

Thymoquinone Potentiates Methotrexate Mediated-Apoptosis in Saos-2 Osteosarcoma Cell Line

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ABSTRACT

Introduction Recently, various studies have concentrated on the therapeutic potential of thymoquinone (TQ), a natural polyphenol, in various human malignancies, including osteosarcoma. However, the underlying mechanisms in TQ-mediated anti-cancer effects are not yet fully understood. Therefore, the present study investigated the effect of TQ on methotrexate (MTX)-induced apoptosis in Saos-2 cells.

Methods Saos-2 cells were treated with MTX, TQ, and a combination of both, and cell viability was assessed by MTT assay. mRNA expression of apoptotic markers, including Bax, Bcl-2, and caspase-3, was assessed using quantitative real-time polymerase chain reaction (qRT-PCR).

Results MTX resulted in significant inhibition of cell proliferation in a dose-dependent manner. The combination of TQ and MTX inhibited proliferation compared to single treatments ($P < 0.05$). TQ also induced apoptosis by regulating pro-apoptotic markers including Bax and caspase-3 and reducing anti-apoptotic mediators including Bcl-2. In addition, TQ increased MTX-induced apoptosis in Saos-2 cells.

Conclusion The findings of the present study highlight new insights into understanding the role of TQ as a potential therapeutic agent in osteosarcoma by increasing MTX-induced apoptosis.

Introduction

Osteosarcoma is the most common type of cancer in bone tissue, which is more common in children and adolescents (between 10 and 30 years) and is also seen in about 10 % of cases among people over 60 years [1]. This type of cancer accounts for only 2 % of cancers in children. Osteosarcoma originates from bone mesenchymal cells that become malignant osteoids [2]. The pathophysiology of osteosarcoma includes the inactivation of p53 and the Rb signaling pathway. The incidence rate of osteosarcoma is commonly high

in males compared to females [3]. Currently, surgery in combination with a chemotherapeutic regimen that includes methotrexate (MTX) and tomudex (TDX) has been standardized as the most effective therapeutic strategy for patients with osteosarcoma, resulting in a 5-year survival rate of 65 percent to 75 percent due to the ameliorative effects of therapeutic interventions on symptoms. [4, 5].

Nevertheless, similar to other kinds of human cancerous, most patients with osteosarcoma represent a low level of response to

chemotherapeutic drugs, which eventually results in tumor recurrence and clinically poor consequences [6]. The emergence of drug resistance is a major factor in the failure of therapeutic interventions [7]. As a result, it is critical to unravel and comprehend the precise molecular pathways behind the acquisition of resistance in osteosarcoma [7]. Recently, much attention has been paid to nutritional interventions such as polyphenols in preventing and treating cancer [8]. Thymoquinone (TQ) is a key member of the polyphenol family and is found mainly in various vegetables and fruits such as wheat, onions, berries, and apples [9]. An accumulating number of previous studies have illustrated promising anti-cancer effects of TQ in numerous human malignancies, including breast, ovarian, prostate, colon, and other cancer types and osteosarcoma [10–13]. The anti-cancer features of TQ are due to the different cellular signaling mechanisms and its ability to inhibit the enzymes responsible for activating cancer. Therefore, we aimed to investigate the role of TQ in increasing the rate of apoptosis alone or in combination with MTX on the Saos-2 osteosarcoma cancer cell line.

Material and Methods

Cell culture

The Saos-2 osteosarcoma cell line was provided by the Pasteur Institute (Tehran, Iran). Saos-2 cells were grown in RPMI-1640 media, including 10% fetal bovine serum (FBS; Gibco) and 1% streptomycin/penicillin solution. The cells were incubated at 37 °C with 5% CO₂. The cells were collected with trypsin–EDTA and passaged every 2–3 days to continue exponential growth.

Evaluating cell proliferation

3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT; Sigma Aldrich) assay was employed to assess the cell viability subsequent treatment with MTX (0–60 μM), thymoquinone (0–100 μM), and a combination of MTX with TQ. After seeding Saos-2 cells (1 × 10⁴ cells per well) at 96-well plates, they were exposed to different concentrations of MTX and thymoquinone alone or in combination. After 48 h, media containing drugs was removed, and cells were incubated with MTT solution at 37 °C. Then the formed formazan crystals were solubilized using dimethylsulfoxide (DMSO). A microplate reader was used to measure the absorbance of each well at 570 nm.

qRT-PCR

BASED ON THE MANUFACTURER'S GUIDELINES, total RNA was isolated from the cells via Trizol reagent. Then, complementary DNA templates (cDNA) were synthesized using a cDNA synthesis kit. Finally, using SYBR Green master mix and Mic qPCR Cycler, generated cDNA was subjected to duplicate quantitative real-time PCR (qRT-PCR). To standardize the level of mRNA expression in all samples, β-actin was utilized as the reference gene. The 2^{−ΔΔC_T} method was used to calculate the qRT-PCR data. Sequences of primers used for amplification of Bax, Bcl-2, Caspase-3, and β-actin are listed in ► Table 1.

