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Protective Effects of Green Tea Polyphenols (GTP) on Hydrogen Peroxide (H_2O_2)-Induced Oxidative Stress, Inflammatory Response, and Apoptosis in Bovine Mammary Epithelial Cells (BMECs) in Vitro

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Abstract – **The increase in metabolic demand during the periparturient period is accompanied by increasing rates of oxidative stress, as an inducer of tissue injury, cause inflammatory reaction of udder in transition dairy cows. Green tea polyphenols (GTP) are key antioxidant with potential to regulate oxidative stress and inflammation. The aim of this study was to evaluate the effect of supplementation of GTP on inflammatory response and redox balance during hydrogen peroxide (H_2O_2) stimulation in primary bovine mammary epithelial cells (BMECs). Primary BMECs (n = 3 replicates per treatment) were pre-incubated with or without 100 μg/mL GTP for 12 h, subsequently, cells were challenged with or without H_2O_2 (600 μM) and incubated for 6 h. Data were analyzed using the one-way ANOVA and two-tailed Student's t-test of SAS 9.4. Results demonstrated that H_2O_2 elevated intracellular reactive oxygen species (ROS) levels and phosphorylation of p65, a biomarker of inflammation, and induced cells inflammatory response and apoptosis. Pretreatment of cells with GTP improved the BMECs survival rate and antioxidant effects during challenge with H_2O_2 by activating the expression of nuclear factor erythroid 2-related factor 2 (NFE2L2)/ *heme*oxgenase-1 (HMOX-1) pathway and scavenging intracellular ROS. Treatment GTP attenuated H_2O_2-induced expression of an inflammatory protein, and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) by inhibiting the activation of mitogen-activated protein kinases (MAPK)/nuclear factor-kappa B (NF-κB) pathway. H_2O_2-induced cells apoptosis was strongly inhibited by inhibiting caspase/p53 pathways. Overall, these results support that oxidative stress can produce cellular inflammation and apoptosis in BMECs, and supply of GTP can alleviate the oxidative stress, inflammatory responses and cells apoptosis triggered by H_2O_2 through activating the NFE2L2/HMOX-1 pathway, and controlling the MAPK, NF-κB, and caspase/p53 pathway.**

Keywords – **Oxidative Stress, Inflammation, Bovine Mammary Epithelial Cells, Green Tea Polyphenols.**

I. INTRODUCTION

The increase in metabolic demand during transition period is accompanied by increased generation of reactive oxygen species (**ROS**). When an overload of ROS (e.g., O^{2-}, OH•, H_2O_2) cannot be neutralized by host antioxidant defenses systems, a condition referred to as oxidative stress [1]. Growing evidence has implicated oxidative stress is the primary inducer factor that induces inflammation primarily through the activation of pro-inflammatory signaling pathways, e.g. nuclear factor-kappa B (**NF-κB**) signaling, which increases the expression of pro-inflammatory tumor necrosis factor-α (**TNF-α**), interleukin-1β (**IL-1β**), and interleukin-6 (**IL-6**) [2], and ultimately leads to serious inflammatory reactions and diseases of mammary glands [3-5]. In addition, previous research has indicated that excessive ROS activates caspase signaling by promoting the transcription of apoptosis-related genes and finally causes cells apoptosis [6], while the apoptosis of bovine mammary epithelial cells (**BMECs**) induced by oxidative stress is also modulated by the cellular antioxidant state [7]. Thus, the inhibition of oxidative stress is critical to prevent inflammatory responses and apoptosis of BMECs.

The nuclear factor erythroid 2-related factor 2 (**NFE2L2**)-antioxidant response element (**ARE**) signaling pathway is a critical antioxidant pathway, which mediates the oxidative stress. In the antioxidant cells signaling pathway, NFE2L2, a master regulator of antioxidant enzymes and detoxification genes, is shown to interact with ARE to eliminate oxidative stress and improve overall the antioxidant functions of organisms and cells [8, 9]. Our previous in vitro studies have indicated that pretreatment of BMECs with green tea polyphenol (**GTP**), as a potent free-radical scavengers and antioxidants, protects BMECs against H_2O_2-induced oxidative stress by inhibiting mitogen-activated protein kinases [**MAPK**; e.g., extracellular signal receptor- activated kinase 1/2 (**Erk1/2**), **p38 MAPK**, and c-Jun N-terminal kinase (**JNK**)] pathway [10], which mediate cellular inflammatory responses by activation of NF-κB [11], reducing reactive oxygen species (**ROS**) levels and activating the NFE2L2/hemeoxgenase-1 (**HMOX-1**) signal pathway [12], which is one of downstream genes of NFE2L2 pathway, indicating that the NFE2L2 pathway may be a potential therapeutic target for protecting mammary glands from oxidative damage. However, the exact mechanism of GTP anti-inflammation under oxidative stress remains unclear.

The GTP has been reported to exhibit potent biological properties including antioxidant, anti-inflammation, anticancer, anticardiovascular, antimicrobial, antibacterial, antihyperglycemic, antitumor and antiobesityproper--ties [13, 14]. In addition, GTP has been shown to promote the release of NFE2L2 for nuclear translocation to attenuate oxidative stress and improve survival under oxidative stress [15]. Recently, GTP has been shown to attenuate the cells apoptosis induced by oxidative stress [16, 17], suppress caspase-3 activity and TNF-α-related apoptosis [18]. Therefore, a model of oxidative stress in the BMECs was established in this study, the effect of GTP on BMECs induced oxidative stress and its underlying GTP-protective mechanisms were investigated.

II. MATERIALS AND METHODS

Isolation of BMECs

All animal experiments were performed in accordance with the Guidelines for Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Inner Mongolia Academy of Agricultural and Animal Husbandry Sciences (approval number IMAAAHS#1215000046002373XP, Hohhot, China). Primary BMECs were isolated and cultured from 3 lactating dairy cows (lactation = 200 ± 5 DIM) as described previously [10, 12, 19, 20]. Isolated cells were cultured with the basal medium including 85.74 mL Dulbecco’s modified Eagle medium/ F-12 medium (**DMEM/ F-12**, 12400-024, Gibco, Grand Island, New York, USA) supplemented with 10 mL fetal bovine serum (Gibco, Grand Island, New York, USA), 2 mL double antibody (Gibco), 0.5 mL of 5% insulin transferrin sodium selenium (Gibco), 100 μL of 100 μg/mL of hydrocortisone (Gibco), 100 μL of 100 μg/m L of amphotericin B (Gibco), 10 μL of 10 ng/mL of epidermal growth factor (Gibco), 50 μL of 50 μg/mL of prolactin (Gibco), 1.5 mL of 200 mmol/L of glutamine (Gibco) at 37 °C under an atmosphere containing 5% CO_2. The primary cells were trypsinized at 80% confluence and passaged. Pure mammary epithelial cells were obtained after 3 passages. Immunofluorescence was used to detect the expression of cytokeratin 18 (CK-18) (Supplemental Figure S1; https: //doi.org/10.3168/jds. 2018-15047) [21], which is a marker of BMECs. For cryopreservation, 1 × 10^6 cells/mL was suspended in freezing medium. A supraphysio--logic concentration of 600 µM H_2O_2 was applied to the BMECs to induce oxidative stress [10, 12, 19, 20].

