubated in an incubator at 37 °C with an atmosphere of 5% CO₂. Then, the cells were treated with

various doses of AZD6738 (0.1, 0.3, 1, 2, 3, 4, 8, 10, 16, 30 μM) for 24 and 48 hours alone. A group of cells was co-treated with different concentrations of AZD6738 and the 6 MV X-ray irradiation. At the end of the experiment, 50 μL of the MTT solution was added to each well, and the plate was incubated at 37 °C for 4 hours. Afterward, the cell culture medium was discarded. The resulting formazan crystals were dissolved in 100 μL DMSO, and the optical absorbance of the wells was recorded at a wavelength of 540 nm by a microplate reader. The cell viability was expressed as a percentage and determined by comparison of the optical absorbance of treated cells with untreated cells. By means of the following formula, the cell viability was assessed: Viability = treated cell absorbance/untreated cell absorbance×100.

Colony formation assay

In this assay, cells were cultured in a 6-well plate and treated with 0.3 μM and 1 μM AZD6738 for 48 hours. Next, AZD6738 was removed, and cells were cultured in fresh media at different concentrations for further 10-12 days. The colony formation was evaluated using the staining of the cells with 0.5% crystal violet dissolved in 95% ethanol. The cells were imaged under an XL70 inverted microscope (Olympus).

The surviving fraction was calculated as the number of colonies of treated cells divided by that for the control cells.

The radiation dose-survival curves were fitted to a linear-quadratic (LQ) equation, surviving fraction = exp(-αD - βD²), where D represents a dose of the X-ray. For the evaluation of drug-irradiation interaction the Sensitizer Enhancement Ratio (SER) was utilized and estimated by fitting the ratio to the LQ model as follows;

$$SER_{x\%} = \frac{d_{x\%}(\text{no drug})}{d_{x\%}(\text{drug})}$$

In this equation $d_{x\%}$ (no drug) represents the dose of radiation (Gy) required to gener-

ate $x\%$ cell survival in the absence of a particular drug, while $d_{x\%}$ (drug) represents the dose of radiation (Gy) required to generate $x\%$ cell survival in the presence of a specific drug (e.g., AZD6738). The values of SER were determined at doses in which the surviving fractions were 10% and 50%. The experiments were repeated in triplicate to ensure achieving repeatable results.

Statistical analysis

All of the experiments were performed twice or three times. The error bars denote standard errors of the means obtained from multiple experiments. The statistical analysis was carried out by independent T-test or one-way analysis of variance (ANOVA) where appropriate, followed by Scheffe's post hoc test. The IC50 values were measured using the CalcuSyn software, and SER values were calculated by GraphPad Prism. The level of statistical significance was set at $p < 0.05$.

Results

Cell death evaluation following irradiation and drug treatment

The MTT assay was employed for the assessment of the viability of A375 cells in response to various treatments. For this purpose,

cells were treated with different concentrations of AZD6738 (0.1, 0.3, 1, 2, 3, 4, 8, 10, 16, and 30 μM) and then incubated for 24 and 48 hours. Figure 1 shows the percentage of cell viability after the treatment courses for 24 and 48 hours. As depicted in Figure 1, by increasing the concentration of AZD6738, the cell viability was significantly decreased in comparison with the control group (cells receiving no treatment). Also, such a reduction was more pronounced in cells incubated for 48 hours, than the cells incubated for 24 hours. According to the results of the MTT method, the IC50 values of AZD6738 were 15 μM and 6 μM after 24 and 48 hours of incubation, respectively. For the evaluation of the impact of AZD6738 and irradiation on melanoma cells, the following concentrations of AZD6738 (2, 3, and 4 μM) were applied. Also, According to Figure 2, cells treated with a combination of AZD6738 and X-ray exhibited a greater reduction in cell viability compared with those treated with only AZD6738.

The ability of colony formation by A375 cells treated with AZD6738 and 6 MV X-ray

The effects of AZD6738 in combination with irradiation on the proliferation of A375 cells were determined by the colony formation

