Open Access

SCHOLARENA

Journal of Veterinary and Animal Research

RESEARCH ARTICLE

ISSN: 2639-7315

Lycopene Protects Bovine Mammary Epithelial Cells from Hydrogen Peroxide-Induced Oxidative Stress by Activating Nfe2l2/Hmox-1 Pathways and Inhibiting Inflammation *in Vitro*

Yanfen Ma[1*], Chunhua Zhang[1], Liwen Song[1], Lei Zhao[2] and Hua Bao[1]

[1]*Institute of Animal Nutrition and Feed, Inner Mongolia Academy of Agricultural and Animal Husbandry Sciences, China*
[2]*College of Animal Science and Technology, Jilin Agricultural University, Changchun, China*

[*]**Corresponding author:** Yanfen Ma, Institute of Animal Nutrition and Feed, Inner Mongolia Academy of Agricultural and Animal Husbandry Sciences, China, Tel: +8618647390466, E-mail: ma2999@163.com

Citation: Yanfen Ma, Chunhua Zhang, Liwen Song, Lei Zhao and Hua Bao (2020) Lycopene Protects Bovine Mammary Epithelial Cells from Hydrogen Peroxide-Induced Oxidative Stress by Activating Nfe2l2/Hmox-1 Pathways and Inhibiting Inflammation *in Vitro*. J Vet Ani Res 3: 103

Article history: Received: 06 March 2020, **Accepted:** 24 March 2020, **Published:** 27 March 2020

Abstract

Bovine mammary epithelial cells (BMECs) are likely subjected to redox imbalance and immune imbalance due to the increased oxidative stress and inflammatory response induced by metabolic demand associated with lactation initiation in early lactation of dairy cows. Lycopene (LYC) possesses several types of properties such as anti-oxidation, and anti-inflammatory. This study examined the effects of BMECs treated with LYC on hydrogen peroxide (H_2O_2)-induced oxidative stress and inflammatory response, and the potential underlying mechanisms. The primary BMECs were transfected with nuclear factor erythroid 2 like 2 (NFE2L2)-small interfering RNA (siRNA), and were pretreated with or without LYC (2 μM) for 12 h, and then followed by H_2O_2 (600 μM) or without H_2O_2 (600 μM) for 6 h. The results revealed that LYC treatment active the NFE2L2-ARE pathway and heme oxygenase-1 (HMOX-1), which was one of the downstream genes of the NFE2L2- ARE pathway. Lycopene treatment significantly decreased BMECs-associated oxidative stress via suppression of intracellular reactive oxygen species (ROS) accumulation, whereas increased the BMECs survival rate, the mRNA levels of NFE2L2 and the antioxidant enzymes including HMOX-1 and NAD(P)H: quinone oxidoreductase-1 (NQO1); however, NFE2L2-siRNA interference results indicated that the negative effect of LYC on ROS generation, and the positive effect of LYC on BMECs survival rate, the mRNA abundance of NFE2L2 and downstream gene pathway during challenge with H_2O_2 were impaired by silencing NFE2L2. Furthermore, LYC treatment significantly reduced oxidative stress-associated inflammation and cells apoptosis by down-regulating related inflammatory mediators and apoptosis rate; however, NFE2L2-siRNA interference results indicated that the negative effect of LYC on the mRNA abundance of Tumor necrosis factor-α (TNF-α) and Interleukin-1 β (IL-1β), and cells apoptosis rate during challenge with H_2O_2 were eliminated by silencing NFE2L2. In summary, LYC exerts the anti-oxidative and anti-inflammatory effects by activating the NFE2L2/HMOX-1 pathway suggested that NFE2L2 is an essential antioxidant transcription factor for the protective mechanism induced by LYC, and has the potential for using in the treatment of several oxidative stress-associated inflammatory responses during perinatal period.

Keywords: Lycopene; Bovine Mammary Epithelial Cells; Oxidative Stress; NFE2L2 Pathway; Inflammatory Responses

Introduction

The perinatal period is the critical time for health, performance and welfare of dairy cows. During this time, high-producing dairy cows are subject to extreme metabolic changes and oxidative stress, which often make dairy cows more susceptible to infectious diseases such as mastitis [1]. Compelling evidence indicates that production of reactive oxygen species (ROS) plays a critical role in oxidative stress and can alter intracellular reduction-oxidation (redox) balance in dairy cows [2,3]. Furthermore, a large number of evidences show that the gradual development of oxidative stress in perinatal dairy cows is an important potential factor leading to dysfunctional inflammatory response and dyslipidemia [1,4]. Bovine mammary epithelium, the predominant cell type in the mammary gland, can bind pathogen-associated molecular pattern molecules via activating various pattern recognition receptors [5]. Bovine mammary epithelial cells (BMECs) as the first barrier for mammary glands can resist stress and invading pathogens and play a major role during the initiation of infection [6]. However, enhanced or prolonged oxidative stress after lactation initiation can lead to dysfunctional inflammatory reactions in mammary glands and injury the BMECs [1]. Among the various types of ROS, hydrogen peroxide (H_2O_2) 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 [2,7], evidence indicates that oxidative stress caused by excessive production of H_2O_2 could be an important contributor [8]. Our previous study revealed that BMECs-associated with oxidative stress were induced by superphysiological dose H_2O_2 (600 μM) [9].

The transcription regulator nuclear factor erythroid 2 like 2 (NFE2L2) is an important component of the intracellular antioxidant machinery and considered the master regulator of cellular redox balance. NFE2L2 transactivates genes with antioxidant response elements (ARE), and it coordinates the expression of cytoprotective genes, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), heme oxygenase-1 (HMOX-1), NAD(P)H: quinone oxidoreductase-1 (NQO1) [10,11], to counteract endogenously or exogenously generated oxidative stress [12]. More importantly, our previous study found that the activation of the NFE2L2-ARE pathway protected BMECs against H_2O_2-induced oxidative stress, indicating that the NFE2L2-ARE pathway may be a potential therapeutic target for protecting mammary glands from oxidative damage [13]. Moreover, our another study found that tea polyphenols as an antioxidant protected BMECs from H_2O_2-induced oxidative damage *in vitro* by activating NFE2L2/HMOX-1 pathway [14]. These findings further suggested that exogenous regulators of NFE2L2 might provide theoretical and technical basis for the feasibility study of its practical application in periparturient cows' management. In conclusion, adding antioxidants is one of the main ways to effectively eliminate the excess free radicals generated by the body and reduce the oxidative stress generated by the body, and is also one of the ways to effectively reduce the inflammatory reactions caused by oxidative stress in tissues, organs and cells of the body during the perinatal period.

