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Thymoquinone Augments Methotrexate-Induced Apoptosis on Osteosarcoma Cells

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Keywords
Chemotherapy, Combinational therapy, Polyphenol, Black seed (Nigella sativa), Bone malignancy

received 05.12.2021
accepted 07.02.2022

Bibliography
Drug Res 2022; 72: 220–225
DOI 10.1055/a-1775-7908
ISSN 2194-9379

Georg Thieme Verlag, Rüdigerstraße 14,
70469 Stuttgart, Germany

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ABSTRACT

Background Osteosarcoma (OS) as the most frequent primary bone malignancy in children and adolescents has a short survival rate in advanced stages. Alternative herbal medicines with fewer side effects or the potency to protect common therapy's side effects can be helpful in combinational therapies. Herein, we aim to explore the effects of Thymoquinone (TQ) combined with Methotrexate (MTX) on Saos-2 cells apoptosis.

Methods The effects of TQ and MTX alone or in combination on Saos-2 cell viability were measured by MTT assay. Real-time PCR was applied for the measurement of Bax, BCL-2, and caspase-9 mRNA expression. Apoptosis evaluation was conducted by flow cytometry.

Results TQ improves the cytotoxic effects of MTX on Saos-2 cells proliferation at lower doses. Indeed, the IC50 of MTX decreased from 26 µM to 15 µM when it combined with TQ. TQ and MTX can induce the expression level of pro-apoptotic factors, Bax and caspase-9 while inhibiting anti-apoptotic protein BCL-2. Moreover, the combination of TQ and MTX potentiates apoptosis to 73 %, compared to either TQ (48 %) or MTX (53 %) treated cells.

Conclusion The co-treatment of TQ and MTX is associated with the up-regulation of apoptotic factors and down-regulation of anti-apoptotic factors, conducting apoptosis aggravation and OS cell death.

Introduction

Osteosarcoma (OS) as a pleomorphic tumor of primitive mesenchymal bone-forming cells is common among children aged 0–19 years which has been reported as the eighth highest childhood cancer and characterized by immature bone or osteoid formation [1]. Epidemiological investigation illustrated that 1–5 cases/million people is the incidence rate of OS. Age, gender, socio-economic status, genetics, and other factors are involved in OS development [2]. Besides, the front-line of OS therapeutic strategy relies on surgery, radiotherapy, and chemotherapy because the adequacy of single-agent treatment is not approvable. In this regard, a huge number of studies have introduced other or combinational treatment protocols in order to enhance the effect of radiotherapy and chemotherapy [3, 4].

Methotrexate (MTX) is one of the most widely used anti-cancer, antimetabolite, and antifolate drugs. A considerable number of studies have been reported the effectiveness of this chemotherapeutic agent on OS treatment [5, 6]. However, the administration of MTX

has some negative impacts on OS. First, delayed clearance is one of the attributes of MTX which is directly interconnected to age. Doxorubicin and cisplatin intensity are also negatively affected by MTX delayed clearance [7]. Moreover, high-dose MTX is associated with severe and lethal complications like renal toxicity and oral mucositis [8, 9]. In addition to MTX side effects, OS cells' resistance to MTX has been reported both in-vivo and in-vitro. Drug targets modifications, drug influx or efflux, and metabolic or savage pathways are the main MTX resistance mechanisms in OS [10]. Therefore, MTX alone is not a proper therapeutic approach in OS treatment. In line with this demonstration, a huge number of studies have investigated MTX combinational therapies [11]. For example, a meta-analysis indicated that the combination of methotrexate (M), doxorubicin (D), cisplatin (C), and ifosfamide (I) as MDCI regime is not only effective but also safe for OS therapy [12]. Another meta-analysis also showed that the combination of these agents can improve the 5-year survival rate in OS cases [13]. However, these treatment options are interrelated with side effects. In order to address this problem and increase the safety and efficacy of therapy, herbal agents have gained a lot of attention in recent years.

