

# Tea polyphenols alleviate hydrogen peroxide-induced oxidative stress damage through the Mst/Nrf2 axis and the Keap1/Nrf2/HO-1 pathway in murine RAW264.7 cells

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**Abstract.** Tea polyphenols (TPs) are the major bioactive extract from green tea that have been extensively reported to prevent and treat oxidative stress damage. In previous studies, TPs have been demonstrated to protect cells against oxidative injury induced by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). However, the underlying mechanism remains unclear. The aim of the current study was to investigate whether the protective and regulatory effects of TPs on oxidative stress damage were dependent on the mammalian STE20-like protein kinase (Mst)/nuclear factor (erythroid-derived 2)-like 2 (Nrf2) axis and the Kelch-like ECH-associated protein 1 (Keap1)/Nrf2/heme oxygenase 1 (HO-1) pathway in RAW264.7 cells, a murine macrophage cell line. Maintaining a certain range of intracellular reactive oxygen species (ROS) levels is critical to basic cellular activities, while excessive ROS generation can override the antioxidant capacity of the cell and result in oxidative stress damage. The inhibition of ROS generation offers an effective target for preventing oxidative damage. The results of the present study revealed that pretreatment with TPs inhibited the production of intracellular ROS and protected

RAW264.7 cells from H<sub>2</sub>O<sub>2</sub>-induced oxidative damage. TPs was also demonstrated to attenuate the production of nitric oxide and malondialdehyde and increase the levels of antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase). In addition, following TPs treatment, alterations in Mst1/2 at the mRNA and protein level inhibited the production of ROS and promoted the self-regulation of antioxidation. TPs-induced Keap1 gene downregulation also increased the expression of Nrf2 and HO-1. Collectively, the results of the present study demonstrated that TPs provided protection against H<sub>2</sub>O<sub>2</sub>-induced oxidative injury in RAW264.7 cells.

## Introduction

Tea polyphenols (TPs) are a group of polyphenol compounds that are mainly extracted from green tea, which contain four substances namely (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG) and (-)-epicatechin (EC) (1). Polyphenols have been reported to exert bioactive effects that are antioxidant, anti-obesity, anti-inflammatory, cancer preventative, anti-tumor and regulatory of lipid metabolism (2). As one of the best-studied antioxidants, it has been demonstrated that TPs exerts definitive protective effects against oxidative stress, which is associated with various clinical symptoms and diseases, including age-related, neurological, cardiovascular and cerebrovascular diseases, occurring through the elimination of free radicals and the subsequent regulation of anti-oxidases activity (3,4). With a basic structure of  $\alpha$ -phenyl-benzopyran, the molecular structure of TPs and polyphenolic compounds, particularly the position and number of hydroxyl groups, serve an important role in antioxidant activity (5). Studies have reported that in laboratory experiments and clinical studies, TPs can provide a more robust protective effect against oxidative damage than polyphenolic compounds individually (6,7).

Reactive oxygen species (ROS) are byproducts of normal cellular metabolism. ROS levels are associated with basic cellular activities and cause oxidative stress when ROS

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generation exceeds the antioxidant capacity of the cell (8). Physiologically, a system of oxidant and antioxidant enzymes delicately balance intracellular ROS levels for cellular homeostasis (9,10). The overproduction of ROS can break cell defenses, leading to oxidative stress that induces irreversible damage to the mitochondria, destroying cellular structure and function and causing increased risk for cardiovascular disease, diabetes mellitus, cancer and other diseases (10). Previous studies have shown that TPs exhibit antioxidant effects in the following processes (11,12): Elimination of free radicals via relatively stable phenolic oxygen radicals formed with ROS; inhibition and inactivity of oxidant enzymes and increased production of antioxidant enzymes. A number of epidemiological studies have revealed that the Kelch-like ECH-associated protein-1 (Keap1)/nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling pathway serves an important role in antioxidant function and reduces oxidative stress (12,13). TPs and certain polyphenolic compounds, such as EGCG, stimulate the activity of the heme oxygenase 1 (HO-1) gene by activating the Nrf2/antioxidant response element pathway (13,14).

H<sub>2</sub>O<sub>2</sub>-induced oxidative damage is one of the most widely used cellular models of oxidative stress as the antioxidant effect of potent antioxidants can be evaluated (15). Macrophages are reportedly vulnerable to ROS and their functions are affected by oxidative stress in a direct and indirect manner (16). Macrophages directly kill pathogens through phagocytosis, secreting large quantities of certain bioactive molecules, including ROS and nitric oxide (NO). ROS production is a major defense mechanism against pathogenic infiltration (17,18). However, the sensitivity of macrophages to ROS can lead to cell injury and even cell death (19). H<sub>2</sub>O<sub>2</sub>-induced oxidative damage in macrophages can provide a drug screening platform for identifying potential antioxidants from natural products (20).

A study by Wang *et al.* (21) reveals that the cellular self-protective mechanism of macrophages against oxidative stress involves the mammalian STE20-like protein kinase (Mst)/Nrf2 axis. In the same study, Mst1 and Mst2 maintain cellular redox balance by acting as an ROS sensor and modulating the stability of the antioxidant transcription factor Nrf2. However, the role of the Mst/Nrf2 axis in the protective cellular mechanism against antioxidant damage exerted by TPs remains to be elucidated.

Although a growing number of epidemiological studies (11,12,22) have identified the molecular mechanisms associated with the antioxidant effects exerted by TP, there are a lack of studies assessing the antioxidant effect of TPs in H<sub>2</sub>O<sub>2</sub>-induced oxidative damaged macrophages. The aim of the current study was to evaluate the antioxidant properties and underlying mechanisms of TPs in H<sub>2</sub>O<sub>2</sub>-induced oxidative macrophage injury using RAW264.7 cells.

## Materials and methods

**Cell culture.** Murine macrophage RAW264.7 cells were purchased from the Cell Bank of the Chinese Academy of Sciences and cultured in DMEM (Biological Industries; Sartorius AG) containing 10% fetal bovine serum (FBS; Biological Industries; Sartorius AG), 100 U/ml of penicillin and 100 µg/ml of streptomycin (HyClone; Cytiva), in a

humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. For all experiments, cells were incubated with various concentrations (0.1, 0.5 and 1.0 µg/ml) of TPs (purity ≥98.0%; cat. no. CAS84650-60-2; Beijing Solarbio Science & Technology Co., Ltd.; containing 46.8% EGCG, 17.3% EGC, 5.2% EC and 1.5% ECG; Fig. S1) for 12 h prior to the addition of H<sub>2</sub>O<sub>2</sub> for 12 h. EGCG, EGC, ECG and EC were bought from Aladdin. Methanol of HPLC-grade was purchased from Sigma-Aldrich (Merck KGaA).

**HPLC analysis.** The appropriate amounts of four standard substances (EGCG, EGC, EC and ECG), were accurately weighed and dissolved in 20% methanol (v/v). The tea polyphenol samples were prepared with 20% methanol and injected into the HPLC system for analysis after filtering through 0.22 µm syringe filters.