Lycopene (LYC) is a natural carotenoid and carotenoid pigment, which is abundant in tomatoes, papaya, apricot, pink grapefruit, pink guava, watermelon and other red fruits and vegetables [15]. Lycopene is a relatively rich carotenoid in tomatoes, with 11 linear conjugated double bonds and 2 non-conjugated double bonds, accounting for more than 80% of the total carotenoid content in fully mature fruits. Lycopene is a powerful singlet oxygen physical quencher due to the length of conjugated double bonds system, the physical quenching rate of LYC is two times higher than β-carotene and 10 times higher than α-tocopherol [16]. Furthermore, compared with other well-known antioxidant, such as polyphenols [17] and resveratrol [18], LYC is one of the most powerful natural antioxidants, the antioxidant, anti-inflammatory, and anti-apoptotic effects of LYC have been illustrated both *in vitro* and *in vivo* [19,20]. Its antioxidant effects include a considerable ROS scavenging activity, which allows LYC to prevent protein, lipid peroxidation, DNA damage and related diseases by activating NFE2L2 [21,22]. Furthermore, LYC exerts an important role in a variety of inflammatory diseases as a result of its strong antioxidant properties, as verified in tremendous experiments [23,24]. To our knowledge, the development of BMECs was decreased critically by oxidative damage, inflammatory response and apoptosis during the perinatal period [13,25]. Studies have shown that LYC can activate NFE2L2-ARE pathway to exert antioxidant and anti-inflammatory effects [26]. However, ROS generation, the mRNA expression of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and apoptosis rate of BMECs in NFE2L2-silenced by small interfering RNA (si-NFE2L2), LYC, and si-NFE2L2+LYC groups were not detected. Importantly, the mRNA expression of NFE2L2 pathway and downstream genes HMOX-1 and NQO-1 in all treatment (including Control, si-NFE2L2, H_2O_2, LYC, LYC+H_2O_2, si-NFE2L2+H_2O_2, si-NFE2L2+LYC, si-NFE2L2+LYC+H_2O_2) groups were not investigated. Therefore, in order to further prove that the mechanisms of LYC exerts the antioxidant and anti-inflammatory effects *in vitro* on oxidative stress-induced bovine mammary epithelial cells (BMECs), NFE2L2 factor was knocked out with small interfering RNA (si-RNA) to judge whether LYC exerts antioxidant and anti-inflammatory effects by activating NFE2L2/HMOX-1 pathway and inhibiting inflammation in this study.

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 (IMAAAHS#1215000046002373XP, Hohhot, China). Primary BMECs were isolated and cultured from 5 lactating dairy cows (lactation=200±5 DIM) as described previously [9,13,14,25]. 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 μ L of 10 ng/mL of epidermal growth factor (Gibco), 50 μ L 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) [27], which is a marker of BMECs. For cryopreservation, 1×10^6 cells/mL was suspended in freezing medium. A supraphysiologic concentration of 600 μM H_2O_2 was applied to the BMECs to induce oxidative stress [9,13,14,25].

NFE2L2-Small Interfering RNA

The NFE2L2-small interfering RNA (siRNA, si-NFE2L2) and scrambled-siRNA (control) were designed according to a previous study [10] and synthesized by Shanghai Genechem Co., Ltd (Shanghai, China). The NFE2L2-siRNA primer sequence was sense:

CTGGAGCAAGATTTAGATCAT and antisense: ATGATCTAAATCTTGCTCCAG. Cells were transfected in antibiotic-free medium following the manufacturer's instructions (Shanghai Genechem Co., Ltd), as described previously [13]. The cells were routinely trypsinized and resuspended to 2×10^6 cells per mL in basal medium without antibiotics and cultured for 24 h. After incubation for 5 min at 37 °C, the siRNA and lipofectamine were mixed, incubated at room temperature for an additional 20 min, and then added to each well. The basal medium was replaced by the same fresh medium 12 h after transfection. After 36 h, the siRNA was removed from the cells, and the cells were used for subsequent analysis or treatment.

Cell Culture and Treatments

The BMECs with and without NFE2L2-siRNA cultured in serum- and antibiotic-free medium (either preincubated with 2 μM of LYC for 12 h or not) [26] 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 [9]. LYC (SMB00706, > 98%, Sigma-Aldrich, St. Louis, Missouri, USA) was delivered to cells (10^8 cells/L) using Tetrahydrofuran (THF, Sigma-Aldrich, Missouri, USA) as a solvent, and it contained 0.025% butylated hydroxytoluene to avoid formation of peroxides. The amount of THF added to cells was not greater than 0.5% (V/V). Control cultures received an amount of solvent (THF) equal to that present in LYC-treated ones. Specific treatments were control, NFE2L2-siRNA (no H_2O_2), NFE2L2-siRNA (600 μM H_2O_2), H_2O_2 (600 μM), LYC (no H_2O_2), LYC (600 μM H_2O_2), NFE2L2-siRNA-LYC (no H_2O_2) and NFE2L2-siRNA-LYC (600 μM H_2O_2), which were used for measuring the BMECs survival rate, ROS generation, the mRNA expression of NFE2L2 and downstream genes 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 overnight. Cells were then transfected with si-NFE2L2. At 48 h after transfection, the culture medium was switched to serum-free, antibiotic-free DMEM, and cells were exposed to 2 μM LYC for 12 h, and then exposed to 600 μM H_2O_2 for 6 h. The BMECs survival rate was determined using the MTT [3-(4, 5-di methylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-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_/?catNu=G3582). Briefly, 20 μL/well 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyme thoxyphenyl)-2-(4sulfophenyl)-2H-tetrazolium was added to each well and incubated for 4 h at 37 °C. Subsequently, 150 μL 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 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 cell apoptosis

Cells apoptosis assays were performed as described previously [26]. 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).

ARE Fluorescence Activity

The ARE fluorescence activity was tested in cultured BMECs without or with H_2O_2 and without or with LYC via an ARE reporter gene according to the manufacturer's instruction (Beyotime Institute of Biotechnology, Jiangsu, China). Briefly, BMECs were seeded in a 24-well plate for 18 h to reach 80% confluence. The cells were then co-transfected with ARE-luciferase reporter plasmid pGL6-ARE and pRL-TK plasmid using a Lipofectamine™ 2000 reagent (Beyotime Biotechnology) for 24 h. Subsequently, the cells were treated as indicated and harvested. Luciferase activity was measured using a Biluciferase reporter gene kit (Beyotime Biotechnology). The luciferase activity of ARE was normalized to the Renilla luciferase activity.

RNA Isolation, cDNA Synthesis, and Quantitative 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 [4, 14] to determine the relative mRNA abundance of NFE2L2, HMOX-1, NQO1 and TUBB (tubulin β; internal control). All reactions were run in triplicate. The primers of NFE2L2 and 2 phase-II detoxifying enzyme in the NFE2L2-ARE signaling pathway used for quantitative reverse-transcription PCR are listed in Table 1.

Gene name	Forward primer (5′→3′)	Reverse primer (5′→3′)	Accession no.
NFE2L2	CCAGCACAACACATACCA	TAGCCGAAGAAACCTCATT	001011678.2
HMOX1	GAACGCAACAAGGAGAAC	CTGGAGTCGCTGAACATAG	001014912.1
NQO1	CAACAGACCAGCCAATCA	ACCTCCCATCCTTTCCTC	001034535.1

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)