Thymoquinone (TQ) is the active constituent of black seed (Nigella sativa) which is the homologue of ubiquinone and plays its role in the mitochondrial respiratory chain [14]. Anticancer, antioxidant, and antimicrobial activities are regarded as the main function of TQ [15]. When it comes to cancer therapy, this component plays its anticancer role in different ways including the prevention of oncogenic signaling pathways and the induction of apoptosis. Studies reported that AKT and NFKB signaling pathways are the main targets of TQ to combat cancer progression [14, 16]. In addition to inhibiting proliferative signaling pathways, studies have reported that TQ induces apoptosis through activating apoptotic factors like Bax, Bad, caspase9, caspase 3, and inhibiting BCL2 which overall results in the potentiation of cancers' treatment like breast [17], colorectal [18], and glioma cancers [19]. Thus, investigations revealed that TQ not only has anti-inflammatory and anti-oxidant capacity but also induces cell cycle arrest, apoptosis and stops cell proliferation and angiogenesis in OS [20, 21].

the induction of Bax and Bad and suppression of BCL2 is associated with the release of cytochrome c (cyt c) from mitochondria to the cytosol; because Bax and Bad as proapoptotic factors increase the permeability of the mitochondrial membrane, while BCL2 as an anti-apoptotic factor preserves the stabilization of mitochondrial membrane. Moreover, when cyt c is released into the cytosol, caspase-9 and caspase-3, which are the main players in caspase – apoptotic cascade, are activated leading to apoptosis [22].

Overall, the combinational effects of TQ and MTX on OS are unclear. In this regard, this study aims to investigate the synergism effect of these drugs on Saos-2 cells' fate.

Methods

Cell culture and reagents

The Saos-2 cell line was purchased from Pasteur Institute Cell Culture Collection (Tehran, Iran); Hyclone provided Fetal Bovine Serum (FBS); RPMI-1640 media, PBS, and Trypsin/EDTA were bought from Gibco Life Technologies (Gibco, USA); TQ, MTX, antibiotics, and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich Co. (Sigma Aldrich, USA); Bio Basic Co. provided 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); Saos-2 cells were seeded in RPMI-1640 and cultured in 10 % FBS, 1 % pen/strep at 37 °C with 95 % O_2, and 5 % CO_2 in the incubator. Cells in the log phase were selected for functional analysis.

MTT assay

The sensitivity to MTX, TQ, and their combination were measured by MTT assay. Cells were seeded in a 96 well plate at the density of 2×10^4 cells/200µl/well and then incubated overnight to evaluate Saos-2 cells growth. In the following 48 hours, cells were treated with MTX (0–50 µM) and TQ (0–100 µM) alone or their combination (0–50 µM MTX + 47 µM TQ). 20 µl MTT solution was added to each well for 4 hours, followed by 150 µl DMSO for purpose of formazan crystals solubilization. The cell viability and half-maximal inhibitory concentration (IC50) were detected at 570 nm by ELx808 plate auto-reader.

Quantitative RT-PCR

After Saos-2 cells were exposed to TQ, MTX, and combination regimen, total RNA was extracted from cells using a TRIzol® reagent, following the supplier's protocol (Invitrogen). The RNA concentration was assessed by NanoDrop (Thermo Scientific, USA). Complementary DNA (cDNA) was synthesized using an RT reagent Kit (QIAGEN), following the supplier's protocol. The cDNA was applied to determine the messenger RNA (mRNA) levels of BCL-2, Bax, and caspase-9 using Taq DNA polymerase. The thermal cycle of PCR was conducted at 94 °C for 10 min (initial denaturation) succeeded by forty-five 94 °C cycles for 10 s (denaturation) and 60 °C for 30 s (annealing) and 72 ◦C for 20 s (extension), in the ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The expression level of the target genes was calculated as a ratio to the relative mRNA expression of CT (threshold cycle) value ($2^{-(\Delta\Delta CT)}$ method). The primer sequences were as follows:

Flow cytometry analysis

The apoptotic cells were detected by flow cytometry. After seeding and 24 hours incubating Saos-2 cells in a six-well plate, we treated the cells with TQ, MTX, and their combination. Then, the cells were detached from the plates using 0.25 % trypsin without EDTA and washed with PBS, and then 0.5 % BSA was used to block nonspecific antigen in 1x PBS at 4 ˚C for 30 minutes. In the following, cell pellets were suspended in 10 µl of fluorescein isothiocyanate (FITC) and labeled with Annexin V and propidium iodide (PI) for 10 minutes at dark in RT. Finally, analysis was carried out on the FACScan cytometer (Becton Dickinson, Heidelberg, Germany) (▶ **Table 1**).

Statistical analysis

Data are presented as the mean ± standard deviation (SD), and ($P < 0.05$) was considered statistically significant. All quantitative results were stuck with GraphPad Prism v7.04 software. Student's t-test and one-way ANOVA were used to investigate the significance of differences between groups.