Cell Culture and Treatments

The BMECs cultured in serum- and antibiotic-free medium (either preincubated with 100 µg/mL of GTP for 12 h or not) were treated with 600 µM H_2O_2, a supraphysiological dosage to induce significant oxidative stress, or without H_2O_2 (control) for 6 h [10]. GTP (1298401, ≥ 89%, Sigma-Aldrich, St. Louis, MO) was delivered to cells (10^8 cells/L) using the basal medium (serum-free). Control cultures received an amount of basal medium (serum-free) equal to that present in GTP-treated ones. Specific treatments were control, H_2O_2 (600 µM), GTP (no H_2O_2), GTP+H_2O_2 (600 µM), which were used for measuring the BMECs survival rate, apoptosis rate, ROS and MDA generation, the activity of oxidative stress-relatedenzymes, the mRNA expression of NFE2L2, HMOX-1, TNF-α, IL-1β and IL-6, and the protein expression of IκBα, p65, caspase-3, Bax, Bcl-2, Erk1/2, p38, JNK, and NFE2L2 in cultures.

BMECs Survival Rate Assay

To quantify BMECs survival rate, cells were plated at a density of 2 × 10^6 cells per well in 6-well plates over night. Cells were exposed to 100 µg/mL of GTP for 12 h or not, and then exposed to 600 µM H_2O_2 for 6 h or not. The BMECs survival rate was investigated using an MTT [3- (4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetra zolium bromide; Sigma-Aldrich, St. Louis, MO] according to the manufacturer's instructions (https://www.promega.com/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/celltiter-96-aqueous-one-solution-cell-proliferation-assay-_mts_/?catNu=G3582). Briefly, 20 µL/well MTT was added to each well and incubated for 4 h at 37 °C. Subsequently, 150 µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) was added to each well and incubated for 10 min at 37 °C. Lastly, the absorbance at 490 nm was determined with a microplate reader (Molecular Devices, Sunnyvale, CA).

Measurement of BMECs Apoptosis

BMECs apoptosis assays were performed as described previously [2]. After treatment, the cells were harvested and washed twice with PBS. Cell apoptosis was measured using an annexin V-FITC/propidium iodide apoptosis detection kit (BD Pharmingen, San Jose, CA, USA) according to the manufacturer's instructions. Subsequently, the cells were analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA).

Detection of ROS

Intracellular ROS was detected with the dichlorofluorescein staining assay (Beyotime Institute of Biotechnology, Jiangsu, China). Briefly, BMECs were washed with PBS and incubated with fresh DMEM containing 10 µM dichlorofluorescein at 37 °C for 35 min, then 1 × 10^6 cells were harvested and suspended in PBS. The optical density at 450 nm was recorded with a microplate reader (Molecular Devices).

Measurement of the Oxidative Stress-Related Enzyme Levels

The levels of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), malondialdehyde (MDA), protein carbonyls (PC), 8-iso-prostaglandin F2α (8-iso-PGF2α), and 8-hydroxy-2'-deoxyguanosine (8-OHdG) in BMECs were measured with the detection kit (Beyotime Institute of Biotechnology, Jiangsu, China) in strict accordance with the manufacturer's protocols. The assays were independently performed three times in triplicate.

Western Blotting

In brief, the BMECs were lysed using the RIPA lysis buffer, and then the proteins were quantified using the BCA kit. Thereafter, the protein samples were loaded and separated through SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto the polyvinylidene fluoride (**PVDF**) membranes (Bio-Rad, CA, USA). Afterwards, the membranes were blocked with 5% dry milk in Tris-hydrochloride buffer, and incubated with monoclonal anti-Erk1/2, anti-phospho-Erk1/2, anti-p38, anti-phospho-p38, anti-JNK, anti-phospho-JNK, anti-IκBα, anti-phospho-IκBα, anti-NF-κB p65, anti-phospho-NF-κB p65, anti-phospho-NFE2L2, caspase-3, Bax, Bcl-2 and p53, as well as anti-β-actin antibodies (at 1:1000 dilution, respectively) at 4 °C overnight. On the following day, these membranes were washed with TBST for three times, and further incubated with goat anti-rabbit or mouse IgG antibody for 1 h at room temperature. Then, ECL chemiluminescence was adopted to visualize the protein expression, and the image software was utilized for densitometry analysis of the protein bands.

Real-Time PCR

Total RNA was isolated from BMECs using the miRNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. Samples were treated on-column with DNaseI (Qiagen), quantification was assessed using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE), and RNA quality was measured using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). All samples had an RNA integrity number factor greater than 6.3. The quantitative PCR was performed as described previously [12, 22] to determine the relative mRNA abundance of NFE2L2, HMOX-1, TNF-α, IL-1β, IL-6 and β-actin (internal control). All reactions were run in triplicate. The primers of the genes are shown in Table 1.

Table 1. Primers for mRNA expression analysis of nuclear factor erythroid 2 like 2 (NFE2L2) and 2 phase-II detoxifying enzyme in the nuclear factor erythroid 2 like 2 (NFE2L2)-antioxidant response element signaling pathway in bovine mammary epithelial cells (BMECs).

Gene Name	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Length (bp)
NFE2L2	CCAGCACAACACATACCA	TAGCCGAAGAAACCTCATT	202
HMOX1	GAACGCAACAAGGAGAAC	CTGGAGTCGCTGAACATAG	225
TNF-α	GCCTCCCTCTCATCAGTTCTA	GGCAGCCTTGTCCCTTG	246
IL-1β	ACCTGTGTCTTTCCCGTGG	TCATCTCGGAGCCTGTAGTG	162
IL-6	AGTTGTGCAATGGCAATTCTGA	CCCCAGCATCGAAGGTAGA	223
β-Actin	GCCCTGAGGCTCTCTTCCA	GCGGATGTCGACGTCACA	101

Statistical Analysis

Data are represented as mean ± SEM. Differences between mean values of normally distributed data were assessed with one-way ANOVA (Dunnett′s t-test) and two-tailed Student′s t-test. $^*P< 0.05$ and $^{**}P< 0.01$ compared with control group. Statistical analysis was performed using SAS 9.4 (SAS Institute Inc., Cary, NC).

