Original Research Paper

Nuclear Factor Erythroid-2-Related Factor 2 (Nrf2) Signalling Pathway and Apoptosis Impact on the Effect of Tetramethylpyrazine against H_2O_2-Damage in Human Hepatic Cell Line (LO_2 Cells)

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Abstract: As an active component in traditional Chinese herbal medicine, Tetramethylpyrazine (TMP) played a critical role in the treatment of cardiovascular, cerebrovascular, and eye diseases. In this study, the TMP has obtained from the Chinese herbal medicine Ligusticum chuanxiong and investigated the protective effects of TMP on oxidative damage in human LO_2 cells with the treatment of different concentrations of TMP. The results showed that TMP reserved oxidative damage caused by hydrogen peroxide in LO_2 cells. Further other investigations suggested that TMP protected LO_2 cells from oxidative damage through elevating activities of antioxidant enzymes and glutathione levels. The TMP treatment promoted the nucleus translocation of the nuclear factor erythroid 2–related factor 2 (Nrf2) and further induced expression of antioxidant response element. Finally, it could be concluded that TMP treatment suppressed apoptosis induced by hydrogen peroxide in LO_2 cells. Thus, the TMP might protect LO_2 cells from oxidative damage through antioxidative and anti-apoptotic effects.

Keywords: Tetramethylpyrazine, Antioxidative, Anti-Apoptosis, Nrf2

Introduction

Oxidative damage is caused by a wide range of factors including ultraviolet stress, smoking, medicine, drugs, and so on (Kournoutou *et al.*, 2020). Oxidative stress is the result of an imbalance between Reactive Oxygen Species (ROS) and the antioxidant defense system (LeBel *et al.*, 1992). Excessive production of ROS might change or even inactivate biomolecules in cells, destroy the cell structure and cause organelle dysfunction (Chen *et al.*, 2018; Cheng *et al.*, 2017; Maryam *et al.*, 2019). In the liver, a major consequence of oxidative stress is DNA damage, lipid peroxidation, and protein oxidation, which could contribute to the

pathogenesis of liver damage (Nakamura *et al.*, 2005; Verhagen *et al.*, 1995). Free radicals act on lipids to undergo a peroxidation reaction, thereby leading to the production of Malondialdehyde (MDA), which is an indicator of oxidative stress (Zhang *et al.*, 2020). They also participate in cytochrome c-dependent activation of caspase 3 as a classic apoptotic protease, which is involved in the execution of cell apoptosis and activated by heterologous DNA damage (Liu *et al.*, 1997; Zou *et al.*, 1997). In addition, cellular antioxidant defense systems consisted of Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GSH-Px), and Glutathione (GSH) (Du *et al.*, 2021; Huang *et al.*, 2020; Li *et al.*, 2020a). Except for scavenging free radicals, the

antioxidants in the body could eliminate the products produced by the immune response against oxidative stress (Abdallah *et al.*, 2018; Fang *et al.*, 2002; Halliwell and Gutteridge, 1985; Valko *et al.*, 2006).

Tetramethylpyrazine (TMP), a type of pyrazine, with a roasted nut aroma and sweet aftertaste (Masuda and Mihara, 1988), is the main active alkaloid component in Chinese herbal medicine (Han *et al.*, 2020). It is used as a food flavor additive in baked food, meat, dairy products, Chinese Baijiu, and so on (Burdock, 2016). As the most important active ingredient of Chuan Xiong, TMP plays an important role in the treatment of brain injury (Liao *et al.*, 2004), inflammation (Yrjänheikki *et al.*, 1999), glaucoma (Zhang *et al.*, 2003), cardiovascular disorders (Shuai *et al.*, 2013) and in particular ocular diseases caused by inflammation (Yang *et al.*, 2008). Thus, TMP is recognized as an antioxidant with several beneficial effects on human health (Samad *et al.*, 2016). As an antioxidant, TMP scavenges free radicals and protects cells from oxidative stress, thereby reducing the risk of many diseases that could be caused by oxidation including diseases associated with disturbed iron metabolism (Zhang *et al.*, 2003), diabetes and its complications, and oxidative brain injury (Gao *et al.*, 2011; Li *et al.*, 2010). However, whether TMP affects liver injury caused by oxidative stress such as drugs and alcohol or not, is still unknown.

In this study, the TMP was obtained by purification and the protective effects of TMP on oxidative damage induced hydrogen peroxide (H_2O_2) in LO_2 cells were investigated. Our results demonstrated that TMP protects liver cells from oxidative stress through elevating activities of antioxidant enzymes and promoting the nucleus translocation of Nrf2 to activate the expression of antioxidant response elements. In addition, TMP treatment suppressed the apoptosis induced by H_2O_2 in LO_2 cells. These research findings may improve and apply to the development of functional foods that have effects on liver disease.

