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Studying the Efficacy of Tolmetin Radiosensitizing Effect in Radiotherapy Treatment on Human Clonal Cancer Cells

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Abstract

One of the methods of cancer treatment is radiotherapy. Although radiation resistance and toxicity in normal cells limit the use of radiotherapy in some specific anatomical situations, however, together with radiotherapy, the use of substances that increase the radiation sensitivity of cancer cells and at the same time toxicity can be useful if they do not have cells on normal cells. The present study aimed to investigate the radiosensitizing effect of tolmetin in radiotherapy treatment on human clonal cancer cells. In this study, human clone HT-29 cancer cells in different groups were exposed to X-rays and tolmetin drug and were compared with each other and with the control group by micronucleus evaluation method and nuclear division index. The effect of cytotoxicity was evaluated with the index of nuclear division and genotoxicity of cells with the method of evaluating the number of micronuclei. Based on the results, the number of micronuclei increased significantly in the radiation-receiving group compared to the control group. In the group receiving tolmetin with 75 and 100 µM concentrations, the number of micronuclei also increased compared to the control group. A significant increase in the number of micronuclei was observed in all groups treated with tolmetin that received radiation, and this increase was more noticeable in concentrations of 100 and 150 µM. Meanwhile, tolmetin did not change the nuclear division index in the studied concentrations. The present study showed that tolmetin has a radiosensitizing effect on HT-29 human colon cancer cells, which is dependent on tolmetin concentration. In addition, tolmetin does not have a cytotoxicity effect on the mentioned cell line.

Keywords: Radiotherapy, Treatment, Cancer cells, Human clone

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Introduction

Radiotherapy is one of the common methods of cancer treatment, which is used alone or together with chemotherapy. Ionizing radiation used in radiation therapy is a physical agent used to destroy cancer cells. High-energy radiation damages the genetic material (DNA) of cells, thereby blocking their ability to further divide and reproduce. Although radiation causes damage to both normal cells and cancer cells, the goal of radiation therapy is to destroy as many cancer cells as possible with minimal damage to healthy tissues. It should be noted that cancer cells are not efficient in regenerating radiation damage compared to healthy cells. Resistance to radiation is considered one of the inherent defects of the treatment system, and there is still a difference of opinion between its therapeutic benefits and physiological disadvantages. There are multiple approaches to increase the

effectiveness of radiation therapy while reducing toxicity, which include increasing the radiation protection of healthy tissue cells, lack of resistance to radiation in cancerous tissue, and increasing the radiation sensitivity of cancerous tissue [1, 2].

Ionizing radiations are among the factors that cause structural damage to chromosomes [3, 4]. Micronucleus (micronucleus) is a piece of a chromosome or a whole chromosome that does not enter the nucleus of the daughter cell during nuclear division during anaphase (separation of chromosomes). Ionizing radiation is known as a factor that induces the formation of micronucleus in cells, which is a sign of genetic damage caused by radiation. Currently, this method is used as a biological marker to estimate the absorbed radiation dose. An increase in the formation of micronuclei in cells has also been observed after the consumption of many sensitizing compounds against radiation [1].

The role of damage to the genetic material and chromosomes in the development of cancer is a topic that has attracted many studies [5]. For example, several chromosomal structural abnormalities have been observed in solid tumors, including colon cancer [6-8]. Colon cancer is known as the fourth cause of death from cancer [9]. The connection between inflammation and cancer is well known. Non-steroidal anti-inflammatory drugs (NSAIDs) are a good class of anti-inflammatory drugs that inhibit cyclooxygenase (COX) enzyme [10]. Many studies show the ability of many anti-inflammatory agents, especially NSAIDs, to inhibit tumor growth. In addition, a significant reduction in colon cancer mortality has been reported in association with the use of NSAIDs [11-13]. Also, the apoptotic effect and inhibition of tumor growth of some compounds derived from NSAIDs have been reported [14-17].

