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Original Article

Protective effects of dimethylthiourea against hydrogen peroxide-induced oxidative stress in hepatic L02 cell

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Abstract: Context: Oxidative stress and mitochondrial dysfunction are hypothesized to contribute to the pathogenesis of chronic cholestatic liver diseases. Objective: This study aims to explore the effects of dimethylthiourea (DMTU) preconditioning on human hepatocytes L02 cells against hydrogen peroxide (H_2O_2) -induced oxidative stress. Methods: Preconditioned or non-preconditioned human hepatic L02 cells were exposed to H_2O_2 with certain concentration and the cell viability was detected by MTT assay. Lactate dehydrogenase (LDH), superoxide dismutase (SOD), glutathione (GSH-Px), and malondialdehyde (MDA) levels were evaluated with corresponding ELISA kits. Caspase-3 activity was determined with Caspase-Glo 3/7 assay kit. Bax/Bcl-2 ratio was measured by Western blot. Results: DMTU preconditioning could significantly ameliorate L02 cells viability loss, promote the decreased SOD and GSH-Px levels and attenuate the cascade of MDA level and LDH leakage induced by H_2O_2. DMTU administration could also reinforce the activity of caspase-3 and increase the Bax/Bcl-2 ratio to inhibit apoptosis and improve the L02 cells activity. Conclusions: These results indicated that DMTU could protect the hepatic L02 cells against H_2O_2-induced liver injury by alleviating oxidative stress and apoptosis process, and DMTU might be potential hepatoprotective medicine.

Keywords: Apoptosis, dimethylthiourea, hepatic L02 cell, hepatoprotective, oxidative stress

Introduction

Production of reactive oxygen species (ROS) is implicated in normal aerobic cellular metabolism [1]. ROS is a general term including a wide set of molecules and free radicals, such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($HO^{\bullet}$) [2, 3]. Generally, ROS production is counterbalanced by antioxidant defense system to maintain an appropriate redox balance [4, 5]. Excessive production of ROS can lead to oxidative stress, which is a physiological status whereby intracellular free radicals exceed the antioxidant abilities. As the major organ responsible for the regulation of extensive physiological processes, the liver is a representative and target organ of the toxicity of drugs and xenobiotics due to its continuous exposure to these toxicants [6, 7]. Most hepatotoxic chemicals can increase the production of free radicals that cause oxidative stress, which has been recognized as a key factor in the pathogenesis of several chronic liver diseases, such as hepatitis, alcoholic and non-alcoholic fatty liver diseases [8, 9]. Therefore, antioxidant therapy may be one of the strategies to correct the imbalance between oxidants and antioxidants in development of these liver diseases and prevent hepatocytes from excessive exposure to oxidative stress.

Dimethylthiourea (DMTU), a hydroxyl radical scavenger, is capable of crossing cell membranes and functioning at the intracellular site of oxygen radical production [10]. It was reported that DMTU was capable of preventing gastric injury by scavenging the hydroxyl radical [11]. Some researches demonstrated that pretreatment with DMTU could afford protection against lung injury [12, 14]. Also, as a hydroxyl radical scavenger, DMTU could prevent the renal mitochondrial damage to protect kidney [15]. The antioxidant characteristic may give the evidence for the hypotheses that DMTU might protect the liver tissue from oxidative stress. This study aims to explore the protective effects of

