Pakistan J. Zool., pp 1-9, 2021. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20200421040417

Lipopolysaccharide Induced Oxidative Stress and DNA Damage in Bovine Mammary Epithelial Cells

Yawang Sun, Yongjiang Wu, Jingbo Chen, Zili Wang, Juncai Chen, You Yang and Guozhong Dong*

College of Animal Science and Technology, Southwest University, No.1 Tiansheng Rd, Beibei District 400716, Chongqing, China.

Article Information
Received 21 April 2020
Revised 03 June 2020
Accepted 20 June 2020
Available online 11 September 2020

Authors' Contribution
YS and GD conceived and designed the study. YS, YW and GD performed the experimental work. YS and JC validated the date. YS and ZW analyzed the date. YS, YY were involved in date curation. YS and GD prepared the original draft of the article. YS and YW helped in the preparation of the article. GD supervised the research work.

Key words
Lipopolysaccharide, Bovine, Mammary epithelial cell, Oxidative stress, DNA damage

ABSTRACT

This study investigated oxidative stress and the consequent DNA damage induced by lipopolysaccharide (LPS) in bovine mammary epithelial cell line MAC-T cells. Cells were cultured for 48 h with different LPS levels (0, 0.1, 0.5, 2.5, 12.5, and 100 ng/mL). The results showed that cell viability was negatively correlated with LPS concentrations. The production of reactive oxygen species increased in a dose-depend manner. Cellular concentrations of oxidative damage markers were positively correlated with applied LPS concentrations. Total antioxidant capability and the activity of superoxide dismutase (SOD) increased with increasing LPS concentrations. Gene expression of antioxidants including SOD, NADPH-quinone oxidoreductase 1, and hemeoxygenase 1 were significantly increased at the LPS concentrations of 12.5 and 100 ng/mL. Both Fanconi Anemia complementation group D2 protein and Fanconi Anemia complementation group L had significantly higher gene expression at 100 ng/mL LPS level. The protein expression of phosphorylated histone 2AX showed a linear rise in the range of LPS levels from 0 to 12.5 ng/mL, then significantly declined at 100 ng/mL LPS level. Oxidative stress and oxidative damage to protein and DNA were induced by LPS treatments, although the antioxidative defense was not impaired under LPS treatments. Upregulation of the Fanconi Anemia pathway mediated genes for activating cellular DNA repair pathway alleviated DNA damage at high LPS levels.

INTRODUCTION

Lipopolysaccharide (LPS) is a component of gram-negative bacterial cell wall and it can elicit innate immune response through binding with the Toll-like receptor 4 (TLR4), resulting in activation of transcription factors, such as nuclear factor-κB (NF-κB) (Iwasaki and Medzhitov, 2004). Subsequently, inflammatory mediators like interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor-α (TNF-α) were released in an increasing amount. The inflammatory mediators, especially TNF-α, promote the production of reactive oxygen species (ROS) (Jin *et al*., 2016). Moreover, high level of LPS can directly stimulate the production of ROS in some immune cells including neutrophils and Kupffer cells (Abaker *et al*., 2017). Normally, ROS will be eliminated by the antioxidant defense system that can effectively scavenge ROS to maintain a dynamic equilibrium in organisms. However, over-production of ROS may result in oxidative stress (Sordillo and Aitken, 2009). The abundance of ROS under oxidative stress could damage major cellular biomolecules, resulting in protein modification, lipid peroxidation and DNA damage (Dröge, 2002). DNA damage includes base pair loss, mismatch, deletion or insertion as well as single- or double-strand breaks and intra- and inter-strand cross-linkage. ROS primarily induces single or double strand breaks and nucleotide modifications (Altieri *et al*., 2008). Cellular DNA damage can result in cell cycle arrest, mutation and apoptosis, depending on the extent of the damage, which has implications in carcinogenesis, genetic disorders and aging (Shukla *et al*., 2010).

The primary function of mammary epithelial cells (MEC) is to synthesize milk protein, fat and other components. MEC can also serve as immune cells with the ability to bind LPS to initiate inflammation (Porcherie *et al*., 2012). In practical dairy production, the bovine MECs (bMECs) are frequently exposed to LPS. It has been a common practice in the dairy industry that dairy cows are fed high-concentrate diets to achieve high milk yield. However, high-concentrate diet feeding can lead to subacute ruminal acidosis (SARA), defined as a ruminal pH level of lower than 5.6 for more than 3 hours daily (Gressley *et al*., 2011). Decreased ruminal pH during SARA promotes the lysis of gram-negative bacteria, resulting in a large amount of LPS release in the rumen.

