·Basic Research·

Effects of different concentrations of tetramethylpyrazine, an active constituent of Chinese herb, on human corneal epithelial cell damaged by hydrogen peroxide

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Received: 2013-08-19 Accepted: 2014-04-21

Abstract

• AIM: To discuss the effects of different concentrations of tetramethylpyrazine (TMP), an active constituent of Chinese herb, on damaged Shandong human corneal epithelial cell (SDHCEC) induced by hydrogen peroxide.

• METHODS: We detected the combined effects of TMP with concentrations ranging from 4 mg/mL to 0.03 mg/mL and 800 μM hydrogen peroxide on SDHCEC. The methyl thiazolyl tetrazolium (MTT) assay was processed at 3, 6 and 12h separately while the detection of cell apoptosis at 6h only by flow cytometry.

• RESULTS: The viability of SDHCEC with 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL and 0.06 mg/mL TMP joint with 800 μM hydrogen peroxide at 3h and 6h was significantly higher than that with 800 μM hydrogen peroxide only, $P<0.05$. However, except 0.25 mg/mL, TMP with other concentrations joint with 800 μM hydrogen peroxide at 12h could not significantly inhibit decreased SDHCEC viability induced by 800 μM hydrogen peroxide. At 12h, TMP of 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL and 0.06 mg/mL could significantly inhibit SDHCEC early apoptosis induced by 800 μM hydrogen peroxide, most remarkable at 0.25 mg/mL TMP, $P<0.05$.

• CONCLUSION: Our results suggested that hydrogen peroxide can induce apoptosis related damage to SDHCEC. TMP can protect SDHCEC from the damage, and the protective effects may be associated with its anti-apoptosis mechanism.

• **KEYWORDS:** human corneal epithelial cell; cell viability; apoptosis; hydrogen peroxide
DOI:10.3980/j.issn.2222-3959.2014.06.06

Li N, Deng XG, Zhang SH, He MF, Zhao DQ. Effects of different concentrations of tetramethylpyrazine, an active constituent of Chinese herb, on human corneal epithelial cell damaged by hydrogen peroxide. *Int J Ophthalmol* 2014;7(6):947-951

INTRODUCTION

In recent years, reports about the effects of oxidative stress in biomedical fields are abundant, such as aging, infertility, tumor, diabetes, neurodegenerative disease, and so on [1]. Eyes, which are continuously exposed to light, air, chemicals in the environment, *etc*. are vulnerable to oxidative stress. Researchers have proved that oxidative damage are related to many eye diseases, such as age-related macular degeneration, senile cataract, actinic keratoconjunctivitis induced by ultraviolet radiation, and so on. Of course, the corneal injury [2-4] which we are going to talk about. Especially for corneal epithelium, which is directly exposed to outside ambient, the antioxidants mainly exist in the tear film and epithelial tissue itself[5-9].

Active oxygen species, such as hydroxyl group, superoxide anion, hydrogen peroxide, *etc*. are produced by cell metabolism process. Though activity of hydrogen peroxide itself is not strong, it can be decomposed into hydroxyl radical of perfect antioxidant effect [10]. Besides, hydrogen peroxide is a stable medium oxidant produced biologically, which has been widely applied in studies. Recent evidence showed that active oxygen species can induce cell apoptosis *via* mitochondrial function damage, and there into hydrogen peroxide may attack some enzymes, consume adenosine triphosphate (ATP), decrease the level of nicotinamide adenine dinucleotide phosphate, and finally lead to cell apoptosis [11]. Recently, several studies showed that hydrogen peroxide can lead to injury of corneal epithelial cell and corneal endothelium[2,4]. However, there are no reports found about corneal epithelial cell apoptosis induced by hydrogen peroxide.