Tolmetin is a nonsteroidal anti-inflammatory drug (NSAID) that inhibits the synthesis of prostaglandins, and the regulatory effect of tolmetin in anticancer drug therapy has been studied and it has been shown that tolmetin and other NSAIDs inhibit ß-catenin [18], increase the toxicity effect of anticancer drugs [19]. For this reason, tolmetin is known as the developer of new anti-cancer agents [18]. As mentioned, a selective radiosensitizer is needed to control tumors without increasing toxicity in normal tissue. Several new agents have shown promising activity in preclinical studies, but few have progressed beyond the early stages of clinical development [20].

Since the high level of ionizing radiation causes a high level of oxidative stress, which is also toxic to the normal tissue around the tumor, it is desirable to use a sensitizing agent along with ionizing radiation to increase the toxicity in cancer cells and at the same time reduce the exposure. It is slow in normal cells [21]. Based on this, this study aims to investigate the sensitization effect of tolmetin radiation in radiotherapy treatment on the HT-29 colon cancer cell line. In this regard, the micronucleus estimation test, which is approved as an acceptable method in genotoxicity studies, will be used [22] and the nuclear division index (NDI) will be used to evaluate the cytotoxic effect of the drug [23].

Materials and Methods

Cell culture

Cells were cultured in RPMI medium (Dacell) containing 10% fetal bovine serum (Gibco), streptomycin 200 µg/ml, and penicillin (Gibco) 500 units/ml. Finally, they were kept inside the incubator with a temperature of 37 C°, 5% CO2, and 95% humidity. After the start of cell culture, depending on the number of cells and the volume of the culture medium, periodic replacement of the cell culture medium (once every 3 days) was done to prevent color change and increase its acidity, so that the cells at a cell density of 80% trypsinized from 0.25% trypsin solution (Gibco) and passaged.

Treatment of cells

HT-29 cells were cultured at 300,000-400,000 cells in 12-well plates. Concentrations of 75, 100, and 150 µM were prepared from the Tolmetin drug. The cells were divided into 8 groups; including: group 1 (control), group 2 (irradiation), group 3 (contains tolmetin 75 µM), group 4 (contains tolmetin 100 µM), group 5 (contains tolmetin 150 µM), group 6 (radiation + tolmetin 75), group 7 (radiation + tolmetin 100), group 8 (radiation + tolmetin 150). 3 hours before irradiation, the cells were exposed to the determined concentrations of tolmetin. So that based on the mentioned groups, 100 microliters of tolmetin solution with a specific concentration were added to the culture medium of the cells.

Irradiation and retreatment

Shinva Company's linear accelerator was used for irradiation. X-rays were irradiated to the exposed groups with a dose of 4 Gy and a dose rate of 1.96 Gy/min and maintaining a distance of 60 cm from the radiation source. After receiving radiation, the plates were transferred to the incubator at 37 37 C°, 5% CO2, and 95% humidity. 48 hours after irradiation, the cells were treated with 100 µL cytochalasin B (Sigma-Aldrich) at a concentration of 6 µL/mL to stop proliferation and binucleation [1].

Micronucleus test

Cells were harvested 28 h after cytolysin B addition. The cell suspension was centrifuged at 5000 rpm for 7 minutes. After fixing the cells with acetic acid/methanol solution with a ratio of 1.6, the cells were transferred to clean and cold slides and dried for 24 hours at room temperature. Staining was done with Giemsa 10%. In each sample, for every 500 binucleated cells, the number of binucleated cells with micronuclei was counted and the average

number of micronuclei in each group was defined as an index. To determine the nuclear division index (NDI), 500 cells from each sample were counted, and the number of one, two, three, and four nuclear cells was determined. The NDI index is calculated using Eq. 1.

$$NDI = \frac{(m1 + 2\,(m2) + 3\,(m3) + 4\,(m4))}{N} \quad (1)$$

In the above relationship, m1, m2, m3, and m4 are the number of cells with one, two, three, and four nuclei, respectively, and N is the total number of cells [1].