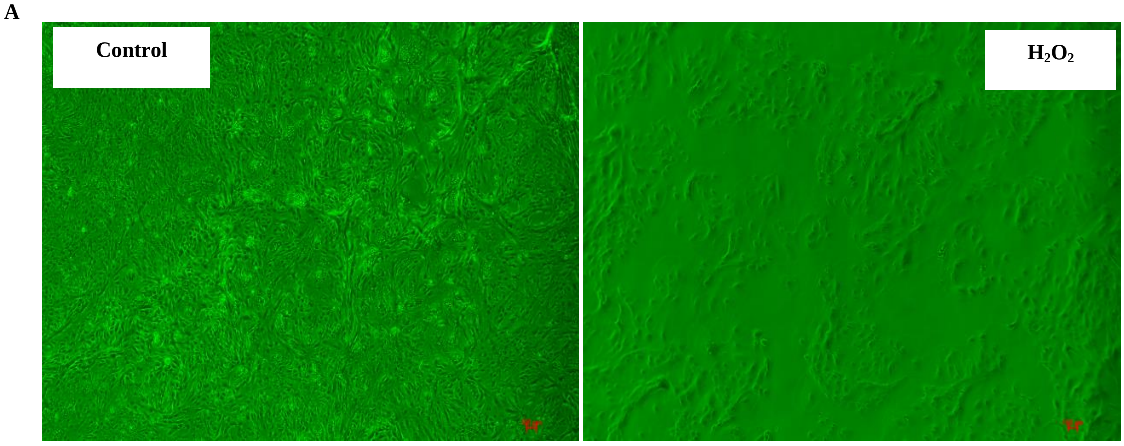
III. RESULTS

GTP Improved Cells Viability

The survival rate of BMECs was measured after the pretreatment with 100 μg/mL of GTP (no GTP) for 12 h, followed by 600 μM H_2O_2 (no H_2O_2) challenge for 6 h. The BMECs viability was decreased significantly and was the lowest when was treated with H_2O_2 (600 μM) compared with all treatments (Figure 1A-B; $P< 0.01$). Treatment with GTP improved the BMECs viability significantly ($P< 0.01$), and was as great as the survival rate of cells in control culture. Compared with BMECs treated with H_2O_2 alone, pretreatment with 100 μg/mL of GTP for 12 h followed by a 600 μM H_2O_2 challenge for 6 h (GTP+H_2O_2) increased survival rate of BMECs ($P< 0.05$). These results further suggested that treatment with GTP could improve the BMECs survival rate during challenge with H_2O_2.

GTP Attenuated H_2O_2-Induced Oxidative Stress

The activities of antioxidant enzymes (including SOD and GSH-Px) were analyzed to evaluate the antioxidant capacities. MDA, an end product of lipid peroxidation in biochemical assays, plays a critical role in monitoring and indicating the degree of peroxidative damage. The data indicated that treatment with H_2O_2 decreased the activity of SOD (Figure 1A; $P< 0.05$) and GSH-Px (Figure 1B; $P< 0.05$), while enhanced the level of intracellular ROS (Figure 1C; $P< 0.05$) and the content of MDA (Figure 1D; $P< 0.01$) compared with the control group. In contrast, the co-treatment of cells with GTP or/and H_2O_2 led to a significant increase in the activity of SOD ($P< 0.01$) and GSH-Px ($P< 0.01$). In a similar fashion, ROS ($P< 0.01$) and MDA ($P< 0.01$) production were decreased by GTP compared to H_2O_2 treatment alone. The crucial biological molecules such as PC, 8-OHdG and 8-iso-PGF2α, were analyzed to evaluate the oxidative injury of proteins, DNA, and lipids, respectively. The data indicated that treatment with H_2O_2 increased the content of PC (Figure 1E; $P< 0.05$), 8-OHdG (Figure 1F; $P< 0.05$) and 8-iso-PGF2α (Figure 1G; $P< 0.05$) compared with the control group. However, the co-treatment of cells with GTP or/and H_2O_2 led to a significant decrease in the content of PC ($P< 0.01$), 8-OHdG ($P< 0.01$) and 8-iso-PGF2α ($P< 0.01$) compared to H_2O_2 treatment alone, indicating that GTP attenuated the oxidative injury of proteins, DNA, and lipids injury in BMECs.



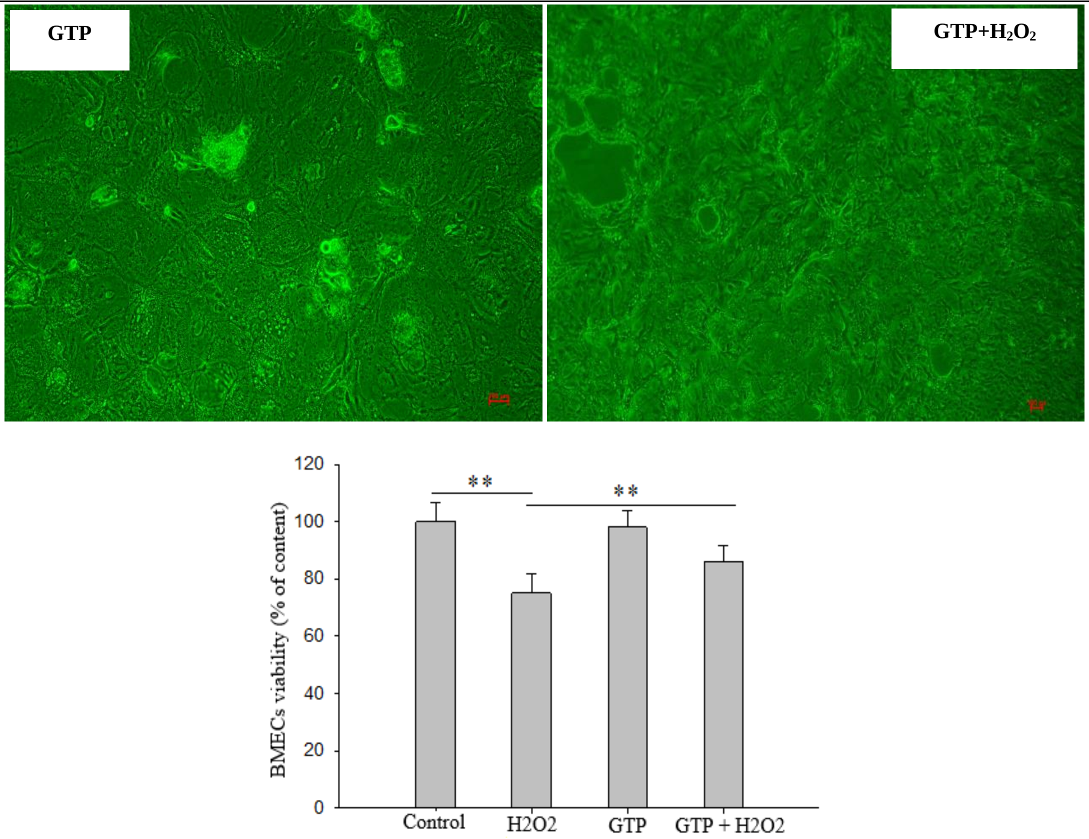
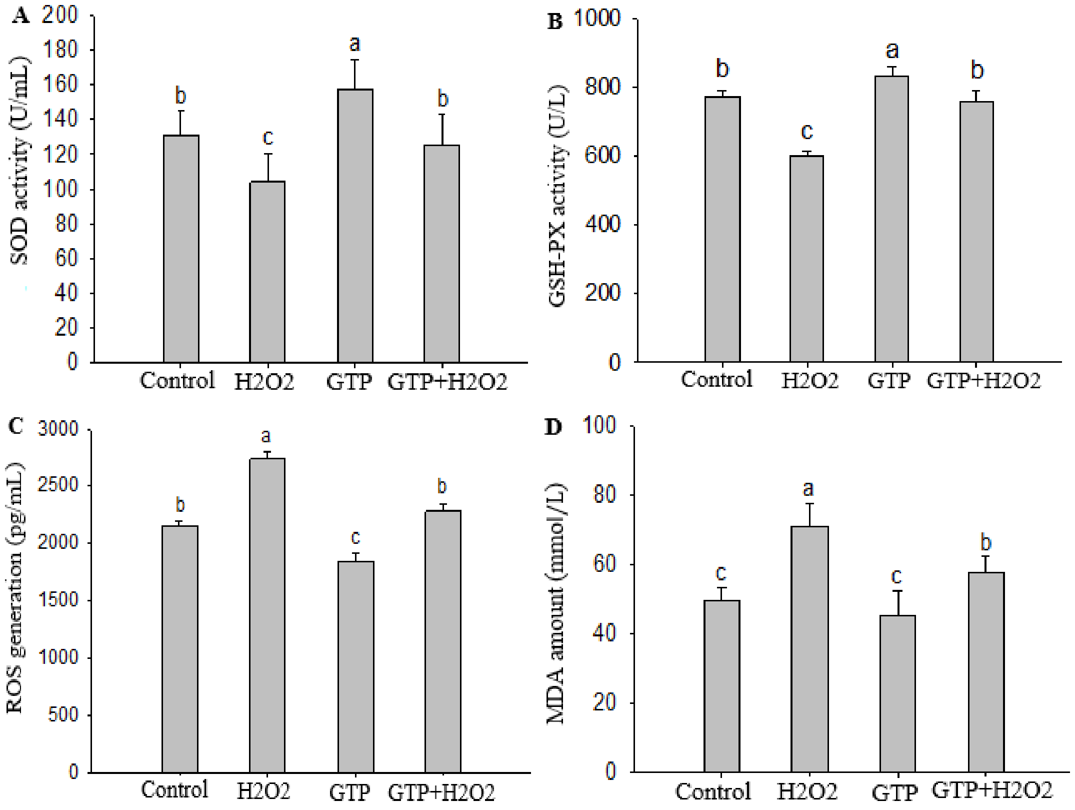