Traditional Chinese medicine represents a worldwide resource for potential treatments of many diseases. Recent studies show that tetramethylpyrazine (TMP), extracted from the Chinese herbal medicine Ligusticum wallichii franchat (chuan xiong in Chinese), is a significant anti-lipid-

peroxidation, anti-free radical, anti-apoptosis[12-15] and calcium antagonist agent[16]. And TMP has been widely used in clinic for treatment of cardiovascular and cerebrovascular diseases and various diseases of retina [17,18]. Therefore, TMP is a potentially useful treatment for ocular surface disorders. In the present study, we aimed to observe effects of different concentrations of TMP against damage of human corneal epithelial cells induced by hydrogen peroxide from the aspects of antiapoptotic *in vitro*, which no one ever discussed before.

SUBJECTS AND METHODS

Our study followed the principles outlined in the Declaration of Helsinki (2008).

Cell Culture Shandong human corneal epithelial cells (SDHCEC) (provided by Prof. Zhi-Chong Wang in Zhongshan Ophthalmic Center of Sun Yat-sen University) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose supplemented with 10% heat-inactivated fetal bovine serum, 100 000 U/L penicillin, 100 mg/L streptomycin, 2 g/L sodium bicarbonate, 0.01 mg/L human epidermal growth factor, 0.292 g/L L-Glutamine, 5 mg/L insulin, 5 mg/L human transferrin, 400 ng/L hydrocortisone, 1× nonessential amino acids in CO_2 incubator (37℃, 5% CO_2; Thermo Fisher Scientific, America). We firstly observed the influence of hydrogen peroxide with different concentrations (1600 μM, 800 μM, 400 μM, 200 μM, 100 μM) on SDHCEC to choose an appropriate concentration. And then we detected the combined effects of TMP with different concentrations (4 mg/mL, 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.06 mg/mL, 0.03 mg/mL) and 800 μM hydrogen peroxide on SDHCEC, while simultaneously we observed TMP with different concentrations (4 mg/mL, 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.06 mg/mL, 0.03 mg/mL) alone on SDHCEC. The methyl thiazolyl tetrazolium (MTT) assay was processed at 3, 6 and 12h separately while the detection of cell apoptosis with annexin-V-FITC and propidium iodide (PI) double staining at 6h only.

Cell Viability Assay Cell viability analysis was based on the capacity of mitochondrial enzymes to transform MTT to MTT formazan. The formazan production would be decreased when mitochondrial redox function was impaired or cellular reactive oxygen species (ROS) was enhanced. Therefore, MTT assay may be used as an index of cell viability responses to experimental interventions. In short, cells were cultured in 96-well plates (0.5×10^5 cells/mL, 200 μL) for 24h and treated as described above. We set normal control wells in which we cultured cells only with serum-free high-glucose DMEM and positive control wells in which we cultured cells with serum-free high-glucose DMEM containing hydrogen peroxide (terminal concentration was 800 μM). After the treatment with different medium conditions for 3, 6 and 12h separately, 20 μL of MTT (5 mg/mL) was added to each well and cells were incubated at 37℃ for 4h. Then culture medium with dye was removed and 200 μL of dimethyl sulfoxide (DMSO) per well was added for formazan solubilization. The absorbance of converted dye was measured at wavelengths of 570 nm and 630 nm using Multiskan MK3 (Thermo Fisher Scientific, America) and the difference values were used to calculate the cell viability. The computational formula: absorptance (tested well)/ absorptance (normal control well)×100%.

Analysis of Cell Apoptosis by Flow Cytometry The apoptotic rate of SDHCEC was detected using an Annexin-V FITC/PI apoptosis detection kit. After the drug treatment, cells were harvested with 0.25% trypsinase, washed with serum-free high-glucose DMEM, removed supernatant after centrifugating at 1500 r for 10min and double-stained with Annexin-V FITC and PI in the dark at room temperature for 15min. After that, samples were analyzed using a FACSaria flow cytometer (Becton, Dickinson and Company, America).

Statistical Analysis Statistical analysis was performed using SPSS version 16.0 (SPSS, Inc, Chicago, IL, USA). A one-way analysis of variance followed by either the Bonferroni or Dunnett T3 test were used to compare the mean value of all items. A significance level was set at $P<0.05$.