Results

The combination of TQ and MTX inhibits Saos-2 cell proliferation

MTT assay was applied to measure the inhibitory effect of MTX and TQ, either as a sole agent or in combination, on Saos-2 cells proliferation. As presented in ▶ **Fig. 1a, b**, the viability of Saos-2 cells treated with either MTX (0–60 µM) or TQ (0–100 µM) reduced dose-dependently. IC50 values for TQ and MTX were 47 µM and 26 µM, respectively. 47 µM TQ was combined with different concentrations of MTX, which resulted in reducing MTX IC50 from 26 µM to 15 µM ($P < 0.05$). 47 µM TQ and 15 µM MTX killed 50 % of Saos-2 cells (▶ **Fig. 1c**). Hence, TQ and lower doses of MTX can reduce Saos-2 cells proliferation.

TQ plus MTX increases Bax and caspase-9 expression in the Saos-2 cell line

The impact of TQ and MTX single treatment and co-treatment on the expression level of apoptotic factors was evaluated. Our data showed that TQ and MTX enhanced Bax and caspase-9 expression. However, the expression level of these factors in drugs' co-treatment was significantly higher than that of single therapies. Overall, the combination of TQ and MTX has more powerful effects on promoting the expression level of apoptotic factors in the Saos-2 cell line (* $P < 0.05$) in comparison to control. (# $P < 0.05$) in comparison to mono treatments (▶ **Fig. 2 a, b**).

▶ **Table 1** The primer sequences of target genes.

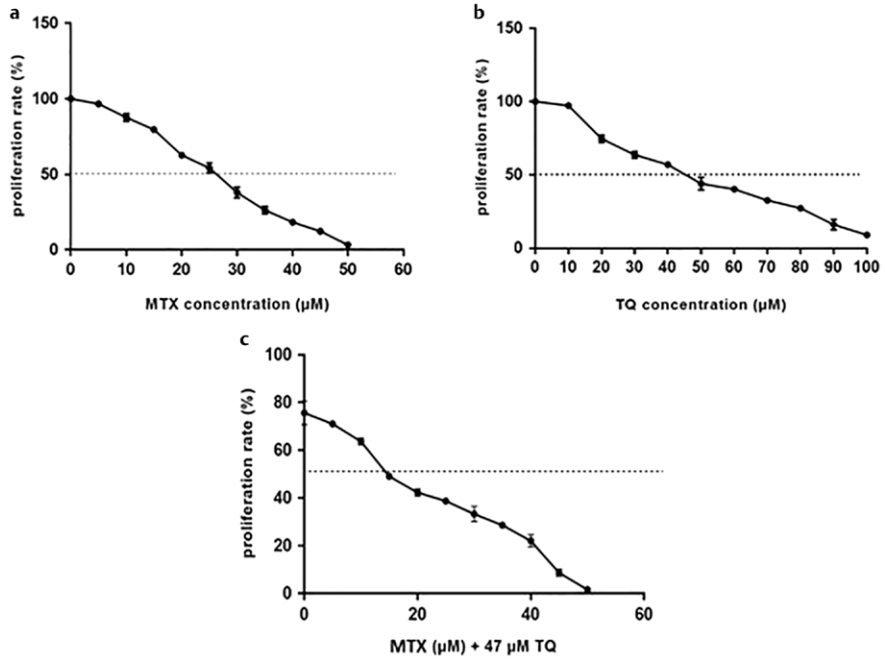
Gene	Primer sequence
BCL-2	F 5′-GAGCGTCAACCGGGAGATGTC-3′
	R 5′-TGCCGGTTCAGGTACTCAGTC-3′
BAX	F 5′-GACTCCCCCCGAGAGGTCTT-3′
	R 5′-ACAGGGCCTTGAGCACCAGTT-3′
Caspase-9	F 5′-TTGTTGTTTGGGTAATGTATTGA-3′
	R 5′-CAACCCTACTTAACTTAACCCTACTCA-3′
B- actin	F 5′-TCCCTGGAGAAGAGCTACG-3′
	R 5′-GTAGTTTCGTGGATGCCACA-3′

