Tea polyphenols protect bovine mammary epithelial cells from hydrogen peroxide-induced oxidative damage in vitro by activating NFE2L2/HMOX1 pathways

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ABSTRACT

Periparturient dairy cows are likely subject to altered intracellular reduction-oxidation (redox) balance due to the high metabolic rates and physiological adaptations occurring around parturition. Such conditions could induce oxidative damage. In nonruminants, it is well established that nuclear factor erythroid 2 like 2 (NFE2L2) is a critical transcription factor for maintaining cellular redox balance by inducing adaptive responses against oxidative stress (OS) that can otherwise lead to uncontrolled inflammation. Tea polyphenols (TP), the major polyphenolic constituents of green tea, are potent antioxidants that could exert protective effects on bovine mammary epithelial cells (BMEC) by scavenging free radicals. We used NFE2L2 short interfering RNA (siRNA) to downregulate NFE2L2 expression in cultured BMEC to investigate whether TP could inhibit H₂O₂-induced OS by activating the NFE2L2/heme oxygenase-1 (HMOX1) pathway. Isolated BMEC were exposed to H₂O₂ (600 μ M) for 6 h to induce OS. Optimal doses of TP (0, 60, 80, and 100 μ g/mL) were evaluated by pretreatment of BMEC for 0, 2, 4, 6, 8, 12, and 24 h, followed by a H₂O₂ (600 μ M) challenge for 6 h. The BMEC were transfected with NFE2L2-siRNA for 48 h, pretreated with 100 μ g/mL of TP for 12 h, then challenged by 600 μ M H₂O₂ for 6 h. Results revealed that after H₂O₂ exposure a concentration of TP of 100 μ g/mL during a 12-h incubation led to greater cell viability, protein, and mRNA abundance of NFE2L2, and lower intracellular reactive oxygen species (ROS) accumulation. In addition, transfection with NFE2L2-siRNA decreased abundance of NFE2L2 and HMOX1 in spite of exogenous TP supplementation,

whereas ROS production was increased in response to exogenous H₂O₂ (600 μ M). Overall, TP had beneficial effects on redox balance in BMEC, slowing down cellular OS-related injury through decreasing the production of ROS and enhancing mechanisms controlled at least in part by the NFE2L2/HMOX1 pathway.

Key words: tea polyphenol, oxidative stress, bovine mammary epithelial cell

INTRODUCTION

Physiologic changes during the periparturient (transition) period often render cows more susceptible to health disorders, and a growing body of data underscore that the regulation of those tissue-level processes occurs at the molecular (mRNA, protein) and enzymatic level (Loor, 2010; Loor et al., 2013). Among the most critical challenges facing dairy cows around parturition, controlling oxidative stress (OS) is paramount because it could contribute to dysfunctional inflammatory responses (Sordillo and Raphael, 2013; Abuelo et al., 2016) that could impair efficiency (Bertoni et al., 2008).

In dairy cows, compelling evidence indicates that production of reactive oxygen species (ROS) plays a critical role in OS and can alter intracellular reduction-oxidation (redox) balance (Bertoni et al., 2008; Zhou et al., 2016a). In fact, work with nonruminant species has verified that overproduction of intracellular ROS is a crucial determinant in the mechanism by which various stressors might injure cells leading to diverse pathologic disorders (Soberanes et al., 2006). Among the various types of ROS, hydrogen peroxide (H₂O₂) is particularly important from a physiologic standpoint because it can be generated by normal cellular processes, including oxidative metabolism. Although the etiology of metabolic disorders in periparturient cows is multifaceted (Zhou et al., 2016a,b), evidence indicates that OS caused by excessive production of H₂O₂ could be an important contributor (Jin et al., 2016). In addition, the production of malondialdehyde (MDA), 8-iso-prostaglandin

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(**8-iso-PG**), protein carbonyl (**PC**), and 8-hydroxydeoxyguanosine (**8-OHdG**) could be used to evaluate OS status. The levels of MDA (Ljubisavljevic et al., 2013) and 8-iso-PG (Milaneschi et al., 2013) provide information on levels of lipid peroxidation, the content of PC (Weber et al., 2015) reflects the extent of protein damage, and 8-OHdG (Kaneko et al., 1996) content is a marker of DNA damage.

At least in nonruminants, the transcription regulator nuclear factor erythroid 2 like 2 (**NFE2L2**) is considered the master regulator of cellular redox balance, which can induce the expression of antioxidant and detoxification enzymes and downstream phase-II enzymes such as superoxide dismutase (**SOD**), glutathione peroxidase (**GSH-Px**), glutathione-S-transferase, catalase, heme oxygenase-1 (**HMOX1**), NAD(P)H:quinone oxidoreductase-1 (**NQO1**), glutamate-cysteine ligase catalytic subunit (**GCLC**), and glutamate-cysteine ligase modifier subunit (**GCLM**; Nguyen et al., 2003; Denzer et al., 2016). When not stimulated, NFE2L2 is sequestered in the cytoplasm where it associates with Kelch-like ECH-associated protein 1 (**Keap1**; Hayes and McMahon, 2001; Jaiswal, 2004), considered a cytosolic inhibitor of NFE2L2. Disrupting the NFE2L2-Keap1 interaction leads to activation of NFE2L2 (Zhang et al., 2010). Under stressful conditions, however, NFE2L2 is released from Keap1 and translocates to the nucleus where it binds to conserved antioxidant response elements (**ARE**) on target genes (Itoh et al., 1997; Kansanen et al., 2013).