RESULTS

The viability of SDHCEC with 1600 μM and 800 μM hydrogen peroxide at 3, 6 and 12h was significantly lower than that in normal controls, progressing with time, while the viability of SDHCEC with 400 μM hydrogen peroxide at 12h was lower than that in normal controls as well, $P<0.01$ or $P<0.05$ (Figure 1).

The viability of SDHCEC with 4 mg/mL and 2 mg/mL TMP at 3, 6 and 12h was significantly lower than that in normal controls, $P<0.001$, while other concentrations less than or equal to 1 mg/mL showed no significance (Figure 2).

The viability of SDHCEC with 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL and 0.06 mg/mL TMP joint with 800 μM hydrogen peroxide at 3, 6h was significantly higher than that with 800 μM hydrogen peroxide only, most remarkable at 0.25 mg/mL TMP, $P<0.05$. However, except 0.25 mg/mL TMP, TMP with other concentrations joint with 800 μM hydrogen peroxide at 12h could not protect SDHCEC from decreased viability induced by 800 μM hydrogen peroxide. The viability of SDHCEC with 4 mg/mL and 2 mg/mL TMP joint with 800 μM hydrogen peroxide at all the three time points was significantly lower than that with 800 μM hydrogen peroxide only, $P<0.05$ (Figure 3).

Cell apoptosis at 6h was assayed by flow cytometry. SDHCEC detected by Annexin-V FITC/PI apoptosis kit can be classified into normal cells (Q1), early apoptotic cells (Q2), late apoptotic cells (Q3) and necrotic cells (Q4). Our results showed that hydrogen peroxide of 1600 μM and

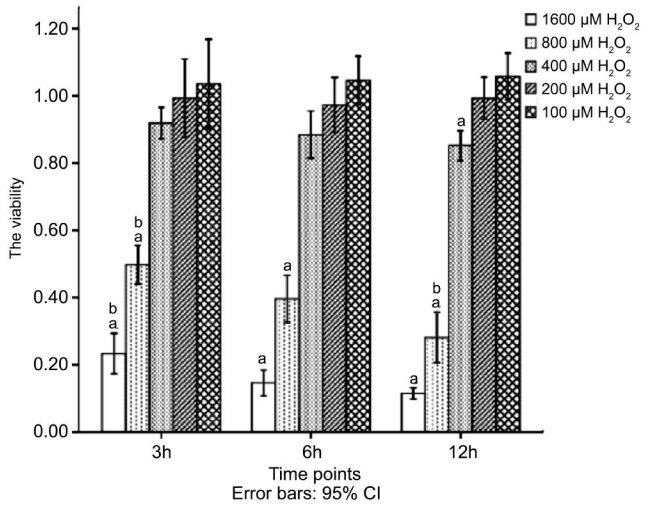

Figure 1 Influence of hydrogen peroxide with different concentrations on SDHCEC viability detected by MTT [a]$P<0.01$ *vs* normal controls; [b]$P<0.05$ *vs* values at 6h at the same group.

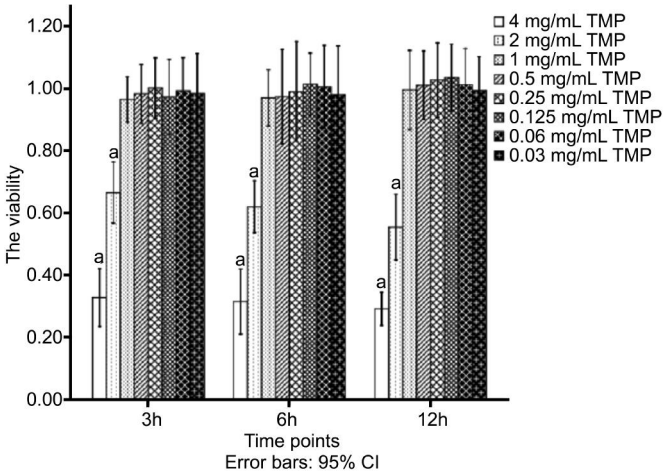

Figure 2 Influence of TMP with different concentrations on SDHCEC viability detected by MTT [a]$P<0.001$ *vs* normal controls.