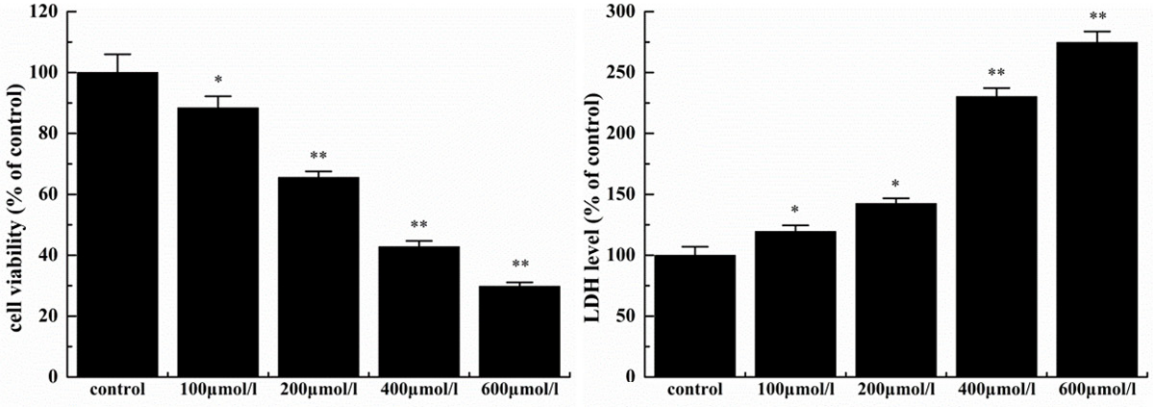

Figure 1. Effect of different concentrations of H_2O_2 on L02 cell viability and LDH leakage. Each column represents the mean ± SD of six individual experiments. *P < 0.05 versus control group; **P < 0.01 versus control group.

DMTU on hepatic cells against the oxidative stress caused by H_2O_2.

H_2O_2 is commonly used to induce oxidative stress to cause apoptosis in various cells, including hepatocytes [16]. The hydroxyl radical derived from H_2O_2 is a powerful oxidizing agent that could chemically modify a number of cellular macro-molecules, such as polyunsaturated fatty acids, DNA, and proteins [17]. In the current study, H_2O_2 was selected to establish oxidative injury model to investigate the potential effect of DMTU on oxidative stress induced by H_2O_2 in hepatic L02 cells and the possible underlying mechanisms.

Material and methods

Materials

Dimethylthiourea was obtained from Shanghai Winherb Medical technology Co., Ltd (Shanghai, China). And the purity is more than 98%.

Cell culture and treatment

L02 cell line was purchased from the cell bank of the Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were cultured in RPMI/1640 medium, supplemented with 10% fetal calf serum, 1% penicillin (100 IU/ml) and 1% streptomycin (100 μg/ml) in a 5% CO_2 humidified atmosphere at 37°C. In order to establish the H_2O_2 injury model of L02 cells, cells were exposed to H_2O_2 with a final concentration of 100, 200, 400 and 600 μmol/l for 12 h in 96-well culture plates (200 μl/well). To investigate the protective effects of DMTU on H_2O_2-induced oxidative stress, L02 cells were treated with different concentrations of DMTU (200, 400 and 600 μmol/l) 1 h before being treated with H_2O_2 (200 μmol/l).

Analysis of cell viability

Cell viability was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazoliumbromide (MTT) assay in 96-well culture plates at a density of 5×10^3 cells/well. 20 μ MTT solution was added to the wells and incubated for 4 h. And then, 150 μl dimethyl sulfoxide (DMSO) was added to dissolve formazan. The absorbance at 570 nm was detected in a microplate reader (Bio-Rad, Hercules, CA). Cell viability was presented as percentage to the control group.

Evaluation of cell apoptosis by cytometry

To delineate the function of DMTU in hepatic L02 cells, cell apoptosis was measured by flow cytometry according to the manufacture's instructions (KeyGen, Nanjing, China). L02 cells were collected and resuspended in 500 μl of binding buffer and 5 μl each of annexin V-FITC and PI was added. The analyses were conducted within 30 min.

Measurement of LDH level

Cell death was assessed by detecting the LDH leakage in the medium [18]. Culture medium was collected to be moved to a new 96-well-culture plate (200 μl/well) and the LDH level was measured by spectrophotometry using a LDH kit (Nanjing Jiancheng Bioengineering

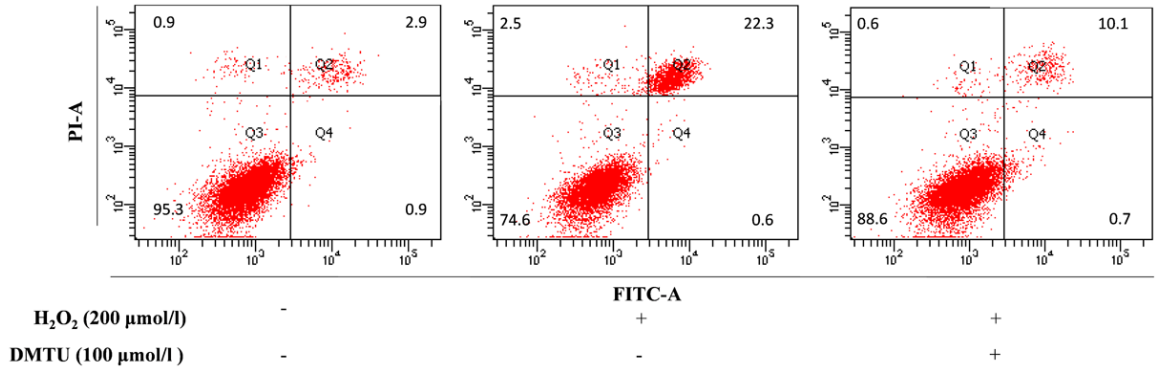

Figure 2. Effect of DMTU on L02 cell apoptosis by flow cytometry.