Activation of the NFE2L2-ARE pathway can affect oxidative status of cells and provides robust protection against oxidative challenge (Kobayashi and Yamamoto, 2006). Previous studies revealed that NFE2L2 not only regulates transcription of genes encoding electrophile-induced phase-II enzymes, but is also critical in the regulation of various antioxidant protein-encoding genes in response to OS (Ishii et al., 2000) and inflammation (Itoh et al., 2004). Among the phase-II detoxifying enzymes, HMOX1, a cytoprotective protein, exerts cellular defense functions against various compounds produced during OS (Ryter and Choi, 2005; Kweon et al., 2006) and was first identified as a target gene of highly expressed transcription regulators in periparturient cow liver (Loor, 2010; Khan et al., 2014). More importantly, previous studies in cultured bovine mammary epithelial cells (**BMEC**) revealed that NFE2L2 controls HMOX1, suggesting that the NFE2L2/ARE pathway is functional in the mammary gland and may confer a mechanism of protection against OS (Ma et al., 2018b). Thus, identifying potential exogenous regulators of NFE2L2 might provide the foundation for future research on their feasibility for practical use in dairy cow management.

Numerous polyphenols that scavenge free radicals have been identified and proposed as therapeutic agents to counteract OS-induced disorders in humans (Vitaglione et al., 2004). Green tea polyphenols (**TP**), which compose 30 to 40% of extractable solids from dried green tea leaves, exhibit significant antioxidant properties (Frei and Higdon, 2003). In addition, polyphenols can interact with cellular defense systems including HMOX1 (Inamdar et al., 1996) and NQO1 (Favreau and Pickett, 1995). Thus, we hypothesize that TP could play an antioxidative role in BMEC during OS challenges at least in part by activating the HMOX1 or NQO1.

Whether TP could inhibit H₂O₂-induced OS in BMEC and the underlying molecular mechanisms is not known. Thus, the aim of the present study was to silence NFE2L2 expression through short interfering RNA (**siRNA**) and investigate whether TP could inhibit H₂O₂-induced OS via specific phase-II enzymes that are controlled through activation of the NFE2L2/ARE pathway. The relevance of this work is underscored by recent data demonstrating not only an increase in phosphorylation status of NFE2L2 in mammary tissue during the periparturient period, but also upregulation of antioxidant gene networks (Han et al., 2018a,b).

MATERIALS AND METHODS

Isolation of BMEC

To ensure that enough viable cells were available, mammary tissue was obtained from five 4-yr-old healthy lactating multiparous grazing Chinese Holstein cows at a similar stage of lactation (200 ± 5 DIM) selected from the Inner Mongolia Academy of Agriculture and Animal Husbandry Sciences. Animals were killed at a local slaughterhouse (Hohhot, China). The mid-point area of left rear side of the udder was clipped and surgically scrubbed. Approximately 150 mg of fresh tissue from each cow was removed and placed in sterilized tubes containing ice-cold Dulbecco's phosphate buffered saline (**DPBS**, Sigma-Aldrich, St. Louis, MO) and immediately transported to the laboratory. The samples were washed 3 times with DPBS and cut into 1-mm³ pieces. Tissue was transferred with tweezers onto sterile, plastic cell culture dishes (Corning Inc., Corning, NY). Mammary tissue with collagenase was kept for 1 h in a humidified incubator at 37°C in an atmosphere of 5% CO₂ and 95% air. Subsequently, tissue extracts were centrifuged at 400 × *g* for 5 min at room temperature in tubes containing an 80-μm nylon mesh strainer and centrifuged. The precipitate was suspended with 1 mL of basal medium and then transferred into culture dishes followed by addition of

Table 1. Primer sequences for the nuclear factor erythroid 2 like 2 (NFE2L2)-short interfering RNA (siRNA) used in the in vitro experiments with bovine mammary epithelial cells (BMEC)

Primer	Primer sequence (5'→3' orientation)	
NFE2L2-siRNA1	Sense	TTGCTCAAAGAAAGAGGAGAA
	Antisense	TTCTCCTCTTTCTTTGAGCAA
NFE2L2-siRNA2	Sense	ACTGGATAAAGAAGTAGGTAA
	Antisense	TTACCTACTTCTTTATCCAGT
NFE2L2-siRNA3	Sense	CTGGAGCAAGATTTAGATCAT
	Antisense	ATGATCTAAATCTTGCTCCAG

4 mL of basal medium and maintained in a humidified incubator at 37°C and 5% CO₂. The basal medium was composed of 95.74 mL of Dulbecco's modified Eagle medium (DMEM; 12400-024, Gibco, Grand Island, NY), 2 mL of penicillin streptomycin (Gibco), 0.5 mL of 0.5% insulin transferrin solution (Gibco), 100 µL of 1 µg/mL hydrocortisone (Gibco), 100 µL of 2.5 µg/mL amphotericin B (Gibco), 10 µL of 10 ng/mL epidermal growth factor (Gibco), 50 µL of 5 µg/mL prolactin (Gibco), and 1.5 mL of 4 mmol/L glutamine (Gibco). Medium was prepared and kept at 4°C until use. The basal medium was replaced with fresh basal medium every 2 to 3 d until the cells were distributed across the bottom of the dish. Subsequently, epithelial cells were enriched by selective detachment with trypsinization using 0.25% trypsin (Gibco). After 3 min of trypsinization, fibroblasts were removed by washing with DPBS. The epithelial cells attached to the dish surface were allowed to grow by addition of fresh medium. The BMEC were continuously purified using the same method. The purified BMEC with positive expression of CK-18 (Supplemental Figure S1; <https://doi.org/10.3168/jds.2018-15047>) demonstrating the epithelial cell lineage (Wang et al., 2014) were seeded at a density of 5×10^5 cells in culture flasks and continuously subcultured up to 4 passages. For cryopreservation, 1×10^6 cells/mL was suspended in freezing medium. A supraphysiologic concentration of 600 µM H₂O₂ was applied to the BMEC to induce OS (Ma et al., 2018a,b).