Protein Extraction and Western Blotting

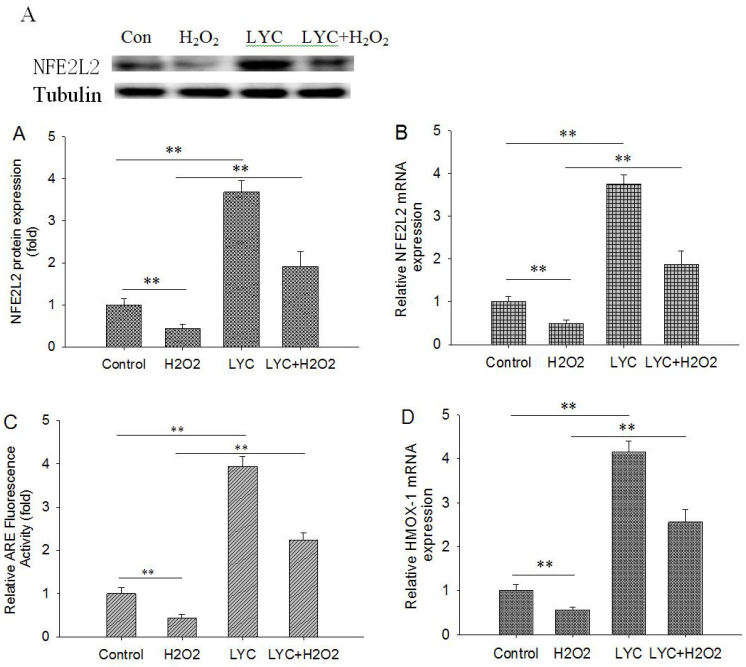
Western blotting was performed as described previously [14]. After treatment, cells were lysed with RIPA protein lysate (Beyotime Institute of Biotechnology, Shanghai, China). The protein was resolved by 10% SDS-PAGE (Bio-Rad, Hercules, CA) and then transferred from the gel to a polyvinylidene difluoride membrane (0.45 μm, Millipore, Billerica, MA). The membranes were then incubated in TBST-containing primary antibodies for NFE2L2 (catalog no. ab137550, Abcam, Cambridge, MA) and Beta-Tubulin (internal control, catalog no. ab56676; Abcam) with gentle agitation at 4 °C overnight, followed by incubation 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). 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

Comparisons among groups were performed using a one-way ANOVA with subsequent Bonferroni correction. In all cases the random effect was BMECs. For the more independent variables, data were analyzed as a 2 × 2 × 2 factorial arrangement of treatments using SAS 9.0 (SAS Institute Inc.). The 3 factors were si-NFE2L2 (siRNA), LYC and H_2O_2, 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: LYC+H_2O_2, si-NFE2L2+LYC, si-NFE2L2+H_2O_2, and si-NFE2L2+LYC+H_2O_2. Least squares means and standard errors were determined using the LSMEANS statement and means were separated using the PDIFF 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±standard error of the means (SEM) for the indicated number of independent experiments performed. Significance was declared at $P \leq 0.05$ and tendencies as $P \leq 0.10$.

Results

LYC activated the NFE2L2-ARE signal pathway



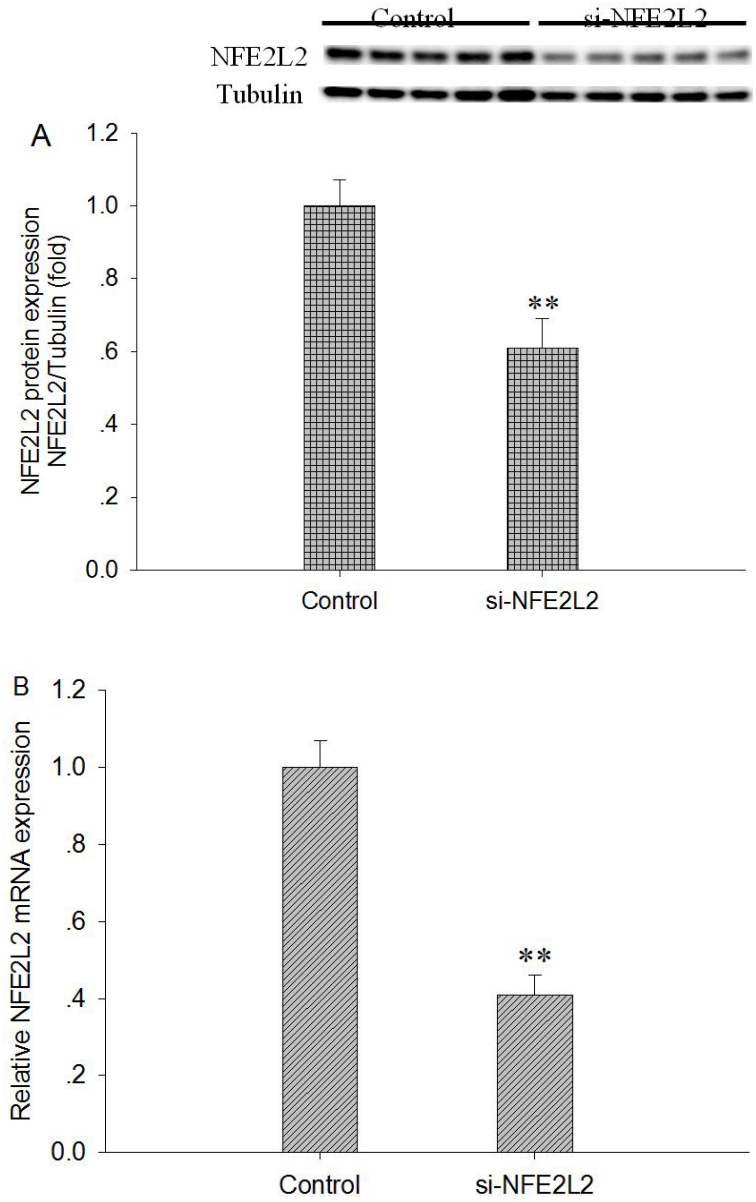
(A) The protein expression of NFE2L2. (B) The mRNA expression of NFE2L2. (C) The relative ARE fluorescence activity. (D) The mRNA expression of heme oxygenase-1 (HMOX-1). The data of the control group were used to normalize the data of each treatment group. The NFE2L2 protein expression, the mRNA expression of NFE2L2, HMOX-1 and NAD(P)H: quinone oxidoreductase-1 (NQO1) were analyzed by Western blot and quantitative real-time PCR. Five replications were done for each experiment and each well (n=5). Values are means, with SE represented by vertical error bars. $^{**}P < 0.01$ (B)

Figure 1: Lycopene (LYC) activated the nuclear factor erythroid 2 like 2 (NFE2L2)-antioxidant response element (ARE) signal pathway. The bovine mammary epithelial cells (BMECs) were pre-treated with or without 2 μM of LYC for 12 h, and then treated with or without 600 μM of hydrogen peroxide (H_2O_2) for 6 h

As shown in Figure 1, H_2O_2 treatment significantly reduced the protein expression of NFE2L2 (Figure 1A) and mRNA expression of NFE2L2 (Figure 1B) compared with the control cultures, while LYC significantly increased the protein expression and mRNA expression of NFE2L2 in the presence or absence of H_2O_2 ($P < 0.01$). Furthermore, the luciferase reporter assay revealed that H_2O_2 treatment reduced the activity of ARE compared with the control culture, while LYC significantly increased the activity of ARE in the presence or absence of H_2O_2 (Figure 1C; $P < 0.01$). Consistent with the increased the level of NFE2L2 and activity of ARE, H_2O_2 decreased the mRNA abundance of HMOX-1, downstream genes of the NFE2L2 pathway, whereas LYC up-regulated the mRNA abundance of HMOX-1 in the presence or absence of H_2O_2 compared with the H_2O_2 treatment group (Figure 1D; $P < 0.01$). These results further reveal that LYC active the NFE2L2-ARE signal pathway and the downstream genes of the NFE2L2-ARE signal pathway.

Si-NFE2L2 Interference

The BMECs transfected with si-NFE2L2 significantly decreased the protein expression of NFE2L2 (Figure 2A) and the mRNA expression of NFE2L2 (Figure 2B; $P < 0.01$) compared with the control group, which is same as our previous results [10]. Therefore, the si-NFE2L2 was chosen as the interference fragment for subsequent experiments.