The analysis was carried out using an LC 15 (Shimadzu Corporation) for the chromatographic determination. The sample was injected into a C18 analytical column (4.6 mm I.D x 150 mm, 3.0 µm particle size; Waters Corporation). The mobile phase was 0.1% formic acid solution (solvent A) and methanol (solvent B). The Diode-Array Detection acquisition wavelength was set at 275 nm. The gradient elution programmer was as follows: ~0-2 min, 2% B; ~2-13 min, 2-90% B; ~13-16 min, 100%. The flow rate was of 0.3 ml/min, and the column temperature was set at 40°C. The injection volume was 5 µl.

**H<sub>2</sub>O<sub>2</sub>-induced oxidative stress model.** RAW264.7 cells were inoculated into 96-well plates at a density of 4x10<sup>3</sup> cells/well at 37°C in a humidified 5% CO<sub>2</sub> incubator for 12 h. Cells were treated with different final concentrations of H<sub>2</sub>O<sub>2</sub> (0, 50, 100, 200, 300, 400, 500, 600, 700 and 800 µM) diluted with complete medium for 0, 4, 8, 12 and 24 h. Cells were subsequently treated with Cell Counting Kit-8 (CCK-8; Shanghai Yeasen Biotech Co., Ltd.) for 3 h. Absorbance was detected at a wavelength of 450 nm and the results were expressed as a percentage.

**Cell viability assay.** TPs cytotoxicity in RAW264.7 cells was analyzed using a CCK-8 assay in accordance with the manufacturer's protocol. RAW264.7 cells were seeded into 96-well plates at a density of 4x10<sup>3</sup> cells/well and left to adhere. Cells were then treated with 0.0, 0.1, 0.5, 1.0, 5.0 and 10.0 µg/ml TPs for 24 h. Subsequently, 10 µl CCK-8 reagent was added to each well and incubated at 37°C for 3 h. The absorbance value at a wavelength of 450 nm was measured using the Varioskan Flash microplate reader (Thermo Fisher Scientific, Inc.). Each evaluation of each concentration was repeated three times.

**Effect of TPs on the proliferation of H<sub>2</sub>O<sub>2</sub>-injured cells.** Cells were seeded into 96-well plates at a density of 4x10<sup>3</sup> cells/well and pre-incubated at 37°C for 24 h. The culture medium supernatant was replaced with an equal volume of solution containing different concentrations of TPs (0.1, 0.5 and 1.0 µg/ml) and incubated at 37°C for 12 h. Subsequently, samples were treated with 400 µM H<sub>2</sub>O<sub>2</sub> at 37°C for a further 12 h. Proliferations was evaluated using the aforementioned CCK-8 method.

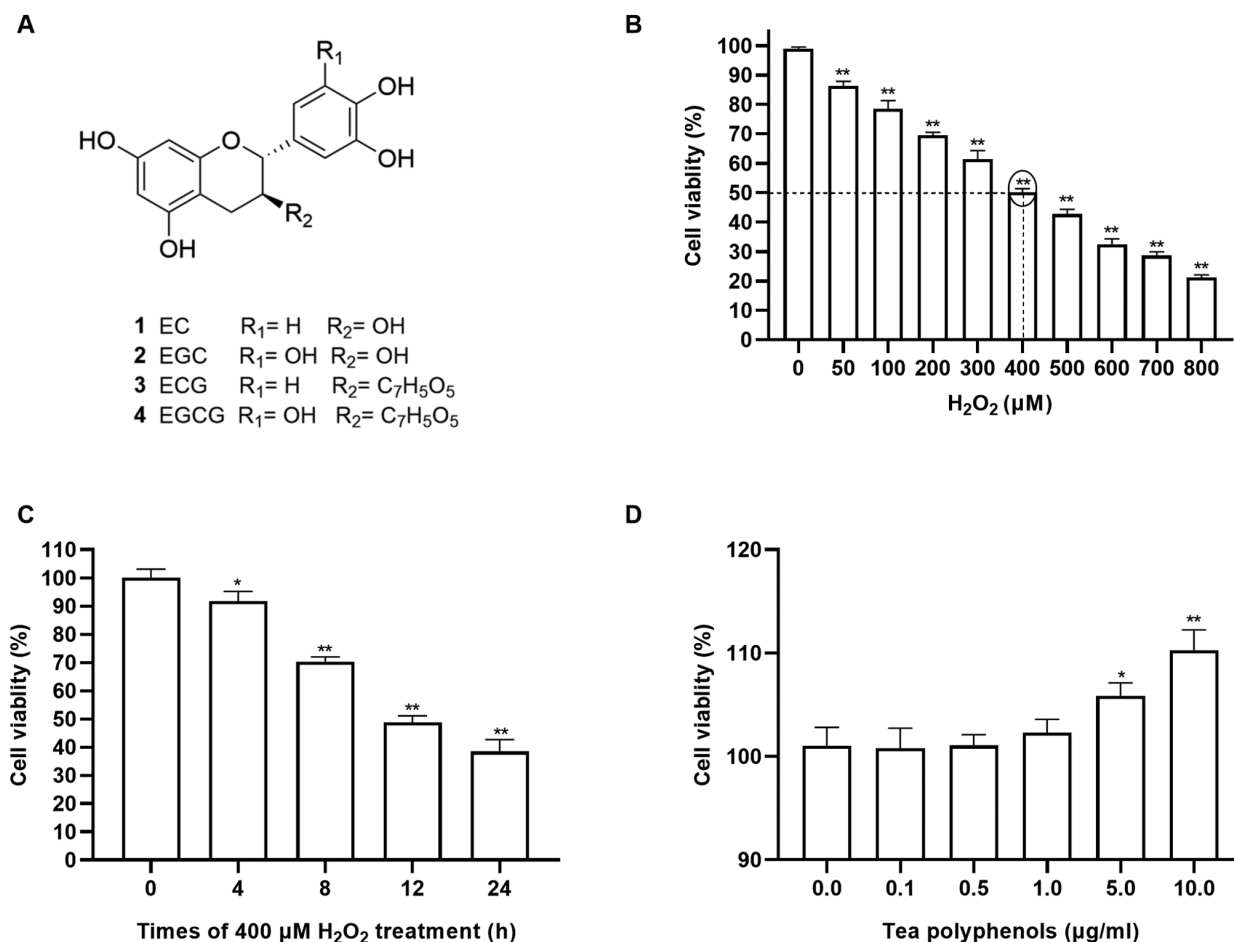


Figure 1. Effect of  $H_2O_2$  and TPs on cell viability of RAW264.7 cells by CCK-8. (A) Chemical structures of major catechins in TPs. (B) RAW264.7 cells were treated with various concentrations of  $H_2O_2$  (0–800  $\mu M$ ) for 12 h and (C) with 400  $\mu M$   $H_2O_2$  in different times and cell viability was analyzed by CCK-8 assay. (D) RAW264.7 cells treated with TPs (0.1, 0.5, 1.0, 5.0 and 10.0  $\mu g/ml$ ) for 24 h. Data are the means  $\pm$  SD from three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  vs. the control group. TPs, tea polyphenols.