Materials and Methods

Chemicals and Reagents

H_2O_2 (30 wt%) was purchased from Beijing Chemical Works (Beijing, China). The primary antibodies against rabbit Nrf2 were purchased from Proteintech Group (Chicago, IL, USA). Primary antibodies against cleaved caspase-3, GAPDH, lamin B2, B-cell lymphoma 2 (Bcl-2), Bcl-2-X-associated protein (Bax), and Horseradish Peroxidase (HRP)–labeled secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). TMP was obtained from J&K Scientific LTD (Beijing, China).

Acquisition of TMP Mixture

3 kg stems and leaves of Chinese herbal medicine-Ligusticum chuanxiong were cut into pieces and added to 6 L 3 mol/L hydrochloric acid solution and extract for 24 h. After filtration, the filtrate was added NaOH to adjust PH = 10. Then filtration, the filter was washed with water to PH = 7. The filtrate was dissolved by 200 mL ethanol, 4000 rpm 20 min. The supernatant was stirred with 400 g D101 macroporous adsorption resin for 48 h. The product was eluted with 3L water, 3L 20% ethanol, 3L 50% ethanol, and 3L 80% ethanol in turn. Final ethanol eluent was collected by blow nitrogen and freeze dry. The resulting mixture is TMP. During the experiment, weigh and configure the appropriate concentration with ddH_2O. The purity of TMP is 90% tested.

Cell Culture

LO_2 cells were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum and antibiotics (100-U/mL penicillin, 100-μg/mL streptomycin) (Hyclone) in a 5% CO_2 incubator at 37°C. Cells were treated with TMP at different concentrations (0–0.8 mg/mL) for 24 h and then treated with 300-μm H_2O_2 for 4 h. LO_2 cells were replaced with a fresh culture medium every two days.

Cell Viability Assay

LO_2 cells were cultured in a 96-well microplate and treated with H_2O_2 or TMP for 24 h as previously described. The viability of the treated LO_2 cells was determined with the Cell Counting Kit-8 (CCK-8; Dojin Laboratories, Kumamoto, Japan). After the pretreatment, the medium was replaced with a 90-μL medium including 10-μL CCK-8 and incubated at 37°C for 2 h. Then, the absorbance was measured at the wavelength of 450 nm using a multi-function reader (SpectraMax iD3; Molecular Devices, San Jose, CA, USA).

Analysis of Intracellular ROS Generation

LO_2 cells were cultured on the coverslips in a 6-well plate. After 24 h, cells were treated with TMP at different concentrations for 24 h. Then, the cells were washed with Phosphate-Buffered Saline (PBS) and treated with 300- μm H_2O_2 for 4 h. The cells were further treated with 10-μm Dichlorofluorescin Diacetate (DCFH-DA) at 37°C for 30 min in the darkness. The cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and observed using a confocal laser scanning microscope (TCS SP8, Leica, Wetzlar, Germany).

Western Blot Analysis

Cells were washed twice with ice-cold PBS, harvested and collected by centrifugation (5000 rpm for 5 min), and lysed in lysis buffer [50 mm Tris-HCl pH 7.5, 150 mm NaCl, 1% Triton X-100, complete protease inhibitor cocktail (Suolaibao, Beijing, China)] for 10 min. Cell lysates were cleared by centrifugation at 15000 rpm for 10 min at 4°C. Supernatants were collected and mixed with sample buffer (Suolaibao, Beijing, China). After boiling for 5 min, the samples were subjected to SDS/Polyacrylamide Gel Electrophoresis (PAGE). The proteins were transferred to PVDF membranes (GE Healthcare, Bucks, UK) using transfer buffer (25 mm Tris base, 190 mm glycine, 20% methanol). The transferred membrane was blocked in 5% skimmed milk which was diluted with TBS-T (25 mm Tris base, 137 mm NaCl, 2.7 mm KCl, 0.1% Tween 20, adjusting pH to 7.4) at room temperature for 1h. After blocking, the membrane was incubated overnight with appropriate dilutions of primary antibody in blocking buffer at 4°C. Then, the membrane was washed three times using TBS-T and incubated at room temperature for 30 min with a 1:5000 dilution of HRP-Conjugated Secondary Antibody (CST) in blocking buffer. The membrane was washed three times and visualized using the ECL Select western blotting detection reagent (GE Healthcare) on a Chemi Scope 6200 Touch detector (Qinxiang, Shanghai, CHINA).