(A) The protein expression of NFE2L2. (B) The mRNA expression of NFE2L2. The data of the control group were used to normalize the data of each treatment group. The NFE2L2 protein and mRNA expression were analyzed by Western blot and quantitative real-time PCR. Five replications were done for each experiment and each well (n=5). Values are means, with SE represented by vertical error bars. $^{**}P < 0.01$

Figure 2: Knockdown of nuclear factor erythroid 2 like 2 (NFE2L2) decreased the NFE2L2 level. The bovine mammary epithelial cells (BMECs) were transfected with NFE2L2-small interfering RNA (siRNA) for 48 h

LYC attenuated H_2O_2-induced BMECs survival rate after si-NFE2L2 Interference

The survival rate of BMECs was measured after the transfection with si-NFE2L2 (no si-NFE2L2) and pretreatment with 2 μM of LYC (no LYC) for 12 h, followed by 600 μM H_2O_2 (no H_2O_2) challenge for 6 h. There was a tendency (P = 0.10) for a si-NFE2L2+LYC+H_2O_2 interaction for BMECs survival rate (Figure 3). Except with the LYC treatment (P = 0.16), compared with the control cultures, the survival rate of BMECs was significantly decreased (P < 0.05) by all treatments. Compared with BMECs treated with H_2O_2 alone (P < 0.01), pretreatment with 2 μM of LYC for 12 h followed by a 600 μM H_2O_2 challenge for 6 h (LYC+ H_2O_2) increased survival rate of BMECs. Transfection with si-NFE2L2, followed by pretreatment with LYC (si-NFE2L2+LYC), did not increase survival rate compared with cells transfected with si-NFE2L2 alone (P = 0.25). The survival rate of BMECs was lowest when cells were transfected with the si-NFE2L2 and treated with H_2O_2 (600 μM; si-NFE2L2+H_2O_2). Transfection with the si-NFE2L2 and pretreatment with LYC followed by H_2O_2 (si-NFE2L2+LYC+H_2O_2) treatment improved survival rate compared with the si-NFE2L2+H_2O_2 treatment (P < 0.01). However, this improvement with LYC was not as great as the survival rate of cells exposed to the LYC+ H_2O_2 treatment. These results further suggested that the positive effect of LYC on BMECs survival rate during challenge with H_2O_2 was impaired by knocking-out NFE2L2.

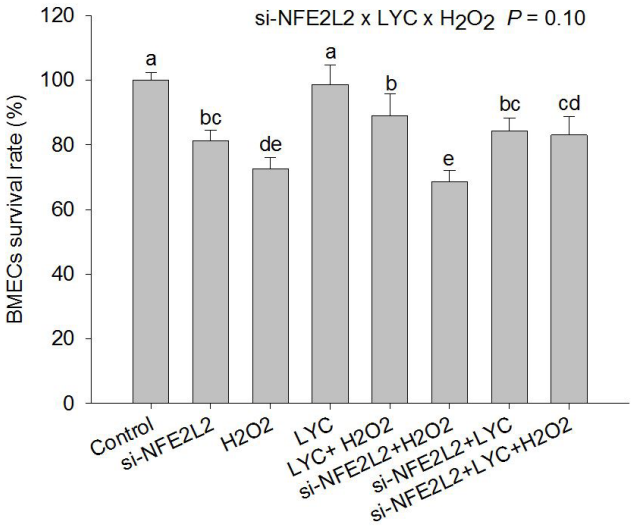


Figure 3: Silence of nuclear factor erythroid 2 like 2 (NFE2L2) attenuated the beneficial effects of lycopene (LYC) on hydrogen peroxide (H_2O_2)-induced bovine mammary epithelial cells (BMECs) survival rate. The BMECs were transfected with NFE2L2-small interfering RNA (siRNA), pretreated with or without 2 μM of LYC for 12 h, and then treated with or without 600 μM H_2O_2 for 6 h. The data of the control group were used to normalize the data of each treatment group. The BMECs survival rate was analyzed by 3-(4, 5-dimethylthiazol-2-yl) 5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium. Means with different letters (a–e) differ (P < 0.05). Five replications were done for each experiment and each well (n=5). Values are means, with SE represented by vertical error bars

LYC attenuated H_2O_2-induced oxidative stress after si-NFE2L2 Interference

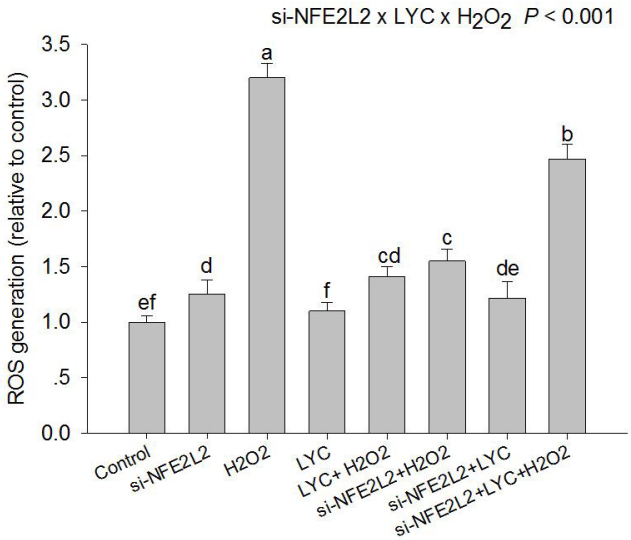
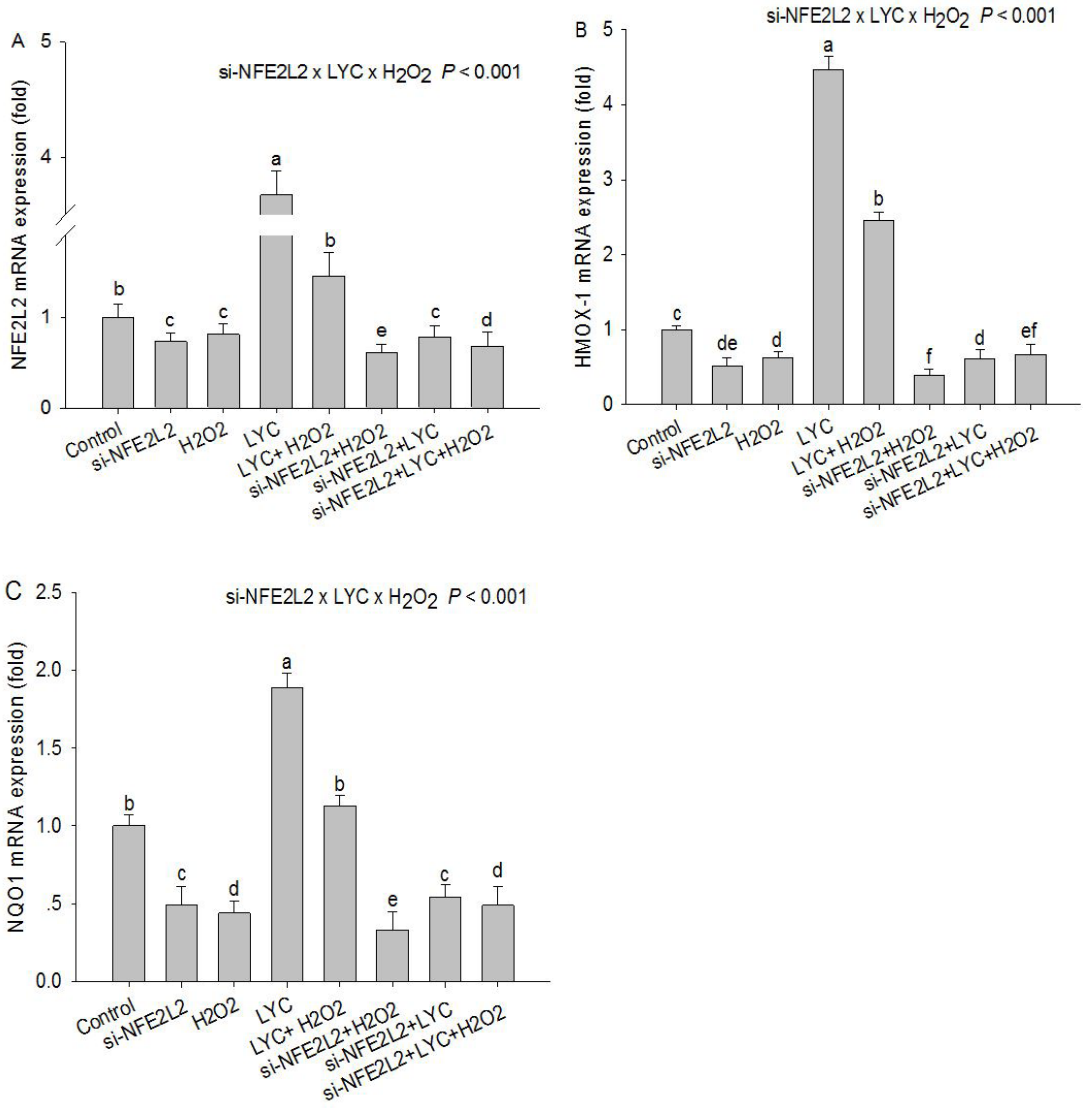