Fig. 1. Green tea polyphenols (GTP) attenuated hydrogen peroxide (H_2O_2)-induced the bovine mammary epithelial cells (BMECs) viability. The BMECs were pretreated with or without GTP (100 µg/mL) for 12 h, then treated with or without H_2O_2 (600 µM) for 6 h. (A) BMECs viability by inverted microscope. (B) BMECs viability. The data of the control group were used to normalize the data of each treatment group. Three replications were done for each experiment and each well (*n* = 3). Values are means, with SE represented by vertical error bars. $^{**}P< 0.01$.



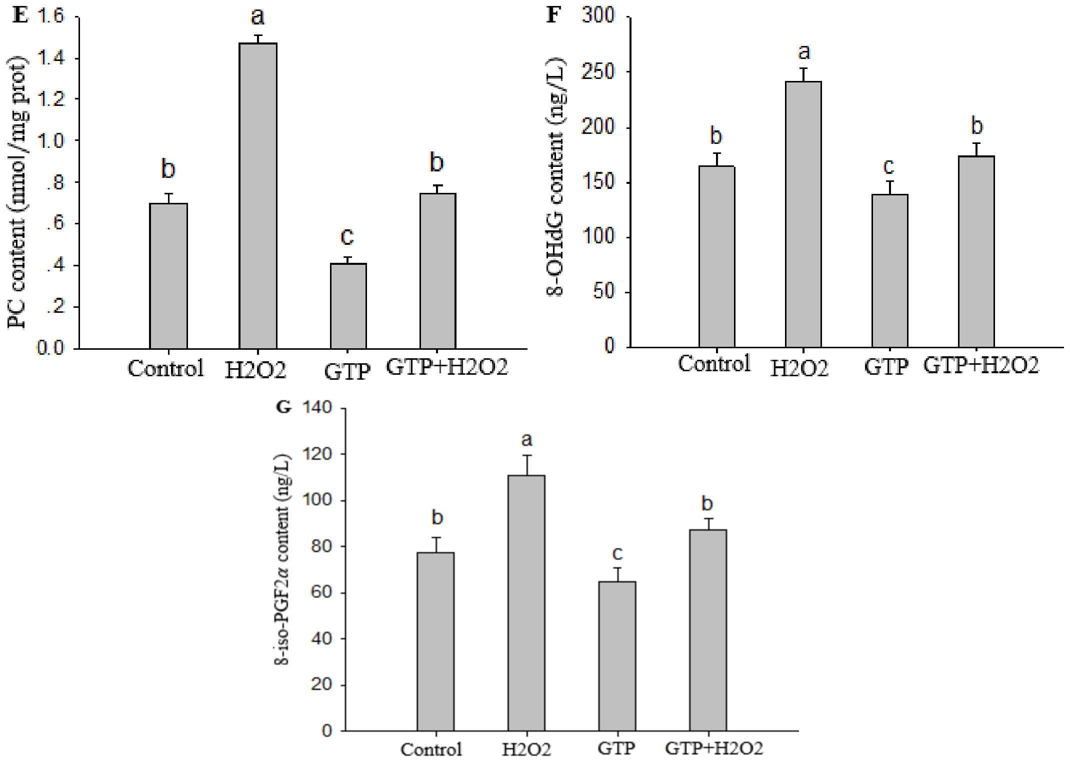
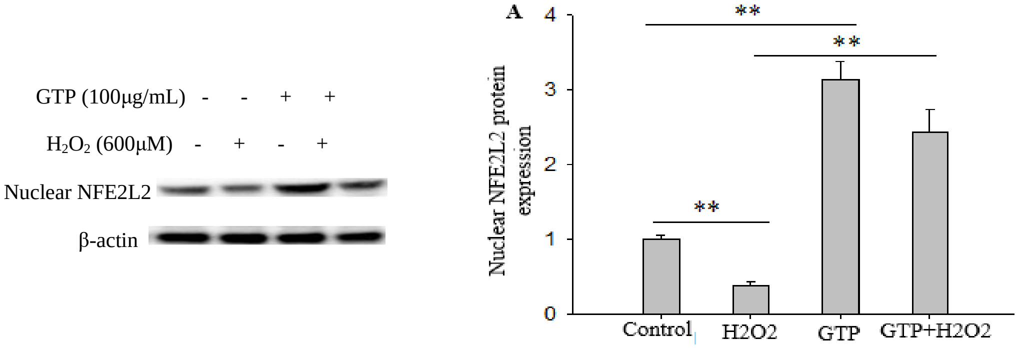


Fig. 2. Green tea polyphenols (GTP) attenuated hydrogen peroxide (H_2O_2)-induced oxidative stress. The bovine mammary epithelial cells (BMECs) were pretreated with or without GTP (100 µg/mL) for 12 h, then treated with or without H_2O_2 (600 µM) for 6 h. (A) Superoxide dismutase (SOD) activity in BMECs. (B) Glutathione peroxidase (GSH-Px) activity in BMECs. (C) Reactive oxygen species (ROS) level in BMECs. (D) Malondialdehyde (MDA) content in BMECs. (E) Protein carbonyls (PC) content in BMECs. (F) 8-hydroxy-2'-deoxyguanosine (8-OHdG) content in BMECs. (G) 8-iso-prostaglandin F2α (8-iso-PGF2α) content in BMECs. The data of the untreated group were used to normalize the data of each treatment group. Three replications were done for each experiment and each well (*n*=3). Values are means, with SE represented by vertical error bars. Means with different letters (a–c) differ ($P< 0.05$).