**ROS assay.** ROS production was estimated using an ROS Assay kit (Beyotime Institute of Biotechnology) as previously described (23). RAW264.7 cells ( $2 \times 10^5$  cells/ml) were cultured in a 24-well plates and incubated at 37°C overnight. Cells were pretreated with different concentrations (0.1, 0.5 and 1.0  $\mu g/ml$ ) of TPs at 37°C for 12 h, after which each well plate was exposed to 400  $\mu M$   $H_2O_2$  at 37°C for a further 12 h. Subsequently, the cells were washed three times with cold PBS and incubated with 5  $\mu M$  DCFH<sub>2</sub>-DA (Gibco; Thermo Fisher Scientific, Inc.) at 37°C for 30 min. RAW264.7 cells were washed with DMEM culture medium and determined the fluorescent intensity was determined using a Bio-Rad ZE5 cell analyzer (Bio-Rad Laboratories, Inc.).

**Antioxidant enzyme activity assays.** RAW264.7 cells were seeded into 6-well plates with a density of  $5 \times 10^5$  cells/cell and cultured at 37°C overnight. Cells were treated with 0.1, 0.5 and 1.0  $\mu g/ml$  of TPs at 37°C for 12 h, followed by 400  $\mu M$   $H_2O_2$  treatment at 37°C for an additional 12 h. At the end of incubation, cells were collected for the detection of NO contents and superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) and glutathione peroxidase (GSH-Px) activity using Griess Regent and test kits (Nanjing Jiancheng Bioengineering Institute), respectively.

**Reverse transcription-quantitative (RT-q)PCR.** Total RNA was extracted from  $5 \times 10^6$  RAW264.7 cells using TRIzol® reagent according to its instructions (Invitrogen; Thermo Fisher Scientific, Inc.) and quantified at 260 nm using a NanoPhotometer (cat. no. P300; Implen GmbH). cDNA was synthesized from 1  $\mu g$  total RNA obtained from each sample using the MonScript RTIII all-in-one Mix according to the manufacturer's protocols (Monad Biotech Co., Ltd.). Subsequently, RT-qPCR was performed to quantify mRNA expression levels using the SYBR Green qPCR Mix (Monad Biotech Co., Ltd.) with the AriaMx RT-PCR system (Agilent Technologies, Inc.). The primer sequences used for PCR were as follows: HO-1 forward, 5'-GAAATCATCCCTTGACGCC-3' and reverse, 5'-CCTGAGAGGTCACCCAGGTA-3'; Keap1 forward, 5'-TGGGTCAAATACGACTGCC-3' and reverse, 5'-ATCATCCGCCACTTCT-3'; Nrf2 forward, 5'-ACATGGAGCAAGTTTGCCAG-3' and reverse, 5'-TGGAGAGGATGCTGCTGAAA-3'; Mst1 forward, 5'-CAGCCTGCACTCAGACCAAC-3' and reverse, 5'-TGGCAGTGGAAGAAGCTATGTC-3'; Mst2 forward, 5'-CCAGGCCCTATGTCCAACAG-3' and reverse, 5'-TGCCTCTCTTCTTCGCATC-3';  $\beta$ -actin forward, 5'-AGTGTGACGTTGACATCCGT-3' and reverse, 5'-AGCTCAGTAACAGTCCGCCTA-3'. The amplification

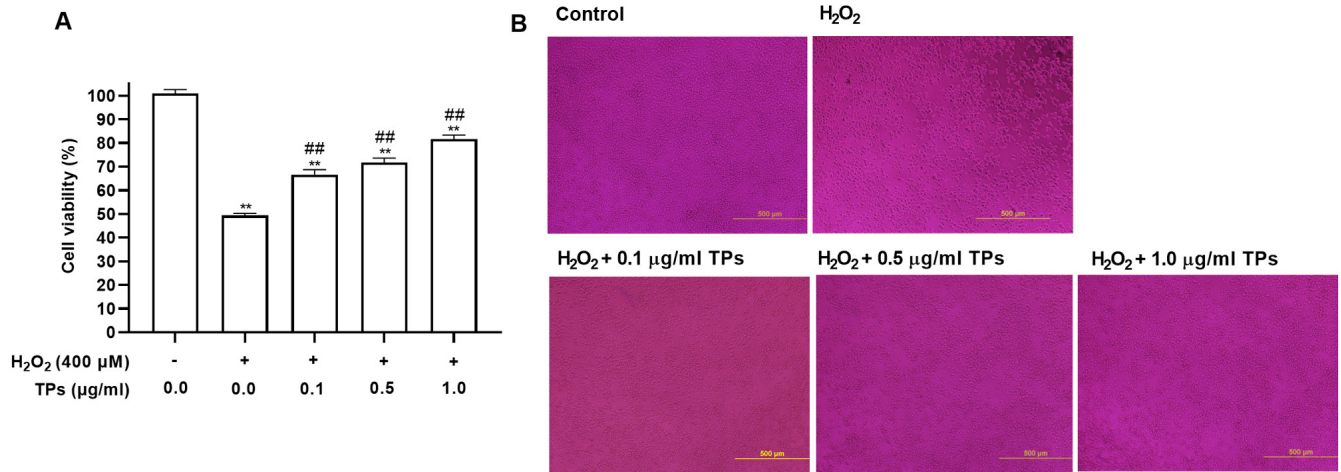


Figure 2. Protective effects of TPs on H<sub>2</sub>O<sub>2</sub>-induced RAW264.7 cells injury. (A) Cell viability and (B) representative images (scale bars = 500 μm; Magnification, x10) of H<sub>2</sub>O<sub>2</sub>-induced RAW264.7 cells pretreated with different concentrations of TPs (0.1, 0.5 and 1.0 μg/ml) for 12 h. The results are expressed as the means ± SD of three independent experiments. \*\*P<0.01 vs. Control, ##P<0.01 vs. the H<sub>2</sub>O<sub>2</sub> group. TPs, tea polyphenols.