The co-treatment of TQ and MTX reduces BCL-2 expression in the Saos-2 cell line

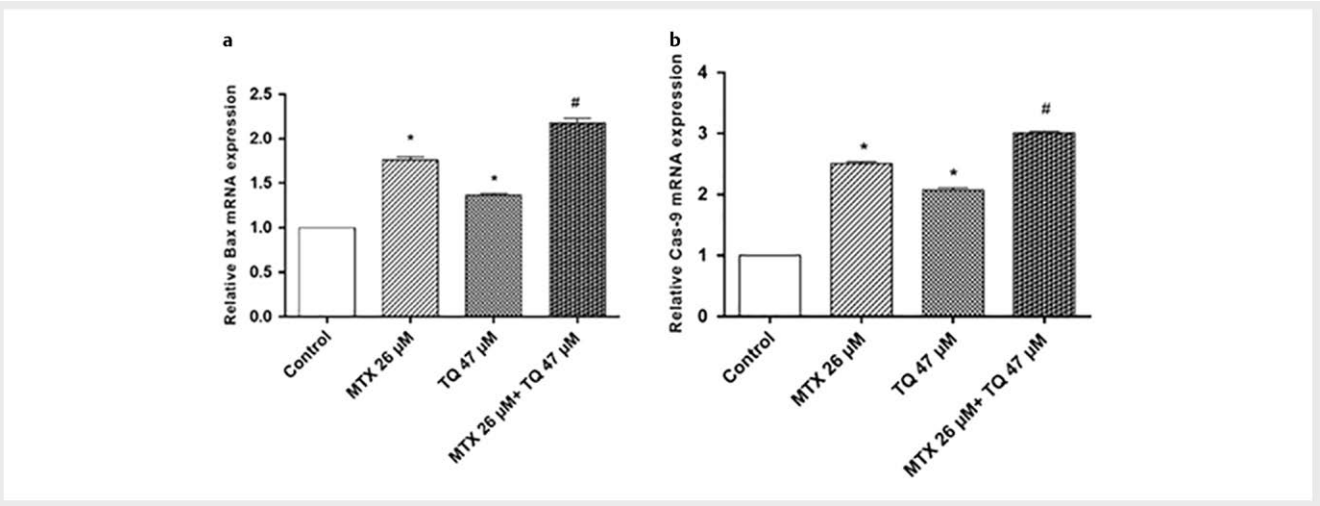
We performed an RT-PCR assay to examine whether the expression level of BCL-2 changed due to TQ and MTX single or combination regimens. As ▶ **Fig. 3** showed, the expression of BCL-2 in all experimental groups was lower than that of the control group in the Saos-2 cell line. Besides, in comparison with a single therapy, the combination regimen significantly suppressed the mRNA level of BCL-2. (* $P < 0.05$) in comparison to control and (# $P < 0.05$) in comparison to mono treatments. In this regard, there is a negative correlation between BCL-2 expression and combined regimen.

TQ plus MTX provokes apoptosis in the Saos-2 cell line

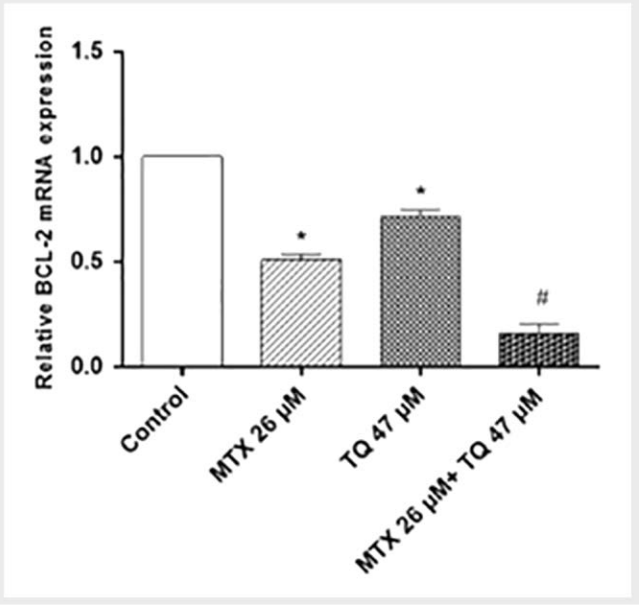
Upon assessing cell cycle changes by flow cytometry analysis, it was shown that the apoptosis rate dramatically enhanced in all experimental groups, compared to untreated cells. The apoptosis rate was 53 and 48 % in MTX and TQ treated cells, respectively, while this percentage for the control group was just 3 %. However, the most significant apoptotic capacity was observed in combination therapy by 73 % ($P < 0.05$) in comparison to control and (# $P < 0.05$) in comparison to mono treatments (▶ **Fig. 4**). As a result, combined treatment significantly induces apoptosis, compared to an individual drug.



▶ **Fig. 1** The effects of TQ and MTX and their co-treatment on cell proliferation. All values are represented as mean ± SD.