RNA Interference

Three siRNA specific for NFE2L2 (NFE2L2-siRNA1, NFE2L2-siRNA2, and NFE2L2-siRNA3) and a scrambled sequence (scrambled siRNA, control) were designed and synthesized by Shanghai Genechem Co., Ltd. (Shanghai, China). The NFE2L2-siRNA1, 2, 3, and primer sequence are presented in Table 1. The BMEC were transfected in antibiotic-free medium following the manufacturer's instructions (Shanghai Genechem Co., Ltd.), as described previously (Ma et al., 2018a,b). Briefly, BMEC were routinely trypsinized and resus-

pended to 2×10^6 cells/mL in basal medium without antibiotics, and cells were then set aside at 37°C. After 24 h, the cells were transfected with NFE2L2-siRNA. After incubation for 5 min at 37°C, the siRNA and lipofectamine were mixed, incubated at room temperature for an additional 20 min, then added to each well. The cell suspension that was previously set aside was transferred to the same wells containing the complex and the cell suspension was mixed by gently rocking the plates back and forth. The basal medium was replaced by the same fresh medium 12 h after transfection. The BMEC were cultured for another 36 h in basal medium to reach 80% transfection efficiency. Transfected cells were then cultured in serum and antibiotic free medium, and then subjected to 600 µM H₂O₂ for 6 h. Protein and mRNA expression of NFE2L2 in NFE2L2-silenced and control cultures was measured to screen for the optimal NFE2L2-specific siRNA.

Cell Culture and Treatments

The BMEC with and without NFE2L2 siRNA cultured in serum- and antibiotic-free medium (either preincubated with 100 µg/mL of TP for 12 h or not) were treated with 600 µM H₂O₂, a supraphysiological dosage to induce significant OS, or without H₂O₂ (control) for 6 h. Specific treatments were control, H₂O₂ (600 µM), TP (no H₂O₂), TP (600 µM H₂O₂), and NFE2L2-siRNA3-TP (600 µM H₂O₂). These were used for measuring BMEC survival rate, production of ROS, and mRNA expression of NFE2L2, and downstream genes in cultures were measured.

BMEC Survival Rate Assay

To quantify BMEC survival rate, cells were plated at a density of 2×10^6 cells per well in 6-well plates overnight. Cells were then transfected with NFE2L2-siRNA3. At 48 h after transfection, the culture medium was switched to serum-free, antibiotic-free DMEM, and cells were then exposed to 600 µM H₂O₂ for 6 h. The BMEC survival rate was determined using the MTT [3-(4,5-di-

methythiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay kit (G3582, Promega, Madison, WI) 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_/?catNum=G3582). Briefly, 20 μ L/well of MTT was added to each well and incubated for 4 h at 37°C. Subsequently, 150 μ L of dimethyl sulfoxide (DMSO, Sigma-Aldrich) 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).

Detection of OS Indicators

The activities of SOD and GSH-Px, and the content of PC and MDA in BMEC were determined by using spectrophotometric diagnostic kits from Nanjing Jiancheng Biotechnology Institute (Nanjing, China) according to the manufacturer's protocols. In brief, BMEC were washed with PBS and incubated with fresh DMEM (Gibco, Grand Island, NY) at 37°C for 35 min, respectively, and then 1×10^6 cells were harvested and suspended in PBS. Absorbance was detected at 560 nm (SOD), 420 nm (GSH-Px), 450 nm (PC), and 532 nm (MDA) and was recorded with a 722N spectrophotometer (Scientific Instrument Co., Ltd., Shanghai, China).

The content of 8-OHdG and 8-iso-PG in BMEC were determined via ELISA kits (Nanjing Jiancheng Biotechnology Institute) according to the manufacturer's protocols. In brief, BMEC were washed with PBS and incubated with fresh DMEM (Gibco) at 37°C for 35 min, respectively, and then 1×10^6 cells were harvested and suspended in PBS. Absorbance was detected at 450 nm (8-OHdG and 8-iso-PG) in a Microplate reader (Multiskan MS352, Labsystems, Helsinki, Finland). The limits of detection of 8-OHdG and 8-iso-PG were 10–300 and 5–150 ng/L, respectively. The assay performance details are included in Supplemental Table S1 (<https://doi.org/10.3168/jds.2018-15047>).

Intracellular ROS Detection

A dichlorofluorescein staining assay was used to detect intracellular ROS. Briefly, BMEC were washed with PBS and incubated with fresh DMEM containing 10 μ M 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).

RNA Isolation, cDNA Synthesis, and Quantitative PCR

Total RNA was isolated from 60 mg of BMEC 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 (Osorio et al., 2014) to determine the relative mRNA abundance of *NFE2L2*, *HMOX1*, *NQO1*, *GCLC*, *GCLM*, and *TUBB* (tubulin β ; internal control). All reactions were run in triplicate. The primers of *NFE2L2* and 4 phase-II detoxifying enzyme in the *NFE2L2*-ARE signaling pathway used for quantitative reverse-transcription PCR are listed in Table 2.

Western Blotting

After treatment, cells were washed twice with 4°C PBS (0.01 M, pH 7.2–7.3) and were then lysed with RIPA protein lysate (Beyotime Institute of Biotechnology, Shanghai, China). Total protein was extracted from BMEC using a tissue protein extraction reagent (catalog no. 78510, Thermo Scientific, Waltham, MA) containing inhibitor cocktail (100 $\times$, catalog no. 78442, Thermo Scientific). Total protein concentration was determined using the BCA Protein Assay Kit (Sigma-Aldrich). Protein samples were boiled for 20 min at 100°C; 50 mg of

Table 2. Primers for mRNA expression analysis of nuclear factor erythroid 2 like 2 (*NFE2L2*) and 4 phase-II detoxifying enzyme in the nuclear factor erythroid 2 like 2 (*NFE2L2*)-antioxidant response element signaling pathway in bovine mammary epithelial cells (BMEC)