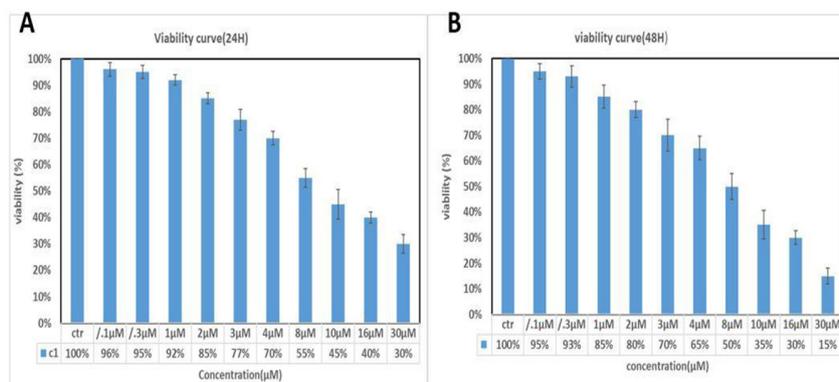


Figure 1: The viability of A375 cells following the treatment with various concentrations of AZD6738 after 24 (A) and 48 (B) hours of incubation. The cell viability was determined by the MTT assay. The obtained values are expressed as the means \pm standard deviation (SD) of 3 independent experiments.

assay. For this purpose, two concentrations of the drug (0.3 and 1 μM) alone and in combination with irradiation were utilized.

Figure 3 represents the survival fraction of cells treated with AZD6738 and radiotherapy. By increasing the concentration of AZD6738, the number of colonies was significantly decreased compared with the control cells (cells receiving no treatment). In cells treated with both the drug and radiotherapy, the ability of the cells to form colonies was more diminished than the cells treated with only AZD6738.

The values of alpha and beta in the LQ equa-

tion are shown in Table 1 in which they were utilized for the calculation of the surviving fraction = $\exp(-\alpha D - \beta D^2)$, where D represents the dose of irradiation. The values of SER_{50} were assessed for the cells treated with AZD6738 at concentrations of 0.3 and 1 μM in the presence of irradiation. The application of combined irradiation and AZD6738 resulted in remarkable radio-sensitizing effects with the SER_{50} values of 1.51 and 1.61, respectively confirming that AZD6738 is an efficient radio-sensitizer agent for inducing cell death in the A375 cell line.

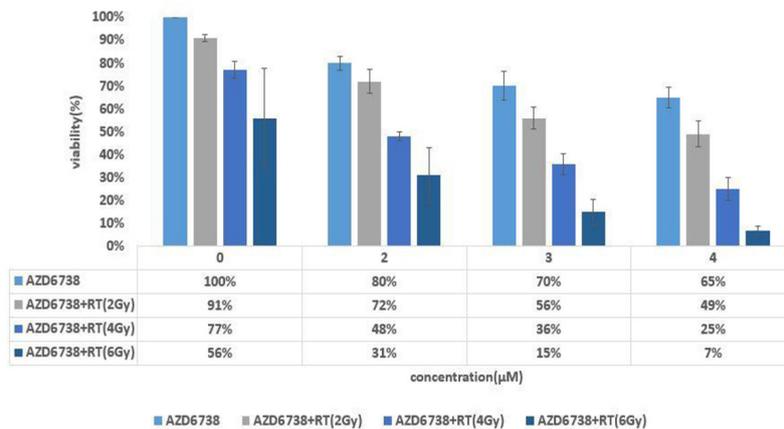


Figure 2: The viability of A375 cells following AZD6738 and radiation treatment. After the incubation with 2, 3, 4 μM AZD6738 for 48 hours, the cells were exposed to 2, 4, and 6 Gy of 6 MV X-ray; then, the cell viability was measured by the MTT assay. The values are expressed as the means \pm standard deviation (SD) of 3 independent experiments.

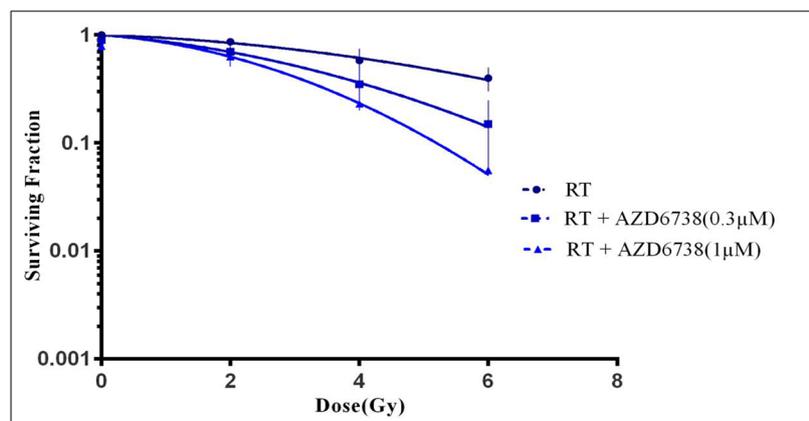


Figure 3: The cell survival curves of irradiation in combination with AZD6738. The A375 cell line was treated with 0.3 and 1 μM AZD6738 and the X-ray at doses of 2, 4 & 6 Gy. Values are shown as the means \pm standard deviation (SD) of 3 independent experiments.