Figure 4: Silence of nuclear factor erythroid 2 like 2 (NFE2L2) attenuated the negative effects of lycopene (LYC) on hydrogen peroxide (H_2O_2)-induced reactive oxygen species (ROS) generation. The bovine mammary epithelial cells (BMECs) were transfected with NFE2L2-small interfering RNA (siRNA) for 48 h, pretreated with or without 2 μM of LYC for 12 h, and then treated with or without 600 μM of H_2O_2 for 6 h. The data of the control group were used to normalize the data of each treatment group. 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 (n=5). Values are means, with SE represented by vertical error bars

A triple interaction was observed among si-NFE2L2, LYC and H_2O_2 ($P < 0.01$) for ROS generation (Figure 4). The results demonstrated that 600 μM H_2O_2 alone increased ROS production significantly compared with all other treatments ($P < 0.01$). Treatment with LYC+ H_2O_2 significantly reduced ROS generation to the same level as si-NFE2L2 and si-NFE2L2+H_2O_2 treatment ($P = 0.01$). The si-NFE2L2+H_2O_2 treatment increased ROS production compared with si-NFE2L2 transfection alone ($P = 0.02$), whereas si-NFE2L2+LYC did not alter ROS generation compared with si-NFE2L2 alone ($P = 0.37$). However, ROS generation was greatly increased in the si-NFE2L2+LYC+H_2O_2 treatment compared with si-NFE2L2+H_2O_2 and LYC+H_2O_2 treatments ($P < 0.05$), suggesting that the decrease in ROS production detected with LYC was eliminated by silencing NFE2L2.

LYC attenuated H_2O_2-induced NFE2L2 pathway after si-NFE2L2 Interference



(A) The mRNA expression of NFE2L2. (B) The mRNA expression of heme oxygenase-1 (HMOX-1). (C) The mRNA expression of NAD(P)H: quinone oxidoreductase-1 (NQO1). The data of the control group were used to normalize the data of each treatment group. The mRNA expression of NFE2L2, HMOX-1, and NQO1 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 (n=5). Values are means, with SE represented by vertical error bars

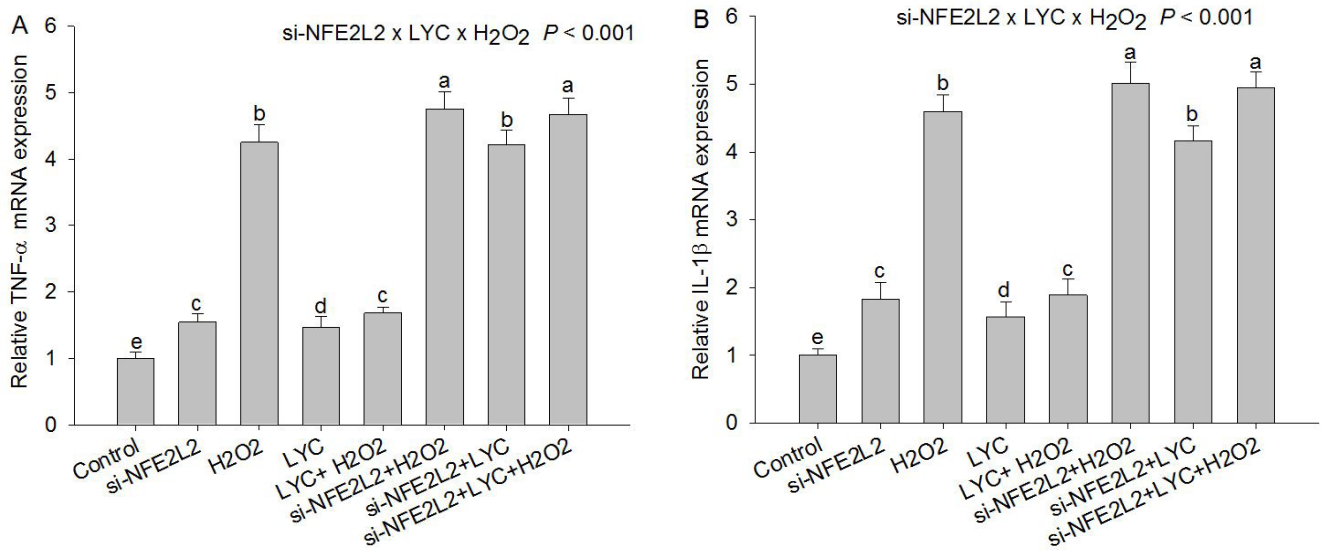
Figure 5: Silence of nuclear factor erythroid 2 like 2 (NFE2L2) attenuated the positive effects of lycopene (LYC) on hydrogen peroxide (H_2O_2)-induced NFE2L2 pathway. The bovine mammary epithelial cells (BMECs) were transfected with NFE2L2-small interfering RNA (siRNA) for 48 h, pretreated with or without 2 μM of LYC for 12 h, and then treated with or without 600 μM of H_2O_2 for 6 h

Triple interactions between si-NFE2L2, LYC and H_2O_2 were observed for the mRNA abundance of NFE2L2 ($P < 0.01$), HMOX-1 ($P < 0.01$) and NQO1 ($P < 0.01$; Figure 5). Exogenous LYC increased the mRNA abundance of NFE2L2 ($P < 0.05$; Figure 5A) under normal conditions compared with the control cultures and all other treatments. Importantly, transfection of si-NFE2L2 under normal conditions decreased the mRNA expression of NFE2L2 compared with control ($P < 0.05$), and the mRNA expression was reduced the lowest when si-NFE2L2-transfected cells were treated with H_2O_2. The lack of change in NFE2L2 mRNA expression when NFE2L2 was silenced with or without LYC. The si-NFE2L2+LYC+H_2O_2 treatment increased NFE2L2 expression compared with the si-NFE2L2+H_2O_2 treatment ($P < 0.05$), but the increase in expression was still lower than observed with the LYC+H_2O_2 treatment (P