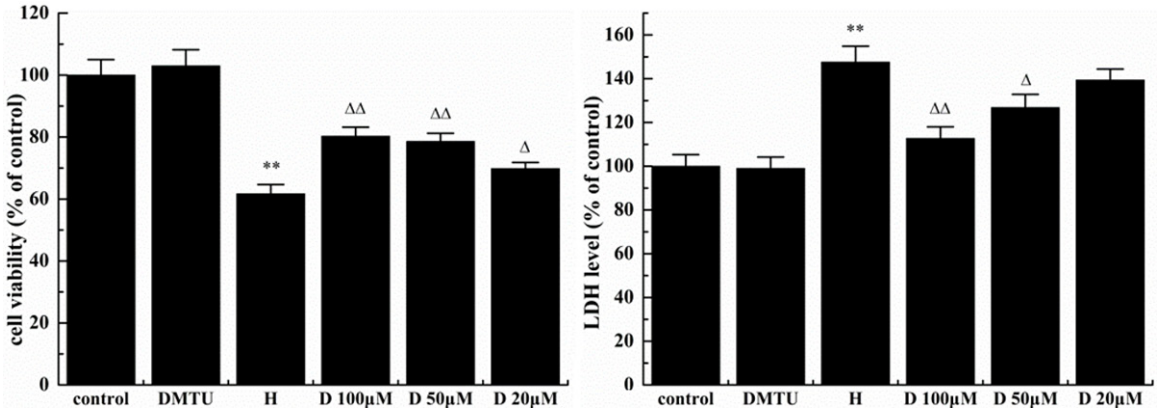

Figure 3. Effect of DMTU on L02 cell viability and LDH leakage. D represents DMTU-H_2O_2 group; H represents H_2O_2 group. Each column represents the mean ± SD of six individual experiments. *P < 0.05, **P < 0.01 versus control group; $^{\Delta}$P < 0.05, $^{\Delta\Delta}$P < 0.01 versus H_2O_2 group.

Institute, Nanjing, China), according to the manufactory instructions.

Measurement of intracellular ROS production

The production of intracellular ROS was measured using the ROS-specific fluorescent dye 2,7-dichlorofluorescein diacetate (DCFH-DA; Beyotime, Haimen, China). After the indicated administration, L02 cells were washed with cold PBS and incubated with DCFH-DA (10 μM) for 30 min at 37°C in darkness. The measurement was done at an excitation wavelength of 488 nm and an emission wavelength of 530 nm using a microplate reader (Bio-Rad, Hercules, CA).

Measurement of SOD, GSH-Px, MDA levels

To analysis influences of increased ROS level on the intracellular antioxidant and oxidant, SOD, GSH-Px and MDA levels were detected in this study. L02 cells were collected, centrifuged, washed and resuspended in saline solution, and then dissolved by sonication. The cell suspension was taken to detect the levels of SOD, GSH-Px and MDA with corresponding kits (Nanjing Jiancheng), according to the manufactory instructions.

Measurement of Caspase-3 activity

To quantify the apoptosis, caspase-3 activity was detected by Caspase-Glo 3/7 assay kit (Nanjing Jiancheng), according to the kit instructions.

Western blot analysis

After indicated administration, cells were collected and the protein were extracted and

Table 1. Effect of DMTU on SOD, GSH-Px and MDA levels in L02 cells

	SOD (U/mg pro)	GSH-Px (nmol/mg pro)	MDA (nmol/mg pro)
Control Group	25.9 ± 2.3	69.2 ± 6.7	0.7 ± 0.1
DMTU (1000 μmol/l)	25.1 ± 1.9	68.9 ± 5.9	0.8 ± 0.1
H_2O_2 (200 μmol/l)	10.6 ± 0.9**	41.5 ± 3.6**	2.1 ± 0.2**
MDTU (500 μmol/l) + H_2O_2	12.9 ± 0.8	48.1 ± 4.5	1.9 ± 0.3
MDTU (750 μmol/l) + H_2O_2	16.3 ± 1.2$^{\Delta\Delta}$	57.2 ± 3.4$^{\Delta\Delta}$	1.6 ± 0. 3$^{\Delta}$
MDTU (1000 μmol/l) + H_2O_2	18.1 ± 1.7$^{\Delta\Delta}$	67.8 ± 6.6$^{\Delta\Delta}$	1.1 ± 0.2$^{\Delta\Delta}$