Data analysis

Results were shown as mean ± SD in at least three separate experiments. Statistical analysis was performed using SPSS software and

Graph Pad Prism V6 through t-test or ANOVA, and a *P*-value < 0.05 was considered statistically significant.

Results

The effect of methotrexate on the proliferation rate of the Saos-2 cell line

The cytotoxic effects of methotrexate on Saos-2 cells were evaluated using an MTT assay. After treating the cells with different concentrations of methotrexate and incubating them for 48 h, the cytotoxicity was calculated using the formula (► Fig. 1). The IC₅₀ value was calculated by entering the values of cell proliferation percentage versus inhibitor concentration and plotting. As shown in ► Fig. 1, the cytotoxicity of methotrexate and its inhibitor effects on cell proliferation increased with increasing drug concentration. In the Saos-2 cell line, the IC₅₀ of methotrexate was 26 ± 1 μM after 48 h of incubation.

The effect of TQ on the proliferation rate of the Saos-2 cell line

The proliferation rate of Saos-2 cells in the presence of thymoquinone was also examined by MTT assay. As shown in ► Fig. 2, compared to the control group, treatment of cells with TQ after 48 h significantly reduced cell proliferation in a dose-dependent manner. The IC₅₀ value for TQ is 47 ± 1 μM.

The effect of MTX and TQ combination on the proliferation of Saos-2 cell line

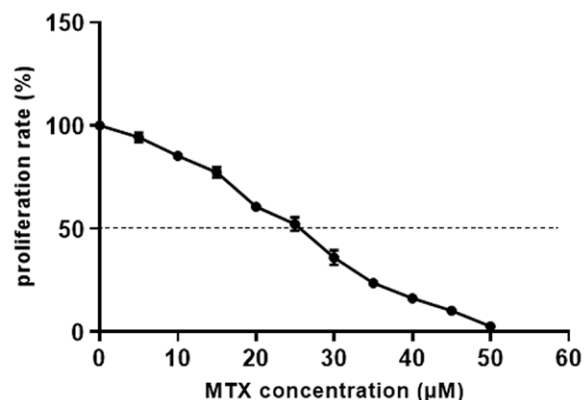
In addition, the combined effects of methotrexate and TQ on cell proliferation were also evaluated in this study. Combination therapy further reduced cell proliferation compared with methotrexate or thymoquinone mono-therapy (► Fig. 3). Combining concentrations less than 20 μM MTX with 47 μM thymoquinone could not induce 50% cytotoxicity in cells. Concentrations above 20 μM showed a synergistic interaction in Saos-2 cells after 48 h of incubation (► Fig. 3). In other words, the combination of MTX and TQ reduced the IC₅₀ levels of MTX from 47 μM to 14 μM in this cell line. In fact, the combination of MTX with TQ increases the cytotoxicity of MTX.

The effects of MTX and TQ on the apoptosis of Saos-2 cell lines

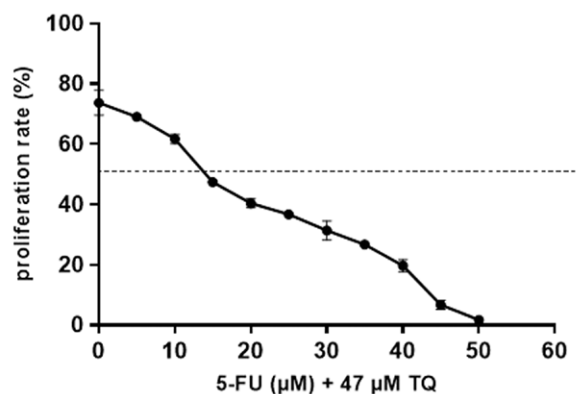
To investigate changes in apoptotic factors including Bax, Bcl-2, and caspase-3 in different groups treated with methotrexate, thymoquinone, and a combination of the two, cells were first divided into four groups: Group 1: Saos-2 cells without any treatment and as a control group; Group 2: Saos-2 cells treated with 26 μM methotrexate; Group 3: Saos-2 cells treated with 47 μM thymoquinone;

► Table 1 Primer sequences.