< 0.01). The mRNA expression of HMOX-1 followed a similar pattern as NFE2L2. The expression was increased with LYC compared with control and all other treatments ($P < 0.05$; Figure 5B). The addition of LYC+H_2O_2 increased HMOX-1 expression compared with treatment with H_2O_2 alone ($P < 0.01$). However, when cells were exposed to the si-NFE2L2+LYC+H_2O_2 treatment, the mRNA expression of HMOX-1 was significantly lower than expression with the LYC+H_2O_2 treatment ($P < 0.01$), and was not different from the expression observed with the si-NFE2L2+H_2O_2 treatment ($P = 0.26$). For NQO1, the mRNA expression was greatest in cells treated with LYC compared with control and all other treatments ($P < 0.01$; Figure 5C). When cells were exposed to the si-NFE2L2+LYC+H_2O_2 treatment, NQO1 expression was significantly lower than expression with LYC+H_2O_2 ($P < 0.01$), and NQO1 expression tended to be altered compared with the si-NFE2L2+ H_2O_2 treatment ($P < 0.05$). A significant LYC+H_2O_2 interaction was observed for the mRNA expression of NQO1 ($P < 0.01$), expression was greater with LYC+H_2O_2 treatment compared with H_2O_2 alone ($P < 0.01$). A si-NFE2L2+LYC interaction was also observed for NQO1 where si-NFE2L2+LYC tended to have a same expression as si-NFE2L2 alone, but was lower than LYC alone ($P < 0.01$). Additionally, there was a tendency for si-NFE2L2+H_2O_2 interaction for the mRNA expression of NQO1 where the addition of si-NFE2L2+H_2O_2 significantly reduced the expression of NQO1 compared with addition of H_2O_2 alone ($P < 0.05$). These results further suggested that the positive effect of LYC on the mRNA abundance of NFE2L2 and downstream gene pathway was eliminated by silencing NFE2L2.

LYC attenuated H_2O_2-induced inflammatory responses after si-NFE2L2 Interference

Triple interactions among si-NFE2L2, LYC and H_2O_2 were observed for the mRNA abundance of TNF-α ($P < 0.01$; Figure 6A) and IL-1β ($P < 0.01$; Figure 6B). The results revealed that the treatment with H_2O_2 alone, si-NFE2L2+H_2O_2, si-NFE2L2+LYC and si-NFE2L2+LYC+H_2O_2 increased the mRNA abundance of TNF-α and IL-1β significantly compared with all other treatments ($P < 0.05$), whereas the treatment with si-NFE2L2+H_2O_2 and si-NFE2L2+LYC+H_2O_2 significantly increased the mRNA abundance of TNF-α and IL-1β compared with the treatment with H_2O_2 alone and si-NFE2L2+LYC ($P < 0.05$). Furthermore, the mRNA abundance of TNF-α, and IL-1β were reduced by LYC treatment compared with that with si-NFE2L2 and LYC+H_2O_2 treatment ($P < 0.05$). However, the mRNA abundance of TNF-α and IL-1β were greatly increased in the si-NFE2L2+H_2O_2 and si-NFE2L2+LYC+H_2O_2 treatment compared with si-NFE2L2 and LYC+H_2O_2 treatments ($P < 0.01$), suggesting that the decrease in the mRNA abundance of TNF-α, and IL-1β detected with LYC was eliminated by knocking-out NFE2L2.



(A) The mRNA expression of Tumor necrosis factor-α (TNF-α). (B) The mRNA expression of Interleukin-1 β (IL-1β). The data of the control group were used to normalize the data of each treatment group. The mRNA expression of TNF-α and IL-1β was analyzed by quantitative reverse-transcription PCR. Means with different letters (a–e) differ ($P < 0.05$). Five replications were done for each experiment and each well (n=5). Values are means, with SE represented by vertical error bars.

Figure 6: Silence of nuclear factor erythroid 2 like 2 (NFE2L2) attenuated the negative effects of lycopene (LYC) on hydrogen peroxide (H_2O_2)-induced inflammatory factors. The bovine mammary epithelial cells (BMECs) were transfected with NFE2L2-small interfering RNA (siRNA) for 48 h, pretreated with or without 2 μM of LYC for 12 h, and then treated with or without 600 μM of H_2O_2 for 6 h

LYC attenuated H_2O_2-induced apoptosis after si-NFE2L2 Interference

Triple interactions among si-NFE2L2, LYC and H_2O_2 were observed for the cells apoptosis ($P < 0.01$; Figure 7). The results suggested that the treatment with si-NFE2L2+H_2O_2, si-NFE2L2+LYC and si-NFE2L2+LYC+H_2O_2 increased the BMECs apoptosis rate significantly compared with all other treatments ($P < 0.05$), whereas the treatment with si-NFE2L2+H_2O_2 and si-NFE2L2+LYC+H_2O_2 significantly increased the cells apoptosis compared with the si-NFE2L2+LYC treatment ($P < 0.05$). In contrast, the cells apoptosis rate were greatly reduced by LYC treatment compared with si-NFE2L2 ($P < 0.05$), H_2O_2 alone ($P < 0.01$) and LYC+ H_2O_2 treatments ($P < 0.05$), and the cells apoptosis reduced by LYC treatment was the same level as control culture. These results further suggested that the negative effect of LYC on the cells apoptosis rate was eliminated by silencing NFE2L2.

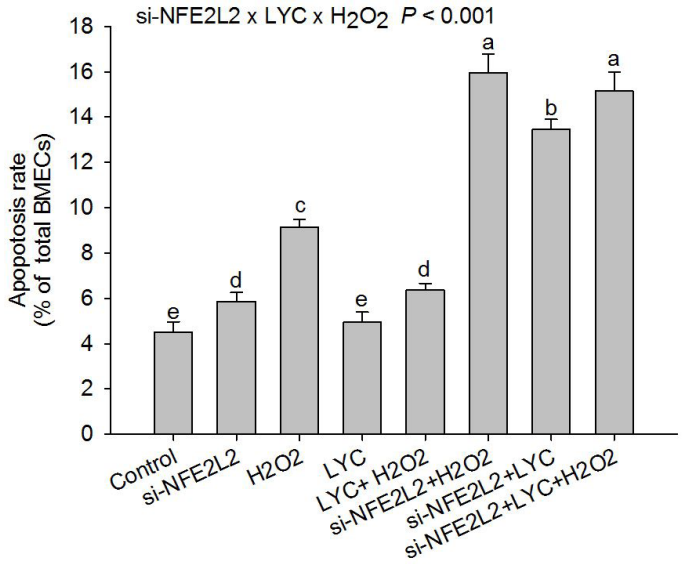


Figure 7: Silence of nuclear factor erythroid 2 like 2 (NFE2L2) attenuated the negative effects of lycopene (LYC) on hydrogen peroxide (H_2O_2)-induced apoptosis rate. The bovine mammary epithelial cells (BMECs) were transfected with NFE2L2- small interfering RNA (siRNA) for 48 h, pretreated with or without 2 μM of LYC for 12 h, and then treated with or without 600 μM of H_2O_2 for 6 h. The data of the control group were used to normalize the data of each treatment group. Means with different letters (a–e) differ ($P < 0.05$). Five replications were done for each experiment and each well (n=5). Values are means, with SE represented by vertical error bars