▶ **Fig. 2** TQ, MTX, and their combination effects on the induction of Bax and caspase-9 expression in Saos-2 cell line. The results were expressed as mean ± standard deviation (n = 3); * P < 0.05 in comparison to control.



▶ **Fig. 3** The effects of TQ and MTX single or co-treatment on the mRNA expression level of BCL-2 in Saos-2 cell line. The results were expressed as mean ± standard deviation (n = 3).

Discussion

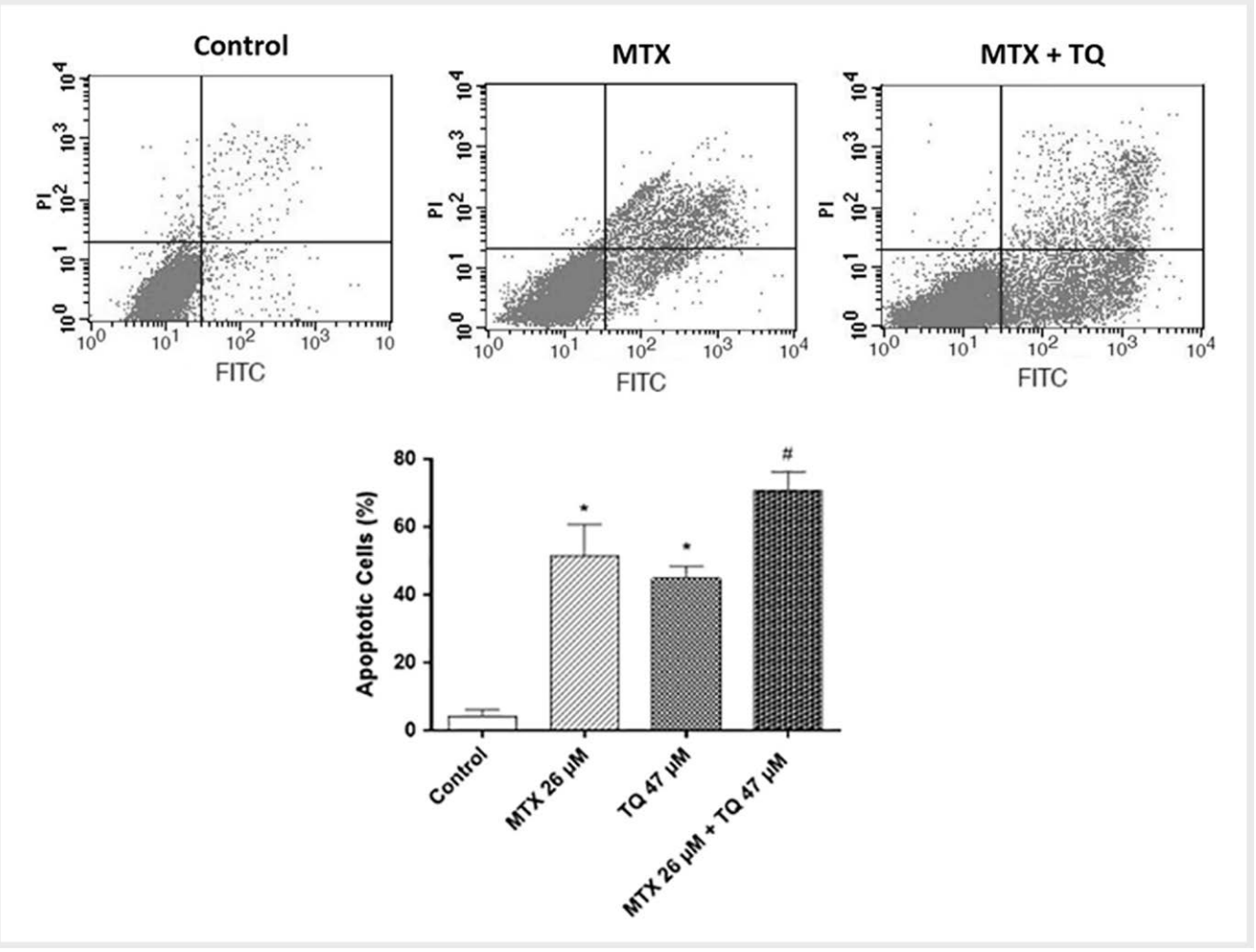
There are two peaks for OS incidence; primary (ages between 10 and 30) and secondary (over 70). The overall incidence of OS is low while its mortality rate and metastasis are high in adolescents. Clinically, a metaphyseal region in long bones is involved in OS which causes severe pain and discomfort [23]. As we discussed above, the combination of chemotherapeutic agents like methotrexate-doxorubicin-cisplatin (MAP) has been studied widely for OS therapy to improve the therapeutic index [24]. However, tumor resistance, drug side effects, and other factors are the main causes of OS recurrence or metastasis which are associated with low survival rates in these cases [25].