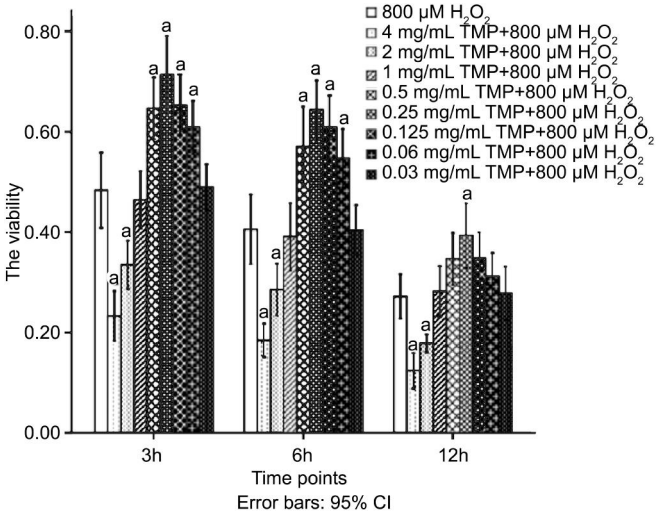

Figure 3 Influence of TMP with different concentrations on decreased SDHCEC viability induced by 800 μM hydrogen peroxide detected by MTT [a]$P<0.05$ *vs* 800 μM H_2O_2.

800 μM could induce SDHCEC early apoptosis, while 1600 μM hydrogen peroxide could induce SDHCEC late apoptosis and necrosis, $P<0.001$ (Figure 4).

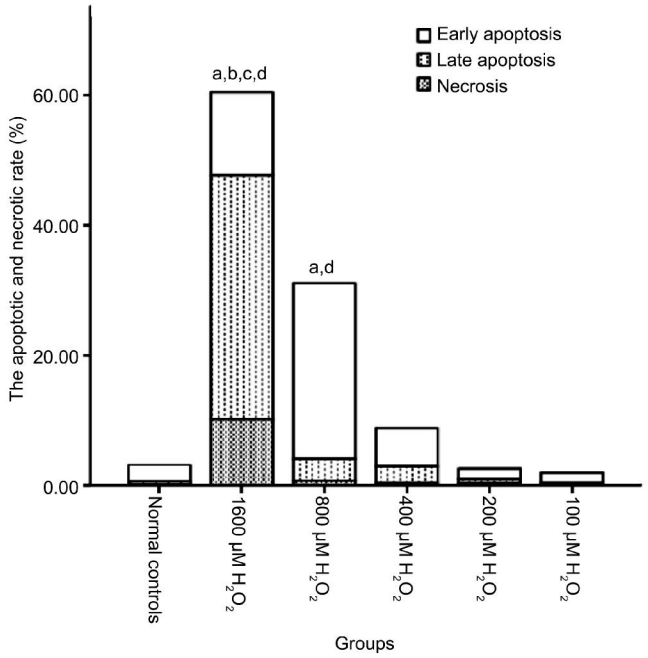

Figure 4 Influence of hydrogen peroxide with different concentrations on the apoptotic rate of SDHCEC detected by flow cytometry [a]$P<0.001$ *vs* normal controls in early apoptosis; [b]$P<0.001$ *vs* normal controls in late apoptosis; [c]$P<0.001$ *vs* normal controls in early necrosis; [d]$P<0.001$ *vs* normal controls in all abnormal cells.

Compared with normal control group, TMP of 4 mg/mL could induce SDHCEC early apoptosis, late apoptosis and necrosis separatly while TMP of 2 mg/mL could only induce SDHCEC early apoptosis, $P<0.001$ or $P<0.01$. There was no significance between TMP with concentrations less than or equal to 1 mg/mL and normal controls, $P>0.05$ (Figure 5). TMP of 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL and 0.06 mg/mL at 6h could significantly inhibit SDHCEC early apoptosis induced by 800 μM hydrogen peroxide, most remarkable at 0.25 mg/mL TMP, $P<0.001$ or $P<0.05$. TMP of 4 mg/mL could significantly increase SDHCEC early apoptosis induced by 800 μM hydrogen peroxide (Figures 6, 7).

