Anticancer effects of methotrexate in combination with α-tocopherol and α-tocopherol succinate on triple-negative breast cancer

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Abstract. Triple-negative breast cancers (TNBCs) lack the estrogen receptor, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Therefore, hormone or targeted therapies are not effective in the treatment of TNBC and thus the development of novel therapeutic strategies is crucial. Methotrexate (MTX), a folate antagonist, has been used in the treatment of various types of cancer; however, the anticancer effects of MTX treatment on breast cancer have thus far been ineffective. Vitamin E variants and derivatives have been applied for cancer therapy. Previous studies have indicated that vitamin E variants and derivatives exert distinct anticancer effects on different types of cancer. However, whether MTX plus vitamin E variants or its derivatives can inhibit TNBC remains unclear. The aim of the present study was to examine the anticancer effects and mechanisms of action of MTX in combination with vitamin E variants (α-tocopherol) and derivatives (α-tocopherol succinate) on TNBC. In the present study, MTT assay and western blot analysis were used to determine the cell survival rates and protein levels. The results demonstrated that combination treatment with MTX and α-tocopherol suppressed TNBC cell proliferation. In addition, various concentrations of MTX exerted distinct cytotoxic effects on α-tocopherol succinate-treated cells. Furthermore, high-dose MTX enhanced α-tocopherol succinate-induced anticancer activity; however, low-dose MTX inhibited α-tocopherol succinate-induced anticancer activity. The present study also demonstrated that caspase-3 activation and poly(adenosine diphosphate-ribose) polymerase cleavage were observed in the α-tocopherol succinate/MTX-treated cells. In conclusion, the findings of the present study demonstrated that high-dose MTX enhanced anticancer activity in α-TOS-treated TNBC, while low-dose MTX reduced anticancer activity in α-TOS-treated TNBC.

Introduction

Breast cancer is a common type of non-skin malignant tumor affecting women (1). A previous study revealed that ~1.67 million breast cancer cases are diagnosed each year worldwide (2). Breast cancers with or without estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) can be divided into hormone-dependent and -independent breast cancers (3,4). It is considered that ~15-20% of breast cancer cells lack ER, PR and EGFR2, and thus are classed as triple-negative breast cancers (TNBCs) (5,6). As TNBCs lack hormone receptors, hormone or targeted therapies are not effective in the treatment of TNBCs in clinical practice (7,8). Thus, the development of novel treatment strategies for TNBCs is of utmost importance.

Methotrexate (MTX) is known as a folate antagonist used widely in the treatment of rheumatoid arthritis and cancer (9,10). Low-dose MTX has been shown to exert anti-inflammatory effects when used in the treatment of rheumatoid arthritis; however, high-dose MTX has been shown to have anti-proliferative cytotoxic activities with a number of side-effects, such as renal damage (9,11,12). Previous studies have suggested that MTX can induce cell cytotoxicity which is related to increases in reactive oxygen species (ROS) production (13,14). Furthermore, a number of studies have demonstrated that MTX can inhibit cell proliferation in various types of cancers including lung cancer, lymphoma, leukemia and hepatoma (15-19). However, to date, to the best

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of our knowledge, no previous study has demonstrated that MTX alone can exert sufficient anticancer effects on breast cancer. In order to enhance the anticancer effects of MTX on breast cancer, combination treatment with MTX with other agents has been considered (4,20,21).