Statistical analysis

In the present study, the values of quantitative variables were shown as mean ± standard deviation. One-way analysis of variance (and Tukey's post hoc test) was used to compare the average of the studied variables between the groups. The data was analyzed using Prism version 9 software, and a significance level of 0.05 was considered.

Results and Discussion

Measuring the sensitivity of tolmetin radiation on HT-29 cells using the micronucleus test

To measure the radiation sensitivity of tolmetin, concentrations of 75, 100, and 150 µM of tolmetin were incubated with HT-29 cells for 3 hours. The cells of the control group and the radiation group (the group that is only exposed to ionizing radiation) were cultured at the same time. Then, one group of each concentration and radiation group was exposed to 4 Gy of X-ray radiation. Cytoclaisin B was used to stop the production of binucleated cells. After harvesting and staining the cells, the micronucleus test was performed for all groups. The results of 3 repetitions are shown in **Table 1**.

Table 1. Mean percentage of micronucleus and NDI produced in 29-HT cells.

No.	Treated groups	Mean ± SD	
		Micronuclei in binucleated HT-29 cells	NDI in binucleated HT-29 cells
1	Control	0.11 ± 0.01	1.35 ± 0.10
2	Irradiated group	0.63 ± 0.03	1.01 ± 0.07
3	Tolmetin 75 µM	0.13 ± 0.01	1.20 ± 0.19
4	Tolmetin 100 µM	0.15 ± 0.01	1.06 ± 0.04
5	Tolmetin 150 µM	0.12 ± 0.01	1.25 ± 0.08
6	Tolmetin 75 µM + radiation	0.68 ± 0.02	0.99 ± 0.02
7	Tolmetin 100 µM + radiation	0.91 ± 0.01	0.90 ± 0.04
8	Tolmetin 150 µM + radiation	0.79 ± 0.01	1.01 ± 0.03

The average percentage of micronucleus index in 8 groups was 0.11 ± 0.01, 0.63 ± 0.03, 0.13 ± 0.01, 0.15 ± 0.01, 0.12 ± 0.01, 0.68 ± 0.02, 0.091 ± 0.01, and 0.79 ± 0.01, respectively.

The results of the effect of tolmetin on the production of micronuclei in HT-29 cells without receiving radiation showed that there is a significant difference between the groups treated with tolmetin concentrations of 75 and 100 µM and the control group ($P<0.05$). In all groups receiving radiation, a significant difference was observed with the radiation group only ($P<0.05$), which was more evident in the groups treated with 100 and 150 µM tolmetin concentrations, in addition, the difference between the radiation group and the control group was also significant ($P<0.05$).

Measuring the cytotoxicity of tolmetin on HT-29 cells by evaluating the nuclear division index

To measure the cytotoxicity of tolmetin on HT-29 cells, concentrations of 75, 100, and 150 µM of tolmetin were incubated with the cells for 3 hours. The cells of the control group and the radiation group were cultured simultaneously. Then one group of each concentration and radiation group was exposed to 4 Gy of X-ray radiation. Cytoclaisin B was used to stop the production of binucleated cells. After harvesting and staining the cells, the nuclear division index was evaluated by counting one, two, three, and four-nucleated cells for all groups. The results of 3 repetitions are shown in **Table 1**. The average percentage of NDI in the studied groups was 1.35 ± 0.10, 1.01 ± 0.07, 1.20 ± 0.19, 1.06 ± 0.04, 1.25 ± 0.08, 0.99 ± 0.02, 0.90 ± 0.04, and 1.01 ± 0.03, respectively. The effect of tolmetin on changes in the nuclear division index in HT-29 cells without receiving radiation is shown in **Figure 1**.