CONCLUSION

Our results showed that hydrogen peroxide of 1600 μM and 800 μM at 3, 6 and 12h could significantly decrease SDHCEC viability, inducing cell early apoptosis. Similarly, previous studies have showed that hydrogen peroxide could induce oxidative damage and apoptosis of various cells, such as endothelial cells, pheochromocytoma 12 cells, cardiac muscle cells, endometrial carcinoma cells, human embryonic stem cells, retina cells, and so on [11-15,19-21]. In our study, TMP of 4 mg/mL and 2 mg/mL could induce SDHCEC oxidative damage while TMP with concentrations less than or equal to 1 mg/mL showed no significant influence on SDHCEC. TMP of 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL and 0.06 mg/mL joint with 800 μM hydrogen peroxide at 3h and 6h could significantly inhibit oxidative damage induced by hydrogen peroxide, most remarkable at 0.25 mg/mL TMP. Results by

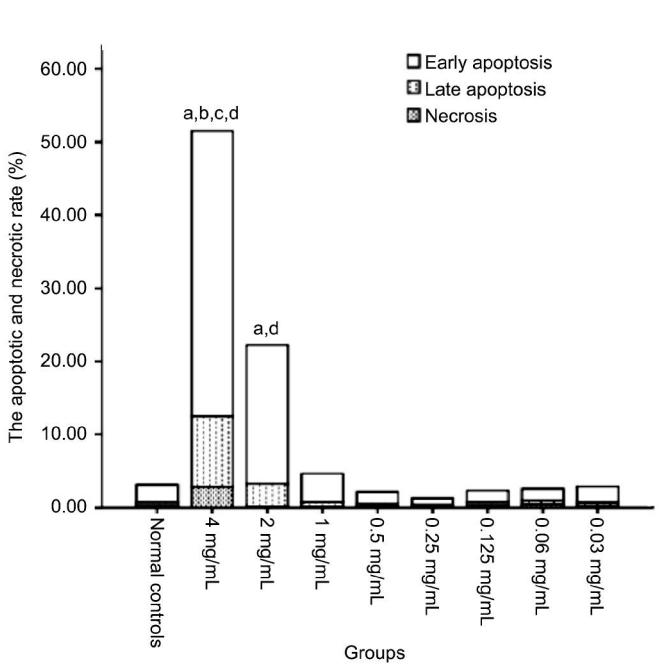

Figure 5 Influence of TMP with different concentrations on the apoptotic rate of SDHCEC detected by flow cytometry [a] $P<0.001$ *vs* normal controls in early apoptosis; [b] $P<0.001$ *vs* normal controls in late apoptosis; [c] $P<0.01$ *vs* normal controls in early necrosis; [d] $P<0.001$ *vs* normal controls in all abnormal cells.