Methotrexate competitively suppresses dihydrofolate reductase (DHFR) which is associated with the impairment of tetrahydrofolate regeneration. This event results in preventing purine and thymidine synthesis and consequently cell apoptosis [26]. Although studies have investigated the application of MTX in OS therapy [27, 28], the drug mechanism of action on OS cells is unclear. In the present study, we illustrated that MTX can reduce cell proliferation and induce cell apoptosis through the up-regulation of Bax and caspase-9 expression and the down-regulation of BCL-2 expression.

However, drug resistance, delayed clearance, drug side effects, and cytotoxicity are the main concerns over the application of MTX alone in OS [10, 29–31]. Therefore, it has been suggested that to address these obstacles, it is essential to change and find safe and practical multidisciplinary treatment management [32].

Furthermore, in recent years, researchers have focused on evaluating the positive effects of natural agents like TQ or their combination with chemotherapy drugs on cancers treatment [15]. There is some evidence that TQ has positive effects against OS progression. For example, Martin Roepke et al reported that TQ induces intrinsic or mitochondrial apoptosis pathways in OS cell lines. Mechanistically, in the mitochondrial respiratory chain, TQ is soluble in the inner layer and, as an ubiquinone homologue, it can be found in three forms; oxidized, semi-reduced, and reduced. When TQ is reduced, it generates oxidative stress leading to cytochrome C, caspase 9 and 3 activation, and finally p53-independent cell apoptosis. In this condition, ROS accumulation is associated with DNA damage and cell death. Moreover, TQ appears to stimulate Bax expression as an apoptotic factor while prohibiting Bcl2 expression as an anti-apoptotic factor. In this regard, the upregulation of the Bax/Bcl-2 ratio by TQ results in cell apoptosis [21].

Furthermore, it has been reviewed that the application of TQ on OS cell lines can induce apoptosis and reduce cells migration and angiogenesis through the suppression of NF-κB, ERK1/2, and PI3K signalings and also VEGF [14]. Both in-vivo and in-vitro examina-



▶ **Fig. 4** The effects of single or combinational regimen on Saos-2 cell apoptosis.

tions by lei peng et al showed that TQ plays its protective effects against OS in dose-dependent manner. This study also revealed that NF-κB inhibition is exerted by TQ which results in the suppression of OS tumor growth and angiogenesis. Our data confirmed previous results and showed that TQ plays its protective role from OS progression via Bax and caspase-9 overexpression and BCL-2 down expression which overall leads to cell proliferation suppression and apoptosis induction [20].

Regarding the onset of combinational therapies, natural agents have attracted a lot of attention to be combined with chemotherapeutic drugs and enhance the overall therapeutic index [15]. For example, experimental analysis in 2016 investigated the combinational effect of TQ and 5FU or OXA on the MG63 OS cell line. Encouraging evidence showed that lower doses of chemotherapeutic agents (5FU or OXA) are required when they are combined with TQ, compared to the time they have been applied alone [33]. On this basis, TQ not only gives rise to apoptosis and prohibits cells proliferation but also might reduce chemoresistance, drug toxicity, and side effects.

In our investigation, we found that when TQ and MTX are combined, their potency enhances to reduce cell proliferation and improve apoptosis in OS cells. Moreover, in TQ and MTX combination therapy, lower doses of MTX are required to protect against OS progression. Therefore, we think that this combined regimen might reduce the MTX side effects and toxicity.

Conclusion

in the current study, we investigated the mechanism of the MTX-induced apoptosis of TQ in Saos-2 cells. The co-treatment of TQ and MTX attenuates cell survival and improves apoptosis via increasing the expression of pro-apoptotic factors (Bax and caspase-9) and reducing the expression of anti-apoptotic factor BCL-2. The IC50 of MTX decreases when it combines with TQ. Thus, this combination might alleviate the adverse effects of MTX. Further research is required to prove this assertion.

Acknowledgment

The authors would like to thank Shohada Clinical Research Development Unit, Shohada Hospital, Tabriz University of Medical Science, Tabriz, Iran (Ethical code: IR.TBZMED.REC.1400.434).

Conflict of Interest

The authors declare that there are no conflicts of interest.

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