Vitamin E is known as a fat-soluble vitamin with anti-oxidative function (22). Vitamin E has 8 variants, including 4 tocopherols (α-, β-, γ- and δ-tocopherols) and 4 tocotrienols (α-, β-, γ- and δ-tocotrienols); it has also been demonstrated to lower cancer risk (23-25). α-tocopherol is a major variant found in vitamin E and has anti-oxidative functions (26,27). The majority of studies have suggested that γ- and δ-tocopherol and γ- and δ-tocotrienol can inhibit breast cancer growth, while α-tocopherol does not exert obvious anticancer activity in breast cancer (25,28). Studies have demonstrated that γ- and δ-tocopherol only inhibit ER-positive (ER$^+$) breast cancer, while γ- and δ-tocotrienol can inhibit both ER$^+$ breast cancer and TNBC (23,28). In addition, a previous study also revealed that high-dose (100 μM) α-tocopherol inhibited cell proliferation in ER$^+$ breast cancer, including MCF-7 and T47D cells in a dose-dependent manner (29). Previous studies have also demonstrated that only α-tocopherol succinate (α-TOS) has potent antioxidant activities, while γ- and δ-tocotrienol do not (22-27). Clinical MTX treatment can induce oxidative stress resulting in side-effects (9,11,12); therefore, perhaps MTX-induced oxidative stress can be decreased by the use of α-TOS. Therefore, whether α-tocopherol has potent anticancer activities in breast cancer warrants further investigation.

α-TOS is an analogue of α-tocopherol with anticancer activity (30-32). Previous studies have revealed that α-TOS has anticancer activities in various hormone-dependent breast cancers, such as MCF-7, MDA-MB-435, 4T1 and MDA-MB-453 cells (33-36). Another previous study demonstrated that α-TOS induced the apoptosis of TNBC cells, such as MDA-MB-231 and SKBR-3 cells (37). However, that study only demonstrated that apoptotic characteristics and Fas signals were induced in α-TOS-treated cells. The other mechanisms associated with α-TOS treatment in TNBC warrant further investigation.

Based on the above-mentioned studies, MTX, vitamin E and its analogue have been previously administered in the treatment of breast cancers. The present study aimed to determine whether combination treatment with MTX and vitamin E or its analogue may have more potential for use in the treatment of TNBCs. The anticancer effects on TNBC were determined following treatment with MTX, α-tocopherol, α-TOS, MTX/α-tocopherol and MTX/α-TOS.

Materials and methods

Materials, reagents and antibodies. Fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM), non-essential amino acids, L-glutamine and penicillin/streptomycin were obtained from Gibco/Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The MTT assay kit was obtained from Bio Basic Canada Inc. (Markham, OT, Canada). Luminol, lucigenin, α-tocopherol, α-TOS and Hoechst 33342 were purchased from Sigma-Aldrich/Merck KGaA (Darmstadt, Germany). Anti-tubulin (1:1,000; cat. no. BS1699) primary rabbit polyclonal antibody was acquired from Bioworld Technology, Inc.

(Louis Park, MN, USA). Anti-cleaved poly(adenosine diphosphate-ribose) polymerase (PARP; 1:2000; cat. no. 9544) and anti-caspase-3 (1:1000; cat. no. 9665) primary rabbit polyclonal antibodies and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (1:2,000; cat. no. 7074) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cells and cell culture. The TNBC cell lines, MDA-MB-231 and MDA-MB-468, were purchased from the Bioresource Collection and Research Center (Shin Chu, Taiwan). The cells were cultured with DMEM supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids, 2 mM L-glutamine and 100 IU/ml penicillin/streptomycin and maintained at 37˚C with a humidified atmosphere containing 5% CO_2.

Cell survival rate assay. The cell survival rate was determined using an MTT assay. The cells were cultured in 96-well dish ($3x10^3$ cells/well). Every 24 h, the control and experimental groups (MTX, vitamin E, MTX/vitamin E treatments) were treated with the MTT kit. The cells were incubated with MTT solution for 3 h at 37˚C and the formazan product was produced. The formazan product was dissolved and the absorbance was determined at 570 nm (A570) using a Multiskan™ FC Microplate Photometer (Molecular Devices LLC, Sunnyvale, CA, USA). The survival rate (%) was calculated as [(A570 experimental group)/(A570 control group)] x 100%.

Measurements of intracellular H_2O_2. The production of cellular H_2O_2 was determined using the lucigenin-amplified chemiluminescence method (38,39). The control and experimental samples (200 μl) were treated with 0.2 mmol/ml of luminol solution (100 μl) to determine the H_2O_2 levels. All samples were analyzed and observed for 5 min using a chemiluminescence analyzing system (CLA-FSI; Tohoko Electronic Industrial Co., Ltd., Sendai, Japan).

