

# Vitamin C enhances anticancer activity in methotrexate-treated Hep3B hepatocellular carcinoma cells

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**Abstract.** Methotrexate (MTX) has been widely used for rheumatoid arthritis therapy for a long time. MTX is also used as an anticancer drug for various tumors. However, many studies have shown that high-dose MTX treatment for cancer therapy may cause liver and renal damage. Although the mechanisms involved in MTX-induced liver and renal damage require further research, many studies have indicated that MTX-induced cytotoxicity is associated with increases in oxidative stress and caspase activation. In order to reduce MTX-induced side-effects and increase anticancer efficiency, currently, combination treatments of low-dose MTX and other anticancer drugs are considered and applied for various tumor treatments. The present study showed that MTX induces increases in H<sub>2</sub>O<sub>2</sub> levels and caspase-9/-3 activation leading to cell death in hepatocellular carcinoma Hep3B cells. Importantly, this study is the first to demonstrate that vitamin C can efficiently aid low-dose MTX in inducing cell death in Hep3B cells. Therefore, the present study provides

a possible powerful therapeutic method for tumors using a combined treatment of vitamin C and low-dose MTX.

## Introduction

Methotrexate (MTX) is one of the most popular and safe antirheumatic drugs under the applied treatment dose (1,2). In order to obtain a better curative effect in clinical cases, MTX is also used in combination with other drugs for rheumatoid arthritis treatment (1,3,4). In addition, MTX is also used as an anticancer drug (5). Recently, MTX has been widely applied for the treatment of various cancers, such as hepatoma, osteosarcoma, leukemia, lymphoma, gastric, breast, head and neck cancers (5-9). Many studies have demonstrated that MTX induces cancer cell death via apoptotic death pathways (10-14). Apoptotic death pathways can be divided into caspase-dependent and caspase-independent cascades (15,16). Concerning the MTX-induced apoptotic pathways, most studies have shown that MTX induces apoptosis via caspase-dependent cascades in many cancer cell lines (17-21). However, some studies have indicated that MTX can induce apoptosis via caspase-independent cascades in osteosarcoma cells (22,23). The present study found that MTX-induced apoptosis in Hep3B cells is via the caspase-dependent cascade, similar to most other studies (17-21).

Two major caspase cascade pathways have been reported (24-26). One is the caspase-8/-3 cascade, known as the extrinsic death receptor pathway (CD95/APO-1/Fas receptor) (27-29). Another is the caspase-9/-3 cascade, known as the intrinsic mitochondrial death pathway (27,30,31). Some studies have shown that MTX-induced apoptosis is mediated by the caspase-9/-3 cascade pathway in choriocarcinoma, breast cancer, oral squamous carcinoma and hepatoma cells (18,19,21,32,33). In contrast, some studies demonstrated that MTX-induced apoptosis is mediated through the caspase-8/-3 cascade pathway in breast cancer, hepatoma and leukemia

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cells (17,33,34). The present study showed that MTX activates the caspase-9/-3 cascade in Hep3B cells, but not the caspase-8/-3 cascade.

Previously, many studies have shown that high-dose MTX treatment can induce increased oxidative stress, resulting in renal and liver damage (3,35-37). However, the specific reactive oxygen species (ROS) induced by MTX treatment have not been identified.  $O_2^-$  and  $H_2O_2$  are ROS families generally existing in many cells. By using the lucigenin-amplified method (38-40), our results are the first to demonstrate that MTX can induce increases in  $H_2O_2$  levels, but not  $O_2^-$  levels.

Considering that high-dose MTX treatments can cause renal and liver damage (35-37), combination treatments of low-dose MTX and other anticancer drugs are suggested and applied during clinical cancer therapy in order to enhance the anticancer effects and decrease MTX-induced side-effects (9,10,12,18,41). However, not all anticancer agents can enhance the anticancer effects of low-dose MTX. A recent study showed that aspirin can antagonize the MTX-induced cytotoxic effect on lung cancer cells (42). Alternatively, there have been many reports on the antioxidant activities of vitamin C (43-47). Moreover, some studies have demonstrated that vitamin C can exert anticancer activities in various cancer cells (48-52). The present study demonstrated that vitamin C can diminish MTX-induced increases in  $H_2O_2$  levels. On the other hand, it is worth noting that vitamin C can help low-dose MTX exert a cytotoxic effect on Hep3B cells. Taken together, the study demonstrated that MTX activates the caspase-9/-3 cascade and induces increased  $H_2O_2$  levels, causing cell cytotoxicity in Hep3B cells, while more importantly, the present study is the first to demonstrate that vitamin C enhances the anticancer efficiency in MTX-treated Hep3 cells.