Values represent the mean ± SD of six individual experiments. *P < 0.05, **P < 0.01 versus control group; $^{\Delta}$P < 0.05, $^{\Delta\Delta}$P < 0.01 versus H_2O_2 group.

quantified by bicinchoninic acid (BCA) kit (Biocolor, Shanghai, China). Denatured protein (30μg) was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a poyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific, Waltham, MA). After being blocked by 5% bovine serum albumin for 1.5 h, membranes were blocked with primary antibodies of Bax (1:1000 dilutions; Epitomics Biotechnology Co., Burlingame, VT), Bcl-2 (1:1000 dilutions; Epitomics), β-actin (1:1000 dilutions; Santa Cruz Biotechnology, Santa Cruz, CA), and caspase-3 (1:1000 dilutions, Cell Signaling Technology, Boston, MA) followed by incubation with the secondary antibodies (anti-mouse, 1:5000 dilutions; Santa Cruz), correspondingly. Quantification of protein level was done by using aBio-Rad ChemiDoc XRS system (Bio-Rad, Hercules, USA) and ImageJ software (NIH, Bethesda, MD).

Statistical analysis

Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Dunnett's test by SPSS 16.0 statistic software. The data were expressed as mean ± SD. A probability level of less than 0.05 was considered to be statistically significant.

Results

The establishment of H_2O_2 injury model

To investigate the dose-dependence of H_2O_2 toxicity to L02 cells, L02 cells were treated with H_2O_2 of different concentrations (100, 200, 400 and 600 μmol/l) and the viability and LDH leakage were detected. As shown in **Figure 1**, H_2O_2 inhibited the cell viability and increased the LDH leakage. And the variation trends were in a dose-dependent manner. After being treated with 100 μmol/l H_2O_2, the cell viability was 88.4 ± 3.8% contrasting to the control group, the damage of which was feeble. When treated with 400 and 600 μmol/l H_2O_2, the cell viability were respectively 42.8 ± 1.9% and 29.8 ± 1.3%, which were too low to investigate the effects of DMTU to L02 cells. Therefore, the final concentration of H_2O_2 used for further study was determined as200 μmol/l, which led to modest cell viability and LDH leakage (65.57 ± 1.97% and 142.50 ± 4.29%, respectively).

Effects of MDTU on L02 cell apoptosis

As shown in **Figure 2**, DMTU remarkably protected hepatic L02 cells from H_2O_2 induced apoptosis. Besides, **Figure 3** presented a remarkable decrease in the viability of cells treated with H_2O_2 compared with the control group, which was blocked by the administration of DMTU. The viability of cells at different DMTU concentrations of 20, 50 and 100 μmol/l were 69.8 ± 2.0%, 78.6 ± 2.6% and 80.3 ± 2.9% contrasting to the control group, respectively. The viability data clearly indicated that DMTU could inhibit the L02 cells damage induced by H_2O_2, which was confirmed by the increased LDH leakage in the culture medium of H_2O_2 group (143.48 ± 4.62%) and the decreased LDH concentration in DMTU-H_2O_2 groups (106.59 ± 3.20%, 120.27 ± 3.61% and 139.81 ± 4.19% respectively at the 100, 50 and 20 μmol/l). Furthermore, comparison of DMTU group and the control group in the viability and LDH release indicated DMTU barely had toxic effects to L02 cells.

Effect of MDTU on SOD, GSH-Px and MDA levels in L02 cells

As demonstrated in **Table 1**, a remarkable decline of antioxidant enzymes (SOD and GSH-