Discussion

The transition period is particularly important for health and subsequent performance of dairy cows, which are exposed to drastic physiological changes and metabolic stress [28]. Metabolic stress and physiological changes experienced by dairy cows after delivery will produce excessive ROS. As the first defense barrier after lactation initiation, BMECs will also experience oxidative stress, and long-term oxidative stress will lead to inflammatory response and apoptosis of BMECs. Thus, it is very important to supplement antioxidants for enhancing the antioxidant ability of cells and eliminating excess ROS during perinatal period. Lycopene, a potent antioxidant and free radical scavenger, can protect lipids, proteins and DNA against being oxidized by blocking the damage from ROS. Lycopene can ameliorate oxidative stress and inflammatory cytokine production by scavenging oxidative stress biomarkers, including malondialdehyde (MDA) and ROS, and blocking the activation of inflammatory factors, such as tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), and C-reactive protein (CRP) [26,29]. In the present study, we revealed that LYC could protect BMECs against oxidative stress. Exogenous LYC activates NFE2L2/HMOX-1 pathway in part by upregulating the steady-state level of NFE2L2 protein and mRNA, and the level of HMOX-1 mRNA. These results are consistent with *in vitro* observations that lycopene, a carotenoid and carotenoid pigment, can enhance NFE2L2 transcription activity, upregulate the mRNA and protein expression of NFE2L2, and increase the expression of HMOX-1 and NQO1 [26]. In summary, these effects of LYC might coordinate the antioxidant response when BMECs are challenged with ROS including H_2O_2. These results indicated that LYC may be a potential therapeutic agent for ruminants to resist oxidant damage.

NFE2L2 plays a key role in cytoprotection by detoxifying and eliminating ROS and MDA, NFE2L2 also mediates antioxidant responses by activating the downstream phase-II detoxifying genes such as HOMX-1 and NQO1 [13]. Our previous studies revealed that activation of NFE2L2 expression enhanced BMECs survival rate under oxidative stress [13,14,30], and NFE2L2 activation was central to the upregulation of antioxidant enzyme expression [14,31]. The present results are consistent with those findings and further underscore the antioxidant function of LYC for suppressing oxidative stress-induced injury by activating the NFE2L2/HMOX-1 pathway [26, 32-34].

A central role for LYC and the NFE2L2 and downstream genes (HMOX-1 and NQO1) in the antioxidant response was confirmed when silencing of NFE2L2 led to a strong inhibition of the antioxidant function of LYC (i.e., the survival rate of BMECs, the mRNA expression of NFE2L2 and downstream genes were decreased while ROS generation was increased). The lack of change in BMECs survival rate, HMOX-1 and NQO1 mRNA expression, and ROS generation when NFE2L2 was silenced with or without LYC along with the failure of LYC to decrease ROS production when NFE2L2 was silenced under oxidative stress status further indicated that the NFE2L2 pathway was very important for LYC to protect BMECs against oxidative stress induced by H_2O_2. That is to say, silencing of NFE2L2 failed to activate the antioxidant functions of LYC. Taken together, these data illustrated clearly that NFE2L2 was a very important factor of the antioxidant response induced by LYC. Further research will have to be performed to evaluate these mechanisms *in vivo*.

Furthermore, our data revealed that excess generation of ROS caused by the mammary glands breaks mammary epithelial cells antioxidant systems, thereby contributing to BMECs oxidative injury and initiating pro-inflammatory signaling such as NF-κB pathway as well as diverse cytokines [26], and increasing BMECs apoptosis [13,26]. In addition, inhibition of an antioxidant enzyme like HMOX-1 and NQO1 further aggravated H_2O_2-induced inflammatory response and apoptosis of mammary epithelial cells [26]. The role for LYC and NFE2L2 pathway was confirmed again when knocking-out of NFE2L2 led to a strong inhibition of the antioxidant function of LYC, i.e., increased ROS generation, significantly increased the mRNA expression of TNF-α and IL-1β and apoptosis rate of BMECs. The failure of LYC to decrease the mRNA expression of TNF-α and IL-1β, and apoptosis rate of BMECs when NFE2L2 was silenced along with the failure of LYC to decrease the mRNA expression of TNF-α and IL-1β, and apoptosis rate of BMECs when NFE2L2 was silenced under oxidative stress status further indicated that the NFE2L2 pathway is very important for LYC to protect BMECs against inflammatory response and apoptosis of cells induced by H_2O_2. In other words, knocking-out of NFE2L2 failed to activate the anti-inflammation and anti-apoptosis functions of LYC. Taken together, these data further illustrated that NFE2L2 was a very important factor of the antioxidant response induced by LYC. Further research will have to be performed to evaluate these mechanisms *in vivo*. From a mechanistic standpoint, and similar to nonruminant and ruminant studies, the data indicate that LYC pretreatment elicits an antioxidant response via increasing the activation of NFE2L2/HMOX-1 pathway as our previous concluded [14,26,35]. 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 LYC via dietary supplementation.

Overall, LYC showed not only the modulation of redox balance by activating NFE2L2/HMOX-1 pathway expression, but also the anti-inflammatory effects which are presumably secondary to its regulation of the release of some endogenous inflammatory endocoids namely, TNF-α and IL-1β, which together can prevent possible oxidative stress-induced apoptosis of mammary epithelial cells in mammary tissue. Two mechanisms possibly concerted the protective role of H_2O_2-induced mammary inflammation or apoptosis. In conclusion, our studies provide some clue that LYC may have a potential in either affording antioxidative defense system or suppressing BMECs inflammation in the diseased mammary gland. However, a clinical trial is needed to confirm these findings in dairy cows with mastitis induced by oxidative stress.

Conclusion

Lycopene *in vitro* can exert antioxidant function by activating NFE2L2/HMOX-1 pathway. The fact that silencing of NFE2L2 failed to activate antioxidant, anti-inflammatory and anti-apoptosis mechanisms, even when LYC was added, underscored its importance in the overall control of oxidative stress, inflammatory response and apoptosis. Hence, the control of NFE2L2 and downstream target genes and proteins constitute important mechanisms for LYC action against oxidative stress, inflammatory response and apoptosis.

Acknowledgment

This work was supported by the Agricultural & Animal Husbandry Science and Technology Innovation Foundation of Inner Mongolia (No. 2019CXJJM06) and Natural Science Foundation of Inner Mongolia (no. 2019MS03033).

Reference

1. Sordillo LM, Aitken SL (2009) Impact of oxidative stress on the health and immune function of dairy cattle. Vet Immunol Immunopathol 128: 104-9.

2. Zhou Z, Bulgari O, Vailati-Riboni M, Trevisi E, Ballou MA, et al. (2016) Rumen-protected methionine compared with rumen-protected choline improves immunometabolic status in dairy cows during the peripartal period. J Dairy Sci 99: 8956-69.

3. Bertoni G, Trevisi E, Han X, Bionaz M (2008) Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. J Dairy Sci 91: 3300-10.

4. Osorio JS, Trevisi E, Ji P, Drackley JK, Luchini D (2014) Biomarkers of inflammation, metabolism, and oxidative stress in blood, liver, and milk reveal a better immunometabolic status in peripartal cows supplemented with Smartamine M or MetaSmart. J Dairy Sci 97: 7437-50.

5. Porcherie A, Cunha P, Trotereau A, Roussel P, Gilbert FB, Rainard P, and Germon P (2012) Repertoire of Escherichia coli agonists sensed by innate immunety receptors of the bovine udder and mammary epithelial cells. Vet Res 43: 14: 14-22.