## Materials and methods

**Chemicals and materials.** Methotrexate was purchased from Pfizer Inc. MTT assay kit was purchased from Bio Basic Canada Inc. Hoechst 33342, vitamin C, lucigenin and luminol were purchased from Sigma. Caspase-3 like substrate (Ac-DEVD-pNA), caspase-8 substrate (Ac-IETD-pNA) and caspase-9 substrate (Ac-LEHD-pNA) were purchased from AnaSpec, Inc. (San Jose, CA, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), non-essential amino acid, L-glutamine and penicillin/streptomycin were purchased from Gibco-BRL.

**Cell cultures.** Hep3B cells were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin, and 0.1 mM non-essential amino acids. The cells were cultured at 37°C in a humidified atmosphere containing 5%  $CO_2$ .

**Cell viability assay.** Hep3B cell viability was assessed using the MTT assay method according to the manufacturer's instructions. In brief, Hep3B cells were maintained in each well of 96-well culture plates. Every 24 h, the control group and experimental groups were subjected to the MTT assay kit. After 3 h of incubation, absorbance at 570 nm for each well containing Hep3B cells was detected under a multi-well ELISA reader (Molecular Devices). Cell viability was

calculated using the following formula:  $A_{570} \text{ experimental group} / A_{570} \text{ control group} \times 100\%$ .

**Nuclear condensation and DNA fragmentation.** Apoptotic cells were identified by nuclear condensation and DNA fragmentation using Hoechst 33342 staining. Cells were treated with 10  $\mu\text{g/ml}$  Hoechst 33342 for 10 min. Nuclear condensation and DNA fragmentation were observed under a fluorescence microscope (excitation, 352 nm; emission, 450 nm) (53,54).

**Caspase activity assay.** Caspase activity assays were executed according to previous studies (55,56). In brief, Hep3B cells were lysed with a lysis buffer (50 mM Tris-HCl, 120 mM NaCl, 1 mM EDTA, 1% NP-40, pH 7.5) and protease inhibitors. After centrifugation (15,000  $\times$  g, 30 min, 4°C) cell pellets were collected. The working solutions containing 40  $\mu\text{l}$  cell lysates (80  $\mu\text{g}$  total protein), 158  $\mu\text{l}$  reaction buffer (20% glycerol, 0.5 mM EDTA, 5 mM dithiothreitol, 100 mM HEPES, pH 7.5) and 2  $\mu\text{l}$  fluorogenic caspase substrate (Ac-LEHD-pNA, Ac-DEVD-pNA or Ac-IETD-pNA) were incubated at 37°C for 6 h. Fluorogenic substrate cleavage was determined at 405 nm in an ultra-microplate reader (BioTek Instruments). The fold increase in caspase activity was calculated using the following formula:  $(A_{405} \text{ experimental group} - A_{405} \text{ control group}) / A_{405} \text{ control group}$ .

**Determination of  $H_2O_2$  and  $O_2^-$  levels.**  $H_2O_2$  and  $O_2^-$  levels were examined by using lucigenin-amplified chemiluminescence according to the lucigenin-amplified method (57,58). In brief, for  $H_2O_2$  levels, the sample (200  $\mu\text{l}$ ) was mixed with 0.2 mmol/l luminol solution (100  $\mu\text{l}$ ). After that, the mixture was measured with a chemiluminescence analyzing system (CLA-FSI; Tohoko Electronic Industrial Co., Ltd., Miyagi, Japan) for determination. For  $O_2^-$  levels, 200  $\mu\text{l}$  of the sample was mixed with 0.1 mmol/l of lucigenin solution (500  $\mu\text{l}$ ), and was then measured by the CLA-FSI chemiluminescence analyzing system.