Gene name	Forward primer (5'→3')	Reverse primer (5'→3')	Accession no.
<i>NFE2L2</i>	CCAGCACAAACATACCA	TAGCCGAAGAAACCTCATT	001011678.2
<i>HMOX1</i>	GAACGCAACAAGGAGAAC	CTGGAGTCGCTGAACATAG	001014912.1
<i>NQO1</i>	CAACAGACCAGCCAATCA	ACCTCCCATCCTTTCTC	001034535.1
<i>GCLC</i>	ATTGGGTGGAGAGTGGA	ACAGCGGGATGAGAAAGT	001083674.1
<i>GCLM</i>	CCGATGAAAGAGAAGAAATG	CAACAGGAGGTGAAGCAA	001038143.1

total protein per lane was resolved by 10% SDS-PAGE (Bio-Rad, Hercules, CA) and then transferred from the gel to a polyvinylidene difluoride membrane (0.45 μ m, Millipore, Billerica, MA). PageRuler Plus Prestained Protein Ladder (Fermentas, Hanover, MD) was used to confirm protein electrophoresis. Membranes were blocked in Tris-buffered saline (TBST; 50 mM Tris, pH 7.6, 150 mM NaCl, and 0.1% Tween 20), which contains 5% (wt/vol) nonfat dry milk for 2 h at room temperature with gentle agitation. The membranes were then incubated in TBST-containing antibodies to NFE2L2 (catalog no. ab137550, Abcam, Cambridge, MA), HMOX1 (catalog no. ab13248, Abcam), NQO1 (catalog no. ab 28947, Abcam), GCLC (catalog no. ab 80841, Abcam), and GCLM (catalog no. ab81445, Abcam) with gentle agitation at 4°C overnight. After incubating with primary antibody, the membranes were washed and incubated with HRP-conjugated secondary antibodies (HRP-labeled sheep anti-mouse, 1:50,000, and HRP-labeled sheep anti-rabbit, 1:50,000; catalog no. ab6721; Abcam) in TBST for 1 h at room temperature. The membranes were washed and then incubated with ECL reagent (catalog no. 170–5060, Bio-Rad). Beta-Tubulin (catalog no. ab56676; Abcam) was used as an internal control. The images were captured using Chemi DOC MP (Bio-Rad). The intensities of the bands were measured with Image-Pro Plus 6.0 software (Media Cybernetics Inc., Rockville, MD).

Statistical Analysis

In the preliminary experiments, the effects of time (0, 6, 8, 12, and 24 h) and dose of TP (0, 60, 80, and 100 μ g/mL) were analyzed using 1-way ANOVA followed by a Student-Newman-Keuls multiple-comparison with the MIXED procedure of SAS 9.0 (SAS Institute Inc., Cary, NC). The fixed effects in the model were time and dose of TP. For subsequent experiments evaluating the effect of TP (100 μ g/mL) we used an ANOVA followed by Student-Newman-Keuls multiple-comparison using SAS 9.0 (SAS Institute Inc.). The fixed effect was treatment. In all cases the random effect was BMEC. For the more independent variables, data were analyzed as a $2 \times 2 \times 2$ factorial arrangement of treatments using SAS 9.0 (SAS Institute Inc.). The 3 factors were TP, H₂O₂, and NFE2L2-siRNA (siRNA), each containing 2 levels (“yes,” it was added/silenced; or “no,” it was not added/silenced). The model tested the effects of the 3 factors, and the interactions between the 3 factors: TP $\times$ H₂O₂, siRNA $\times$ TP, siRNA $\times$ H₂O₂, and siRNA $\times$ TP $\times$ H₂O₂. Least squares means and standard errors were determined using the LSMEANS statement and means were separated using the PDIF statement. Data for significant triple interactions are presented in graphs.

Five replicate cultures were done for each treatment in each experiment. All data are expressed as means $\pm$ standard error of the means for the indicated number of independent experiments performed. Significance was declared at $P \leq 0.05$ and tendencies as $P \leq 0.10$.

RESULTS

Screening for TP Optimum Conditions

Pretreatment of BMEC with 0 to 100 μ g/mL of TP for 0 to 12 h elicited a time- and dose-dependent increase of cell survival rate, with the highest response when the cells were pretreated with 100 μ g/mL of TP for 12 h ($P < 0.01$; Table 3). To further verify whether 100 μ g/mL of TP was the optimum concentration to protect BMEC against OS and assess whether TP elicits antioxidant effects, BMEC were pretreated with 60, 80, and 100 μ g/mL for 12 h, followed by H₂O₂ (600 μ M) challenge for an additional 6 h. We then analyzed cell survival rate (Figure 1A), SOD (Figure 1B), and GSH-Px (Figure 1C) activity, ROS (Figure 1D) and MDA (Figure 1E) production, caspase-3 activity (Figure 1F), PC (Figure 1G), 8-OHdG (Figure 1H), and 8-iso-PG (Figure 1I) content in control and oxidative damaged BMEC. The performance characteristics of the assays of 8-OHdG and 8-iso-PG were detected (Supplemental Table S1; <https://doi.org/10.3168/jds.2018-15047>).

Results revealed that compared with the control, the treatment of cells with 600 μ M H₂O₂ for 6 h decreased the cell survival rate ($P < 0.01$) and SOD and GSH-Px activity ($P < 0.01$), and enhanced ROS and MDA production ($P < 0.01$), caspase-3 activity ($P < 0.01$), PC, 8-OHdG, and 8-iso-PG content ($P < 0.01$). However, after pretreatment of cells with 60, 80, and 100 μ g/mL of TP for 12 h followed by H₂O₂ (600 μ M) challenge for 6 h, the cell survival rate ($P < 0.05$), SOD and GSH-Px activity ($P < 0.05$) induced by H₂O₂ were significantly increased, whereas ROS and MDA production ($P < 0.05$), caspase-3 activity ($P < 0.05$), PC, 8-OHdG, and 8-iso-PG content ($P < 0.05$) induced by H₂O₂ were significantly decreased compared with the control.

The cell survival rate increased to a maximum (95.5%) when the concentration of TP was 100 μ g/mL, which was significantly higher than 60 μ g/mL (77.0%; $P < 0.01$) and 80 μ g/mL of TP group (81.8%; $P < 0.05$). Activity of SOD and GSH-Px (175.5 U/mL and 785.9 U/L) in the cells pretreated with 100 μ g/mL of TP was higher than in cells pretreated with 60 μ g/mL of TP (115.2 U/mL, 698.7 U/L; $P < 0.01$) and 80 μ g/mL of TP (136.7 U/mL, 742.5 U/L; $P < 0.01$), respectively.