Determination of Enzyme Activities

GSH-Px, CAT, and SOD and the levels of MDA and GSH (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were determined according to the manufacturer's instructions.

Statistical Analysis

All data are presented as the mean ± standard deviation of at least three replicates for each prepared sample. Analysis of variance was performed by SPSS 23 (Taka *et al.*, 2013). Significant differences were calculated according to the Bonferroni post hoc test. $P<0.05$ was considered statistically significant.

Results

Effects of TMP on the Viability of LO₂ Cells

To investigate the effects of TMP on cell viability, LO_2 cells were treated with TMP at final concentrations of 0, 0.1, 0.2, 0.4, and 0.8 mg/mL for 24 h. As shown in Fig. 1A, increasing TMP concentration reduced cell viability from 100 to 80%, suggesting that TMP (0–0.8 mg/mL) did not affect the viability of LO_2 cells significantly. H_2O_2 is commonly used as a ROS generator because it affects mitochondrial function and increases mitochondrial oxidative damage (Hirata 2009; Choi *et al.*, 2010;

Konno *et al.*, 2021; Li *et al.*, 2020b; Xu *et al.*, 2020). Therefore, the effects of H_2O_2 on LO_2 cell viability were also determined. As shown in Fig. 1B, H_2O_2 had less effect on LO_2 cells when the concentration was lower than 300 μm. Thus, TMP (0–0.8 mg/mL) and H_2O_2 (300 μm) were selected for further analyses. To determine whether TMP can protect against cell death induced by H_2O_2 or not, LO_2 cells were pretreated with TMP for 24 h, followed by H_2O_2 treatment for an additional 4 h at a concentration of 300 μm. Compared with H_2O_2 treatment, LO_2 cell viability increased to some extent with pretreatment of TMP (Fig. 1C). These results suggest that TMP may protect cells from oxidative damage induced by H_2O_2 treatment.

Effects of TMP on ROS Generation in LO₂ Cells

The DCFH-DA assay was employed to observe ROS generation induced by H_2O_2 treatment. As shown in Fig. 1D, compared with the control group, H_2O_2 treatment significantly increased green fluorescence intensity in a dose-dependent manner, suggesting that intracellular oxidative stress is correlated with H_2O_2 concentration. According to the effects of H_2O_2 on cell viability (Fig. 1B), 300-μm H_2O_2 was selected for inducing oxidative stress in subsequent assays. Pretreatment with TMP for 24 h markedly decreased the intensity of DCFH-DA fluorescence induced by H_2O_2 treatment (Fig. 1E). In conclusion, these results showed that TMP could protect LO_2 cells from oxidative damage.

TMP Maintain the Redox Balance in LO₂ Cells

Antioxidant systems have been developed in cells to protect against cellular toxicity and modulate the physiological effects of ROS. There are many essential systems that clear free radicals including SOD, CAT, and GSH-Px. To evaluate the potential protective efficacy of TMP in LO_2 cells, the transcriptional levels of SOD, CAT, GPX, and GSTP1 were detected by Quantitative Real-time PCR (qRT-PCR). The transcription levels of these enzymes were significantly upregulated by the treatment of TMP in LO_2 cells. As shown in Fig. 2A–2D, compared with the control group, H_2O_2 treatment significantly ($p<0.05$) reduced mRNA expression levels of SOD, CAT, GPX, and GSTP1. Furthermore, the contents of MDA, as well as the activities of SOD, CAT, and GSH, were monitored with TMP treatment. As shown in Fig. 2E–2G, TMP treatment gradually reversed SOD, CAT, and GSH activities, which were down-regulated by H_2O_2. MDA is the main product of lipid hydroperoxides. TMP treatment significantly reduced the MDA level, which was induced by H_2O_2 in a dose-dependent manner. These results suggested that TMP exerts its anti-oxidative effects on LO_2 cells by up-regulating the activities of SOD, CAT, and GSH and reducing the MDA level.