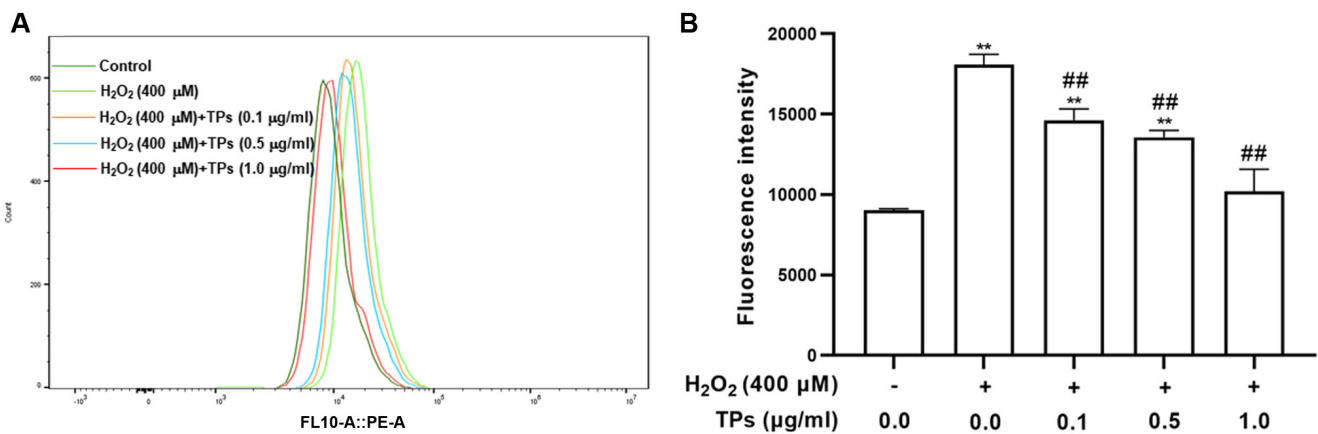


Figure 3. TPs inhibited intracellular ROS generations of RAW264.7 cells under oxidative stress. (A) Flow cytometry of RAW264.7 cells stained for 30 min with ROS dye DCFH2-DA. RAW264.7 cells were incubated with or not for TPs (0.1, 0.5 and 1.0 μg/ml) for 12 h followed by 400 μM H<sub>2</sub>O<sub>2</sub> for another 12 h. (B) Statistical analysis of the ROS per group. \*\*P<0.01 vs. the control group, ##P<0.01 vs. the H<sub>2</sub>O<sub>2</sub> group. TPs, tea polyphenols.

program included an initial denaturation step at 95°C for 30 sec, followed by 40 cycles of denaturation at 94°C for 5 sec, annealing at 58°C for 30 sec and extension at 70°C for 5 sec. The experiments were repeated in triplicate using independent samples. Relative gene expression was analyzed using the 2<sup>-ΔΔC<sub>q</sub></sup> method with β-actin as an internal control (24).

**Western blot analysis.** Cells were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology) containing 1% phenyl-methylsulfonyl fluoride, protease inhibitors and phosphatase inhibitors. The concentration of protein was subsequently quantified using a BCA protein estimation kit (Beyotime Institute of Biotechnology). A total of 40 μg protein was loaded onto 12% SDS-PAGE gels and transferred onto a nitrocellulose membrane (EDM Millipore). The membrane was then blocked with 5% nonfat milk in PBST (PBS containing 0.05% Tween-20) at room temperature for 2 h and incubated with the following primary antibodies for 14 h at 4°C: Keap1 (cat. no. sc-515432; 1:500; Santa Cruz Biotechnology, Inc.), Mst1 (cat. no. ab51134; 1:1,000; Abcam), Mst2 (cat. no. ab70546; 1:1,000; Abcam), Nrf2 (cat. no. ab92946; 1:1,000; Abcam), HO-1 (cat. no. ab52947;

1:1,000; Abcam) and β-actin (cat. no. ab8226; 1:1,000, Abcam). After washing three times with PBST, the membrane was incubated for 1 h with HRP-conjugated IgG (cat. nos. ab6721 and ab6728; 1:10,000; Abcam) at room temperature. The resultant signals were detected using the LumigenTMA-6 kit (Cytiva). The relative protein levels were normalized to β-actin, which was used as the internal control. Quantity One software (Bio-Rad Laboratories, Inc.) was used for densitometry.

**Statistical analysis.** All data were presented as the mean ± SD of three independent experiments and analyzed using GraphPad Prism 8.0.1 software (GraphPad Software, Inc.). Multiple comparisons were analyzed via one-way ANOVA followed by Tukey's comparison post hoc test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Effects of H<sub>2</sub>O<sub>2</sub> and TPs on RAW264.7 cells.** To investigate the cytotoxicity of TPs and H<sub>2</sub>O<sub>2</sub> in RAW264.7 cells, the viability of RAW264.7 cells was evaluated following treatment with

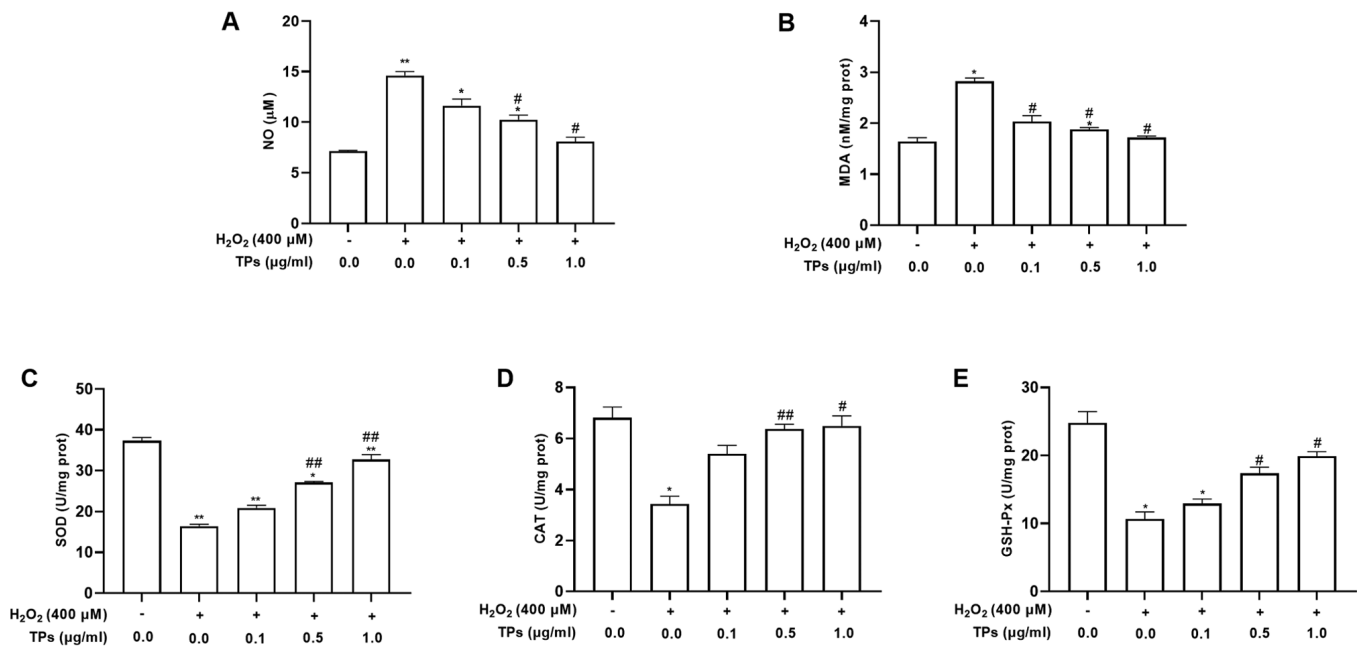


Figure 4. Evaluation of NO level and activities of the antioxidant enzymes. The effects of TPs on the cellular concentrations of (A) NO and (B) MDA. The effects of TPs on the activities of (C) SOD, (D) CAT and (E) GSH-Px. The results are expressed as the mean  $\pm$  SD of three independent experiments. \*P<0.05 and \*\*P<0.01 vs. the control group, #P<0.05 and ##P<0.01 vs. the H<sub>2</sub>O<sub>2</sub> group. NO, nitric oxide; TPs, tea polyphenols; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase.