SDS electrophoresis and western blot analysis. The control and experimental cells were treated with lysis buffer (radio-immunoprecipitation assay buffer; cat. no. 20-188; EMD Millipore, Billerica, MA, USA). Following centrifugation (16,000 x g; 4˚C) for 30 min, proteins were obtained from the supernatant layer. The protein concentration was determined using a protein assay kit (cat. no. 23200; Thermo Fischer Scientific, Inc.). A total of 40 μg protein was loaded and separated on 13.3% SDS-PAGE under 80 volts. Separated proteins were transferred onto PVDF membranes (EMD Millipore). The membranes were firstly blocked with 5% non-fat milk at room temperature for 2 h. After washing with phosphate-buffered saline (PBS) for 15 min (3 times), the membranes were treated with primary antibodies for 4 h at room temperature, and the membranes were then washed with PBS for 15 min (3 times). The membranes were subsequently incubated with anti-rabbit HRP-conjugated secondary antibodies (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) at room temperature for 1 h. Finally, immunolabeled proteins were added and incubated with Western Lightning® Chemiluminescence Plus reagent (PerkinElmer, Inc., Waltham, MA, USA). The protein band was observed

and analyzed with a Luminescence Image Analysis system (LAS-4000; FUJIFILM Electronic Materials Taiwan Co., Ltd., Taiwan) and ImageJ 1.51j8 by Wayne Rasband (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All data were obtained and calculated from 3 or 4 independent experiments. Values are expressed as the means ± SD and analyzed using one-way ANOVA (SPSS for Windows, version 10; SPSS, Inc., Chicago, IL, USA) followed by Tukey's test for the comparisons of group means. All statistical analyses were performed using SAS for Windows, version 9.4. The level of significance was set at a P-value <0.05.

Results

Combined treatment with MTX and α-tocopherol suppresses the proliferation of TNBC cells. MTX and α-tocopherol were used to treat the TNBC MDA-MB-231 cells. The survival rates were ~90% following 72 h of treatment with 0.1 μM MTX, 10 μM MTX and 5 μM α-tocopherol in the MDA-MB231 cells (Fig. 1). These results indicated that MTX alone and α-tocopherol alone did not effectively suppress the proliferation of the MDA-MB-231 cells. Combined treatment with MTX and α-tocopherol was then applied in the MDA-MB-231 cells. The survival rate was ~75% following treatment of the MDA-MB231 cells with 0.1 μM MTX plus 5 μM α-tocopherol (Fig. 2A). In addition, the survival rate was below 70% following treatment of the MDA-MB231 cells with 10 μM MTX plus 5 μM α-tocopherol (Fig. 2B). As shown in Fig. 2, when compared with the group treated with MTX alone, the group treated with MTX plus α-tocopherol (MTX/α-tocopherol) exhibited significantly lower survival rates at 48 and 72 h. These data suggested that MTX/α-tocopherol treatment suppressed the proliferation of the MDA-MB-231 cells. However, the survival rate was above 60% in the MTX/α-tocopherol-treated group following 72 h of treatment. Thus, it was considered that MTX/α-tocopherol may only attenuate cell proliferation and thus MTX/α-tocopherol treatment may be not be an effective strategy for clinical treatment.