The concentrations of ROS and MDA decreased to a minimum (1,282 pg/mL, 38.0 mM) when TP was sup-

plied at 100 µg/mL, which was significantly less than 60 µg/mL of TP (2,672.9 pg/mL, 66.3 mM; $P < 0.01$) and 80 µg/mL of TP (2,353.8 pg/mL, 56.9 mM; $P < 0.01$), respectively. The concentration of PC, 8-OHdG and 8-iso-PG decreased to a minimum (0.8 nmol/mg, 144 ng/L, 67.2 ng/L) when the concentration of TP was 100 µg/mL, which was significantly less than 60 µg/mL of TP (1.0 nmol/mg, 234.2 ng/L, 98.6 ng/L) ($P < 0.01$) and 80 µg/mL of TP (0.8 nmol/mg, 182.6 ng/L, 85.3 ng/L; $P < 0.01$), respectively. Similarly, the activity of caspase-3 (144.0) in the cells pretreated with 100 µg/mL of TP was significantly less than that in cells pretreated with 60 µg/mL of TP (185.4; $P < 0.05$) and 80 µg/mL of TP (169) ($P < 0.05$). Thus, the pretreatment of cells with 100 µg/mL of TP for 12 h was the optimum amount chosen for subsequent experiments to assess the antioxidant effects of TP in BMEC.

Cytoprotective Effects of TP Against OS in BMEC

To examine the cytoprotective effect of TP against OS in BMEC, we further evaluated whether TP was able to induce NFE2L2 expression (Figure 2). The results revealed that TP significantly upregulated NFE2L2 mRNA expression in a dose-dependent manner under OS, and NFE2L2 mRNA expression induced by TP was highest when the content of TP was 100 µg/mL under OS ($P < 0.01$). The results also revealed that the treatment of cells with 600 µM of H₂O₂ for 6 h reduced the mRNA expression of NFE2L2 by 61% compared with the control cultures ($P < 0.05$). However, compared with the control, after the pretreatment of the cells with 100 µg/mL of TP for 12 h, followed by H₂O₂ (600 µM) challenge for 6 h, the mRNA expression of NFE2L2 significantly increased by 2.7-fold ($P < 0.01$).

Without OS, the treatment of cells with 100 µg/mL of TP for 12 h led to a 3.2-fold increase in mRNA expression of NFE2L2 ($P < 0.01$) compared with control cultures (Figure 3A). In addition, after pretreatment of cells with 100 µg/mL of TP for 12 h followed by H₂O₂ (600 µM) challenge for 6 h, the protein expression of NFE2L2 was increased compared with the control and H₂O₂ cultures ($P < 0.01$; Figure 3B). Therefore, TP can increase the protein and mRNA expression of NFE2L2 in BMEC with or without OS.

Verification and Screening of NFE2L2-siRNA Interference Fragment

Compared with the control cultures, the BMEC transfected with NFE2L2-siRNA1, NFE2L2-siRNA2, and NFE2L2-siRNA3 led to lower abundance of both NFE2L2 protein (Figure 4A) and mRNA (Figure 4B) with the best interference efficiency achieved with NFE2L2-siRNA3 (>50%; $P < 0.01$). Therefore, NFE2L2-siRNA3 was chosen as the interference fragment for subsequent experiments.

Effect of H₂O₂ on Survival Rate of BMEC After NFE2L2-siRNA3 Interference

The survival rate of BMEC was measured after the pretreatment of cells with 100 µg/mL of TP for 12 h, followed by H₂O₂ (600 µM) challenge for 6 h. There was a tendency ($P = 0.10$) for a siRNA × TP × H₂O₂ interaction for BMEC survival rate (Figure 5). Except with the TP treatment ($P = 0.19$), compared with the control cultures, the survival rate of BMEC was significantly decreased ($P < 0.05$) by all treatments. Compared with cells treated with H₂O₂

Table 3. Effect of different concentrations and incubation times with tea polyphenols (TP) on the proliferation rate of bovine mammary epithelial cells (BMEC)¹

TP concentration (µg/mL)	Time (h)					SEM	P-value
	0	6	8	12	24		
0	100.00 ^{a,A}	100.00 ^{a,A}	100.000 ^{b,A}	100.000 ^{b,A}	100.000 ^{b,A}	0	—
60	101.278 ^{a,D}	103.068 ^{a,CD}	110.168 ^{a,BC}	119.384 ^{a,A}	115.714 ^{a,AB}	2.834	0.0007
80	103.192 ^{a,B}	106.068 ^{a,B}	115.048 ^{a,A}	119.432 ^{a,A}	117.090 ^{a,A}	2.458	0.0004
100	105.514 ^{a,B}	107.740 ^{a,B}	117.264 ^{a,A}	120.060 ^{a,A}	119.476 ^{a,A}	3.046	0.0054
120	90.438 ^{b,A}	92.430 ^{a,A}	94.106 ^{b,A}	97.064 ^{b,A}	95.224 ^{b,A}	6.102	0.9491
SEM	2.616	5.582	2.919	2.33	2.942		
P-value	0.0065	0.3527	<0.0001	<0.0001	<0.0001		

^{a,b}Different lowercase letters for means in the same column denote significant differences compared with other concentrations of tea polyphenols (TP) ($P < 0.05$).

^{A-D}Different uppercase letters for means in the same row denote significant differences compared with other incubation times ($P < 0.05$).

¹Bovine mammary epithelial cell proliferation rate in response to increasing concentrations of tea polyphenols (TP; 0–120 µg/mL) without hydrogen peroxide. The BMEC were pretreated with 0 to 120 µg/mL of TP for 0 to 24 h before analysis of the proliferation rate. Proliferation rate was measured with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT). Five replications were done for each experiment and each well.