TMP Ameliorates the Antioxidant Capacity Via Nrf2-Antioxidant Response Element Pathway

Nrf2 is a well-known key transcription factor in the antioxidant defense system. To determine whether TMP up-regulates anti-oxidative activity, we evaluated the Nrf2 protein expression level and translocation in LO_2 cells with TMP treatment. Compared with the control, treatment with H_2O_2 alone increased the cytoplasmic Nrf2 level and decreased the nuclear-localized Nrf2, suggesting that H_2O_2 treatment suppressed the nuclear translocation of Nrf2 (Fig. 3A-C). In contrast, pretreatment with TMP caused the Nrf2 level in the

nucleus to gradually increase, indicating that TMP treatment promotes the nuclear translocation of Nrf2. Meanwhile, it suggested that Nrf2 uncouple with keap1 and enter into the nuclear to enhance antioxidant capacity. With the increase of TMP concentration, the fluorescence of nucleus Nrf2 gradually increased (Fig. 3D). The increase of nucleus Nrf2 increases the expression of downstream molecules related to the Nrf2-oxidation pathway, including HO-1, NQO1, and GCLC. The related expression of HO-1, NQO1, and GCLC at the mRNA level was increased by Nrf2 ((Fig. 3E-G). Taken together, the above results suggested that TMP elevates antioxidant capacity via the Nrf2-Antioxidant Response Element (ARE) signaling pathway.
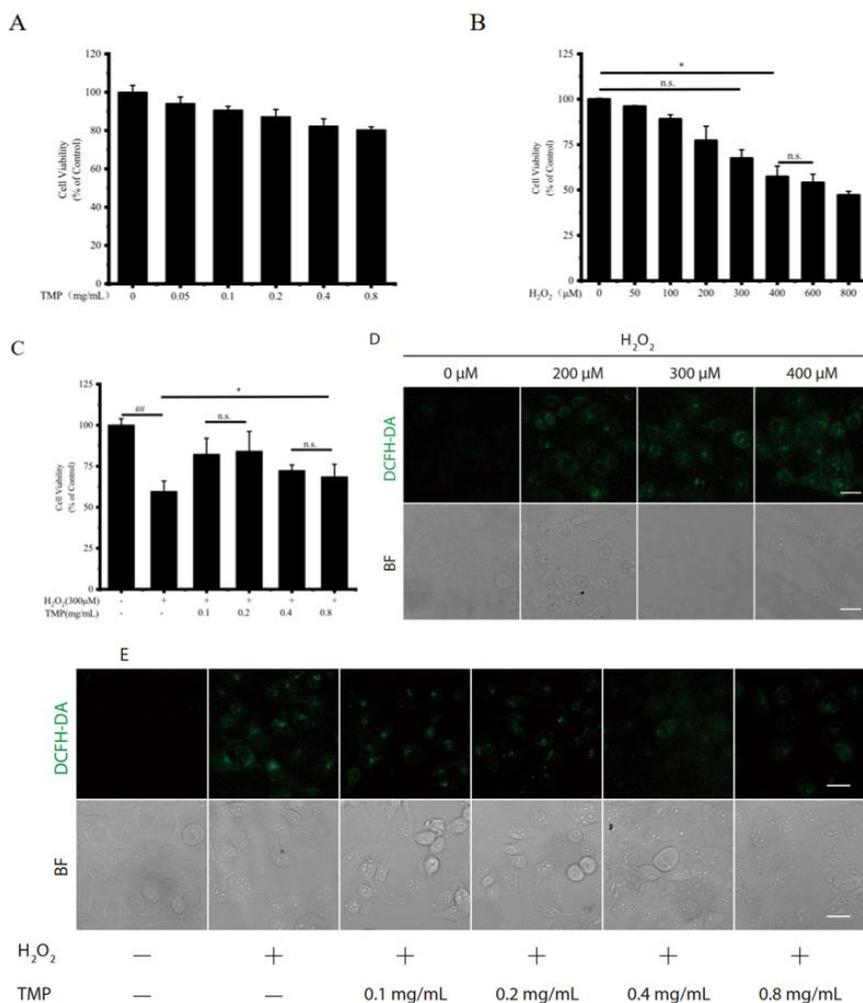