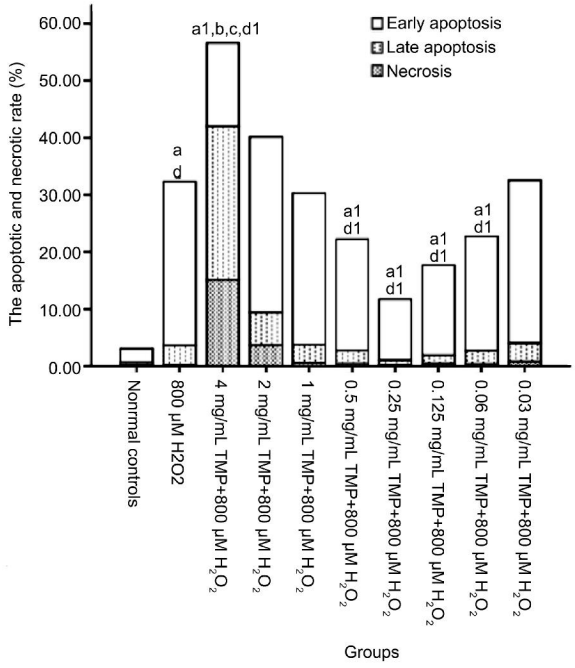

Figure 6 Effects of TMP with different concentrations on SDHCEC apoptosis induced by 800 μM hydrogen peroxide detected by flow cytometry [a] $P<0.001$ *vs* normal controls in early apoptosis; [a1] $P<0.05$ *vs* 800 μM H_2O_2 in early apoptosis; [b] $P<0.001$ *vs* normal controls in late apoptosis; [c] $P<0.001$ *vs* normal controls in early necrosis; [d] $P<0.001$ *vs* normal controls in all abnormal cells; [d1] $P<0.05$ *vs* 800 μM H_2O_2 in all abnormal cells.

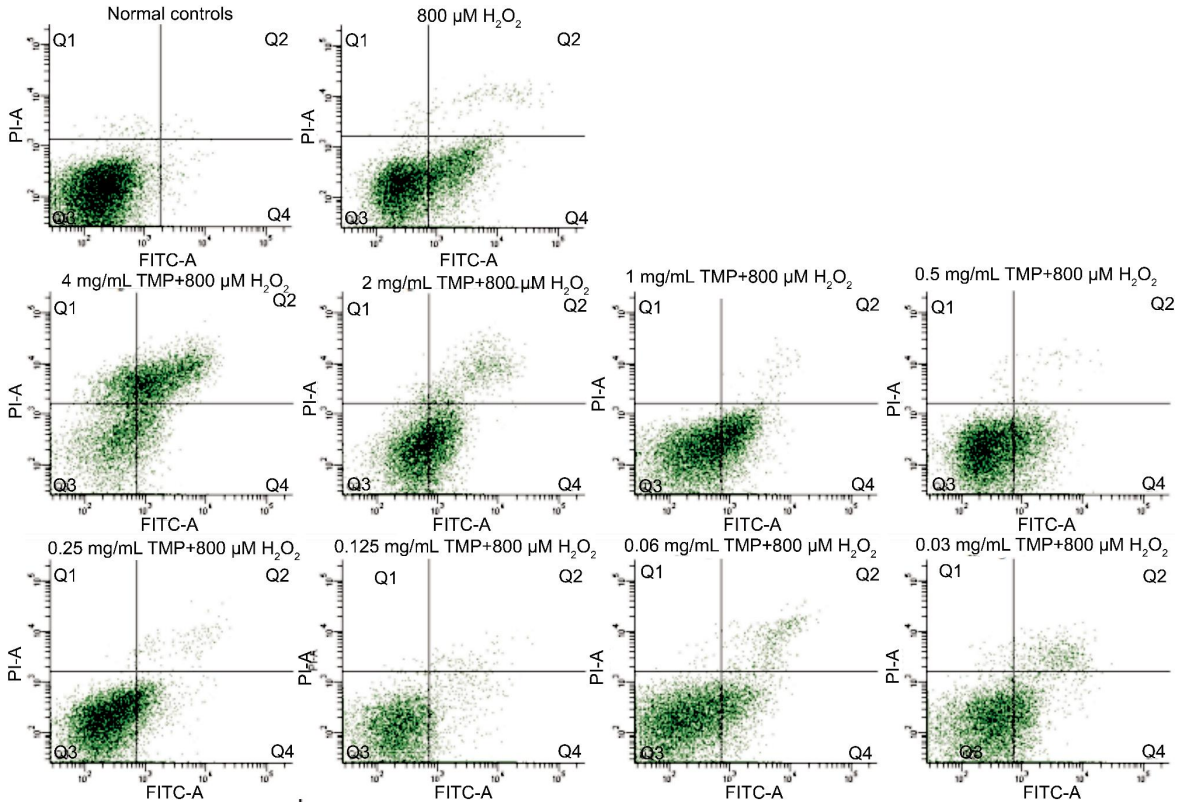

Figure 7 The images of SDHCEC about the effects of TMP with different concentrations on SDHCEC apoptosis induced by 800 μM hydrogen peroxide detected by flow cytometry.