Figure 1. Screening for optimum conditions of tea polyphenol (TP) supplementation. Panels A–I: Cell survival rate, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity, reactive oxygen species (ROS), and malondialdehyde (MDA) production, caspase-3 activity, protein carbonyl (PC), 8-hydroxydeoxyguanosine (8-OHdG), and 8-iso-prostaglandin (8-iso-PG) content in bovine mammary epithelial cells (BMEC) incubated without (control) or various levels of TP (0–100 $\mu\text{g/mL}$) with H_2O_2 . The BMEC were pretreated with TP (0–100 $\mu\text{g/mL}$) for 12 h, followed by H_2O_2 (600 μM) challenge for 6 h. Cell survival rate, SOD and GSH-Px activity, ROS and MDA production, caspase-3 activity, content of PC, 8-OHdG, and 8-iso-PG were determined. Cell survival rate was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) assay. Superoxide dismutase and GSH-Px activity, ROS and MDA production, caspase-3 activity, and content of PC, 8-OHdG, and 8-iso-PG were measured using commercial ELISA according to manufacturer's protocols. Means with different letters (a–c) differ ($P < 0.05$). Five replications were done for each experiment and each well. Values are means, with SE represented by vertical error bars.

alone ($P < 0.01$), pretreatment with 100 $\mu\text{g/mL}$ of TP for 12 h followed by a 600 μM H_2O_2 challenge for 6 h (TP+ H_2O_2) increased survival rate. Transfection with NFE2L2-siRNA3, followed by pretreatment with TP (siRNA+TP), did not increase survival rate compared with cells siRNA transfection alone ($P = 0.37$). The survival rate of BMEC was lowest when cells were

transfected with the NFE2L2-siRNA3 and treated with H_2O_2 (600 μM ; siRNA+ H_2O_2). Transfection with the NFE2L2-siRNA3 and pretreatment with TP followed by H_2O_2 (siRNA+TP+ H_2O_2) treatment improved survival rate compared with the siRNA+ H_2O_2 treatment ($P < 0.01$). However, this improvement with TP was not as great as the survival rate of cells exposed to

the TP+H₂O₂ treatment. These results further suggest that the positive effect of TP on BMEC survival rate during challenge with H₂O₂ was impaired by silencing NFE2L2.

Effect of H₂O₂ on ROS in BMEC After NFE2L2-siRNA3 Interference

A triple interaction was observed between siRNA, TP, and H₂O₂ ($P < 0.01$) for ROS production (Figure 6). The results demonstrated that 600 μ M H₂O₂ alone increased ROS production significantly compared with all other treatments ($P < 0.05$). Treatment with TP+H₂O₂ significantly reduced ROS generation to the same level as siRNA and siRNA+H₂O₂ treatment ($P = 0.01$). The siRNA+H₂O₂ treatment increased ROS production compared with siRNA transfection alone ($P = 0.02$), whereas siRNA+TP did not alter ROS generation compared with siRNA alone ($P = 0.47$). However, ROS generation was greatly increased in the siRNA+TP+H₂O₂ treatment compared with siRNA+H₂O₂ and TP+H₂O₂ treatments ($P < 0.05$), suggesting that the decrease in ROS production detected with TP was eliminated by silencing NFE2L2.

Figure 2. Cytoprotective effects of tea polyphenols (TP) on H₂O₂-induced nuclear factor erythroid 2 like 2 (NFE2L2) mRNA expression in bovine mammary epithelial cells (BMEC). The BMEC were pretreated with TP (0–100 μ g/mL) for 12 h and then treated with H₂O₂ (600 μ M) for 6 h. The mRNA expression of NFE2L2 was analyzed by quantitative real-time PCR. Means with different letters (a–d) differ ($P < 0.05$). Five replications were done for each experiment and each well. Values are means, with SE represented by vertical error bars.

Figure 3. Effect of tea polyphenols (TP) on H₂O₂-induced nuclear factor erythroid 2 like 2 (NFE2L2) protein and mRNA expression. Panels A and B: Expression of NFE2L2 protein and mRNA in bovine mammary epithelial cells (BMEC) without (control) or with H₂O₂ and TP with or without H₂O₂. The BMEC were pretreated with or without 100 μ g/mL of TP for 12 h and then treated with or without 600 μ M of H₂O₂ for 6 h. The NFE2L2 protein and mRNA expression were analyzed by Western blot and quantitative real-time PCR. Means with different letters (a–d) differ ($P < 0.05$). Five replications were done for each experiment and each well. Values are means, with SE represented by vertical error bars.

Effect of H_2O_2 on *NFE2L2* and Downstream Genes in BMEC After *NFE2L2*-siRNA3 Interference

Triple interactions between siRNA, TP, and H_2O_2 were observed for the mRNA abundance of *NFE2L2* ($P < 0.01$), *HMOX1* ($P < 0.01$), and *GCLC* ($P < 0.01$; Figure 7).

Exogenous TP increased the mRNA abundance of *NFE2L2* ($P < 0.05$) under normal conditions compared with the control cultures, and all other treatments. Importantly, transfection of *NFE2L2*-siRNA3 under normal conditions decreased the mRNA expression of *NFE2L2* compared with control ($P < 0.01$), and expression was reduced the lowest when siRNA-transfected cells were treated with H_2O_2 . Treatment with siRNA+TP increased the expression of *NFE2L2* compared with siRNA alone ($P = 0.01$). The siRNA+TP+ H_2O_2 treatment increased *NFE2L2* expression compared with the siRNA+ H_2O_2 treatment ($P < 0.01$), but the increase in expression was still lower than observed with the TP+ H_2O_2 treatment ($P < 0.01$).

The mRNA expression of *HMOX1* followed a similar pattern as *NFE2L2*; expression was increased with TP compared with control and all other treatments ($P <$

Figure 4. Screening for efficacy of nuclear factor erythroid 2 like 2 (*NFE2L2*)-short interfering RNA (siRNA) fragments. Panels A and B: protein and mRNA expression of *NFE2L2* after siRNA treatment. Protein and mRNA expression of *NFE2L2* after siRNA treatment was detected by Western blot and quantitative reverse-transcription PCR. Means with different letters (a–c) differ ($P < 0.05$). Five replications were done for each experiment and each well. Values are means, with SE represented by vertical error bars.