various concentrations of TPs or H<sub>2</sub>O<sub>2</sub>. The chemical structures of major catechins in TPs were shown in Fig. 1A. As presented in Fig. 1B, the viability of cells exposed to H<sub>2</sub>O<sub>2</sub> decreased in a dose-dependent manner within 12 h. Additionally, treatment with 400  $\mu$ M H<sub>2</sub>O<sub>2</sub> exhibited 50% inhibition within 12 h (Fig. 1B and C) and as such was selected for use in subsequent experiments. In present study, CCK-8 assays showed that TPs did not have significant effect on cellular viability after 24 h treatment with 0.1, 0.5 and 1.0  $\mu$ g/ml (Fig. 1D). Therefore, TPs of 0.1, 0.5 and 1.0  $\mu$ g/ml were adopted as the suitable concentrations for the subsequent experiments.

*TPs attenuate H<sub>2</sub>O<sub>2</sub>-induced cell injury in an oxidative stress model.* H<sub>2</sub>O<sub>2</sub> can induce mitochondrial injury and membrane structure disruption due to its excessive generation (25). CCK-8 analysis was performed to evaluate the protective effects of TPs on the viability of cells exposed to H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity. As presented in Fig. 2, TPs reduced H<sub>2</sub>O<sub>2</sub>-induced cytotoxic effects. Additionally, pretreatment with TPs at 0.1, 0.5 and 1.0  $\mu$ g/ml for 24 h, followed by exposure to 400  $\mu$ M H<sub>2</sub>O<sub>2</sub> markedly increased cell viability in a TPs dose-dependent manner (Fig. 2A). Morphological observations revealed that the number of RAW264.7 cells in the H<sub>2</sub>O<sub>2</sub> group was decreased, while pretreatment with TPs could prevent this effect (Fig. 2B). The results indicated that TPs could protect cells against H<sub>2</sub>O<sub>2</sub>-induced injury.

*TPs inhibit ROS production in an oxidative stress model.* Given the essential requirement for cellular homeostasis, ROS are generated in various metabolic processes; however, external H<sub>2</sub>O<sub>2</sub> can lead to the excessive production of ROS, which causes damage to cell lipids, proteins and organelles (26). The current

study measured intracellular ROS content via flow cytometry using DCFH2-DA. As presented in Fig. 3A and B, following cell exposure to H<sub>2</sub>O<sub>2</sub> for 12 h, ROS levels were significantly increased in the H<sub>2</sub>O<sub>2</sub> treated group compared with the control group (P<0.05). However, ROS levels were significantly decreased in the three TPs treated groups compared with the H<sub>2</sub>O<sub>2</sub> group (P<0.05). The results indicated that TPs enhanced RAW264.7 cell ROS elimination in a dose-dependent.

*TPs alter the H<sub>2</sub>O<sub>2</sub>-induced expression of antioxidant enzyme activity in RAW264.7 cells.* According to previous studies, TPs, or the main contents, can reduce levels of NO and MDA, simultaneously increasing the production of SOD, GSH-Px and CAT (27,28). To determine the antioxidant effect of TPs under oxidative stress, the activities of NO and antioxidant enzymes (SOD, CAT, MDA and GSH-Px) were assessed in TPs-pretreated cells. The results revealed that increased NO and MDA levels induced by H<sub>2</sub>O<sub>2</sub> were significantly reduced in TPs pretreated groups (P<0.05; Fig. 4A and B). In addition, the three groups treated with TPs demonstrated significantly declined NO and MDA activities compared with the oxidative injure group (P<0.05). As presented in Fig. 4C-E, the H<sub>2</sub>O<sub>2</sub> treated group demonstrated significantly decreased activities of SOD, CAT and GSH-Px compared with the control group (P<0.05). In addition, significant increased activities of SOD, CAT and GSH-Px were detected in all TPs-pretreated groups compared with the H<sub>2</sub>O<sub>2</sub> group (P<0.05).

*Mst/Nrf2 axis and Keap1/Nrf2/HO-1 pathway-related gene and protein expressions.* Recent studies have demonstrated that ROS activates the Mst/Nrf2 axis and Keap1/Nrf2/HO-1 pathway to initiate cellular self-protective mechanisms against oxidative damage (19,21). To clarify the underlying antioxidant