Fig. 1: Effect of TMP on the viability in LO_2 cells. (A) LO_2 cells were treated with different concentrations (0–0.8 mg/mL) of TMP for 24 h. (B) LO_2 cells were treated with 0-800 μM H_2O_2 for 4 h. (C) Cells were pretreated with different concentrations (0–0.8 mg/mL) of TMP for 24 h, and then treated with 300 μm H_2O_2 for 4 h. Cell viability was measured by CCK-8 assay. Data are reported as mean ± S.D. (n = 3) for each group. ##P<0.01 vs. control group, *P<0.05, **P<0.01 vs·H_2O_2-treated group, n.s. means no significant difference at P<0.05. (D) The LO_2 cells were treated with 0-400 μm H_2O_2 for 4h and exposed to DCFH-DA for 30 min. The fluorescence intensity was evaluated using fluorescent microscopy. (E) TMP inhibited ROS generation and apoptotic-related proteins in H_2O_2-treated LO_2 cells. Cells were pretreated with different concentrations (0–0.8 mg/mL) of TMP for 24 h, and treated with 300 μm H_2O_2 for 4 h. Cells were exposed to DCFH-DA for 30 min. The fluorescence intensity was evaluated using fluorescent microscopy
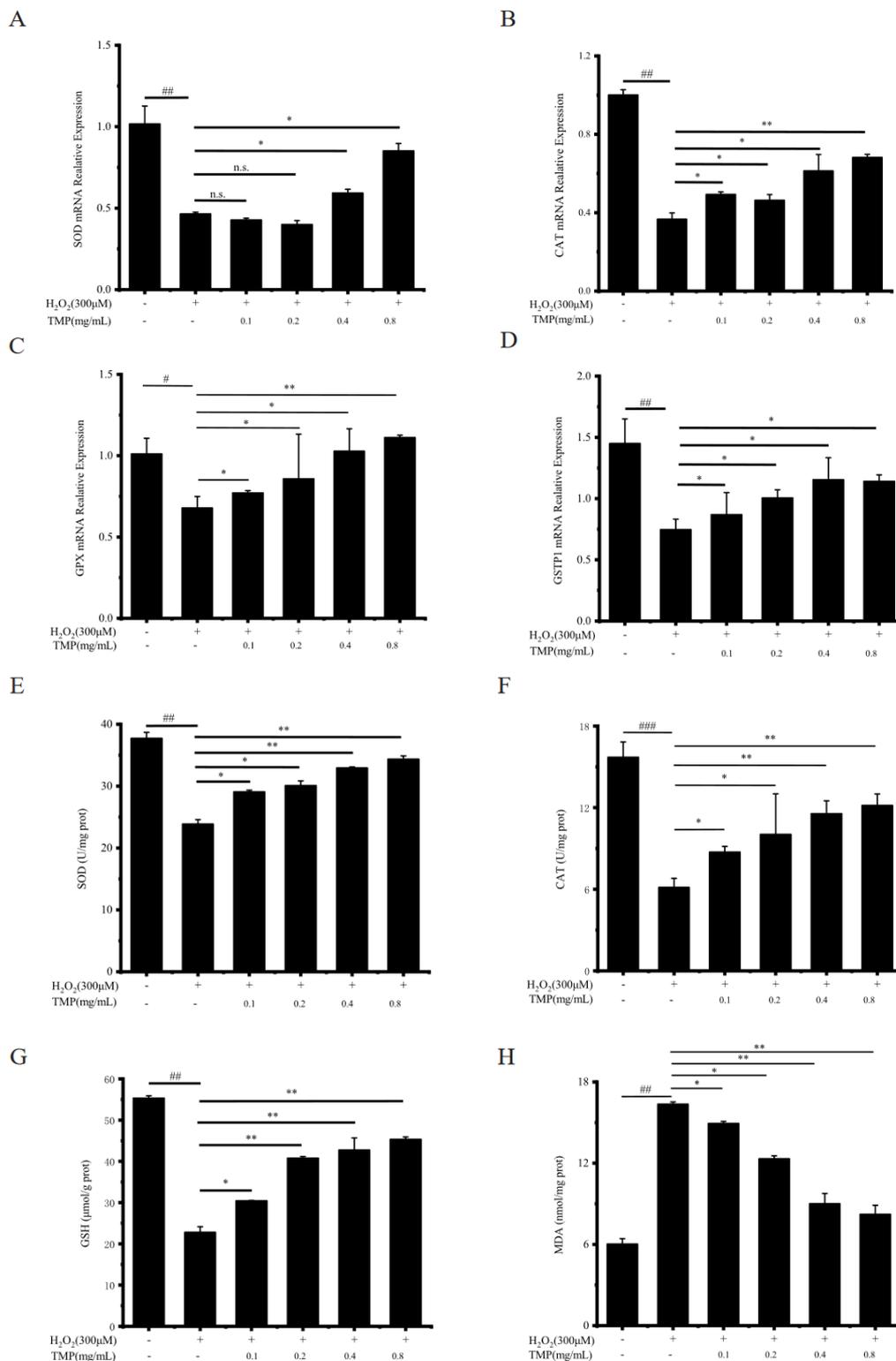
Fig. 2: TMP enhanced the level of the antioxidative defense system in H_2O_2-treated LO2 cells. (1) The mRNA expression levels of (A) SOD; (B) CAT; (C) GPX and (D) GSTP1 (2) The levels of (E) SOD activity; (F) CAT activity; (G) GSH level and (H) MDA level were measured using a microplate reader. Data presented as mean ± S.D. (n = 3) for each group. ##P<0.01, ###P<0.001 vs. control group, *P<0.05, **P<0.01 vs·H_2O_2-treated group, n.s. means no significant difference at P<0.05