**Statistical analysis.** Experimental data were calculated from three independent triplicate experiments and are presented as the mean values of the chosen triplicate groups. These experimental data are shown as means with standard deviations.

## Results

**MTX exerts dose-dependent and time-dependent anticancer effects on Hep3B cells.** In clinical cases, 10-25 mg/week MTX ( $\sim 0.1 \mu\text{M/day}$ ) is a safely applied dose for rheumatoid arthritis treatment (1,2,59). In the present study, 0.1  $\mu\text{M}$  (treatment-dose), 0.01  $\mu\text{M}$  (low-dose) and 10  $\mu\text{M}$  (high-dose) MTX were used for studying the anticancer effects on Hep3B cells. Hep3B cell viability decreased in the 0.1 and 10  $\mu\text{M}$  MTX treatment groups, but did not decrease in the 0.01  $\mu\text{M}$  treatment group (Fig. 1). In addition, the 10  $\mu\text{M}$  MTX treatment group showed a stronger cytotoxic effect in the Hep3B cells than the 0.1  $\mu\text{M}$  MTX treatment group. These data suggest that MTX exerts a dose-dependent anticancer effect on Hep3B cells. In addition, cell viability was observed over different MTX incubation times, with results showing that the cell viability decreased incrementally in the 0.1 and 10  $\mu\text{M}$  MTX groups. The present

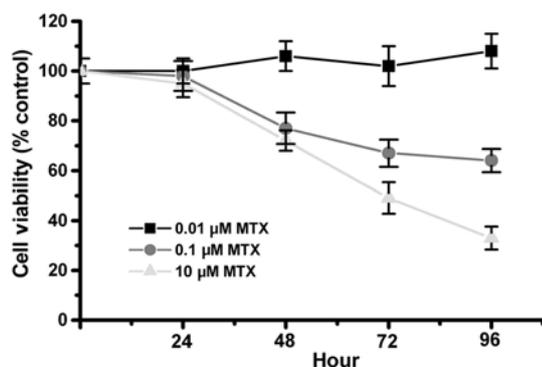


Figure 1. Cell viability. Hep3B cells were treated with 10, 0.1 and 0.01  $\mu\text{M}$  MTX. Cell viability was measured with MTT assay every 24 h and presented as A570 experimental group/A570 control group  $\times$  100%. Data were collected from 4 independent experiments and are presented as mean  $\pm$  SD.

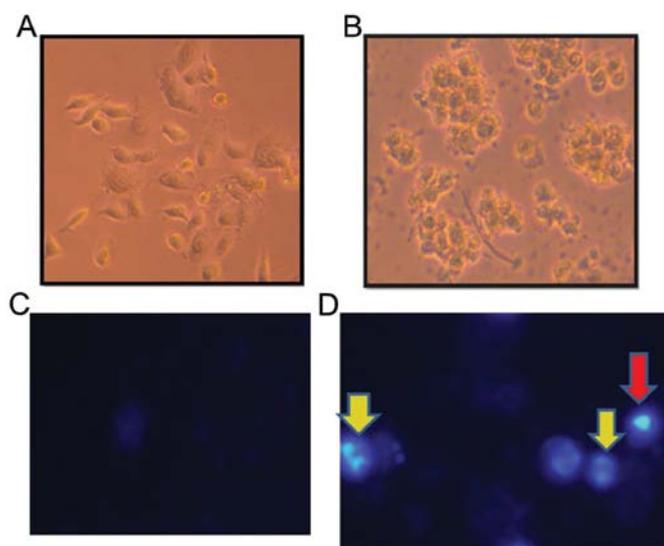


Figure 2. Cell morphology, nuclear condensation and DNA fragmentation. (A) Control cells, (B) MTX-treated cells. Hep3B cells were treated with or without 10  $\mu\text{M}$  MTX for 96 h, and cell morphology was observed under a phase-contrast microscope. (C) Control cells, (D) MTX-treated cells. Cells were treated with or without 10  $\mu\text{M}$  MTX for 96 h, and nuclear condensation and DNA fragmentation were observed using Hoechst 33342 staining. Note that nuclear condensation (red arrow) and DNA fragmentation (yellow arrow) were noted in the MTX-treated cells.

study indicates that MTX exerts a dose-dependent and time-dependent anticancer effect on Hep3B cells.