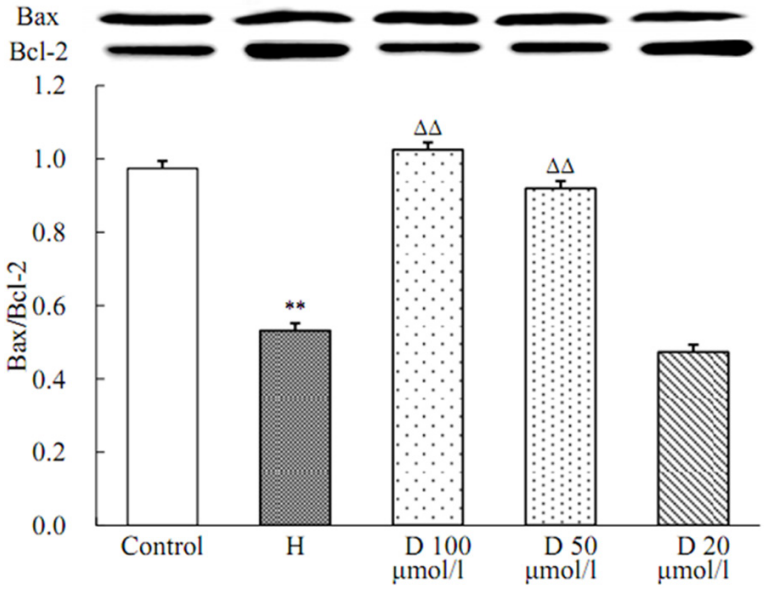

Figure 4. Effect of DMTU on L02 cells' Bax/Bcl-2 ratio. D represents DMTU-H_2O_2 group; H represents H_2O_2 group. Each column represents the mean ± SD of six individual experiments. **P < 0.01 versus control group; $^{\Delta}$P < 0.05, $^{\Delta\Delta}$P < 0.01 versus H_2O_2 group.

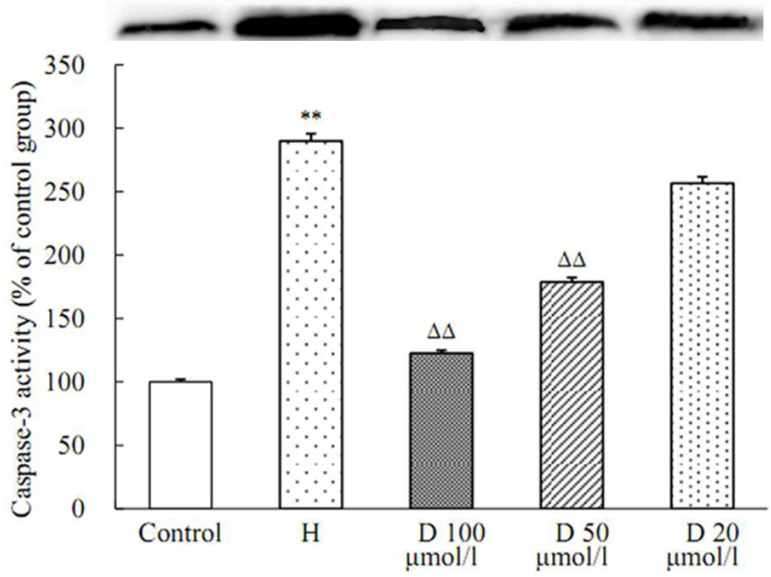

Figure 5. Effects of DMTU on the increase of caspase-3 activity induced by H_2O_2. D represents DMTU-H_2O_2 group; H represents H_2O_2 group. Each column represents the mean ± SD of six individual experiments. **P < 0.01 versus control group; $^{\Delta}$P < 0.05, $^{\Delta\Delta}$P < 0.01 versus H_2O_2 group.

Px) level and a significant elevation of MDA level were observed in the H_2O_2 group compared with the control group. And in the DMTU-H_2O_2 groups, the differences were reversed after being treated with DMTU.

In the control group, the level of SOD and GSH-Px were 25.9 ± 2.3 U/mg and 69.2 ± 6.7 nmol/mg, which were significantly decreased to 10.6 ± 0.9 U/mg and 41.5 ± 3.6 nmol/mg in H_2O_2 group, respectively (P < 0.01). Moreover, the MDA level was also significantly increased to 2.1 ± 0.2 nmol/mg from the 0.7 ± 0.1 nmol/mg in the control group (P < 0.01). However, DMTU reversed the tendency. The SOD level significantly rose to 16.3 ± 1.2 and 18.1 ± 1.7 U/mg after the pretreatment with 50 and 100 μmol/l DMTU, respectively (P < 0.01). Similarly, the GSH-Px level picked up to 57.2 ± 3.4 and 67.8 ± 6.6 nmol/mg, which were significant versus the H_2O_2 group (P < 0.01). While the MDA level were significantly brought down to 1.6 ± 0. 3 and 1.1 ± 0.2 nmol/mg by 50 and 100 μmol/l DMTU (P < 0.05, P < 0.01, respectively). What's more, the SOD, GSH-Px and MDA level of DMTU group had no significant differences compared with the control group (P > 0.05).