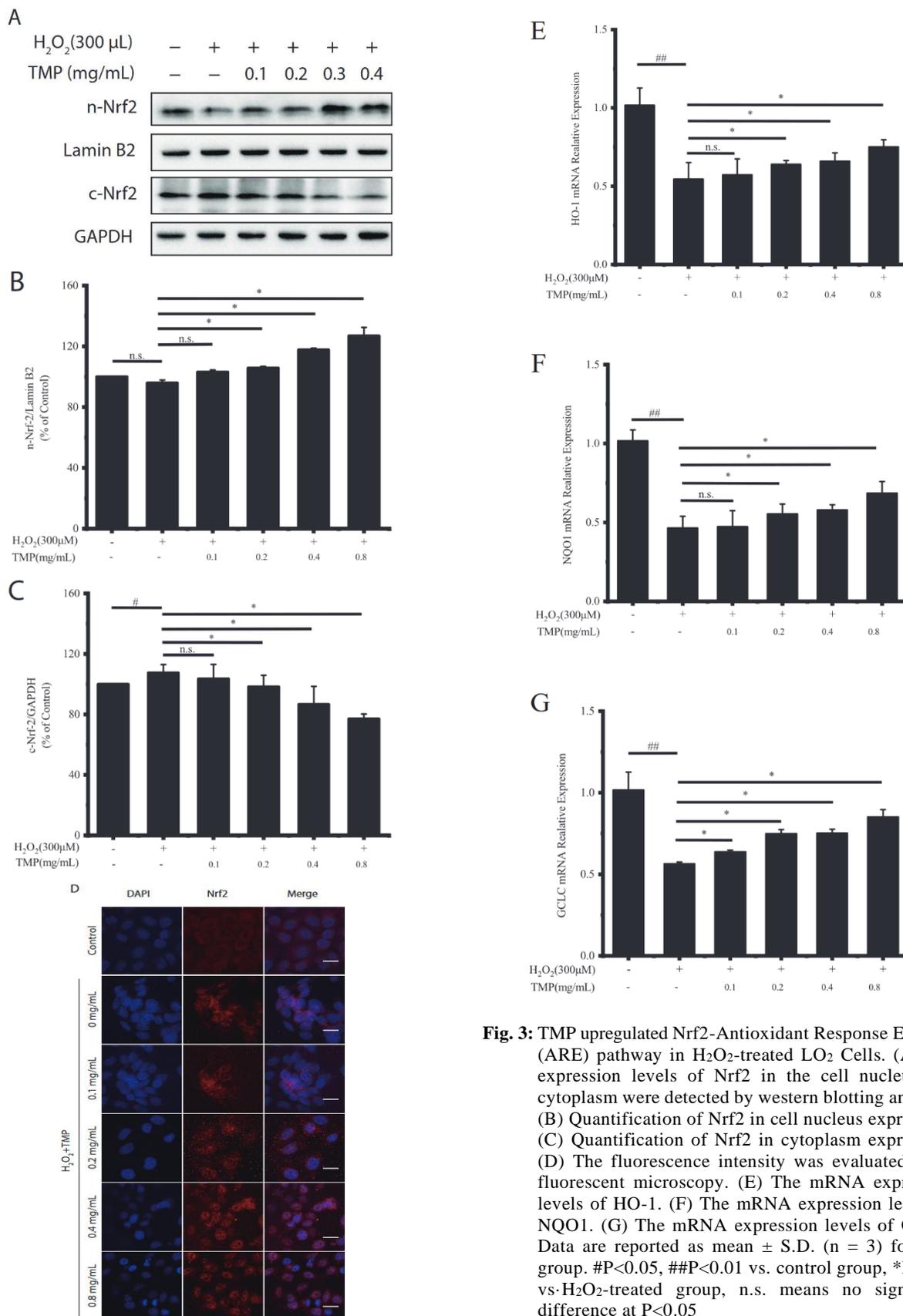
Fig. 3: TMP upregulated Nrf2-Antioxidant Response Element (ARE) pathway in H_2O_2-treated LO_2 Cells. (A) The expression levels of Nrf2 in the cell nucleus and cytoplasm were detected by western blotting analysis. (B) Quantification of Nrf2 in cell nucleus expression. (C) Quantification of Nrf2 in cytoplasm expression. (D) The fluorescence intensity was evaluated using fluorescent microscopy. (E) The mRNA expression levels of HO-1. (F) The mRNA expression levels of NQO1. (G) The mRNA expression levels of GCLC. Data are reported as mean ± S.D. (n = 3) for each group. #P<0.05, ##P<0.01 vs. control group, *P<0.05 vs·H_2O_2-treated group, n.s. means no significant difference at P<0.05
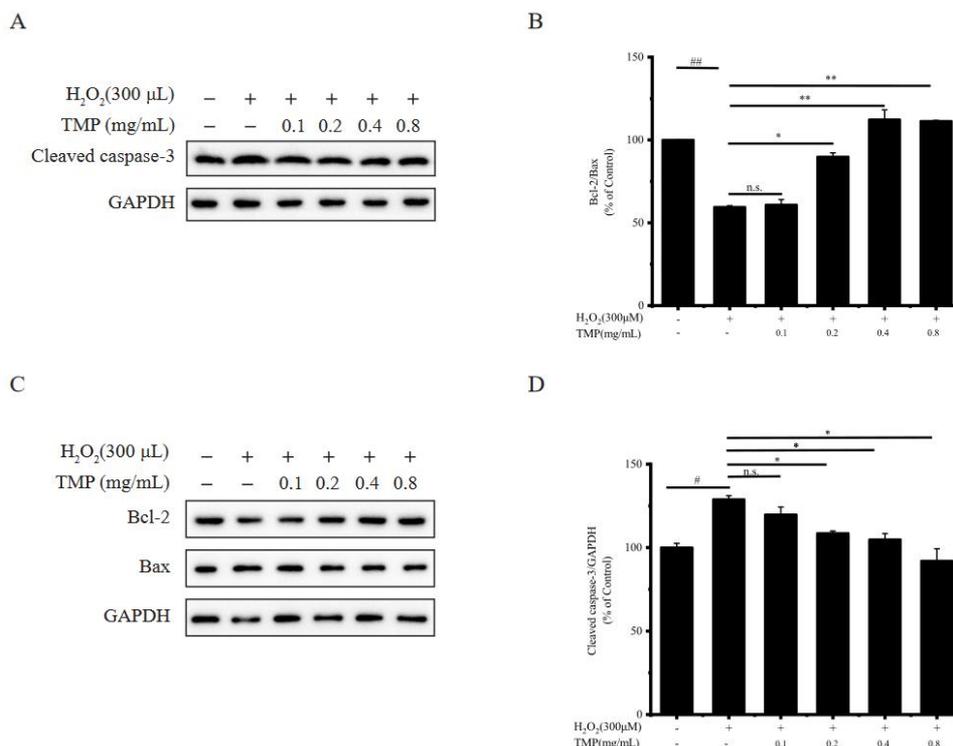
Fig. 4: (A) The expression levels of cleaved caspase-3 were detected by western blotting analysis. (B) Quantification of cleaved caspase-3/GAPDH. (C) The expression levels of Bcl-2 and Bax were detected by western blotting analysis. (D) Quantification of cleaved Bcl-2/Bax. Data are reported as mean ± S.D. (n = 3) for each group. #$P<0.05$, ##$P<0.01$ vs. control group, *$P<0.05$, *$P<0.01$ vs·H_2O_2-treated group, n.s. means no significant difference at $P<0.05$