Figure 5. Effect of tea polyphenols (TP) on H_2O_2 -induced bovine mammary epithelial cells (BMEC) survival rate in BMEC without TP, H_2O_2 , or nuclear factor erythroid 2 like 2 (*NFE2L2*)-short interfering RNA (siRNA; control) with H_2O_2 , TP without or with H_2O_2 , *NFE2L2*-siRNA3 without or with TP, *NFE2L2*-siRNA3 without or with H_2O_2 , and *NFE2L2*-siRNA3 with TP and H_2O_2 . The BMEC were transfected with *NFE2L2*-siRNA3, pretreated with or without 100 μ g/mL of TP for 12 h, and then treated with or without 600 μ M H_2O_2 for 6 h. The BMEC survival rate was analyzed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) assay. Means with different letters (a–c) differ ($P < 0.05$). Five replications were done for each experiment and each well. Values are means, with SE represented by vertical error bars.

0.05; Figure 7). The addition of TP+ H_2O_2 increased *HMOX1* expression compared with treatment with H_2O_2 alone ($P < 0.01$). However, when cells were exposed to the siRNA+TP+ H_2O_2 treatment, the mRNA expression of *HMOX1* was significantly lower than expression with the TP+ H_2O_2 treatment ($P < 0.01$), and was not different from the expression observed with the siRNA+ H_2O_2 treatment ($P = 0.38$).

For *GCLC*, mRNA expression was greatest in cells treated with TP+ H_2O_2 compared with control and all other treatments ($P < 0.05$; Figure 7). When cells were exposed to the siRNA+TP+ H_2O_2 treatment, *GCLC* expression was significantly lower than expression with TP+ H_2O_2 ($P < 0.01$), and *GCLC* expression tended to be altered compared with the siRNA+ H_2O_2 treatment ($P = 0.07$).

A significant TP $\times$ H_2O_2 interaction was observed for the mRNA expression of *NQO1* ($P < 0.01$; Table 4); expression was greater with TP and H_2O_2 treatment compared with H_2O_2 alone ($P < 0.01$). An siRNA $\times$ TP interaction was also observed for *NQO1* ($P = 0.01$) where siRNA and TP tended to have a greater expression than siRNA alone ($P = 0.94$) but was lower than TP alone ($P < 0.01$). Additionally, there was a tendency for an siRNA $\times$ H_2O_2 interaction for the mRNA

Figure 6. Effect of tea polyphenols (TP) on H₂O₂-induced reactive oxygen species (ROS) generation in bovine mammary epithelial cells (BMEC) without TP, H₂O₂, or nuclear factor erythroid 2 like 2 (NFE2L2)-short interfering RNA (siRNA; control) with H₂O₂, TP without or with H₂O₂, NFE2L2-siRNA3 without or with TP, NFE2L2-siRNA3 without or with H₂O₂, and NFE2L2-siRNA3 with TP and H₂O₂. The BMEC were transfected with NFE2L2-siRNA3, pretreated with or without 100 µg/mL of TP for 12 h, and then treated with or without 600 µM of H₂O₂ for 6 h. The generation of ROS was analyzed with a commercial kit following the manufacturer's recommendations. Means with different letters (a–f) differ ($P < 0.05$). Five replications were done for each experiment and each well. Values are means, with SE represented by vertical error bars.

expression of *NQO1* ($P = 0.07$) where the addition of siRNA and H₂O₂ significantly reduced the expression of *NQO1* compared with addition of H₂O₂ alone ($P < 0.01$).

A significant siRNA $\times$ TP interaction was also observed for the mRNA expression of *GCLM* ($P < 0.01$; Table 4). The expression of *GCLM* was greater with TP alone versus siRNA or siRNA with TP ($P < 0.01$), and expression was not different between siRNA and siRNA with TP ($P = 0.47$). A significant TP $\times$ H₂O₂ interaction was also observed for the mRNA expression of *GCLM* ($P < 0.01$; Table 4) where TP+H₂O₂ increased the expression of *GCLM* compared with H₂O₂ alone ($P = 0.01$). These results further suggested that the positive effect of TP on the mRNA abundance of *NFE2L2* and 4 main phase-II detoxifying enzyme was eliminated by silencing *NFE2L2*.

DISCUSSION

In the present study, we demonstrated that TP could play an important role in antioxidant protection of BMEC. Exogenous TP activates NFE2L2 and the HMOX1 and NQO1 pathway in part by upregulating the steady-state level of NFE2L2 protein and mRNA.

Figure 7. Effect of tea polyphenols (TP) on H₂O₂-induced mRNA expression of *NFE2L2*, *HMOX1*, and *GCLC* in bovine mammary epithelial cells (BMEC). Panels A–E: The mRNA expression of *NFE2L2*, *HMOX1*, and *GCLC* in BMEC without TP, H₂O₂, or nuclear factor erythroid 2 like 2 (NFE2L2)-siRNA (control) with H₂O₂, TP without or with H₂O₂, NFE2L2-siRNA3 without or with TP, NFE2L2-siRNA3 without or with H₂O₂, and NFE2L2-siRNA3 with TP and H₂O₂. The BMEC were transfected with NFE2L2-siRNA3, pretreated with or without 100 µg/mL of TP for 12 h, and then treated with or without 600 µM of H₂O₂ for 6 h. The mRNA expression of *NFE2L2*, *HMOX1*, *NQO1*, *GCLC*, and *GCLM* was analyzed by quantitative reverse-transcription PCR. Means with different letters (a–f) differ ($P < 0.05$). Five replications were done for each experiment and each well. Values are means, with SE represented by vertical error bars.