Table 1: The values of radiobiological parameters. The mean values of α , β , and Sensitizer Enhancement Ratio (SER)₅₀ for A375 cells, as estimated by fitting the cell survival rate to the linear-quadratic (LQ) model.

Treatment	$\alpha \pm SD$	$\beta \pm SD$	SER ₅₀
X-ray	0.0717±0.003	0.018±0.01	-
X-ray + AZD6738 (0.3 μ M)	0.09±0.01	0.06±0.04	1.51
X-ray + AZD6738 (1 μ M)	0.11±0.002	0.03±0.002	1.61

SD: Standard Deviation, SER: Sensitizer Enhancement Ratio

Discussion

Patients suffering from melanoma may undergo surgery, chemotherapy, biological treatment, and radiotherapy, or sometimes may receive a combination of these therapeutic strategies. The most conventional method for the treatment of melanoma is surgery. Radiotherapy is also performed as major or adjuvant therapy [5-10]. Melanoma tumors are among the most resistant types of cancer to irradiation. Furthermore, after surgery, tumors can invade other parts of the body, which may cause the tumor recurrence [12-16]. The use of chemotherapeutic agents for patients with melanoma may lead to the development of cytotoxicity and emerging some side effects such as nausea, vomiting, and alopecia [17, 18].

In addition, in spite of numerous treatment methods, there is still no definitive treatment strategy for this type of skin cancer. Thus, to improve the treatment of melanoma tumors, applying new methods to be able to sensitize cells to irradiation would be required. One of these strategies is the utilization of the combination of radiosensitizing drugs/chemotherapeutic agents and radiotherapy. In this study, we used the combination AZD6738, an ATR inhibitor, and radiotherapy. ATR and ATM (Ataxia Telangiectasia Mutant) proteins belong to a group of protein kinases that are involved in DNA damage signaling. These proteins phosphorylate several thousands of different substrates. The kinase activity of ATR is activated in one-ended DNA double-strand breaks (DSBs) and damaged replication forks. The inhibitors of ATR are prescribed in

the early-phase clinical trials alone or combined with DNA-damaging agents and irradiation, as well as in combination with other newly-discovered chemotherapeutic molecules. Hence, we examined an orally active specific inhibitor of ATR, AZD6738, which is used in phase I of clinical trials [21, 27, 33, 34].

As demonstrated in Figure 1, in parallel with an increase in the concentration of AZD6738, the cell viability is significantly declined in comparison with the control cells (cells receiving no treatment). Furthermore, a reduction in cell viability is time-dependent. Our data showed that by increasing the treatment time from 24 h to 48 h at the highest concentration, the cell viability is decreased from 30% to 15%. Consequently, it is inferred that the drug is absorbed in a longer period, resulting in higher degrees of toxicity. So, the optimal time for the drug absorption for the cell is 48 hours. Also, according to Figure 2, in cells treated with AZD6738 and X-ray, a higher reduction was observed in the cell viability compared with those treated with AZD6738 alone, denoting that irradiation caused higher rates of cytotoxicity, leading to an increased rate of cell death. As shown in Figure 2, a strong inhibitory effect of 4 μ M AZD6738 in combination with the X-ray (6 Gy) on melanoma cells, which may be owing to the cell cycle arrest at the G2/M phase. Because of this inhibitory effect, the cell viability rate in combination therapy reached 7%. Therefore, it is concluded that by increasing the drug concentration, the cell death rate would be increased significantly. However, such a cell death rate

would be greater in combination therapy. These findings were in agreement with the results of Kim et al. They investigated the effect of monotherapy with AZD6738 on 13 different breast cancer cell lines. They found that by increasing the drug concentration, the cell viability is dramatically decreased [26]. Moreover, Checkley et al. demonstrated that different concentrations of AZD6738 along with the application of the X-ray at a dose of 6 Gy decreased the tumor size of colon cancer tissues to 0.1 cm³, while such a rate was 0.4 cm³ when only the drug was applied. Their results were in line with the findings of our study [35]. In order to confirm the MTT assay results, colony formation assay was examined. Figure 3 indicated that by increasing the dose of the X-ray from 2 Gy to 6 Gy, the percentage of SF for the A375 cell-line was remarkably decreased so that the SF rate was diminished from 1 (for control cells) to less than 0.1 (for cells treated with 1 μM AZD6738 in combination with irradiation). However, there was no significant difference between cells treated with either 0.3 μM or 1 μM AZD6738 at lower doses. According to Table 1, the values of SER₅₀ for AZD6738 at the concentrations of 0.3 μM and 1 μM in combination with radiotherapy was calculated to be 1.51 and 1.61, respectively. So, the radio-sensitizing effect was markedly tangible when the cells were treated with AZD6738 and 6 MV X-ray. Also, there was no significant difference in radio-sensitization effects when different concentrations of AZD6738 were used. Indeed, AZD6738 inhibits the activation of proliferative signals [20-27]. These results were consistent with the findings obtained by Dillon et al. They demonstrated that radio-sensitization of the cells with the application of AZD6738 and single radiation fractions is independent of both p53 and BRCA2 when multiple cancer cell lines were examined. They concluded that utilizing AZD6738 as monotherapy is p53-independent. Also, their clonogenic assay results showed the radio-sensitization of both p53