Effect of MDTU on the L02 cells apoptosis induced by H_2O_2

As shown in **Figure 4**, Bax/Bcl-2 ratio was significantly downregulated to 0.53 ± 0.02 in L02 cells after being treated with H_2O_2 compared with the control group (0.97 ± 0.05). And the value difference between H_2O_2 and control group was narrowed down by DMTU, which had a correlation with the concentration of MDTU. The ratios were respectively 0.91 ± 0.04 and 1.02 ± 0.04 at the dosage of 50 and 100 μmol/l. The caspase-3 activity was also detected presented in **Figure 5**. The activ-

ity of the H_2O_2 group ascended to 2.89 ± 0.06 times of control group, which was significantly decreased to 1.88 ± 0.04 and 1.22 ± 0.04 respectively by50 and 100 μmol/l DMTU.

Discussion

Oxidative stress is a situation whereby intracellular ROS levels overwhelm the cellular antioxidant abilities and is reported to be involved in various diseases [19]. Oxidative stress-induced cell injury has been involved in various disorders, including liver cells dysfunction and liver disease [20, 22]. H_2O_2, which can induce oxidative stress and damage to various cells, has been extensively used as an indicator of oxidative stress in vitro model [23]. Thus, we selected H_2O_2 to establish oxidative injury model. The present study showed that H_2O_2 administration significantly decreased L02 cell viability in a dose-dependent manner, which is consistent with the previous study [24]. Nevertheless, pretreatment of DMTU significantly increased the tolerance of cells to H_2O_2 exposure.

LDH, a steady cytoplasmic enzyme in normal cells, will be released to medium when the membrane is damaged. The LDH level in medium reflected the impaired degree of the cells [25]. In this study, the effects of DMTU on the LDH leakage were inspected and the results indicated that DMTU could attenuate the damage of L02 cells induced by H_2O_2, which was dosage-related.

GSH-Px and SOD are significant antioxidant enzymes in cells. SOD plays the role of primary defense line against cell injury caused by ROS, which can transform intracellular superoxide anion to H_2O_2. And GSH-Px is widely regarded as a second defense line by decomposing H_2O_2 with catalase into oxygen and water through enzymatic reactions [26, 27]. Moreover, the toxicity induced by H_2O_2 was accompanied with increasing lipid peroxides. MDA, a product of lipid peroxidation, could reflect the injury level caused by ROS [26]. In this study, H_2O_2 significantly decreased the intracellular level of SOD and GSH-Px, and increased the MDA level, which led to cell damage. Nevertheless, the pretreatment with MDTU apparently reversed the changes in DMTU-H_2O_2 groups, which indicated that DMTU might protect the L02 cells via increasing the SOD and GSH-Px levels and decreasing the MDA level.

In order to investigate the protective mechanisms DMTU, the expression of some apoptosis-related proteins were determined by western blot in L02 cells. Bax and Bcl-2 are involved with apoptosis under physiological and pathological conditions, which are considered as major roles in determining cell survival or death after apoptotic inducement [28]. It is extensively known that Bax homodimer dominating will cause cell death, whereas if Bcl-2 and Bax heterodimerization predominates, cells will survive [29]. In this study, combining with a decrease in cell viability loss induced by H_2O_2, pretreatment with DMTU increased the expression of Bax, and reduced the Bcl-2 expression, which resulted in an increase of Bax/Bcl-2 ratio. It might be reasonably demonstrated that DMTU could attenuate L02 cell apoptosis induced by H_2O_2. Furthermore, it is well-known that caspase-3 activity can be aroused by Bax and inhibited by Bcl-2 [30]. The activation of caspase-3 is a vitally important step in the execution phase of apoptosis and the inhibition hinders apoptosis [31]. In this study, the caspase-3 activity was detected to support our findings. The results showed that H_2O_2 induced the activation of caspase-3, which was attenuated by DMTU.

Conclusion

In conclusion, the pretreatment with DMTU significantly attenuated the apoptosis via recovering the activity of SOD and GSH-Px and downregulating MDA level and LDH leakage induced by H_2O_2. DMTU could also reinforce the L02 cells viability, caspase-3 activity and increase the Bax/Bcl-2 ratio to improve the L02 cells activity. Thus, we suggested that DMTU might be a potential hepatoprotective medicine.

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Disclosure of conflict of interest

None.

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