flow cytometry assay also showed that TMP of 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL and 0.06 mg/mL joint with 800 μM hydrogen peroxide at 6h could significantly inhibit cell apoptosis induced by hydrogen peroxide, indicating that TMP could protect SDHCEC from oxidative damage induced by hydrogen peroxide, which might be related to anti-apoptosis

effect of TMP. The results were similar to reports that TMP can protect damaged cells *via* anti-oxidation and/or anti-apoptosis effects. Cheng *et al* [13] reported in 2007 that TMP could inhibit oxidative damage of PC12 cells induced by hydrogen peroxide *in vitro*, which was related to anti-apoptosis effect of TMP. Li *et al* [14] reported in 2009 that TMP could inhibit oxidative damage of human umbilical vein endothelial cells induced by hydrogen peroxide *in vitro via* anti-oxidation and anti-apoptosis effects. Ou *et al* [11] also reported in 2010 the similar effect of TMP and its extract. Some researches about eye diseases also showed similar effects. Yang *et al* [12] proved that TMP could protect retina cells from oxidative damage induced by hydrogen peroxide, which was related to anti-oxidation and anti-apoptosis effects of TMP. Our previous studies also showed that TMP could inhibit lens opacity induced by sodium selenite *via* anti-oxidation and calcium antagonistic effects *in vivo* and *in vitro* [22,23].

MTT results at 12h showed that except 0.25 mg/mL TMP, TMP with other concentrations could not significantly increase the viability of SDHCEC damaged by 800 μM hydrogen peroxide, indicating that persistence of hydrogen peroxide led to irreversible damage of SDHCEC.

In conclusion, our results showed that TMP could protect damaged SDHCEC induced by hydrogen peroxide, which might be associated with anti-apoptosis mechanism of TMP. The study provides a basis for local application of TMP on cornea correlated diseases.

ACKNOWLEDGEMENTS

Foundation: Supported by Guangdong Administration of Traditional Chinese Medicine (No.2007095)

Conflicts of Interest: Li N, None; **Deng XG,** None; **Zhang SH,** None; **He MF,** None; **Zhao DQ,** None.

REFERENCES

1 Shoham A, Hadziahmetovic M, Dunaief JL, Mydlarski MB, Schipper HM. Oxidative stress in diseases of the human cornea. *Free Radic Biol Med* 2008;45(8):1047-1055

2 Atilano SR, Chwa M, Kim DW, Jordan N, Udar N, Coskun P, Jester JV, Wallace DC, Kenney MC. Hydrogen peroxide causes mitochondrial DNA damage in corneal epithelial cells. *Cornea* 2009;28(4):426-433

3 Memarzadeh F, Shamie N, Gaster RN, Chuck RS. Corneal and conjunctival toxicity from hydrogen peroxide: a patient with chronic self-induced injury. *Ophthalmology* 2004;111(8):1546-1549

4 Hudde T, Comer RM, Kinsella MT, Buttery L, Luthert PJ, Polak JM, George AJ, Larkin DF. Modulation of hydrogen peroxide induced injury to corneal endothelium by virus mediated catalase gene transfer. *Br J Ophthalmol* 2002;86(9):1058-1062

5 Imamura Y, Noda S, Hashizume K, Shinoda K, Yamaguchi M, Uchiyama S, Shimizu T, Mizushima Y, Shirasawa T, Tsubota K. Drusen, choroidal neovascularization, and retinal pigment epithelium dysfunction in SOD1-deficient mice: a model of age-related macular degeneration. *Proc Natl Acad Sci USA* 2006;103(30):11282-11287

6 Khandhadia S, Lotery A. Oxidation and age-related macular degeneration: insights from molecular biology. *Expert Rev Mol Med* 2010;12:e34