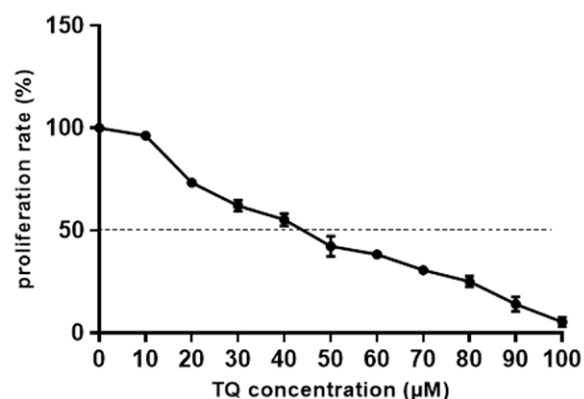
Gene	Forward primer (5'–3')	Reverse primer (5'–3')
Bax	GGTTGTGCGCCCTTTCTA	CGGAGGAAGTCCAATGTC
Bcl-2	GATGTGATGCCCTGCGAAG	CATGCTGATGCTCTGGAATCT
Caspase-3	GTGGAAGTACGATGATATGCC	CGCAAAGTGACTGGATGAACC
β-actin	TCGTGCGTGACATTAAGGAG	AGGAAGGAAGGCTGGAAGAG



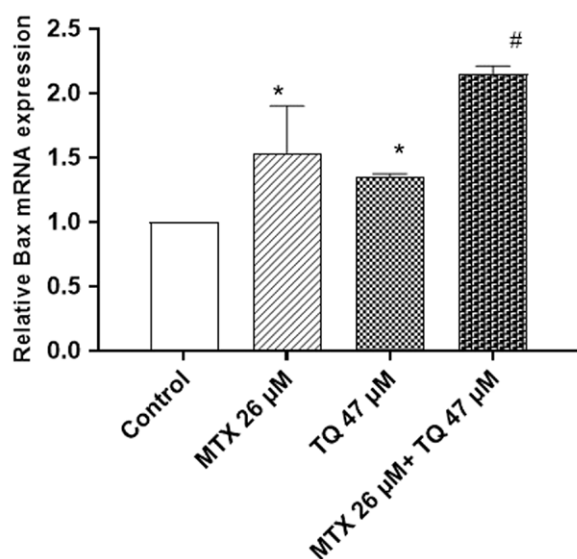
► **Fig. 1** The effect of methotrexate on Saos-2 cell line proliferation rate. The results are shown as mean $\pm$ SD after three repetitions of experiments.



► **Fig. 3** The effect of methotrexate and thymoquinone combination on Saos-2 cell line proliferation. The results are shown as mean $\pm$ SD after three repetitions of experiments.



► **Fig. 2** The effect of thymoquinone on Saos-2 cell line proliferation. The results are shown as mean $\pm$ SD after three repetitions of experiments.



► **Fig. 4** Bax expression changes in the treated group. The results are shown as mean $\pm$ SD after three repetitions of experiments.

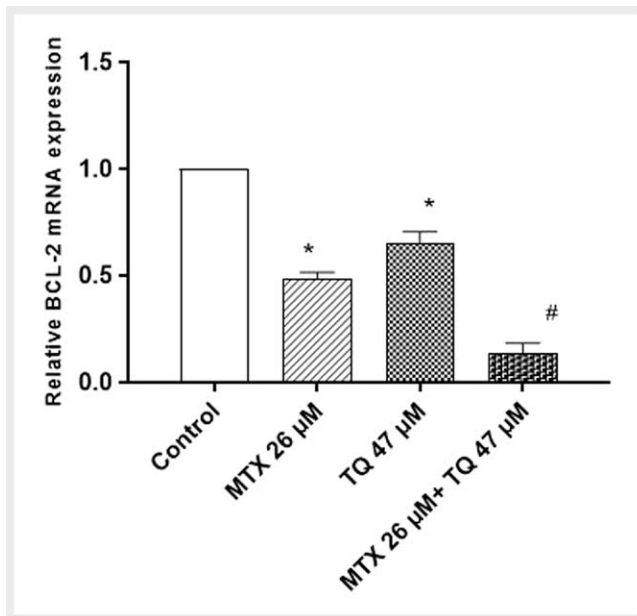
Group 4: Saos-2 cells treated with 26 μ M methotrexate and 47 μ M thymoquinone (MTX + TQ).