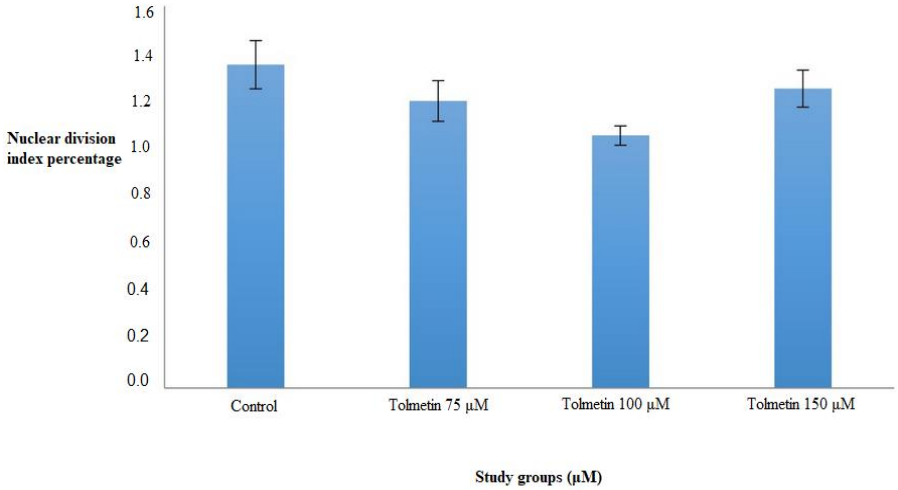

Figure 1. Cytoplasmic toxicity effect caused by tolmetin (at concentrations of 75, 100, and 150 µM) in HT-29 cells by measuring nuclear division index (NDI) in groups without radiation.

As it is clear from the results, there was no significant difference in the average nuclear division index between all groups treated with Tolmetin compared to the control group. In the groups receiving radiation **(Figure 2)**, in all groups treated with tolmetin, there was no significant difference in the percentage of NDI compared to the radiation group. Only a significant difference was observed between the group receiving radiation alone and the control group (P<0.05).

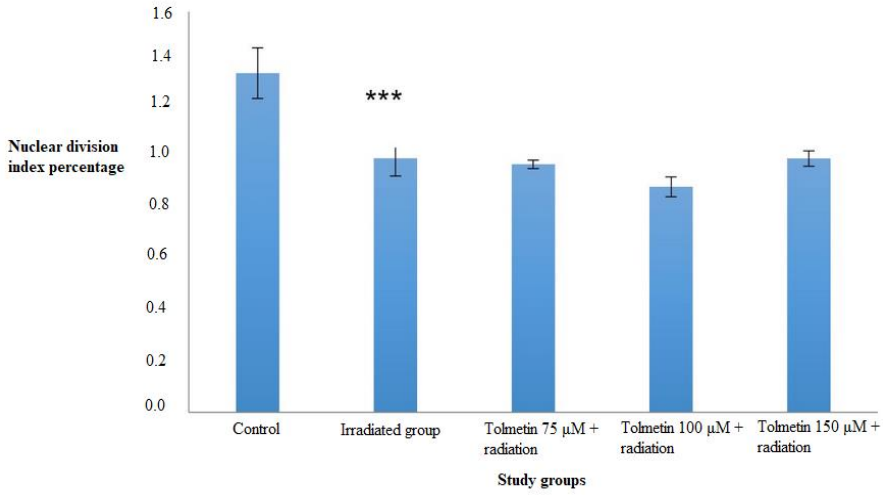

Figure 2. Cytoplasmic toxicity effect caused by tolmetin (at concentrations of 75, 100, and 150 µM) in HT-29 cells by measuring nuclear division index (NDI) in groups without radiation; *** Significant difference with the control group (P<0.001).

Along with chemotherapy, radiation therapy plays a prominent role in the treatment of most cancers. The goal of radiation therapy is to deliver the maximum amount of radiation to the tumor tissue with the least amount of damage to the healthy cells so that while destroying the cancerous masses or shrinking them, the surrounding healthy cells are not damaged. The resistance of cancer cells to ionizing radiation is a challenge in radiotherapy regimens in patients. Cancer cells activate different signaling pathways that lead to resistance to radiation-induced cell death [24]. Therefore, the use of agents or compounds that can sensitize tumor cells to radiation and also maximize the radiation dose to the patient will be considered. These compounds should have minimal side effects, no cytotoxicity, and be cheap and available to the public.