wild-type and p53 mutant cells when treated with AZD6738. Also, the SER₅₀ values were calculated to be 1.54 and 1.43 for A549 and FaDu cell lines, respectively [33]. Vendetti et al. showed that by increasing the concentration of AZD6738 from 0.3 to 1 μM, the survival fraction and cell proliferation rate of four various lung cancer cell lines was declined [36]. In addition, Clack et al. investigated the impact of this drug in combination with irradiation on mice breast tumors and concluded that the tumor volume was decreased in parallel with an increase in the concentration of the drug. Also, such a reduction in tumor volume was greater for chemotherapy when combined with irradiation [37]. These findings were in accordance with the results of the present study. On the other hand, Min et al. examined different doses of AZD6738 on gastric cancer cells. They concluded that the drug prevented DNA damage repair and it can increase DSBs cause the cell cycle arrest at the S phase, and inhibit tumor growth, thereby inducing apoptosis [38]. Consequently, by means of detailed mechanistic in-vitro analysis, we demonstrated the radio-sensitizing effect of AZD6738 on the A375 cell-line in which the cell viability and the ability of colony formation were decreased. Table 2 compares the results of our study with the recent findings obtained from other studies conducted on the cytotoxicity of AZD6738 on cancer cells.

Conclusion

Cellular repair mechanism plays an essential role in the treatment of cancer. The results of our study showed that AZD6738, as an ATR inhibitor drug, can decrease the viability of melanoma cells alone, and exhibited a greater reduction when combined with 6 MV X-ray. Furthermore, the degree of a decrease in the cell proliferation rate of cells treated by AZD6738 or irradiation only was significantly lower than cells treated with the combination of both. Therefore, our results indicate that the combination of AZD6738 with irradiation

Table 2: A comparison between our study and recent in-vitro and in-vivo studies performed on the role of AZD6738 and radiotherapy.

Reference	Cell line	Protocol	
[37]	gastric, breast (mouse)	AZD6738 + 2Gy (X-ray)	Tumor volume: Control= 1.8 cm ³ 2 Gy X-ray= 0.6 cm ³ AZD6738= 0.4 cm ³ drug+ X-ray=0.02 cm ³
[35]	Lovo (colon) & mouse (colon)	AZD6738 (0.3-30 μM) + 2Gy (X-ray)	Percentage of tumor volume decrease: X-ray alone: 50% Drug alone: 75% Drug + 2Gy X-ray: 87.5%
[26]	BT-474 (breast)	AZD6738 drug (0.125-1 μM)	Cell viability: 0.125 μM = 95% 0.25 μM = 93% 0.5 μM = 85% 1 μM = 70%
[33]	FaDu (hypopharyngeal), A549 (lung)	AZD6738 (0.25 μM) + 250Kv X-ray (1, 2, 3, 4Gy)	SER ₅₀ (A549) =1.54 SER ₅₀ (Fa Du)=1.43
Our study	A375 (melanoma)	AZD6738 (0.1-30 μM) + 2,4&6 Gy X-ray(6 Mv)	SER ₅₀ =1.51-1.61

SER: Sensitizer Enhancement Ratio

improved the effect of radiotherapy on melanoma cancer cells.

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Authors' Contribution

A. Neshastehriz conceived the idea. Introduction and manuscript of the paper was written by M. Talebpoor, A. Neshastehriz and S. Eynali. The method implementation and experimental studies was carried out by M. Talebpoor. Results and Analysis was carried out by M. Talebpoor, A. Neshastehriz, S. Eynali and F. Koosha. The research work was proofread and supervised by A. Neshastehriz, S. Eynali and F. Koosha. All the authors read, modified, and approved the final version of the manuscript.

Ethical Approval

The Ethics Committee of Iran University of Medical Sciences approved the protocol of the study (Ethic cod: IIR.IUMS.REC 1395.28726).

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Conflict of Interest

None

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