After cell treatment, RNA was extracted from the cells. Then cDNA was synthesized and the expression of all three specific genes that are markers of apoptosis was measured using specific primers. Bax is a pro-apoptotic gene that promotes apoptosis. As shown in ► **Fig. 4**, treatment of cells with methotrexate and TQ alone increased Bax expression in cells compared with the control group ($P < 0.05$). Methotrexate and TQ combined have a more significant effect on increasing the expression of this gene in the Saos-2 cell line ($P < 0.05$).

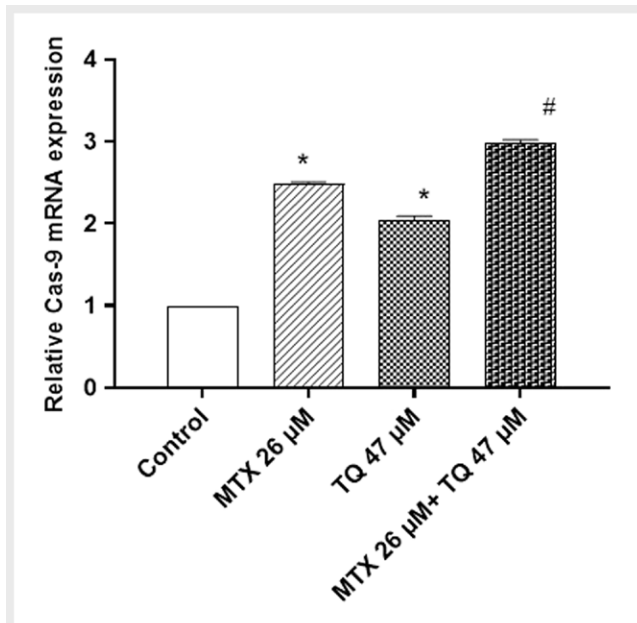
Bcl-2, unlike Bax, is an anti-apoptotic gene that prevents apoptosis. Herein, the expression level of Bcl-2 in cells treated with methotrexate and TQ alone was significantly lower than in the control group ($P < 0.05$) (► **Fig. 5**). On the other hand, the expression of this anti-apoptotic gene in cells treated with methotrexate and TQ was much lower than in the treated groups alone ($P < 0.05$).

The next gene to be studied in our study is caspase-3, which is an essential pro-apoptotic gene. In this study, treatment of cells with methotrexate and TQ alone increased the expression of caspase-3 in cells compared to the control group (► **Fig. 6**) ($P < 0.05$). Methotrexate and TQ, combined with each other, have a greater effect on increasing the expression of this gene in the Saos-2 cell line ($P < 0.05$).

In general, methotrexate and TQ increase the expression of pro-apoptotic genes and decrease the expression of anti-apoptotic genes. But combining the two has a more substantial effect on promoting apoptosis in the Saos-2 osteosarcoma cell line. In other words, TQ increases the sensitivity of Saos-2 cells to methotrexate-induced apoptosis.



► **Fig. 5** Bcl-2 expression changes in the treated group. The results are shown as mean $\pm$ SD after three repetitions of experiments.



► **Fig. 6** Caspase-3 expression in the treated group. The results are shown as mean $\pm$ SD after three repetitions of experiments.

Discussion

Osteosarcoma is one of the leading factors of cancer death worldwide [14]. Despite recent advances in diagnosing and treating this disease, the prognosis is still poor [15]. Treatment for osteosarcoma includes neoadjuvant chemotherapy followed by surgical removal and adjuvant chemotherapy [16]. The goal of neoadjuvant chemotherapy is first to damage the tumor cells at the initial site to reduce the size of the tumor before surgery. It also allows the

eradication of micrometastases as well as the evaluation of tumor tissue response to chemotherapy. Most of the molecules used in chemotherapy protocols are a combination of cisplatin, doxorubicin, methotrexate, and ifosfamide [17, 18].

Chemotherapy has a variety of modes of action. Thus, combining their action methods makes it probable to target cancer cells at different levels. Methotrexate inhibits the proliferation of rapidly dividing cells by impeding folic acid depletion, preventing cell proliferation and thus cell division [19]. However, current therapies have limitations, such as inadequate efficacy for high-risk patients and low survival rates for patients with metastasis at diagnosis. In addition, some patients developed resistance to chemotherapy, which in some cases explained tumor recurrence and progression. In fact, about 25 percent of patients who are classified as good responders to chemotherapy will still have a recurrence. In addition, weak respondents can quickly develop metastases that lead to death. Two types of resistance must be illustrious: internal and acquired [20]. Internal resistance exists because resistant cells are present in the tumor before any treatment. When chemotherapy is prescribed, sensitive cells are killed by the toxic impacts of the drugs.