GTP Activated the NFE2L2-ARE Pathway

Treatment with H_2O_2 decreased the nuclear protein and mRNA expression of NFE2L2 compared with the control cultures. However, Exogenous GTP significantly increased the nuclear protein and mRNA expression of NFE2L2 in the absence or presence of H_2O_2 compared with the H_2O_2 alone (Figure 3A-B; $P< 0.01$). In addition, data revealed that the H_2O_2 treatment reduced the activity of ARE, while GTP significantly increased the activity of ARE in the presence or absence of H_2O_2 compared with the control cultures (Fig. 3C; $P< 0.01$). Consistent with the increased expression of NFE2L2 and activity of ARE, H_2O_2 decreased the mRNA abundance of HMOX-1, a downstream gene of the NFE2L2 pathway, while GTP up-regulated the mRNA abundance of HMOX1 induced by H_2O_2 compared with the H_2O_2-treated alone (Figure 3D; $P< 0.01$).



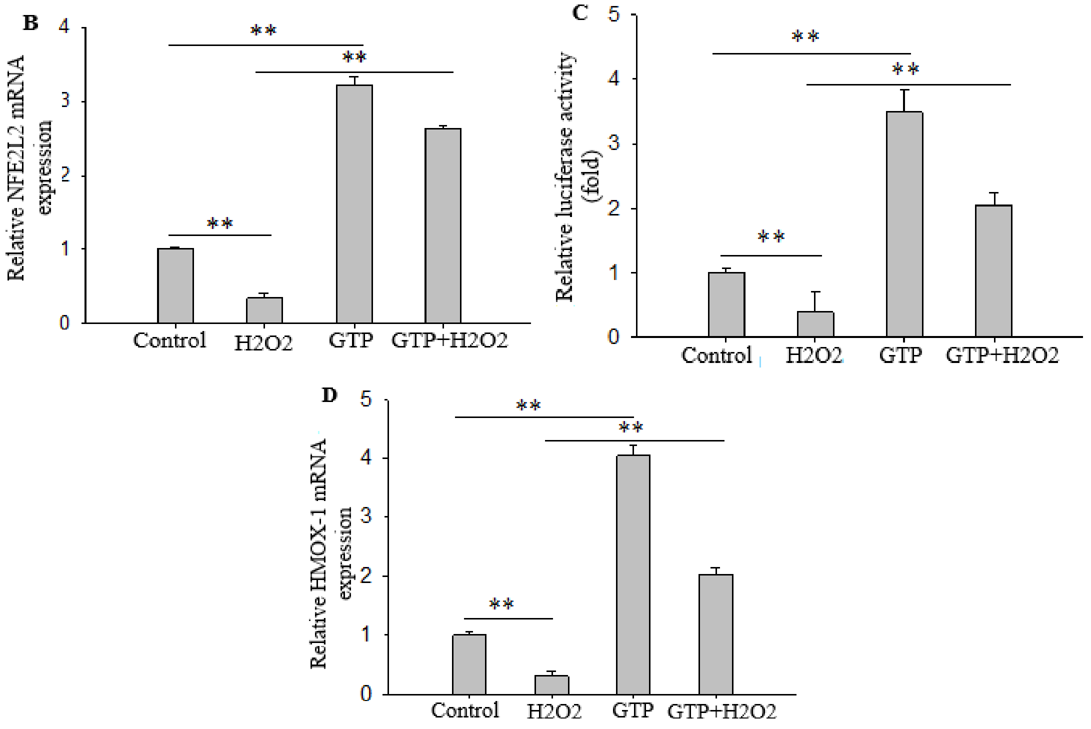


Fig. 3. Green tea polyphenols (GTP) activated the nuclear factor erythroid 2 like 2 (NFE2L2)-antioxidant response element (ARE) pathway. The bovine mammary epithelial cells (BMECs) were pretreated with or without GTP (100 μg/mL) for 12 h, then treated with or without H_2O_2 (600 μM) for 6 h. (A) Nuclear NFE2L2 protein level in BMECs. (B) NFE2L2 mRNA abundance in BMECs. (C) Relative luciferase activity of ARE in BMECs. (D) Hemeoxgenase-1 (HMOX-1) mRNA abundance in BMECs. The data of the untreated group were used to normalize the data of each treatment group. Three replications were done for each experiment and each well (*n* = 3). Values are means, with SE represented by vertical error bars. $^{**}P< 0.01$.

GTP Inhibited the Activation of NF-κBPathway and H_2O_2-mediated Inflammatory Responses

To illustrate the signaling mechanism that results in inflammatory mediators decrease upon H_2O_2-treated in the presence of GTP, NF-κB pathway was assessed, a major signaling pathway involved in the transcription of pro-inflammatory cytokines and enzymes. Our data showed that H_2O_2-stimulated phosphorylation of IκBα (**p-IκBα**) was hypophosphorylated in the presence of GTP compared with the control cells (Figure 4A). Similarly, GTP also attenuated H_2O_2-stimulated p65 phosphorylation that was accompanied by an increase in total p65 (Figure 4B). That is, the H_2O_2-stimulated ratio of p-IκBα/IκBα and p-p65/p65 was attenuated with GTP-treated compared with the H_2O_2 alone (Figure 4A–B; $P< 0.01$). Taken together, these results demonstrated that the NF-κB signaling pathway was one molecular target contributing to the anti-inflammatory effects of GTP.

Inflammatory factors have been recognized as the most direct indicators of the severity of inflammation. Our data revealed that the H_2O_2-stimulated mRNA expression of TNF-α, IL-1β and IL-6 was increased, while GTP-treated dramatically inhibited the H_2O_2-induced mRNA expression of inflammatory cytokines (Figure 4C–E; $P< 0.01$). Conclusively, these results suggested that GTP played indispensable roles for protection against H_2O_2-induced inflammatory responses.