α-tocopherol decreases the H_2O_2 levels in MTX-treated cells. Previous studies have demonstrated that MTX can induce an increase in ROS in cells, particularly H_2O_2 levels, causing cell cytotoxicity (14,19). In the present study, we wished to determine whether MTX also induces an increase in H_2O_2 levels in MDA-MB-231 cells. Compared to the control group, treatment with 10 μM MTX significantly increased the H_2O_2 levels in the MDA-MB-231 cells, while treatment with 0.1 μM MTX did not markedly increase the H_2O_2 levels (Fig. 3A). These results indicated that MTX increases the H_2O_2 levels in a dose-dependent manner. α-tocopherol is known to have anti-oxidative activities (26,27). In the present study, whether α-tocopherol can inhibit the MTX-induced increase in H_2O_2 levels was investigated. The results revealed that α-tocopherol decreased the H_2O_2 levels in the MTX-treated cells (Fig. 3B). Notably, as shown in Figs. 2 and 3, the H_2O_2 levels were not associated with cell proliferation in the MTX-treated and MTX/α-tocopherol-treated MDA-MB-231 cells.
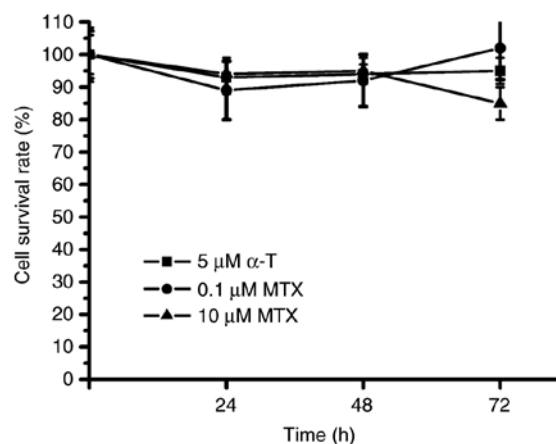

Figure 1. MTX alone and α-tocopherol alone do not induce cytotoxicity. MDA-MB-231 cells were incubated with 0.1 and 10 μM MTX and 5 μM α-tocopherol for 72 h. Every 24 h, the samples were treated with an MTT kit. Survival rates were calculated as (A570 experimental group/A570 control group) x 100%. Data were analyzed from 4 independent experiments and are presented as the means ± standard deviation. MTX, methotrexate; α-T, α-tocopherol.

α-TOS induces cell cytotoxicity in TNBC in a time- and concentration-dependent manner. A previous study indicated that α-TOS induces the apoptosis of and Fas activation in breast cancer cells (37). In the present study, the concentration of α-TOS and the incubation time were further investigated in the α-TOS-treated MDA-MB-231 cells. As shown in Fig. 4, the cell survival rate was ~90% following treatment of the MDA-MB-231 cells with 5 and 20 μM α-TOS for 72 h; however, the cell survival rate at 72 h was ~50% following treatment with 40 μM α-TOS and below 50% following treatment of the MDA-MB-231 cells with 60 and 80 μM α-TOS. These results indicated that α-TOS induced cytotoxicity in a concentration-dependent manner. Furthermore, following treatment with 40, 60 and 80 μM α-TOS, the survival rates of the MDA-MB-231 cells gradually decreased during the 72-h treatment period. Thus, α-TOS induced cytotoxicity in a time-dependent manner.