Furthermore, tumor-resistant cells that show pre-existing genetic mutations or activate different signaling pathways can increase despite the presence of chemotherapeutic agents [21]. In contrast, acquired resistance is induced by agents and appears after treatment. During treatment, the anti-cancer effect of the drugs is gradually reduced. In fact, the activation of proto-oncogenes, mutations, changes in the expression level of transport proteins or therapeutic targets, and alterations in the tumor microenvironment are all mechanisms that result in tumor cell development [22]. As a result of these changes, tumor cells are able to withstand chemotherapy. As a result of genomic instability, resistant clones are selected following chemotherapy treatment. Specific chemicals or molecular signaling pathways in operating system cells cause both types of resistance [23]. One biological process that leads to treatment failure is resistance, which requires more practical and immediate treatments to overcome. Hence, a better understanding and decoding of conventional chemotherapy resistance's molecular mechanisms is essential to develop new strategies and adapt therapies for patients and thus improve survival. Although disease-free survival has improved dramatically with the advent of chemotherapy, only 50 to 60 percent of tumors are sensitive to chemotherapy [24].

There is ample evidence (mainly preclinical studies) showing that TQ, combined with other mainstream chemotherapeutic agents, can significantly inhibit cancer progression and increase the burden of tumors on various malignancies by altering tumor pathways. A study by Timokinon et al. showed that TQ synergistically increased the anti-cancer activity of temozolomide (TMZ) in the glioblastoma U87MG cell line by inhibiting autophagy [25]. In another recent study, Khazaei et al. reported that apoptotic cell death was associated with synergistic glioblastoma cells with combination therapy with TQ and TMZ [26]. Gurung et al. observed a similar finding with glioblastoma cells, in which TQ was found to reduce growth and produce DNA damage, cell cycle arrest, and apoptosis [27]. This group of researchers showed that TQ could promote telomere shortening in GBM cells by inhibiting telomerase

activity. This effect was more pronounced in GBM cells that express DNA-PKcs *in vivo* [26]. There is also a report in which TQ inhibits tumor growth and enhances the preventive effect of 5-fluorouracil in the early model of colorectal tumors in rats [28]. Azoxymethane (AOM) was used to induce colorectal neoplasia in this model. Treatment with 5-FU/TQ combined reduced AOM-induced colorectal malignancies and large ectopic crypt foci [28]. A recent study by Fröhlich et al. showed increased ROS production levels and concomitant DNA damage in human colon cancer cells treated with a new hybrid of TQ and artemisinin [29]. In another study by Lei et al., it was found that TQ sensitizes 5-FU in the treatment of gastric cancer by increasing the induction of apoptosis and growth inhibition [30]. In general, the results obtained from our study are in line with previous studies and are consistent with them. Our study showed that combining MTX and TQ decreased the IC₅₀ levels of methotrexate, or in other words, increased the cytotoxicity of methotrexate.

On the other hand, MTX and TQ enhance the expression of proapoptotic genes and alleviate the expression of anti-apoptotic genes. But combining these two anti-cancer agents has a stronger effect on promoting apoptosis in the Saos-2 osteosarcoma cell line. In other words, TQ increases cell sensitivity in osteosarcoma cells.

Conclusion

As discussed above, preclinical research results encourage TQ application in clinical settings. Significant amounts of information about TQ on molecular anti-cancer activity, drug toxicity, bioavailability and pharmacokinetics, and new drug delivery approaches are now available to researchers. Our results generally emphasize the durable potential of targeting apoptosis as a therapeutic approach to improve the methotrexate response in osteosarcoma. However, the choice of drug to be combined with methotrexate is very important because we have shown that TQ is an up-and-coming therapeutic agent in preclinical cancer models. These findings reveal TQ's sound effects on enhancing the sensitivity of osteosarcoma cancer cells to conventional chemotherapy medicines, which makes it a suitable option for future clinical trials in cancer patients.

Ethical approval

All experimental procedures were applied in accordance with the approval from the Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1400.362).

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Conflict of Interest

The authors declare that they have no conflict of interest.

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