*MTX induces apoptosis and activates the caspase-9/-3 cascade in Hep3B cells.* The study investigated whether MTX induces apoptosis in Hep3B cells. Cell morphology was observed under a phase-contrast microscope. Hep3B cells survived with morphology intact in the control group (Fig. 2A). However, dead cells were noted in the MTX treatment group (Fig. 2B). In addition, nuclear condensation and DNA fragmentation are apoptotic features and can be observed using a nuclear staining method, as previously described (55,60). Compared with the control group (Fig. 2C), nuclear condensation and DNA fragmentation were noted in the MTX-treated group (Fig. 2D). The results indicate that MTX induced apoptosis in the Hep3B cells. Next, caspase activation was determined in the

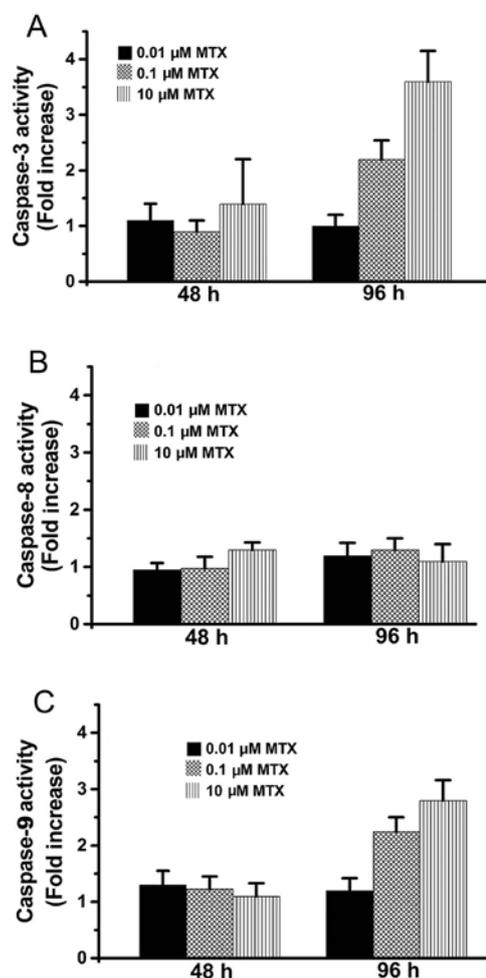


Figure 3. Caspase activity. (A) Caspase-3, (B) caspase-8 and (C) caspase-9 activities were determined at 48 and 96 h in Hep3B cells treated with 0.01  $\mu\text{M}$ , 0.1  $\mu\text{M}$  and 10  $\mu\text{M}$  MTX. Note that caspase-3 and caspase-9 activities were significantly increased in the 0.1 and 10  $\mu\text{M}$  MTX-treated cells. Data were obtained from 3 independent experiments and are presented as means  $\pm$  SD.

MTX-treated Hep3B cells by using a substrate cleavage assay (56,61). As shown in Fig. 3A, caspase-3 activity increased in the Hep3B cells at 96 h following treatment with 0.1 and 10  $\mu\text{M}$  MTX while caspase-3 activity did not increase in Hep3B cells following treatment with 0.01  $\mu\text{M}$  MTX. Caspase-9 activity also increased in the 0.1 and 10  $\mu\text{M}$  MTX-treated Hep3B cells at 96 h but did not increase in the 0.01  $\mu\text{M}$  MTX-treated cells (Fig. 3C). However, there was no obvious increase in caspase-8 activity among the MTX-treated Hep3B cells (Fig. 3B). These results suggest that MTX (10 and 0.1  $\mu\text{M}$ ) induced apoptosis in the Hep3B cells via the caspase-9/-3 cascade but not via the caspase-8/-3 cascade.