High- and low-dose MTX induces distinct cytotoxicity in α-TOS-treated TNBC cells. Our data demonstrated that MTX/α-tocopherol treatment suppressed TNBC cell proliferation, although MTX/α-tocopherol induced-anticancer activity was ineffective (Fig. 2). The present study further investigated the anticancer effects on MDA-MB-231 cells following treatment with MTX plus α-TOS, or α-tocopherol derivatives. The 72-h cell survival rate was ~50% following treatment of the MDA-MB-231 cell with 40 μM α-TOS alone (Fig. 5). Notably, the 72-h MDA-MB-231 cell survival rate was ~35% in the group treated with 40 μM α-TOS plus 10 μM MTX; however, it was ~60% in the 40 μM α-TOS plus 0.1 μM MTX group (Fig. 5). During the 72-h treatment period, the survival rate curve of the group treated with 40 μM α-TOS plus 10 μM MTX was below that of the group treated with 40 μM α-TOS only. However, the survival rate curve of the group treated with 40 μM α-TOS plus 0.1 μM MTX was above that of the group treated with 40 μM α-TOS alone. Therefore, these results indicated that high-dose MTX enhanced anticancer activity
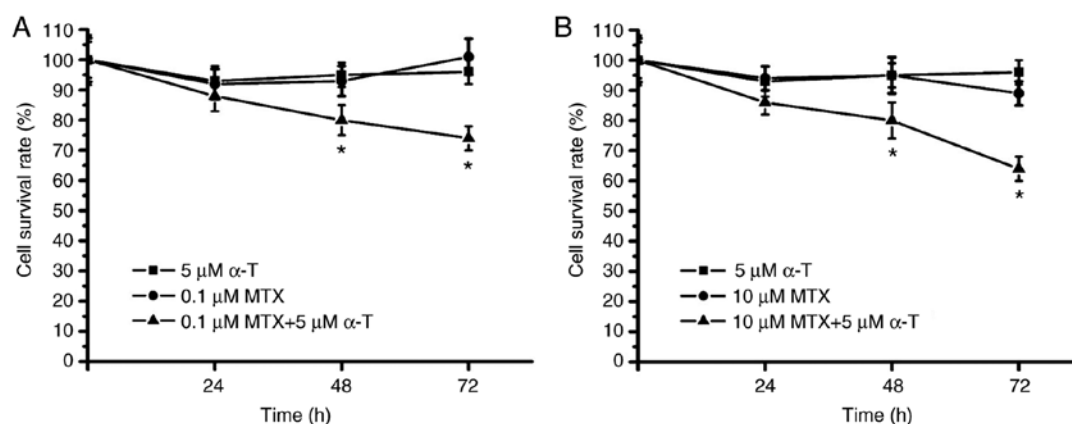
Figure 2. Combined treatment with MTX and α-tocopherol exerts a synergistic effect. (A) MDA-MB-231 cells were incubated with 0.1 μM MTX, 5 μM α-tocopherol and 0.1 μM MTX plus 5 μM α-tocopherol, respectively, for 72 h. (B) MDA-MB-231 cells were incubated with 10 μM MTX, 5 μM α-tocopherol and 10 μM MTX plus 5 μM α-tocopherol, respectively, for 72 h. Every 24 h, the samples were treated with an MTT kit. Survival rates were calculated as (A570 experimental group/A570 control group) x 100%. Data were analyzed from 4 independent experiments and are presented as the means ± standard deviation. *P<0.05 vs. the group treated with MTX only. MTX, methotrexate; α-T, α-tocopherol.
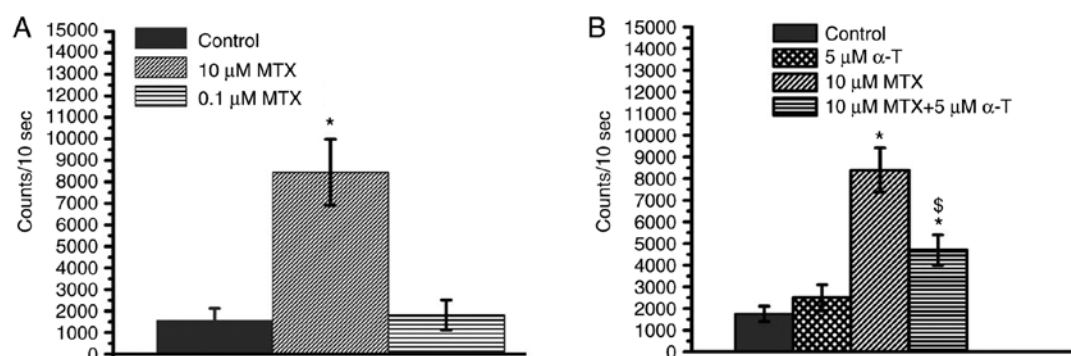