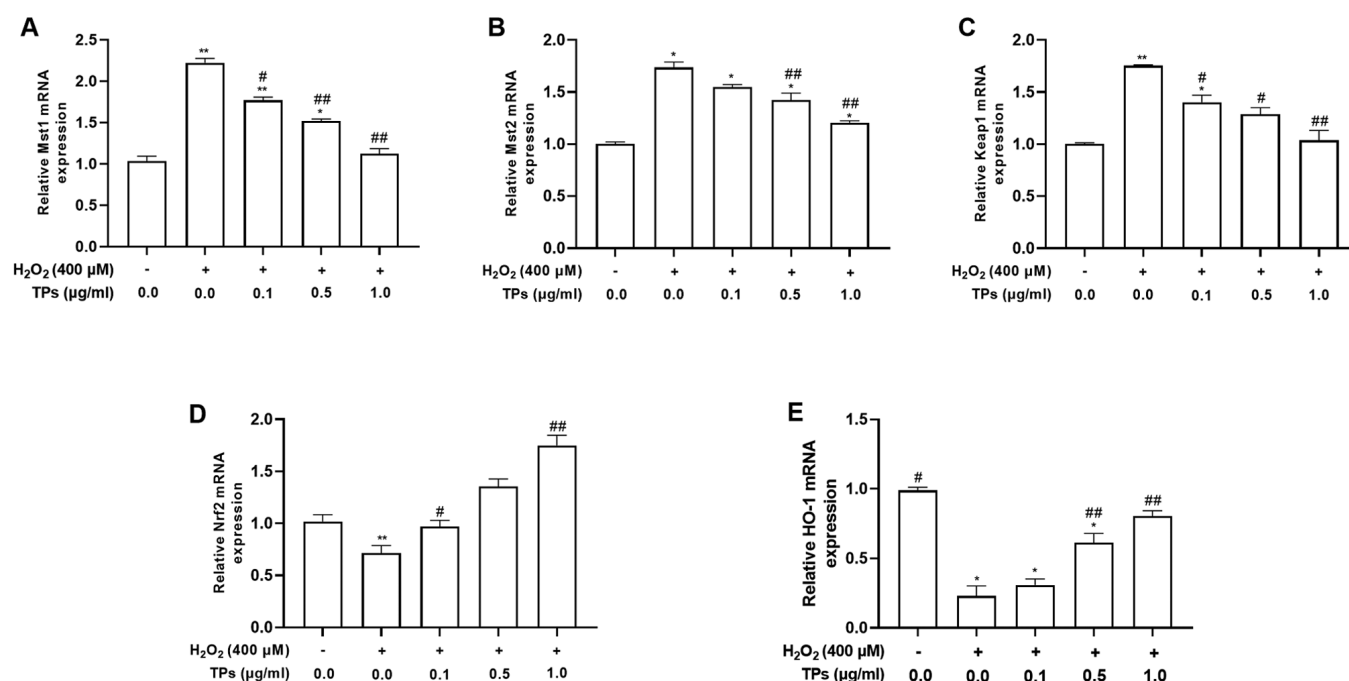


Figure 5. mRNA level changes after RAW264.7 cells were treated with TPs against oxidative damage. The (A) Mst1, (B) Mst2, (C) Keap1, (D) Nrf2 and (E) HO-1 mRNA expression levels were estimated using reverse transcription-quantitative PCR. Each bar represents the mean  $\pm$  SD of three independent experiments. \* $P$ <0.05 or \*\* $P$ <0.01 vs. the control group, # $P$ <0.05 or ## $P$ <0.01 vs. the H<sub>2</sub>O<sub>2</sub> group. TPs, tea polyphenols; Mst, mammalian STE20-like protein kinase; Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; HO-1, heme oxygenase 1.

mechanisms of TP, the mRNA and total protein levels of the Mst/Nrf2 axis and Keap1/Nrf2/HO-1 pathway were measured using RT-qPCR and western blotting, respectively.

After 12 h of H<sub>2</sub>O<sub>2</sub> and/or 24 h of TPs pretreatment, the mRNA expression levels of Mst1, Mst2, Keap1, Nrf2 and HO-1 were evaluated (Fig. 5). The results demonstrated that Mst1, Mst2 and Keap1 mRNA levels were significantly increased ( $P$ <0.05), while Nrf2 and HO-1 mRNA levels were significantly decreased ( $P$ <0.05) in the H<sub>2</sub>O<sub>2</sub> group compared with the control group. TPs treatment reduced the mRNA expression levels of Mst1 and Mst2 in a concentration-dependent manner, but could not fully restore levels to those prior to H<sub>2</sub>O<sub>2</sub> oxidative stress induction (Fig. 5A and B). In addition, the three TPs groups resulted in decreased Keap1 mRNA expression levels compared with the H<sub>2</sub>O<sub>2</sub>-treated group ( $P$ <0.01; Fig. 5C). TPs-pretreated groups also demonstrated significantly increased Nrf2 and HO-1 mRNA expression levels compared with the H<sub>2</sub>O<sub>2</sub> group ( $P$ <0.01; Fig. 5D and E).

In support of the results of RT-qPCR, western blot analysis also revealed that H<sub>2</sub>O<sub>2</sub> treatment significantly affected the Mst/Nrf2 axis and Keap1/Nrf2/HO-1 pathway. As presented in Fig. 6, when compared with the control group, H<sub>2</sub>O<sub>2</sub> treatment resulted in increased expression of Mst1, Mst2 and Keap1 (Fig. 6A-D) and decreased expression of Nrf2 and HO-1 (Fig. 6E and F). These effects were all reversed by preincubation with TP.

## Discussion

Accumulating studies indicate that plant bioactive polyphenols exhibit higher antioxidant activity and lower toxicity than synthetic compounds (29-31). Research in our laboratory (Tsinghua University; Beijing) has focused on the bioactive

effects of natural low molecular weight polyphenols, including lychee, tea, blueberry and other polyphenols derived from edible plants (32-34). As recognized natural antioxidants, TPs and their main four substances have been used in the prevention and treatment of clinical diseases involving oxidative damage. The TPs content was ~1.8-3.6 mg per gram of dry tea leaves; in addition, 75-147 µg/ml of polyphenols was dissolved in 100 ml tea liquor from 1 g leaf (35,36). Frei and Higdon (37) reported that one cup of tea (2 g of tea leaves infused in hot water for ~1-3 min) will provide 0.15~0.2 g of flavonoids. As few as 2-3 cups/day of tea will therefore supply a significant contribution to the total flavonoid intake in most individuals, which is estimated to average 1 g per day. Note that the concentrations of TPs by drinking tea are much higher than the experimental concentrations.

High doses of polyphenols are reported to exert positive effects as well as some negative effects (12). In the pre-experiments of the present study, the effect of ~0-100 µg/ml TPs on the cell viability of RAW264.7 cells was assessed. The experiment results indicated that the non-cytotoxic concentrations were  $\leq$ 1.0 µg/ml for TPs; 5.0 µg/ml and 10.0 µg/ml enhanced cell viability of RAW264.7 cells; 50.0 µg/ml and 100.0 µg/ml inhibited the viability of RAW264.7 cells. Similar experimental results are reported by Lagha and Grenier (38); 62.5 µg/ml green tea extract induces cell viability, and  $\geq$ 62.5 µg/ml inhibits cell proliferation of U937 macrophage-like cells. This finding differs from the fact that TPs inhibits the growth of tumor cells in a concentration-dependent manner. This phenomenon is puzzling and the underlying mechanisms remain unclear. The regulation of TPs on the cell viability in macrophages will become the focus of future research. Experiments performed on animals have shown that high concentrations of green tea extract (~500-2000 mg/kg) and of single tea phenolics, such as EGCG (150 mg/kg/day)