TMP Inhibits Apoptotic-Related Proteins in H_2O_2-Treated LO_2 Cells

The caspase enzyme family is an important class of apoptotic molecules that was discovered in recent years through an in-depth study of cell apoptosis. They play a key role in the process of apoptosis signal transduction. Particularly, caspase-3 is an apoptotic-related protein in the apoptotic system; in most cases, all apoptotic signals converge on caspase-3, which is the final executor of apoptosis. The upstream apoptotic signal induced the cleaving of caspase-3 and makes it enzymatically. Cleaved caspase-3 executes the final apoptosis program, which represents the process of apoptosis. We further determined whether TMP can protect cells from oxidative stress via apoptotic. As shown in Fig. 4A and 4B, the expression levels of cleaved caspase-3 protein in the oxidative damage model group were significantly higher than those in the normal control group. However, the expression levels of cleaved caspase-3 in the TMP pretreatment group gradually decreased, showing a concentration-dependent manner. Subsequently, the expression of Bax and Bcl-2 was detected. As shown in Fig. 4C and 4D, compared with the control group, the Bax level markedly increased after H_2O_2 treatment. The expression levels of Bax gradually decreased with increasing TMP concentration and the expression levels of Bcl-2 had the opposite trend. The ratio of the expression levels of the Bcl-2 to Bax tended to gradually increase with increasing TMP concentration. These results suggest that TMP inhibits cell apoptosis induced by oxidative stress with H_2O_2 treatment.