Figure 3. α-tocopherol decreases MTX-induced H_2O_2 levels. (A) Cells were incubated with 0.1 or 10 μM MTX, respectively. (B) Cells were incubated with 10 μM MTX or 10 μM MTX plus 5 μM α-tocopherol, respectively. Following a 4-h incubation, samples were treated with luminol solution. Data were analyzed from 4 independent experiments and are presented as the means ± standard deviation. *P<0.05 vs. the control group; $P<0.05 vs. the group treated with 10 μM MTX only. MTX, methotrexate; α-T, α-tocopherol.
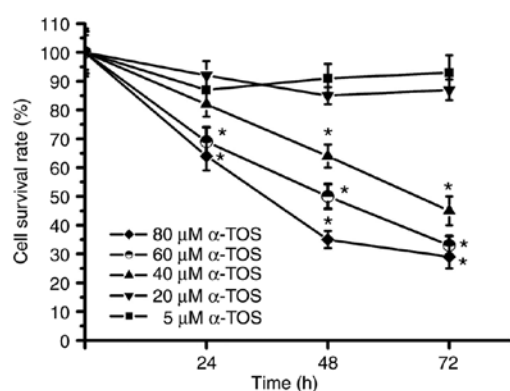

Figure 4. α-TOS exerts a dose-dependent cytotoxicity effect. Cells were incubated with 5, 20, 40, 60 and 80 μM α-TOS, respectively, for 72 h. Every 24 h, the samples were treated with an MTT kit. Survival rates were calculated as (A570 experimental group/A570 control group) x 100%. Data were analyzed from 4 independent experiments and are presented as the means ± standard deviation. *P<0.05 vs. the 5 μM α-TOS group. α-TOS, α-tocopherol succinate.
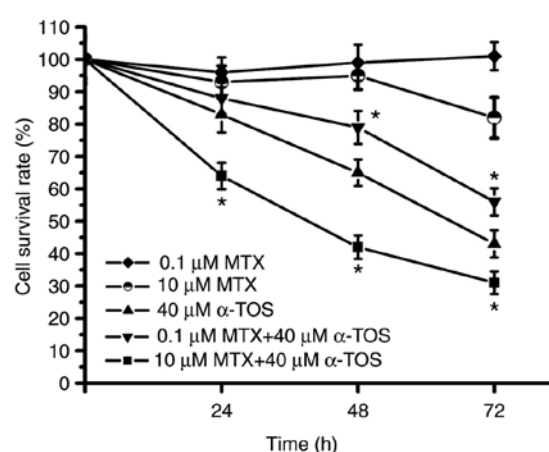
in α-TOS-treated MDA-MB-231 cells, while low-dose MTX reduced anticancer activity in α-TOS-treated MDA-MB-231 cells.

Figure 5. High- and low-dose MTX induce distinct cytotoxicity in α-TOS-treated cells. Cells were incubated with 0.1 μM MTX, 10 μM MTX, 40 μM α-TOS, 0.1 μM MTX plus 40 μM α-TOS and 10 μM MTX plus 40 μM α-TOS, respectively, for 72 h. Every 24 h, the samples were treated with an MTT kit. Survival rates were calculated as (A570 experimental group/A570 control group) x100%. Data were analyzed from 4 independent experiments and are presented as the means ± standard deviation. P-values were evaluated among the 40 μM α-TOS only group, 0.1 μM MTX plus 40 μM α-TOS group and 10 μM MTX plus 40 μM α-TOS group. *P<0.05 vs. the 40 μM α-TOS only group. MTX, methotrexate; α-TOS, α-tocopherol succinate.
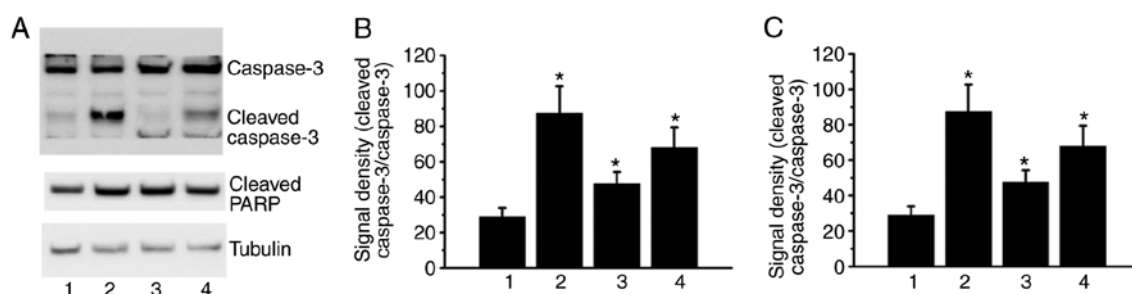
Figure 6. MTX and α-TOS induce casepase-3 activation and PARP cleavage. (A) Western blot analysis, (B) cleaved caspase-3/caspase-3 relative intensity and (C) cleaved PARP/tubulin were observed and determined at 72 h in the control group (lane 1), 40 μM α-TOS group (lane 2), 0.1 μM MTX plus 40 μM α-TOS group (lane 3) and 10 μM MTX plus 40 μM α-TOS group (lane 4). Data were determined from 3 independent experiments and are presented as the means ± standard deviation. *P<0.05 vs. the control group. MTX, methotrexate; PARP, poly(adenosine diphosphate-ribose) polymerase; α-TOS, α-tocopherol succinate.