6. Swanson KM, Stelwagen K, Dobson J, Henderson HV, Davis SR, et al. (2009) Transcriptome profiling of Streptococcus uberis-induced mastitis reveals fundamental differences between immune gene expression in the mammary gland and in a primary cell culture model. J. Dairy Sci 92: 117–29.

7. Zhou Z, Vailati-Riboni M, Trevisi E, Drackley JK, Luchini DN, et al. (2016) Better postpartal performance in dairy cows supplemented with rumen-protected methionine compared with choline during the peripartal period. J Dairy Sci 99: 8716-32.

8. Jin X, Wang K, Liu H, Hu F, Zhao F (2016) Protection of Bovine Mammary Epithelial Cells from Hydrogen Peroxide-Induced Oxidative Cell Damage by Resveratrol. Oxid Med Cell Longev 2016: 2572175.

9. Ma Y, Zhao L, Gao M, Loor JJ (2018) Tea polyphenols protect bovine mammary epithelial cells from hydrogen peroxide-induced oxidative damage in vitro. J Anim Sci 96: 4159-72.

10. Nguyen T, Sherratt P, Pickett C (2003) Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. Annu Rev Pharmacol Toxicol 43: 233-60.

11. Denzer I, Münch G, Friedland K (2016) Modulation of mitochondrial dysfunction in neurodegenerative diseases via activation of nuclear factor erythroid-2-related factor 2 by food-derived compounds. Pharmacol. Res. 103: 80-94.

12. Itoh K, Ye P, Matsumiya T, Tanji K, Ozaki T (2015) Emerging functional cross-talk between the Keap1-Nrf2 system and mitochondria. J Clin Biochem Nutr 56: 91-7.

13. Ma YF, Wu ZH, Gao M, Loor JJ (2018) Nuclear factor erythroid 2-related factor 2 antioxidant response element pathways protect bovine mammary epithelial cells against H2O2-induced oxidative damage in vitro. J Dairy Sci 101: 5329-44.

14. Ma YF, Zhao L, Coleman DN, Gao M, Loor JJ (2019) Tea polyphenols protect bovine mammary epithelial cells from hydrogen peroxide-induced oxidative damage in vitro by activating NFE2L2/HMOX1 pathways. J Dairy Sci 102: 1658-70.

15. Mortensen A (2006) Carotenoids and other pigments as natural colorants. Pure Appl Chem 78: 1477-91.

16. Di Mascio P, Kaiser S, Sies H (1989) Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Arch Biochem Biophys 274: 532-8.

17. Colitti M, Stefanon B (2006) Effect of natural antioxidants on superoxide dismutase and glutathione peroxidase mRNA expression in leukocytes from periparturient dairy cows. Vet Res Commun 30: 19-27.

18. Bucak MN, Ataman MB, Başpınar N, Uysal O, Taşpınar M, et al. (2015) Lycopene and resveratrol improve post-thaw bull sperm parameters: sperm motility, mitochondrial activity and DNA integrity. Andrologia 47: 545-52.

19. Lee W, Ku SK, Bae JW, Bae JS (2012) Inhibitory effects of lycopene on HMGB1-mediated pro-inflammatory responses in both cellular and animal models. Food Chem Toxicol 50: 1826-33.

20. Marcotorchino J, Romier B, Gouranton E, Riollet C, Gleize B, et al. (2012) Lycopene attenuates LPS-induced TNF-α secretion in macrophages and inflammatory markers in adipocytes exposed to macrophage-conditioned media. Mol Nutr Food Res 56: 725-32.

21. Abass MA, Elkhateeb SA, Abd El-Baset SA, Kattaia AA, Mohamed EM, et al. (2016) Lycopene ameliorates atrazine-induced oxidative damage in adrenal cortex of male rats by activation of the Nrf2/HO-1 pathway. Environ Sci Pollut Res Int 23: 15262-74.

22. Kelkel M, Schumacher M, Dicato M, Diederich M (2011) Antioxidant and anti-proliferative properties of lycopene. Free Radic Res 45: 925-40.

23. Zhao Q, Yang F, Meng L, Chen D, Wang M, et al. (2019) Lycopene attenuates chronic prostatitis/chronic pelvic pain syndrome by inhibiting oxidative stress and inflammation via the interaction of NF-kappaB, MAPKs, and Nrf2 signaling pathways in rats. Andrology.

24. El-Ashmawy NE, Khedr NF, El-Bahrawy HA, Hamada OB (2018) Suppression of inducible nitric oxide synthase and tumor necrosis factor-alpha level by lycopene is comparable to methylprednisolone in acute pancreatitis. Dig Liver Dis 50: 601-7.

25. Ma YF, Wu ZH, Gao M, Loor JJ (2018) Nuclear factor erythroid 2-related factor 2-antioxidant activation through the action of ataxia telangiectasia-mutated serine/threonine kinase is essential to counteract oxidative stress in bovine mammary epithelial cells. J Dairy Sci 101: 5317-28.

26. Sun X, Jia H, Xu Q, Zhao C, Xu C (2019) Lycopene alleviates H_2O_2-induced oxidative stress, inflammation and apoptosis in bovine mammary epithelial cells via the NFE2L2 signaling pathway. Food Funct 10: 6276-85.

27. Wang M, Xu B, Wang H, Bu D, Wang J, Loor JJ (2014) Effects of Arginine concentration on the in vitro expression of Casein and mTOR pathway related genes in mammary epithelial cells from dairy cattle. PLoS One 9: e95985.

28. Drackley JK (1999) ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? J Dairy Sci 82: 2259-73.

29. Zheng Z, Yin Y, Lu R, Jiang Z (2019) Lycopene ameliorated oxidative stress and inflammation in type 2 diabetic rats. J Food Sci 84: 1194-200.

30. Duan J, Guan Y, Mu F, Guo C, Zhang E, et al. (2017) Protective effect of butin against ischemia/reperfusion-induced myocardial injury in diabetic mice: involvement of the AMPK/GSK-3beta/Nrf2 signaling pathway. Sci Rep 7: 41491.

31. Hyung JH, Ahn CB, Kim BIl, Kim K, Je JY (2016) Involvement of Nrf2-mediated heme oxygenase-1 expression in anti-inflammatory action of chitosan oligosaccharides through MAPK activation in murine macrophages. Eur J Pharmacol 793: 43-8.

32. Wu H, Kong L, Cheng Y, Zhang Z, Wang Y, et al. (2015) Metallothionein plays a prominent role in the prevention of diabetic nephropathy by sulforaphane via up-regulation of Nrf2. Free Radic Biol Med 89: 431-42.

33. Shi J, Zhang M, Zhang L, Deng H (2018) Epigallocatechin-3-gallate attenuates microcystin-LR-induced apoptosis in human umbilical vein endothelial cells through activation of the NRF2/HO-1 pathway. Environ Pollut 239: 466-72.

34. Lan A, Li W, Liu Y, Xiong Z, Zhang X, et al. (2016) Chemoprevention of oxidative stress-associated oral carcinogenesis by sulforaphane depends on NRF2 and the isothiocyanate moiety. Oncotarget 7: 53502-14.

35. Skrzydlewska E, Ostrowska J, Farbiszewski R, Michalak K (2002) Protective effect of green tea against lipid peroxidation in the rat liver, blood serum and the brain. Phytomedicine 9: 232-8.