*MTX causes increases in  $\text{H}_2\text{O}_2$  levels but not  $\text{O}_2^-$  levels in Hep3B cells.* Previous studies have shown that MTX can cause cell cytotoxicity associated with increases in reactive oxygen species (ROS) (35-37). Prior to the present study, the literature has not yet identified which ROS is induced by MTX treatment. Both  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  belonging to ROS commonly exist in cells. Therefore,  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  levels were examined according to the lucigenin-amplified method (57,58). The present study found that MTX did not raise  $\text{O}_2^-$  levels in the Hep3B cells

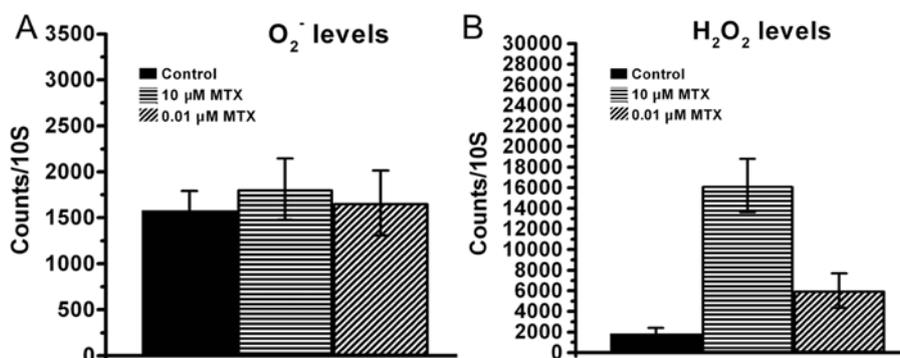


Figure 4. O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> levels. (A) O<sub>2</sub><sup>-</sup> counts and (B) H<sub>2</sub>O<sub>2</sub> counts are presented in the control cells, 0.01 μM MTX-treated cells, and 10 μM MTX-treated cells. O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> levels were determined after treatment for 1 h using a lucigenin-amplified method. Data were collected from 4 independent experiments and are presented as the means ± SD.

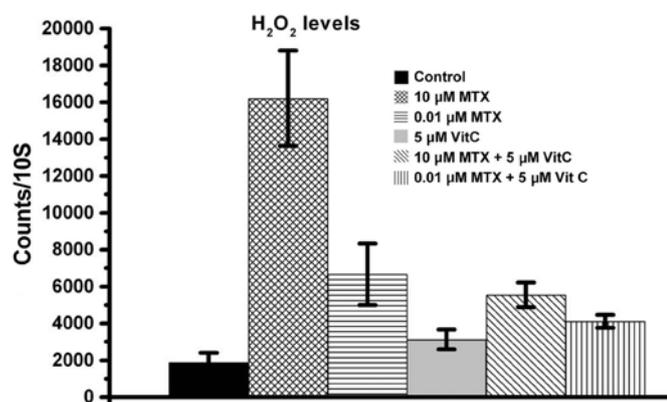


Figure 5. H<sub>2</sub>O<sub>2</sub> levels. H<sub>2</sub>O<sub>2</sub> counts are presented in control cells, MTX-treated cells, vitamin C-treated cells, and MTX plus vitamin C-treated cells. H<sub>2</sub>O<sub>2</sub> levels were determined after treatment for 1 h using a lucigenin-amplified method. Data were collected from 4 independent experiments and are presented as means ± SD.

(Fig. 4A). However, both high-dose MTX and low-dose MTX raised H<sub>2</sub>O<sub>2</sub> levels in the Hep3B cells (Fig. 4B). Therefore, the MTX-induced ROS increase is related to H<sub>2</sub>O<sub>2</sub> levels but not related to O<sub>2</sub><sup>-</sup> levels in the Hep3B cells.