Discussion

Oxidative stress refers to a state in which the oxidative and anti-oxidative effects in the body are out of balance, leading to oxidative stress (Finkel 2003; Martindale and Holbrook, 2002; Sies 1997; Rezayian *et al*., 2019; Zeng *et al*., 2021). As an inducer of oxidative stress, H_2O_2 could lead to cell death including apoptosis.

In this study, we found that pretreatment with TMP reduced cell viability and reduced DCF binding in the nucleus of H_2O_2-treated LO_2 cells (Fig. 1). Oxidative stress is accompanied by changes in mRNA levels of various oxidation-related enzymes and oxidation-related pathways. TMP pretreatment changed the cells' oxidation-related enzyme activity in a concentration-dependent manner, with the greatest effect at 0.8 g/mL (Fig. 2).

According to the mRNA level, maximum expression occurred at 0.8 g/mL (Fig. 2). NRF2 is an indispensable active transcription regulator protein that maintains the oxidation balance. It is usually coupled to Keap1 and anchored in the cytoplasm; when stimulated by an oxidant, it is phosphorylated and decoupled with Keap1 and transferred to the nucleus (Johnson *et al.*, 2008; Kensler *et al.*, 2007; van Muiswinkel *et al.*, 2005; Szklarz, 2013). It can change the expression of proteins related to the oxidation pathway including heme oxygenase 1, NAD(P)H dehydrogenase [quinone] 1, and glutamate-cysteine ligase modifier subunit (Sahin *et al.*, 2010; Walsh, 2003; Yeligar *et al.*, 2010). The absence or activation of NRF2 aggravates the damage of oxidative stress. TMP protects cells from damage induced by oxidative stress by increasing the expression of Nrf2 protein in the nucleus (Fig. 3). In this study, TMP significantly decreased ROS generation in oxidant-induced damage in LO_2 cells (Fig. 4). These results show that TMP can effectively alleviate the damage caused by H_2O_2 to the cell's antioxidant system and exert antioxidant effects.

Apoptosis is a type of programmed cell death, which is the orderly and autonomous death of cells controlled by genes caused by various cell stress stimuli (Johnstone *et al.*, 2002; Liu *et al.*, 2020; Nagata, 1997; Zhou *et al.*, 2019). Oxidative stress can damage cells, proteins, and DNA and is also one of the inducing factors of apoptosis (Chandra *et al.*, 2000; Motallebzadeh *et al.*, 2020). Meanwhile, the expression level of cleaved caspase-3 was reduced by TMP pretreatment (Fig. 4). H_2O_2 induced an increase in the amount of ROS produced by LO_2 cells, which in turn caused hepatocytes to undergo apoptosis. TMP reduced the amount of H_2O_2-induced ROS, reduced Bax/Bcl-2, and stabilized the mitochondrial membrane potential. These findings suggest that TMP protects against oxidative damage by activation of Nrf2 and consequently the antioxidant defense system.

Conclusion

In summary, the present data suggest that TMP might improve the recovery of cells from oxidative damage caused by H_2O_2 at the molecular level. TMP reduced oxidative stress-induced cell damage by affecting the expression of key proteins in the process of apoptosis and exhibited protective effects. TMP prevents oxidative stress damage by changing the cytoplasmic and nuclear expression of Nrf2 protein. Therefore, TMP has antioxidant activity and could provide a promising method for the prevention of oxidative damage.

Authors Contribution

Songbo Guo: Performed the experiment and performed the data analyses and wrote the manuscript.

Xinglin Han: Contributed to the conception of the study.

Shanbin Chen: Performed the experiment and contributed significantly to analysis and manuscript preparation and wrote the manuscript.

Xiaomeng Zhang, Hongjiao Li, Yishu Li, Wenmei Zhao and Liaofei Zhang: Contributed significantly to analysis and manuscript preparation.

Baoguo Sun: Helped perform the analysis with constructive discussions.

Feike Hao and Yefu Chen: Contributed to the conception of the study and helped perform the analysis with constructive discussions

Ethics

All authors read and approved the final version of this manuscript. There are not any ethical issues to declare that could arise after the publication of this manuscript. This article does not contain any studies with human participants or animals performed by any of the authors.

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