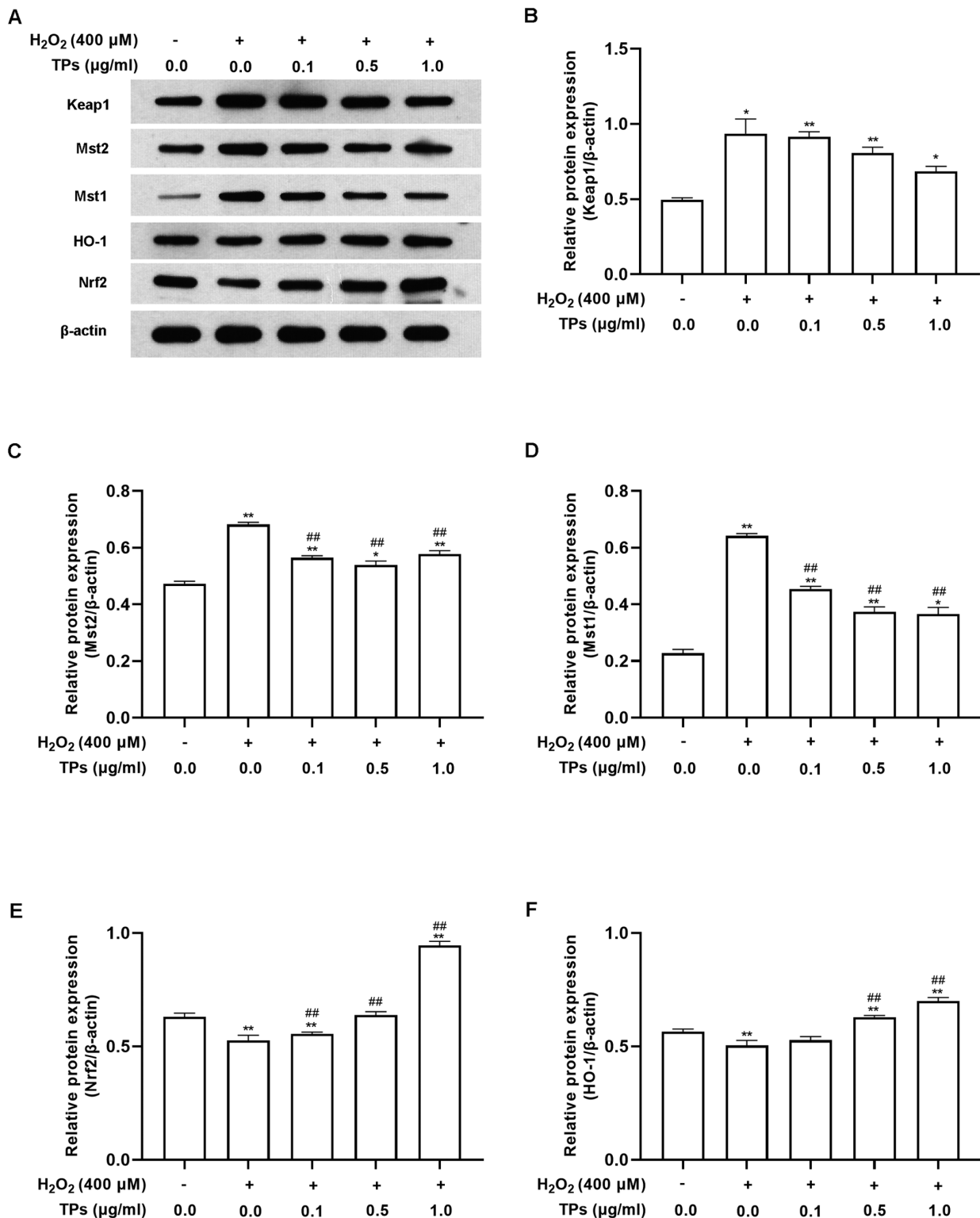


Figure 6. Effects of TPs on the Mst-Nrf2 axis and Keap1/Nrf2/HO-1 pathway protein expression on RAW264.7 cells under oxidative damage. (A) The protein expression levels were tested using western blotting. (B) Keap1, (C) Mst2, (D) Mst1, (E) Nrf2 and (F) HO-1 proteins levels were quantified via densitometry and were normalized to  $\beta$ -actin. Each bar represents the mean  $\pm$  SD of three independent experiments. \* $P$ <0.05 or \*\* $P$ <0.01 vs. the control group, ## $P$ <0.01 vs. the H<sub>2</sub>O<sub>2</sub> group. TPs, tea polyphenols; Mst, mammalian STE20-like protein kinase; Nrf2, nuclear factor (erythroid-derived 2)-like 2; Keap1, Kelch-like ECH-associated protein 1; HO-1, heme oxygenase 1.

produce toxicity in the liver, intestine and kidneys (39,40). Similarly, a previous study revealed that the continuous administration of high-dose plant bioactive polyphenols does not achieve the expected continuous prophylactic and therapeutic effects in the treatment of RAW264.7 cells and oxidative-damaged

RAW264.7 cells. The data from the present study suggested that low doses of TPs exerted effective preventive biological effects. The doses of TPs (0.1, 0.5, 1.0  $\mu$ g/ml) that protected RAW264.7 cells against H<sub>2</sub>O<sub>2</sub>-induced injury were much lower compared with those used in other studies (~10-100  $\mu$ g/ml TPs;

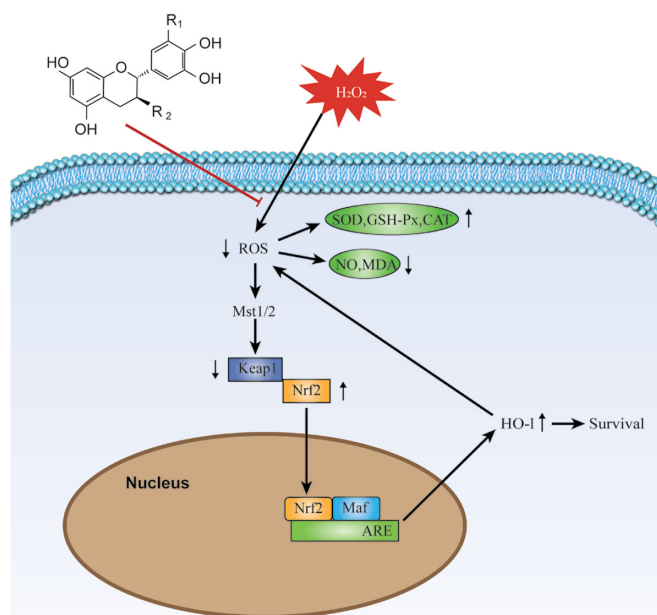


Figure 7. Molecular mechanism of TPs protection against H<sub>2</sub>O<sub>2</sub>-induced RAW264.7 cell injury. The excessive production of ROS in H<sub>2</sub>O<sub>2</sub>-injured RAW264.7 cells is alleviated and the balance of the oxidoreductase system is maintained by TPs. The expression of Nrf2 is directly affected by the change of intracellular ROS levels, following the Mst/Nrf2 axis maintenance of cellular redox balance and via Nrf2 binding to the ARE in the nucleus and thereby activating HO-1 protein expression. Induction via the Mst/Nrf2 axis and the Keap1/Nrf2/HO-1 pathway appears to represent the antioxidant mechanism of TPs. TPs, tea polyphenols; ROS, reactive oxygen species; Nrf2, nuclear factor (erythroid-derived 2)-like 2; Mst, mammalian STE20-like protein kinase; HO-1, heme oxygenase 1; Keap1, Kelch-like ECH-associated protein 1; ARE, antioxidant response element; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; NO, nitric oxide; MDA, malondialdehyde; Maf, musculoaponeurotic fibrosarcoma; ARE, antioxidant response element.