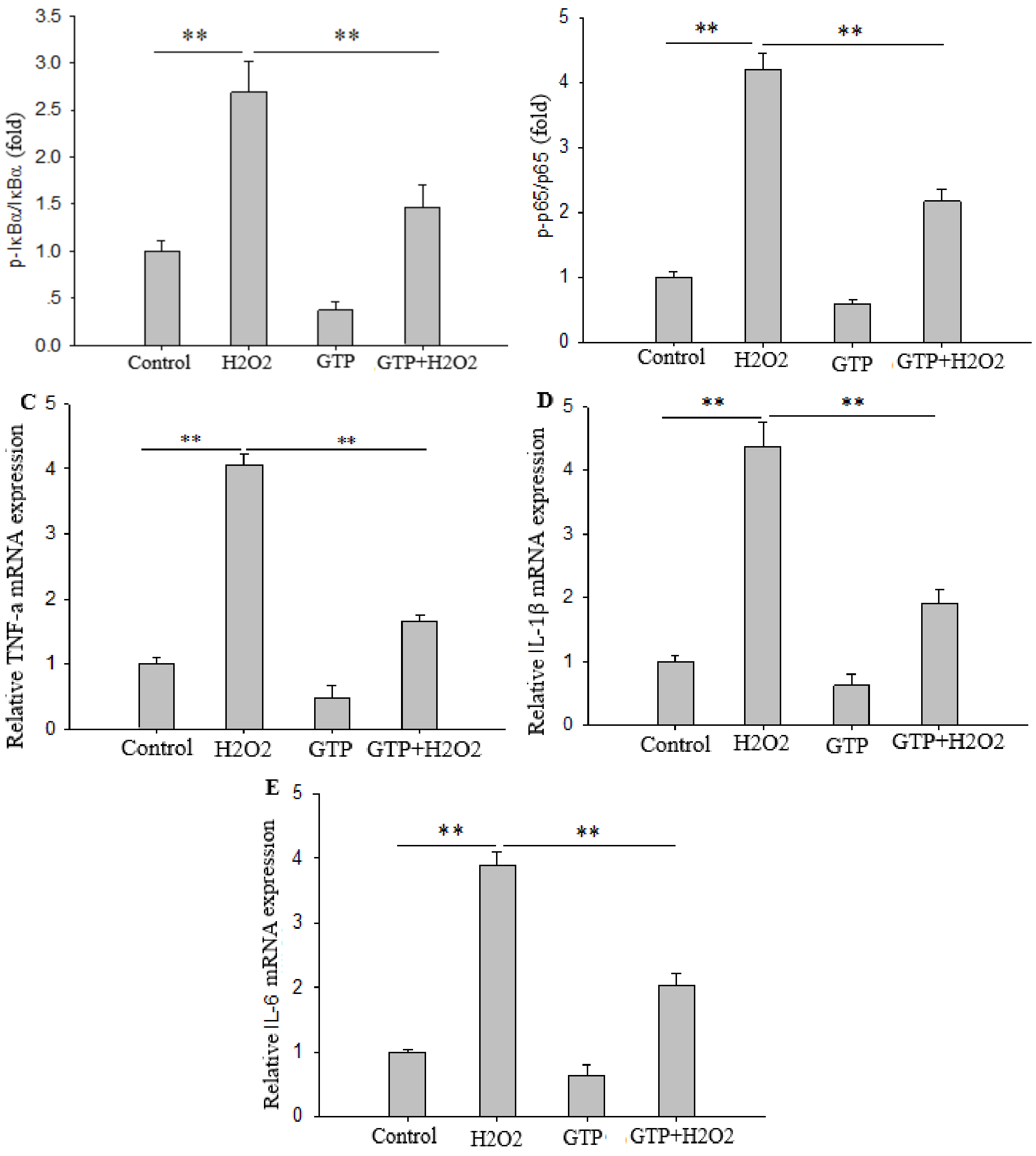


Fig. 4. Green tea polyphenols (GTP) inhibited the activation of nuclear factor-κB (NF-κB) pathway and inflammatory cytokines. The bovine mammary epithelial cells (BMECs) were pretreated with or without GTP (100 μg/mL) for 12 h, then treated with or without hydrogen peroxide (H_2O_2, 600 μM) for 6 h. (A) The protein ratio of p-IκBα/IκBα. (B) The protein ratio of p-p65/p65. (C) Tumor necrosis factor-α (TNF-α) mRNA abundance. (D) Interleukin-1β (IL-1β) mRNA abundance. (E) Interleukin-6 (IL-6) mRNA abundance. The data of the untreated group were used to normalize the data of each treatment group. Three replications were done for each experiment and each well (n=3). Values are means, with SE represented by vertical error bars. **$P< 0.01$.

GTP Inhibited the Activation of MAPK Pathways

To evaluate whether MAPK signaling pathway is also involved in the anti-inflammatory effects of GTP, three MAPK signaling cascades, namely the p38, Erk1/2 and JNK pathways were examined. Our data demonstrated that treatment with GTP significantly inhibited H_2O_2-induced levels of phospho-p38 (**p-p38**), phospho-Erk1/2 (**p-Erk1/2**) and phospho-JNK (**p-JNK**) (Figure 5A-D; $P< 0.01$), whereas the levels of total p38, p-Erk1/2 and p-JNK remained unchanged. The H_2O_2-stimulated ratio of p-38/p38, p-Erk1/2/Erk1/2 and p-JNK/JNK was attenuated with GTP-treated compared with the H_2O_2 alone ($P< 0.01$). Therefore, these results showed that the MAPK pathways were also likely target for the anti-inflammatory effects of the GTP.

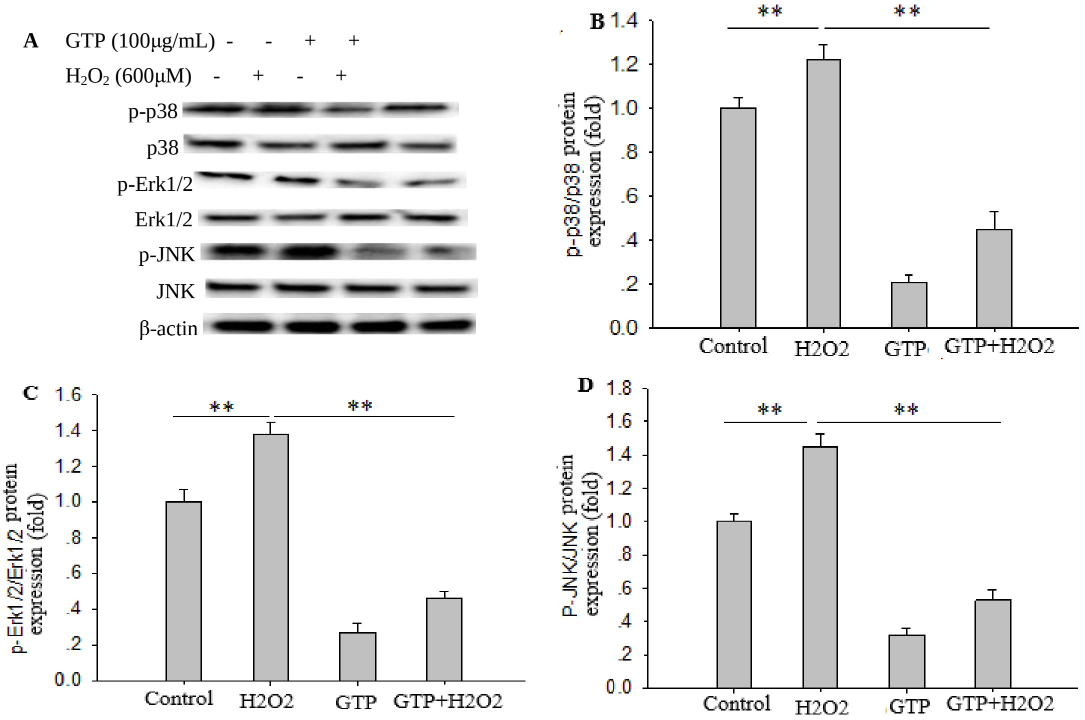


Fig. 5. Green tea polyphenols (GTP) inhibited the activation of mitogen-activated protein kinases (MAPK) pathways. The bovine mammary epithelial cells (BMECs) were pretreated with or without GTP (100 μg/mL) for 12 h, then treated with or without hydrogen peroxide (H_2O_2, 600 μM) for 6 h. (A) The protein ratio of phospho-p38/p38. (B) The protein ratio of phospho-extracellular signal receptor-activated kinase 1/2 (Erk1/2)/ extracellular signal receptor-activated kinase 1/2 (Erk1/2). (C) The protein ratio of phospho-c-Jun N-terminal kinase (JNK)/c-Jun N-terminal kinase (JNK). The data of the untreated group were used to normalize the data of each treatment group. Three replications were done for each experiment and each well (n=3). Values are means, with SE represented by vertical error bars. **$P< 0.01$.