7 Bhuyan KC, Bhuyan DK, Podos SM. Lipid peroxidation in cataract of the human. *Life Sci* 1986;38(16):1463-1471

8 van Kuijk FJ. Effects of ultraviolet light on the eye: role of protective glasses. *Environ Health Perspect* 1991;96:177-184

9 Chen Y, Mehta G, Vasiliou V. Antioxidant defenses in the ocular surface. *Ocul Surf* 2009;7(4):176-185

10 Leonard SS, Harris GK, Shi X. Metal-induced oxidative stress and signal transduction. *Free Radic Biol Med* 2004;37(12):1921-1942

11 Ou Y, Dong X, Liu XY, Cheng XC, Cheng YN, Yu LG, Guo XL. Mechanism of tetramethylpyrazine analogue CXC195 inhibition of hydrogen peroxide-induced apoptosis in human endothelial cells. *Biol Pharm Bull* 2010;33(3):432-438

12 Yang Z, Zhang Q, Ge J, Tan Z. Protective effects of tetramethylpyrazine on rat retinal cell cultures. *Neurochem Int* 2008;52(6):1176-1187

13 Cheng XR, Zhang L, Hu JJ, Sun L, Du GH. Neuroprotective effects of tetramethylpyrazine on hydrogen peroxide-induced apoptosis in PC12 cells. *Cell Biol Int* 2007;31(5):438-443

14 Li WM, Liu HT, Li XY, Wu JY, Xu G, Teng YZ, Ding ST, Yu C. The effect of tetramethylpyrazine on hydrogen peroxide-induced oxidative damage in human umbilical vein endothelial cells. *Basic Clin Pharmacol Toxicol* 2010;106(1):45-52

15 Li L, Fan TJ, Yang XX, Cong RS, Zhao J, Wang J. Effects and Antioxidative Activities of Flavone on Human Corneal Endothelial Cells *in vitro*. *Guoji Yanke Zazhi* 2008;8(5): 881-885

16 Zhu JX, Zhang GH, Yang N, Rowlands DK, Wong HY, Tsang LL, Chung YW, Chan HC. Activation of apical CFTR and basolateral Ca(2+)-activated K+ channels by tetramethylpyrazine in Caco-2 cell line. *Eur J Pharmacol* 2005;510(3):187-195

17 Guo SK, Chen KJ, Qian ZH, Weng WL, Qian MY. Tetramethylpyrazine in the treatment of cardiovascular and cerebrovascular diseases. *Planta Med* 1983;47(2):89

18 Wu YN, Hao J. The mechanism and clinical application of Ligustrazine on the effect of retinal protection. *Zhongguo Zhongyi Yanke Zazhi* 2012;22(1):72-75

19 Wang B, Shravah J, Luo H, Raedschelders K, Chen DD, Ansley DM. Propofol protects against hydrogen peroxide-induced injury in cardiac H9c2 cells *via* Akt activation and Bcl-2 up-regulation. *Biochem Biophys Res Commun* 2009;389(1):105-111

20 Jung EM, Choi KC, Jeung EB. Expression of calbindin-D28k is inversely correlated with proapototic gene expression in hydrogen peroxide-induced cell death in endometrial cancer cells. *Int J Oncol* 2011;38(4):1059-1066

21 Hu ZX, Geng JM, Liang DM, Luo M, Li ML. Hepatocyte growth factor protects human embryonic stem cell derived-neural progenitors from hydrogen peroxide-induced apoptosis. *Eur J Pharmacol* 2010;645(1-3):23-31

22 Li N, Zhu Y, Deng X, Gao Y, Zhu Y, He M. Protective effects and mechanism of tetramethylpyrazine against lens opacification induced by sodium selenite in rats. *Exp Eye Res* 2011;93(1):98-102

23 Zhu YX, Deng XG, Gao Y, Zhu YG. Effect of tetramethylpyrazine on SOD, CAT and MDA of isolated rat lens. *Guoji Yanke Zazhi* 2010;10(3):437-439