In recent years, more and more studies have been conducted in the field of investigating the radiation sensitization effects of NSAIDs. For example, Seyed Jalal Hosseini Mehr and his colleagues in a study in 2018 investigated the sensitizing effects of mefenamic acid radiation with ionizing radiation on the HT-29 clone cancer cell line. The cells were exposed to ionizing radiation with a dose of 4 Gy, and in the groups with the presence of mefenamic acid compared to the group that was not exposed to mefenamic acid, micronuclei

significantly increased [1]. Another study conducted in 2018 by Mir Helal Ahmad and his colleagues in evaluating the effect of oxidative stress, hepatotoxicity, and genotoxicity of naproxen on male Wistar rats showed that naproxen causes a significant increase in DNA damage by increasing the micronucleus; also, treatment with naproxen leads to biochemical imbalance and oxidative stress, which leads to the loss of cell integrity and significant damage to the genetic material and the effect of liver function in male Wistar rats, so naproxen is known as a potential genotoxic agent [25]. In another study, tolmetin was among the drugs that enhanced the killing effect of cyclophosphamide on cancer cells without a significant toxic effect on CFU-GM bone marrow cells [26]. Another study on the effect of NSAIDs, conducted by Aida Poyafer and her colleagues in 2019, investigated the cytotoxic effect of resveratrol and sulindac on a human clone HT-29 cancer cell line. Both of these were shown to inhibit or reduce the survival of human cancer cell clone HT-29 [27].

In 2015, new derivatives of tolmetin were designed and evaluated in laboratory conditions on the human clone HT-29 cancer cell line, and the MTT method was used to determine the growth inhibition and cell viability. The results showed that the anticancer activity of tolmetin derivatives is due to the activation of caspase-8 and caspase-9 involved in the apoptosis pathway [28]. Another study in 2019 investigated the radiation sensitization effect of 5-aminolevulinic acid on human colon cancer cell line HT-29 in in vivo and in vitro conditions. The results of the work showed that, in vitro, cells treated with 5-aminolevulinic acid, which were irradiated in a multi-dose manner, had a significant decrease in their survival rate, and in addition, in vivo, the volume of the xenografted cancer mass after radiotherapy along with The mentioned composition was significantly smaller compared to the masses that did not receive 5-aminolevulinic acid [29]. In this way, the studies clearly show the effectiveness of the radiation-sensitizing compound in the success rate of the radiotherapy treatment process.

In the present study, we investigated the radiosensitizing effect of tolmetin in radiotherapy treatment on human clone HT-29 cancer cells. In the current study, by performing the micronuclei test in HT-29 cancer cells that were not exposed to ionizing radiation, all groups of cells treated with tolmetin increased micronuclei, but only at the concentration of 75 and 100 µM, they caused significant radiation sensitization effects in tumor cells ($P<0.05$). Also, by conducting the micronucleus test in HT-29 cancer cells that were exposed to ionizing radiation, it was found that in addition to the fact that the group receiving radiation had a significant difference from the control group ($P<0.05$), the cells treated with the mentioned drug in all studied concentrations There was a significant difference with the group that received only radiation ($P<0.05$) and caused radiosensitizing effects in tumor cells. Most of the micronuclei were seen in the concentration of 100 µM, which indicates the maximum effect of the drug in this concentration and its optimal effect **(Figure 3)**.