α-TOS- and MTX/α-TOS-induced cytotoxicity is associated with caspase-3 activation and PARP cleavage. Previous studies have demonstrated that both α-TOS and MTX can induce cell cytotoxicity via caspase-3 activation (19,40,41). In the present study, as shown in Fig. 5, treatment with 40 μM α-TOS, 40 μM α-TOS/0.1 μM MTX and 40 μM α-TOS/10 μM MTX induced cell cytotoxicity. Thus, caspase-3 activation and PARP, downstream of caspase-3, were investigated in the present study. As determined by western blot analysis, the ratio of cleaved caspase-3/pro-caspase-3 was increased in the α-TOS- and α-TOS/MTX-treated cells (Fig. 6). In addition, PARP cleavage was also observed in the α-TOS- and α-TOS/MTX-treated cells (Fig. 6). Therefore, these results indicate that α-TOS and α-TOS/MTX may induce cell cytotoxicity via caspase-3 activation in MDA-MB-231 cells.

Discussion

Previous studies have indicated that vitamin E has anti-oxidative functions in cancer chemotherapy, reducing chemical agent-induced side-effects (42,43). Furthermore, vitamin E can prolong the survival of patients with gastric cancer (44) and can attenuate prostate cancer metastasis (45). α-tocopherol is the most common form found abundantly in vitamin E and displays anti-oxidative activity (42,46). α-tocopherol is also used to decrease chemical agent-induced side-effects in cancer therapy (47). The low dose of MTX (0.1 μM) used in the present study, is the concentration that is used in clinical practice for the treatment of rheumatoid arthritis. The high dose of MTX (10 μM) used in the present study is the dose used in clinical practice for cancer therapy. Currently, a dose of ≥10 μM MTX is used in clinical practice for cancer therapy. On the other hand, vitamin E is an antioxidant nutrient, and the dose range of vitamin E used in clinical practice varies widely. Generally, the dose of 5 to 80 μM vitamin E is used for combination treatment in clinical practice for various diseases. Therefore, the dose range of 5 to 80 μM vitamin E was used in the present study. A previous study revealed that α-tocopherol enhances the anti-proliferative effects of gefitinib in cells (48). However, α-tocopherol may attenuate the anticancer activity of some chemical agents, such as tamoxifen and crizotinib (49,50). The present study demonstrated that treatment with α-tocopherol plus MTX decreased the proliferation of MDA-MB-231 cells (Fig. 2). Therefore, it was suggested that α-tocopherol may

have different anticancer effects when used in combination with different chemical agents. However, the mechanisms through which α-tocopherol promotes MTX- and gefitinib-induced cell proliferation and inhibits tamoxifen- and crizotinib-induced anticancer activities remain unclear.