*Vitamin C reduces the increase in H<sub>2</sub>O<sub>2</sub> levels and enhances the anticancer efficacy in MTX-treated Hep3B cells.* Many studies have demonstrated that vitamin C can prevent oxidative stress-induced cell damage (43-47). Considering that MTX induces oxidative stress resulting in cell damage (35-37), this study examined whether vitamin C could decrease H<sub>2</sub>O<sub>2</sub> levels, essentially inhibiting MTX-induced cytotoxicity in Hep3B cells. As shown in Fig. 5, the group receiving a combination treatment of vitamin C and 10 μM MTX had lower H<sub>2</sub>O<sub>2</sub> levels than the 10 μM MTX group. Similarly, the vitamin C and 0.01 μM MTX combination treatment group had lower H<sub>2</sub>O<sub>2</sub> levels than the 0.01 μM MTX group. These data indicate that vitamin C reduced the MTX-induced H<sub>2</sub>O<sub>2</sub> levels. However, to our surprise, vitamin C did not attenuate cell cytotoxicity in the MTX-treated Hep3B cells. On the contrary, our data showed that vitamin C enhanced the anticancer efficacy in MTX-treated Hep3B cells (Fig. 6). As shown in Fig. 6A and B, combination treatments of 5 μM vitamin C and MTX (0.01

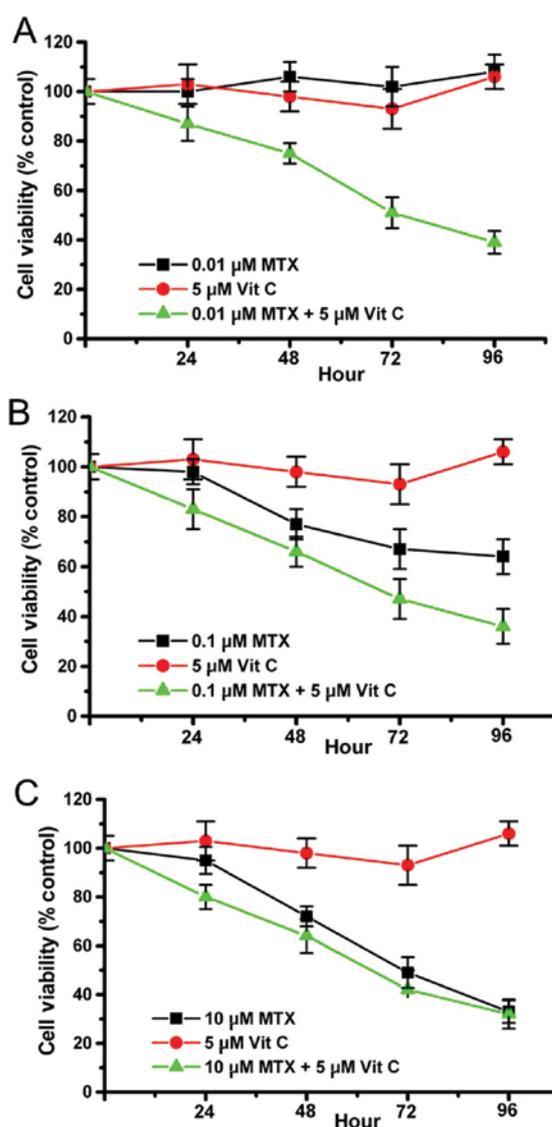


Figure 6. Cell viability. (A) Hep3B cells were treated with 0.01 μM MTX, 5 μM vitamin C, and a combination of 0.01 μM MTX and 5 μM vitamin C. (B) Hep3B cells were treated with 0.1 μM MTX, 5 μM vitamin C or a combination of 0.1 μM MTX and 5 μM vitamin C. (C) Hep3B cells were treated with 10 μM MTX, 5 μM vitamin C or a combination of 10 μM MTX and 5 μM vitamin C. Cell viability was measured with MTT assay every 24 h and is presented as A570 experimental group/A570 control group × 100%. Data were collected from four independent experiments and are presented as means ± SD.

or 0.1  $\mu\text{M}$ ) exerted a stronger anticancer effect on Hep3B cells than MTX treatment alone. It is worth noting that 0.01  $\mu\text{M}$  MTX alone or 5  $\mu\text{M}$  vitamin C alone did not have a significant cytotoxic effect on Hep3B cells, whereas a combination treatment of 0.01  $\mu\text{M}$  MTX and 5  $\mu\text{M}$  vitamin C did induce a cytotoxic effect on Hep3B cells (Fig. 6A). While vitamin C did not enhance the 10  $\mu\text{M}$  MTX-induced cytotoxic effect on Hep3B cells (Fig. 6C), the present study was important in indicating that vitamin C can assist low-dose MTX exert an anticancer effect on Hep3B cells.