~10–50  $\mu$ M EGCG) (41–43). Pharmacokinetics studies in humans or mice show that the peak serum concentrations of EGCG, EGC or ECG are affected by an individual's metabolism and the number of catechins in the ingested type of green tea ranged from 34.7 ng/ml–3.39  $\mu$ g/ml (44–46). Accordingly, the concentrations of TPs and main substances used in the present study were comparable to their serum concentrations.

H<sub>2</sub>O<sub>2</sub> can diffuse freely through the cell due to its membrane permeability (47). When an organism is stimulated by exogenous H<sub>2</sub>O<sub>2</sub>, excessive ROS interferes with the balance of intricate defense mechanisms, triggering oxidative damage (48). Therefore, the H<sub>2</sub>O<sub>2</sub>-induced cell injury model is ideal for *in vitro* antioxidant research (15). Forman *et al.* (49) reveal that H<sub>2</sub>O<sub>2</sub> concentration in blood plasma is ~1–5  $\mu$ M, which would be >100-fold higher than that estimated to occur within cells. An estimated 100-fold concentration gradient from extracellular to intracellular is given for rough orientation and ensures to maintain homeostasis adjacent to normal cells (15). This also suggests that 400  $\mu$ M H<sub>2</sub>O<sub>2</sub> was a reasonable concentration in an injury cell model. Based on the results of flow cytometry analysis in the current study, all three doses of TPs alleviated the excessive production of ROS induced by H<sub>2</sub>O<sub>2</sub>.

The oxidoreductase system, which involves CAT, SOD, GSH-Px and MDA, serves an important regulatory role in H<sub>2</sub>O<sub>2</sub>-induced injury. A number of reports have revealed that

natural antioxidants can increase CAT, SOD and GSH-Px levels and inhibit the production of MDA both *in vivo* and *in vitro*. Similarly, treatment with TPs or its four components can reduce MDA production and increase levels of antioxidant enzymes (3,5,11). In the present study, H<sub>2</sub>O<sub>2</sub>-treated RAW264.7 cells that were exposed to TPs increased the levels of CAT, SOD and GSH-Px and decreased levels of MDA, as well as NO. H<sub>2</sub>O<sub>2</sub> can also increase NO levels in RAW264.7 cells, which in turn further promotes the production of H<sub>2</sub>O<sub>2</sub>, inhibiting catalase activity and creating a cycle of aggravated oxidative damage to cells (25,50,51). The current data demonstrated that TPs efficiently reduced NO release, alleviating oxidative damage to RAW264.7 cells.

To elucidate the underlying protective mechanism of TPs in H<sub>2</sub>O<sub>2</sub>-injured RAW264.7 cells, the expression of the Mst/Nrf2 axis and Keap1/Nrf2/HO-1 pathway was evaluated. The Nrf2 transcription factor is an essential regulator of cellular responses against oxidative stress (52), which mainly activates the antioxidant response and promotes the production of antioxidant proteins and enzymes (53). In the cytoplasm of normal cells, Nrf2 is maintained at low levels by polymerizing into dimers and binding to Keap1, which facilitates polyubiquitination and enables proteasomal degradation of Nrf2 (54). When cells are exposed to excessive endogenous or exogenous ROS under conditions of stress, Nrf2 dissociates from Keap1, migrates to the nucleus and exerts its function (55), ultimately stimulating HO-1 activity (54). In addition, Nrf2 can mediate the expression of certain antioxidant factors including SOD, CAT and NAD(P)H quinone oxidoreductase 1 (54). In both animal and cell line models, the preventive and therapeutic effects of TPs are alleviated via the Nrf2/Keap1/HO-1 pathway (56,57). In the present study, the results of RT-qPCR and western blotting revealed that TPs upregulated Nrf2 and HO-1 expressions, downregulated Keap1 expression and reversed oxidative damage in RAW264.7 cells induced by H<sub>2</sub>O<sub>2</sub>. The results suggested that the possible mechanisms in TPs-alleviated cellular damage may be through the modulation of Nrf2/Keap1/HO-1 pathway.

Recent research revealed that the Mst/Nrf2 axis maintains cellular redox homeostasis when sensing the production of ROS in macrophages (21). ROS recruits and activates the protective kinases Mst1 and Mst2, which induce Keap1 phosphorylation, prevent Keap1 polymerization and block Nrf2 ubiquitination and degradation. The stability of Nrf2 is a major factor for the protection of cells against oxidative damage (54). Consistent with previous studies, the results of the current study also supported the view that Mst1 and Mst2 are markedly activated by H<sub>2</sub>O<sub>2</sub>-induced ROS release, achieving self-protection in RAW264.7 cells. In addition, the production of ROS was downregulated following TPs treatment, with Mst1 and Mst2 exhibiting lower expressions compared with the H<sub>2</sub>O<sub>2</sub>-treated group; however, the levels were still higher compared with the control group. The results suggested that the Mst/Nrf2 axis may be a specific regulatory system for the defense against oxidative damage induced by H<sub>2</sub>O<sub>2</sub>. However, the present study did not conduct verification experiments by intervention strategies, such as inhibitors, activators, some genes and epigenetic modifications, targeting the relative pathways. The promising findings are not conclusive due to some limitations of the present study. The underlying



mechanism needs further investigation and will be addressed in future studies.

A number of epidemiological studies have been conducted to investigate the effects of tea consumption on human benefits with special reference to antioxidant, cancer and cardiovascular diseases. Tea drinking is shown to be associated with a lower risk for several types of cancer (58). During an 11-year follow-up, a prospective cohort study containing 285 males and 203 female cancer patients was conducted with green tea. Individuals who consumed >10 cups of green tea per day showed marked reductions of relative risk for lung, colon and liver cancers than other groups (<3 cups; 4-9 cups) (59). In addition, increasing frequency, duration and quantity of green tea consumed weakened the risk of prostate cancer (60). Therefore, a cell line model consuming TPs continuously will need to be established to simulate regular tea consumption in a follow-up study. In addition, two commonly used human cell lines, THP-1 and U937, will be used in future to identify the most effective dosage and frequency to achieve the maximum human health benefits.

In conclusion, TPs treatment was applied in the current study to induce antioxidant activity, reducing the effects of H<sub>2</sub>O<sub>2</sub>-induced cellular injury. The protective effects of TPs partly involved the reduction of intracellular ROS generation, NO release and MDA levels, along with the restoration of SOD and GSH-Px activity. The underlying mechanism may possibly be associated with the Mst/Nrf2 axis and Keap1/Nrf2/HO-1 signaling pathway for the sensing and regulation of antioxidant defense (Fig. 7). Since the oxidative injury model serves a key role in screening antioxidants and protective substances, the current study provides further evidence for the application of TPs in pro-oxidant ROS-mediated oxidative stress.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

QL, YW and HX performed the experiments and analyzed the data; QL and ZQ obtained the data and wrote the manuscript. XC and YZ analyzed the data. JT, LL and CG conceptualized and guided the research. QL and HX confirmed the authenticity of all the raw data. All authors 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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