A recent study demonstrated that treatment with vitamin C/MTX increased H_2O_2 levels, resulting in cell cytotoxicity (4). A number of studies have also revealed that high H_2O_2 levels are associated with cell cytotoxicity (51-53). However, the present study demonstrated that α-tocopherol attenuated the MTX-induced H_2O_2 levels (Fig. 3B), while treatment with α-tocopherol plus MTX exerted anti-proliferative effects on MDA-MB-231 cells (Fig. 2). Therefore, it was suggested that the anti-proliferative effects of treatment with α-tocopherol/MTX may not be related to the H_2O_2 levels. The mechanisms underlying the anti-proliferative activity of α-tocopherol/MTX require further investigation in the future.

Previous studies have demonstrated that cyclopentenone prostaglandins/α-TOS treatment and MTX/α-TOS treatment have synergistic anticancer effects on oral squamous carcinoma cells (54) and osteosarcoma cells (55). Similar to these studies, the data from the present study also demonstrated that high-dose MTX/α-TOS had a synergistic anticancer effect on MDA-MB-231 cells. The survival rate was below 50% following treatment with high-dose MTX/α-TOS for 48 h (Fig. 5). Compared with the survival rate of the high-dose MTX/α-TOS treatment group, the survival rate was still above 60% following treatment with high-dose MTX/α-tocopherol for 72 h (Fig. 2B). Therefore, it was suggested that high-dose MTX/α-TOS may be a potential treatment for TNBC, while high-dose MTX/α-tocopherol treatment may not be an effective strategy for TNBC treatment.

The present study, as well as previous studies have demonstrated that α-tocopherol/MTX and α-tocopherol/gefitinib treatment exert synergistic anti-proliferative effects (48). However, previous studies have also revealed that α-tocopherol/tamoxifen and α-tocopherol/crizotinib treatment exert antagonistic anti-proliferative effects (49,50). These studies indicate that α-tocopherol in combination with different chemical agents has distinct anti-proliferative effects. However, in the present study, high-dose MTX enhanced α-TOS-induced cell cytotoxicity, while low-dose MTX antagonized α-TOS-induced cell cytotoxicity in MDA-MB-231cells (Fig. 5). This result was similar to that of a previous study (34). This previous

study demonstrated that high-dose sodium selenite promoted α-TOS-induced cell cytotoxicity, while low-dose sodium selenite attenuated α-TOS-induced cell cytotoxicity in MCF-7 cells. Based on these studies, it was suggested that both synergistic and antagonistic effects can arise in the same drug components in a dose-dependent manner. Although the detailed signaling mechanisms require further investigation, the results of the present study suggested that caspase signaling (Fig. 6) had a similar mechanism when comparing the low- and high-dose effects of MTX with α-TOS treatment. However, oxidative stress (such as H_2O_2 levels; Fig. 3) may have different signaling pathways when comparing the low- and high-dose effect of MTX with α-TOS treatment. In addition, as shown in Fig. 5, high-dose MTX slightly reduced the cell survival rate, while low-dose MTX slightly increased the cell survival rate. These results may be related to the synergistic or antagonistic anticancer effects caused by α-TOS/MTX treatment. Both MDA-MB-231 and MDA-MB-468 were examined in the present study. Similar results were obtained with the MDA-MB-468 cells (data not shown).

In conclusion, the present study demonstrated that combined treatment with MTX and α-tocopherol or α-TOS attenuated the cell survival rate of TNBC cells. However, MTX/α-TOS exerted a more potent anti-proliferative effect than MTX/α-tocopherol. In addition, high-dose MTX enhanced the α-TOS-induced cytotoxic effects, while low-dose MTX antagonized the α-TOS-induced cytotoxic effects.

Availability of data and materials

All data generated or analyzed during the present study are included in this published article or are available from the corresponding author on reasonable request.

Authors' contributions

CWW and YLY performed the experiments, analyzed the data and wrote the manuscript. YHC and YTH performed the experiments and analyzed the data. GTY designed the experiments and analyzed the data. All authors approved the final version of the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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