* Corresponding author: gzdong@swu.edu.cn
0030-9923/2021/0001-0001 $ 9.00/0

Meanwhile, decreased ruminal pH together with increased osmotic pressure can damage the epithelial cell integrity and tight junction (Steele *et al*., 2010), and more LPS will translocate into the blood. On the other hand, in practical dairy production dairy cattle are frequently subjected to mastitis or metritis, under which conditions LPS in the blood and the mammary gland increased dramatically (Eckel and Ametaj, 2016). It was reported that the plasma LPS concentrations displayed a large variation ranging from 0.05 to 86 ng/mL under various physiological and health conditions in dairy cows (Bilal *et al*., 2016; Dong *et al*., 2014; Magata *et al*., 2017; Khafipour *et al*., 2009).

Previous studies proved that the presence of LPS in the mammary gland reduced the lactation performance in dairy cows, and the treatment of bMECs with LPS resulted in decreased expression of genes related to milk protein and milk fat synthesis (Chen *et al*., 2019; Zhang *et al*., 2019; Zhou *et al*., 2014). According to these studies, there are several causes for the impaired lactation. The LPS-induced systemic immune response competes with milk component synthesis for substrate supplies, resulting in repartition of milk component precursors away from milk secretion (Zhou *et al*., 2014). LPS inhibits the activity of key enzymes in milk fat synthesis (Khovidhunki *et al*., 2004), and even alters lipid and amino acid metabolic pathways through LPS induced DNA methylation. However, few studies have focus on the LPS provoked oxidative DNA damages in bMECs, which could induce cell dysfunction and apoptosis. Thus, the aim of this experiment was to investigate the effects of LPS on the oxidative stress and oxidative DNA damage in bMECs. In the present study, the LPS doses were designed to cover a wild range of plasma LPS concentrations in dairy cows under various health conditions.

MATERIALS AND METHODS

Cell culture and treatments

The bovine mammary epithelial cell line (MAC-T) used in this experiment was kindly provided by Professors Jianxin Liu and Hongyun Liu at the Institute of Dairy Science, Zhejiang University, China. The cells were cultured in DMEM/F12 medium (90ml, Gibco, Grand Island, NY, USA) supplemented with feta bovine serum (10ml, Gibco, Grand Island, NY, USA) and penicillin-streptomycin solution (1ml, Hyclone, Logan, UT, USA) at 37°C in a CO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA).

MAC-T cells were firstly seeded into the culture medium without LPS until 60% confluence, and then were treated with LPS (*Escherichia coli* O111:B4, Sigma, Saint Louis, MO, USA) for 48 h at the concentrations of 0 (Control), 0.1, 0.5, 2.5, 12.5, and 100 ng/mL, respectively. Culture medium was replaced every 24 h. The cells were harvested at the end of the treatment for further analysis.

Cell viability assay

Cell viability assay was carried out using the cell counting kit-8 (CCK-8) (Dojindo, Kumamoto, Japan). Based on the instruction of manufacture, 1×10^4/mL MAC-T cells were seeded into 96-well culture plates with 100 μL per well, and wells without cells were set as blanks and incubated with the same culture medium. After 48 h incubation, cells and blanks were added with 10 μL of CCK-8 into each well, and incubated for another 2 h before measuring the optical density (OD) at 450 nm using a microplate reader (Bio-Rad, xMark™, USA).

ROS detection

The production of cellular ROS was measured with ROS detection kits using the chemical fluorescence method (Jiancheng Bioengineering Institute, Nanjing, China). Briefly, 2×10^5/mL MAC-T cells were seeded into 6-well plates, and incubated for 48 h with different LPS treatments. Thereafter, culture medium was replaced with phosphate-buffered saline (PBS) containing 10 μM 2,7-dichlorofuorescin diacetate (DCFH-DA), and cells were incubated for another 30 min. Then cells were resuspended in 500 mL PBS, and cellular fluorescence was detected with a flow cytometer (BD Biosciences, SanDiego, CA, USA) using the fluorescein isothiocyanate (FITC) detection method (excitation and emission wavelength at 488 and 525 nm, respectively). The data analysis was performed using the FlowJo software.

Assay for oxidative damage markers and antioxidant activity

MAC-T cells were seeded at 2×10^5/mL in 6-well plates, and treated with different LPS levels for 48 h. After incubation, cells were washed twice with PBS, and digested with 0.25% trypsin/EDTA (Gibco, Grand Island, USA). Harvested cells were then disrupted ultrasonically on ice, and centrifuged at 2500*g* for 5 min at 4°C. The supernatant was collected and stored at -20 °C for oxidative damage markers and antioxidant activity analyses. The contents of malondialdehyde (MDA), protein carbonyl (PC), 8-hydroxy-2'-deoxyguanosine (8-OHdG), and the activities of Glutathione peroxidase (GSH-Px), catalase (CAT), superoxide dismutase (SOD), and total antioxidant capacity (T-AOC) were determined using ELISA kits (Mlbio, Shanghai, China) following the manufacturer's instructions. Cellular protein concentrations were also measured using bicinchoninic acid (BCA) protein assay kit (Sangon Biotech, Shanghai, China) to normalize the above data.