GTP Attenuated H_2O_2-activated Caspase Pathway Activity and Apoptosis

H_2O_2-treated alone enhanced the protein expression of p53, caspase-3, and Bax compared with the control group, while decreased the protein expression of Bcl-2 (Fig. 6A–E; $P<0.01$). In contrast, GTP decreased the protein expression of p53, caspase-3, and Bax, and increased the expression of Bcl-2 in the absence or presence of H_2O_2 ($P< 0.01$). Consistent with the effects of GTP on the caspase pathway, GTP also attenuated H_2O_2-induced cells apoptosis rate (Fig. 5F; $P< 0.01$). Conclusively, these results suggested that GTP played key roles for protection against H_2O_2-induced cells apoptosis.

Effects of GTP on the Oxidative Stress-Related Signaling Pathways

To further investigate the underlying mechanism of GTP in treating oxidative stress, the activities of NFE2L2, NF-κB, caspase, and MAPK were detected by Western blotting. As demonstrated in Figure 3-6, H_2O_2-induced oxidative stress enhanced the activities of the p-p65 subunit of NF-kB, p-IκBα, p53, caspase, Bax, p-p38MAPK, p-Erk1/2, and p-JNK. On the contrary, the activities of p-p65, p-IκBα, p53, caspase, Bax, p-p38MAPK, p-Erk1/2, and p-JNK were suppressed while the activities of p-NFE2L2, and Bcl-2 were enhanced by GTP-treated. In other words, GTP inhibited the phosphorylation of p65 subunit and IκBα, p53 and caspase, up-regulated the protein expression of phosphorylated-NFE2L2 rather than NFE2L2, and restrained the phosphorylation level instead of the total protein amounts of p38 MAPK, Erk1/2, and JNK. Collectively, these results supported that GTP pretreatment strongly inhibited H_2O_2-induced inflammatory response and apoptosis in BMECs by inhibiting the activation of MAPK pathways.

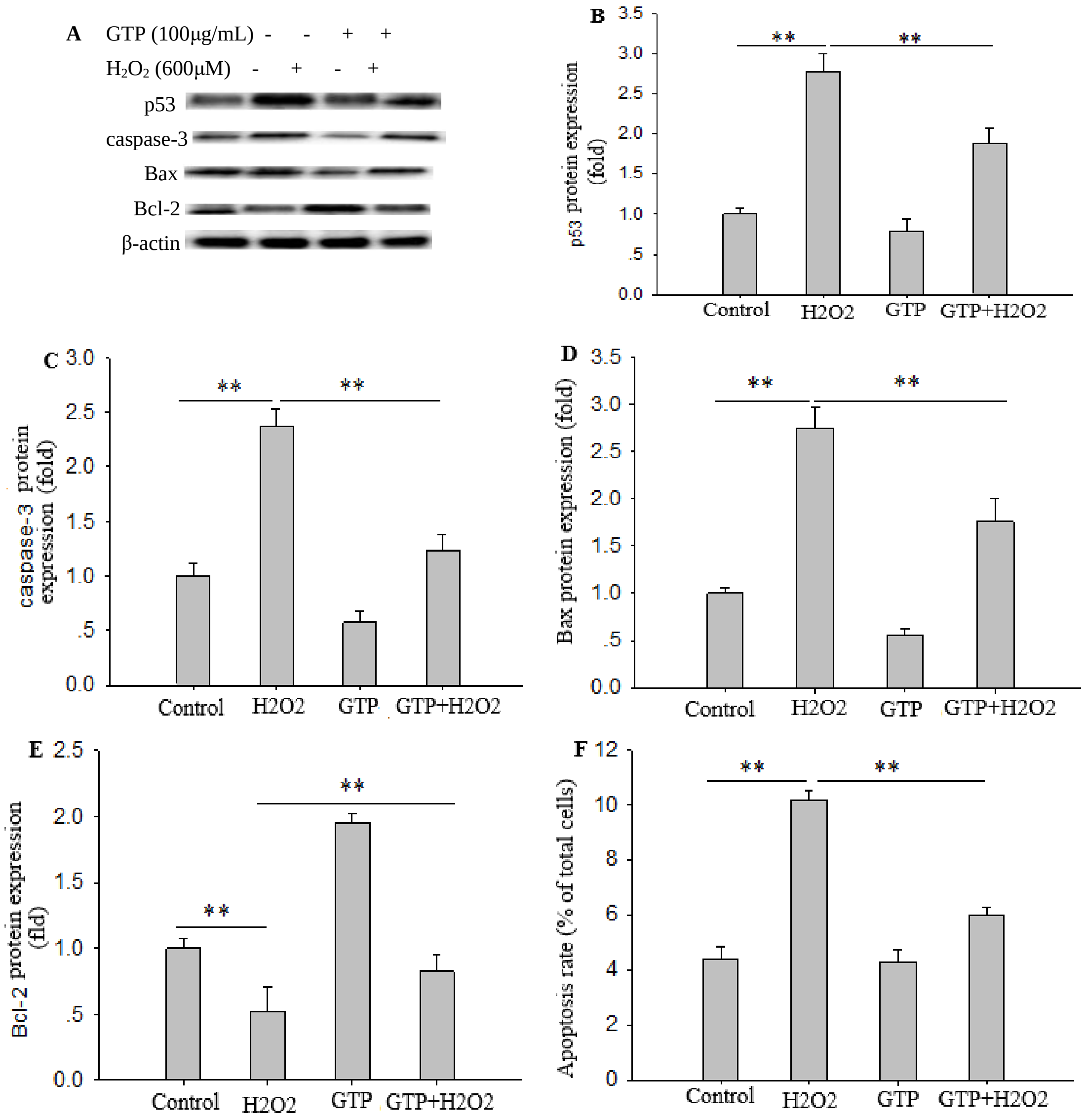


Fig. 6. Green tea polyphenols (GTP) attenuated the hydrogen peroxide (H_2O_2)-activated caspase pathway and apoptosis. The bovine mammary epithelial cells (BMECs) were pretreated with or without GTP (100 μg/mL) for 12 h, then treated with or without H_2O_2 (600 μM) for 6 h. (A) Relative protein level of p53. (B) Relative protein level of caspase-3. (C) Relative protein level of Bax. (D) Relative protein level of Bcl-2. (E) The apoptosis rate of BMECs. The data of the untreated group were used to normalize the data of each treatment group. Three replications were done for each experiment and each well (*n*=3). Values are means, with SE represented by vertical error bars. ***P*< 0.01.

IV. DISCUSSION

Studies have revealed that high-yielding dairy cow is known to produce oxidative stress and may result in mastitis after lactation initiation [23-25]. Our results supported the conclusion that oxidative stress could increase the inflammatory response in BMECs and cause cells apoptosis, while green tea polyphenol, an antioxidant from green tea extracts, could attenuate the inflammatory response by reducing oxidative stress.