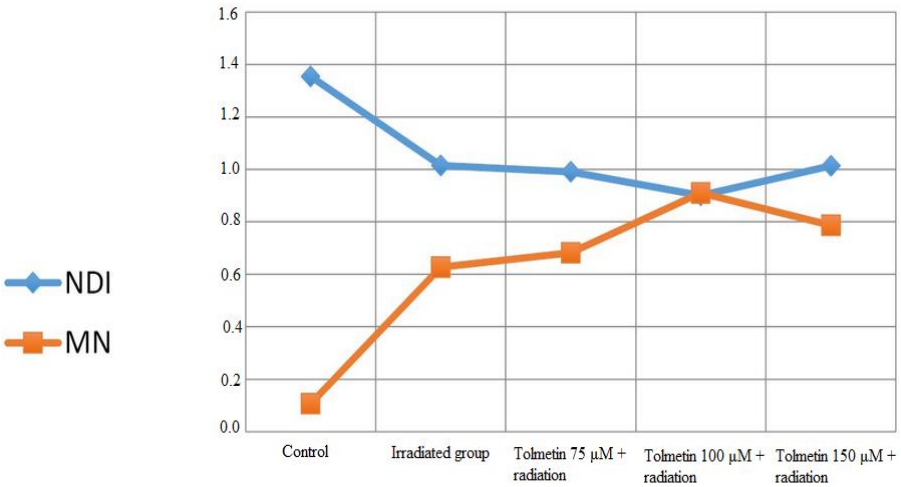

Figure 3. Comparison of the average percentage of the nuclear division index and the average percentage of the number of micronuclei in 29-HT cells in the groups receiving radiation and the groups receiving radiation and tolmetin at the same time (in concentrations of 75, 100, and 150 µM).

In investigating the cytotoxic effects of Tolmetin in HT-29 cancer cells that were not exposed to ionizing radiation, a decrease in the NDI index was seen, but it was not statistically significant. Also, the effect of the Tolmetin drug on cytotoxicity caused by ionizing radiation on the human clone HT-29 cancer cell line was investigated using the NDI index. The results of the study on HT-29 cells in this research show that tolmetin does not have cytotoxicity effects on tumor cells in the studied concentrations.

Based on the results of this study, tolmetin in the studied concentrations has radiosensitizing effects on tumor cells and in addition, tolmetin in the studied concentrations does

not have cytotoxicity effects on tumor cells. Known mechanisms in increasing radiation sensitivity by anti-inflammatory drugs include inhibition of cyclooxygenase-2 enzyme and subsequent reduction or cessation of prostaglandin production, resulting in increased apoptosis and decreased angiogenesis and growth. Also, the cell cycle stop in the G1-S region should be considered as one of the sensitive areas in this cycle. However, the mechanism by which this stop is done is not fully known until now. The mechanism of radiation sensitization of tumor cells in radiotherapy by NSIAD drugs is not fully understood and is not the main subject of our discussion, but in general, it can be said that anti-inflammatory drugs as radiation sensitizers move the radiation response curve to the right. Considering that the study conducted is the first study in the field of the radiation sensitization effect of Tolmetin drug on the amount of acute damage caused by ionizing radiation on the human colon cancer cell line HT-29, considering the positive results of this research, it is suggested that the number of inflammatory factors should also be measured in the next studies measured in these cells, it will also be more appropriate to study in vivo and on mice with colon cancer.

Conclusion

The present study aimed to investigate the radiosensitizing effect of tolmetin in radiotherapy treatment on human clonal cancer cells. Based on the results, the number of micronuclei increased significantly in the radiation-receiving group compared to the control group. In the group receiving tolmetin with 75 and 100 µM concentrations, the number of micronuclei also increased compared to the control group. A significant increase in the number of micronuclei was observed in all groups treated with tolmetin that received radiation, and this increase was more noticeable in concentrations of 100 and 150 µM. Meanwhile, tolmetin did not change the nuclear division index in the studied concentrations. The present study showed that tolmetin has a radiosensitizing effect on HT-29 human colon cancer cells, which is dependent on tolmetin concentration. In addition, tolmetin does not have a cytotoxicity effect on the mentioned cell line.

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Conflict of interest: None

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Ethics statement: None

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