RNA isolation, cDNA synthesis, and quantitative real-time PCR (qPCR)

MAC-T cells in 6-well plates were harvested after treatment, and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. The concentration and purity of RNA were measured using a spectrophotometer (Implen, Munich, Germany). Total RNA (1μg) was prepared for reverse transcription process, and cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The incubation program consisted of 25°C for 5 min, 46°C for 20 min, and 95 °C for 1 min. The reverse transcription product was used as cDNA template in qPCR reaction, and the reaction was conducted in BIO-RAD CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA) using the Ssofast EvaGreen Supermix kit (Bio-Rad, Hercules, CA, USA). The amplification and quantification were performed with the followed cycling conditions: pre-incubation at 95 °C for 30 sec, 40 cycles of denaturation at 95 °C for 5 sec, and annealing at 60 °C for 5 sec. Melting curves were analyzed to ensure the specific amplification, and relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Primers of antioxidant genes and DNA repair genes were designed using Primer Premier 5.0 software (Table I), and synthesized by BGI Co., Ltd (Shenzhen, China). Antioxidant genes include hemeoxygenase 1 (HO-1), SOD, thioredoxin reductase 1 (TXNRD1), NADPH-quinone oxidoreductase 1 (NQO-1), and cysteine uptake transporter (XCT), and the DNA repair genes are Fanconi Anemia complementation group D2 protein (FANCD2), and Fanconi Anemia complementation group L (FANCL). The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used as a housekeeping gene.

Western blot analysis

MAC-T cells in 6-well plates were harvested after treatment, and the total cellular protein was extracted using the Tissue or Cell Total Protein Extraction Kit (Sangon Biotech, Shanghai, China) for the detection of phosphorylated histone 2AX (γH2AX) expression, which is a sensitive marker of DNA damage. Total protein concentrations were measured using the BCA protein assay kit, and 20 μg protein was separated by SDS-PAGE (SurePAGE™, Bis-Tris, Genscript, Nanjing, China) at 140V for 45 min and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Wasukesha, WI, USA). Membranes were blocked for 1 h with 5% skim milk buffer (BioFroxx, KG, Germany), and then incubated overnight at 4 °C with primary antibody against γH2AX (anti-pSer^{139}-H2AX, Enzo Life Sciences International, Plymouth, PA, USA) or GAPDH (Proteintech, Wuhan, China). Subsequently, membranes were washed 3 times with TBST (APPLYGEN,

Table I. Primer sequences used for quantitative real-time PCR.

Gene	Primer sequence	Product size (bp)	Gene Bank association no.
HO-1	F: GGCAGCAAGGTGCAAGA	221	NM_001014912.1
	R: GAAGGAAGCCAGCCAAGAG		
SOD	F: GAGGCAAAGGGAGATACAGTC	197	NM_174615.2
	R: GTCACATTGCCCAGGTCTC		
TXNRD1	F: GTGTTCACGACTCTGTCGGT	240	NM_174625.3
	R: CTGCCTTCCACGAATCACCT		
NQO-1	F: GGTGCTCATAGGGGAGTTCG	235	NM_001034535.1
	R: GGGAGTGTGCCCAATGCTAT		
XCT	F: GATACAAACGCCCAGATATGC	136	XM_002694373.2
	R: ATGATGAAGCCAATCCCTGTA		
FANCD2	F: AGTGAACACGGCAGAACAGT	152	NM_001192429.1
	R: CAGTCCCACCTACGAAGGAAA		
FANCL	F: ACCCCTGGGAATTAGGCTGA	183	NM_001076332.2
	R: TGATCAGGAATGGCACCATCA		
GAPDH	F: GGGTCATCATCTCTGCACCT	177	NM_001034034.2

HO-1, hemeoxygenase 1; SOD, superoxide dismutase; TXNRD1, thioredoxin reductase 1; NQO-1, NADPH-quinone oxidoreductase 1; XCT, cysteine uptake transporter; FANCD2, Fanconi Anemia complementation group D2 protein; FANCL, Fanconi Anemia complementation group L; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Beijing, China) and incubated at room temperature for 1 h with secondary antibody (HRP-conjugated Affinipure Goat Anti-Mouse IgG, Proteintech, Wuhan, China). Then membranes were washed 3 times with TBST, and immunoreactive protein bands on the membranes were developed with the Clarity Western ECL Substrate Reagent (Bio-Rad, Hercules, CA, USA). Finally, the membranes were visualized using the ChemiDoc™ MP System (Bio-Rad, Herculers, CA, USA), and the band density was measured using Image Lab 6.0.1 software (Bio-rad, Herculers, CA, USA).