Tea is a common beverage consumed daily in many parts of the world. The polyphenols in green tea are credited with its significant protective effects against several chronic diseases due to their higher bioavailability and strong antioxidant properties in many epidemiological and clinical studies [14, 26]. In the current study, BMECs were treated with H_2O_2 to produce oxidative stress by generating a great amount of cellular ROS and

MDA, which is consistent with our previous results [10, 12, 19, 20], and in turn, participation of excessive ROS in BMECs damage was strongly supported by the fact that treatment with free-radical scavengers had a beneficial effect in BMECs. These free radicals are harmful to organisms and cells and may cause cell death by damaging components of cells such as proteins, DNA, and lipids. Pretreatment of cells with the antioxidant GTP attenuated the damage H_2O_2-induced by promoting the release of NFE2L2 for nuclear translocation and up-regulating the NFE2L2-ARE signaling pathway, which consequently enhanced the antioxidant enzymes activity of SOD, GSH-Px, and HMOX-1 [27], whereas decreased the ROS and MDA generation in BMECs, and finally promoted antioxidant functions and cells survival under oxidative stress. HMOX-1 is a downstream gene of NFE2L2-ARE signaling pathway that counteracted oxidative stress and inflammatory responses [27]. SOD is the first killer of oxygen free radicals in the body, which converts the superoxide free radicals into hydrogen peroxide and water through carrying out the disproportionation reaction under the action of enzymes. Thereafter, GSH-Px can transform the resultant hydrogen peroxide into water, so as to protect cells from oxidative stress-induced injury. Our results suggested that the enzyme activities of SOD, GSH-Px and the level of MDA were vital indicators of oxidative damage from free radicals and lipid peroxidation [28], and supplementation GTP protected BMECs from oxidative stress by up-regulating the expression of NFE2L2 and enhancing antioxidant enzymatic activity via the activation of NFE2L2-ARE signaling [12]. Therefore, Extracellular supplement of GTP were the redox-protectors and mitigated oxidative stress mainly by activating NFE2L2/HMOX-1 signaling pathway as our previous results [10, 12] and other antioxidant [29]. Furthermore, GTP, as a scavenger of lipid peroxidation free radicals, prevented oxidative injury from crucial biological molecules, such as proteins, DNA, and lipids by decreasing the content of PC, 8-OHdG, and 8-iso-PGF2α, respectively [10]. Therefore, GTP has been used as a key antioxidant *in vivo* and *in vitro* of dairy cows [10, 12, 30].

Moreover, studies have found that activated NF-κB pathway induced by oxidative stress can also decrease SOD and GSH-Px activity, and increase cellular MDA levels under oxidative stress [31], while antioxidant has the ability to block the NF-κB activation. Our study also found that ROS production that exceeds host antioxidant capacity induced inflammatory responses [5] by activating the NF-κB pathway via inducing the phosphorylation and degradation of IκB, as well as the nuclear transport of the liberated NF-κB p65, eventually induced the expression of various inflammatory mediators such as TNF-α, IL-1β, and IL-6. Studies have shown that TNF-α and IL-1β are the vital pro-inflammatory factors, IL-6 is a major immune factor in inflammatory response, which down-regulates the synthesis of TNF-a and IL-1β. NF-κB is comprised of five families and usually in the form of heterodimers, among which, the dimers formed by p65 and p50 are quite common. When cells are in a resting state, NF-κB binds to its inhibitor IκB to form a trimer; however, multiple stimuli can activate NF-κB signaling by degradation of IκB and release of the NF-κB p65–p50 dimer, which thereby transferred into the nucleus to regulate the transcriptional activation of the target genes. As shown in the results of our study, phosphorylation of IκB and p65 was significantly activated by oxidative stress, which subsequently upregulated expression of pro-inflammatory factors. While treatment of BMECs with GTP decreased the phosphorylation of IκB and p65, and down-regulated the expression of pro-inflammatory factors, indicating GTP reduced inflammatory activity of BMECs induced by H_2O_2. These results further illustrated that GTP, as an anti-inflammatory agent, suppressed the activation of H_2O_2-mediated NF-κB signaling pathway, which consequently inhibited H_2O_2-induced inflammatory responses. So inflammatory mechanism plays a key role in the BMECs damage related with increased NF-κB, and inhibiting the activation of NF-κB is protective and develops smaller impairment.

In addition, our results found that cells apoptosis was induced by oxidative stress, which was the same as the previous studied [32, 33], and also found the caspase pathway played a key role in controlling the mitochondrial intrinsic, apoptotic pathway, which included the Bcl-2 family proteins [34]. Bcl-2, as an anti-apoptotic protein, prevents apoptosis by suppressing oxyradical-mediated membrane damage and stabilizing the mitochondrial membrane potential, while Bax, a pro-apoptotic, promotes cells apoptosis by regulating cytochrome-c release [34]. Pretreatment with GTP reversed the H_2O_2-induced cells apoptosis by decreasing the protein expression of Bax and caspase-3 and enhancing the protein expression of Bcl-2, further confirming the protective effect of GTP. Furthermore, study has showed that oxidative stress inhibits growth and induces death of aortic smooth muscle cells (SMCs) by activating p53 and NF-κB signaling pathways [35]. NF-κB has been found to be an essential downstream factor in a p53-mediated apoptotic pathway, expression of p53 lead to increase NF-κB binding and cells apoptosis, while inhibition of NF-κB reduces cell death. Pretreatment with GTP attenuated the cells apoptosis by inhibiting the protein expression of p53 and NF-κB pathway under oxidative stress, further confirming the anti-apoptosis effect of GTP.

Intracellular signaling mechanism linked to oxidative stress-induced inflammation was investigated in BMECs. Phosphorylation of cell signaling molecules such as MAPK is known to be linked to the activation of NF-κB and caspase/p53 pathway [36]. The MAPK-mediated signaling pathway is an important inflammatory signaling pathway, and the basic model is that cells receive stimulus signals and transmit them through the cascade of phosphatase activation. Notably, p38 MAPK, Erk1/2, and JNK are the three subfamilies of MAPK, which can be activated by ROS and TNF-α, and play crucial roles in triggering the phosphorylation of numerous important signaling molecules such as NF-κB and caspase [10, 37]. According to our previous reports, inhibition of pathways for MAPK attenuated H_2O_2-induced NFE2L2 expression in BMECs [10]. Similarly, our results also revealed that phosphorylation of p38 MAPK, Erk1/2, and JNK was increased in cells treated with H_2O_2 and these signaling molecules were responsible for NF-κB pathway, pro-inflammatory factors and pro-apoptosis factors expression. In our experiment, when both phosphorylated and non-phosphorylated antibodies were used to incubate with samples from each group, GTP remarkably suppressed the expression of phosphorylated antibodies associated with MAPK, and NF-κB, but their total protein levels remained unchanged, which demonstrated that GTP prevented the activation rather than the biosynthesis of MAPKs and NF-κB. Furthermore, the abundance of pro-inflammatory factors and the protein expression of pro-apoptosis factors were also reduced, which was ascribed to the inhibition of MAPK, NF-κB, and caspase/p53 activation, and the enhancement of NFE2L2/HMOX-1 activation by GTP. Put together, our results indicate that the anti-inflammatory effects of GTP are initiated by suppression of MAPK, resulting in inhibition of NF-κB, and caspase/p53 activation, and pro-inflammatory factors formation.

V. CONCLUSION

Our results demonstrate that oxidative stress can activate inflammatory signaling cascades such as MAPK (p38MAPK, Erk1/2, JNK) and NF-κB, and ultimately induce cells apoptosis in bovine mammary epithelial cells. Pretreatment with green tea polyphenols ameliorate oxidative stress-induced inflammatory response and cells apoptosis in BMECs. Our research clarified the antioxidant, anti-inflammatory and anti-apoptosis effects of

GTP in BMECs. These results further indicate that reducing oxidative stress may be an effective strategy to reduce the incidence of mastitis in lactating cows.

VI. DECLARATION OF INTEREST

The authors declare no conflict of interest.

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