## Discussion

Previous reports have revealed that MTX-induced cytotoxicity is related to increased reactive oxygen species (ROS) (35-37). However, no study has shown which ROS are induced following MTX treatment. In the present study, two types of ROS,  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$ , were measured.  $\text{H}_2\text{O}_2$  levels in MTX-treated cells rose significantly while  $\text{O}_2^-$  levels did not. In addition, it is well known that glutathione can convert toxic  $\text{H}_2\text{O}_2$  into non-toxic  $\text{H}_2\text{O}$ . We suggest that the increase in  $\text{H}_2\text{O}_2$  levels is a possible and important reason why N-acetyl cysteine (NAC), a clinical drug for glutathione synthesis, is used for MTX-induced cell damage (35,38,39,62). On the other hand, high-dose MTX-induced  $\text{H}_2\text{O}_2$  level increases were higher than low-dose MTX-induced  $\text{H}_2\text{O}_2$  level increases (Fig. 4B). Our data also showed that MTX induced cytotoxicity in a dose-dependent manner (Fig. 1). Taken together, we consider increases in the  $\text{H}_2\text{O}_2$  level to be one factor resulting in the inhibition of cell survival following MTX treatment.

MTX has anticancer effects on various hepatoma cell lines, including HepG2, MHCC97, Huh7 and Morris 5123 cells (6,63-67). Although the mechanisms involved in the MTX-induced cytotoxic effects on different hepatoma cells remain undetermined, a previous study demonstrated that MTX-induced cytotoxic effects on HepG2 cells are related to the CD95 death receptor pathway (caspase-8/-3 cascade pathway), whereas MTX-induced cytotoxic effects on Huh7 and Hep3B cells are not related to death receptor pathways (65). Similarly, the caspase-8/-3 cascade pathway was also found not to be involved in MTX-treated Hep3B cells in the present study (Fig. 3B). This study further demonstrated that MTX-induced apoptosis in Hep3B cells occurred through the caspase-9/-3 cascade pathway (Fig. 3A and C). These previous studies indicate that MTX induces different caspase pathways in different hepatoma cell lines. HepG2 is a p53 wild-type hepatoma cell line, while Hep3B is a p53-deficient hepatoma cell line (68,69). Thus, we suggest that p53 may be a possible reason for why the caspase-8/-3 pathway was activated in the MTX-treated HepG2 cells, while the caspase-9/-3 pathway was activated in the MTX-treated Hep3B cells.

Previous studies have demonstrated that MTX-induced cell cytotoxicity is associated with increases in reactive oxygen species (ROS) (35-37). The present study also indicated that MTX-induced  $\text{H}_2\text{O}_2$  level increases may be one factor resulting in cell growth inhibition. On the other hand, vitamin C can reduce oxidative stress against ROS-induced cell damage (43-47). Here, we also demonstrated that vitamin C did reduce MTX-induced increases in  $\text{H}_2\text{O}_2$  levels. However, vitamin C did not inhibit MTX-induced cell cytotoxicity in

Hep3B cells. On the contrary, vitamin C assisted low-dose MTX to exhibit a strong cytotoxic effect in Hep3B cells. Similarly, recent studies also indicated that vitamin C can enhance anti-cancer agents to exert a strong cytotoxic effect on cancer cells, although the mechanisms remain unknown (48,70-72). Thus, MTX-induced increases in  $\text{H}_2\text{O}_2$  levels may be one of the factors resulting in cytotoxicity noted in MTX-treated Hep3B cells. There are various unclear MTX-induced death signals that remain to be studied. Regardless, a combination treatment of vitamin C and low-dose MTX may be a potential method for hepatoma cancer therapy.

Overall, the present study first demonstrated that MTX induces an increase in  $\text{H}_2\text{O}_2$  levels and activates the caspase-9/-3 cascade pathway to cause apoptosis in Hep3B cells. Importantly, a combination treatment of vitamin C and low-dose MTX exerted a strong anticancer effect in Hep3B cells. This treatment method may be useful for future clinical cancer therapy.

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