Table 4. Least squares means for the effects of tea polyphenols (TP), H₂O₂, and nuclear factor erythroid 2 like 2 (NFE2L2)-short interfering RNA (siRNA) on bovine mammary epithelial cell (BMEC) survival rate, reactive oxygen species (ROS) generation, and mRNA abundance of *NFE2L2*, *HMOX1*, *NQO1*, *GCLC*, and *GCLM*¹

Item	TP				H ₂ O ₂				siRNA				P-value ²			
	No		Yes		No		Yes		No		Yes		SEM	TP	H ₂ O ₂	TP × H ₂ O ₂
	Survival rate (%)	ROS generation ³	Survival rate (%)	ROS generation ³	Survival rate (%)	ROS generation ³	Survival rate (%)	ROS generation ³	Survival rate (%)	ROS generation ³	Survival rate (%)	ROS generation ³				
BMEC survival rate (%)	80.79	86.68	1.16	90.55	76.92	1.16	88.8	78.61	1.16	0.001	0.01	0.001	0.58	0.001	0.01	0.001
ROS generation ³	1.75	1.53	0.04	1.06	2.22	0.04	1.59	1.68	0.04	0.001	0.16	0.14	<0.001	0.07	0.002	0.002
<i>NFE2L2</i>	0.69	1.02	0.03	1.02	0.70	0.03	1.18	0.60	0.03	<0.001	0.01	<0.001	0.07	<0.001	0.11	0.11
<i>HMOX1</i>	0.63	1.80	0.03	1.56	0.88	0.03	1.96	0.47	0.03	<0.001	0.94	<0.001	<0.001	0.01	<0.001	<0.001
<i>NQO1</i>	0.57	0.80	0.04	0.78	0.58	0.04	1.03	0.44	0.05	<0.001	0.03	<0.001	0.01	0.01	0.07	0.07
<i>GCLC</i>	0.51	0.71	0.04	0.62	0.55	0.04	0.94	0.38	0.05	<0.001	0.47	<0.001	0.05	<0.001	0.06	0.06
<i>GCLM</i>	0.78	1.23	0.06	1.12	0.89	0.06	1.33	0.68	0.06	<0.001	0.01	<0.001	<0.001	<0.001	0.45	0.45

¹*HMOX1* = heme oxygenase-1; *NQO1* = NAD(P)H: quinone oxidoreductase-1; *GCLC* = glutamate-cysteine ligase catalytic subunit; *GCLM* = glutamate-cysteine ligase modifier subunit.

²Data for the significant triple interactions of siRNA × TP × H₂O₂ are presented in Figure 7.

³ROS generation is relative to control.

These results are consistent with in vitro observations that quercetin, a polyphenolic compound, can enhance NFE2L2 transcription activity, upregulate the mRNA and protein expression of NFE2L2, and increase the expression of *NQO1* (Tanigawa et al., 2007). Together, these effects of TP might coordinate the antioxidant response when BMEC are challenged with ROS including H₂O₂.

First discovered in China, tea is grown in over 30 countries and aside from water is the most widely consumed beverage in the world (Graham, 1992). In vitro and in vivo evidence with nonruminant cells suggests that TP are effective antioxidants against ROS-induced disorders (Wiseman et al., 1997). A key function of TP is to both scavenge ROS directly and stimulate endogenous cellular defense systems (Shi et al., 2000) with antioxidant roles (Frei and Higdon, 2003; McMahon et al., 2004). Thus, by challenging BMEC with a supraphysiologic dose of H₂O₂ and providing exogenous TP, the present study demonstrated a positive effect on activity of SOD and GSH-Px, accompanied by decreases in concentrations of ROS, MDA, and the activity of caspase-3. The decrease in concentrations of PC, 8-OHdG, and 8-iso-PG under OS conditions also underscored that the positive effect of TP extends to reducing the damage of proteins, DNA, and lipids.

The NFE2L2 can regulate antioxidant responses by activating the downstream phase-II detoxifying genes such as *HOMX-1*, *NQO1*, *GCLC*, and *GCLM*. Previous studies revealed that activation of NFE2L2 expression enhanced cell survival rate under OS (Duan et al., 2017), and NFE2L2 activation was central to the upregulation of antioxidant enzyme expression (Hyung et al., 2016). The present results are consistent with those findings and underscore the potency of TP for suppressing OS-induced injury (Wu et al., 2015; Lan et al., 2016; Shi et al., 2018).

A direct role for TP and components of the NFE2L2 in the antioxidant response was confirmed when silencing of NFE2L2 led to a strong inhibition of the antioxidant function of TP (i.e., the survival rate of BMEC and mRNA expression of NFE2L2 and downstream genes were decreased while ROS generation was increased). The lack of change in BMEC survival rate, *GCLC* and *HMOX1* mRNA expression, and ROS production when NFE2L2 was silenced with or without TP along with the failure of TP to decrease ROS production when NFE2L2 was silenced under OS status further indicated that the NFE2L2 pathway is very important for TP to protect BMEC against OS induced by H₂O₂. In other words, silencing of NFE2L2 failed to activate the antioxidant functions of TP. Taken together, these data illustrated clearly that NFE2L2 was a key component of the antioxidant response induced by TP. Further

research will have to be performed to evaluate these mechanisms *in vivo*.

From a mechanistic standpoint, and similar to non-ruminant studies, the data indicate that TP pretreatment elicits an antioxidant response via increasing the activation of NFE2L2/*HMOX1* pathway (Lee et al., 1995; Skrzydlewska et al., 2002). Therefore, these data provide further support for a biological role of the NFE2L2 pathway in mammary tissue, and emphasize a potential benefit for increasing the supply of TP via dietary supplementation.

CONCLUSIONS

Tea polyphenols *in vitro* can alleviate H₂O₂-induced oxidative damage of mammary epithelial cells by controlling the production of ROS and MDA, PC, 8-OHdG, and 8-iso-PG, and the activity of caspase-3. They do this in part by increasing the activity of antioxidant enzymes such as SOD and GSH-Px, and the protein and mRNA abundance of NFE2L2. The fact that silencing of NFE2L2 failed to activate antioxidant mechanisms, even when TP was added, underscored its importance in the overall control of oxidative stress. Hence, the control of NFE2L2 and downstream target genes and proteins constitute important mechanisms for TP action against oxidative stress.

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