Statistical analysis

Data were expressed as the means ± standard error (SE, n = 6), and statistical analysis was performed using SPSS 19.0 (IBM Inc., Armonk, NY, USA) and GraphPad PRISM 5.0 (Graph Pad Software, La Jolla, CA, USA). Probability value (*P*-value) of < 0.05 was considered statistically significant. One-way analysis of variance (ANOVA) followed with Duncan's multiple comparison was used to determine the statistical significance among variables of different treatments. The statistical correlation was performed using GraphPad PRISM 5.0, and *P*-value and R^2 were computed to evaluate the goodness of fit.

RESULTS

Cell viability and ROS production

Treatment of MAC-T cells with different levels of LPS (0 to 100 ng/mL) resulted in dose-dependent reduction of cell viability (Fig. 1A), and the correlation analysis showed that the cell viability was negatively correlated with LPS concentrations (Fig. 1B). On the contrary, cellular ROS production exhibited a dose-dependent rise under LPS treatments (Fig. 2).

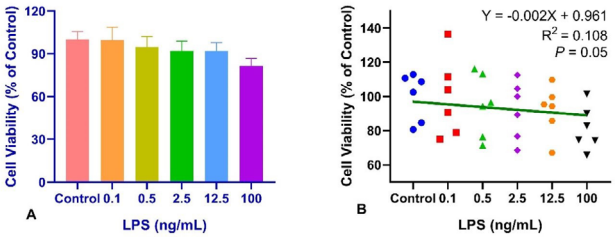

Fig. 1. Effects of different LPS levels on the cell relative viability (A) and correlation analysis between LPS levels and cell viability (B) in MAC-T cells.

Oxidative damage markers' concentrations

As shown in Figure 3 (A, C, and E), treatment with LPS at 100 ng/mL significantly increased ($P < 0.05$) the concentrations of PC and 8-OHdG, while no significant effects of LPS on the concentration of MDA were observed. There existed a significant linear correlation between LPS levels and PC and 8-OHdG concentrations (Figs. 3B, D and F).

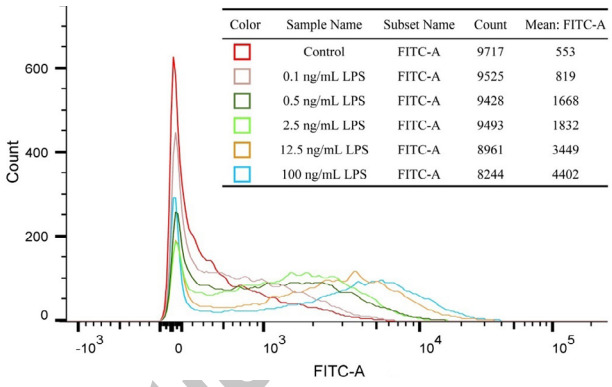

Fig. 2. Effects of different LPS levels on the cellular ROS production in MAC-T cells. The representative flow cytometric results are showed in the histogram, and the line color and its corresponding sample name, total cell count, and mean fluorescence intensity are showed in the upper right table. Mean fluorescein isothiocyanate-A (FITC-A) values represent ROS concentrations.

Activities of antioxidants

The T-AOC was significantly higher ($P < 0.05$) under treatments from 0.5 to 100 ng/mL LPS, compared with control and 0.1 ng/mL LPS treatment (Fig. 4A). The effects of LPS at different levels on the SOD activity displayed an irregular pattern with a higher SOD activity at 0.5 and 2.5 ng/mL of LPS (Fig. 4B). Meanwhile, LPS had no significant effects on GSH-Px and CAT activities.

Gene expression of antioxidants

As presented in Figure 5, the relative gene expression of HO-1 in MAC-T cells treated with 12.5 and 100 ng/mL LPS was significantly upregulated ($P < 0.05$) compared with that in control and treatments with 0.1 and 0.5 ng/mL LPS. The gene expressions of SOD and NQO-1 at 100 ng/mL LPS were significantly higher ($P < 0.05$) than at 12.5 ng/mL LPS. Treatments with LPS did not affect relative gene expression of XCT and TXNRD1.

DNA repair gene expression

Treatment with 100 ng/mL LPS significantly increased ($P < 0.05$) the relative gene expression of FANCD2 compared to control and other treatments (Figure 6A). Treatments with 0.1, 0.5 and 2.5 ng/mL LPS had significantly increased ($P < 0.05$) the relative gene expression of FANCD2 compared with the 12.5

ng/mL LPS treatment. Treatment with 100 ng/mL LPS significantly upregulated ($P < 0.05$) the relative gene expression of FANCL compare with control and other treatments (Fig. 6B).

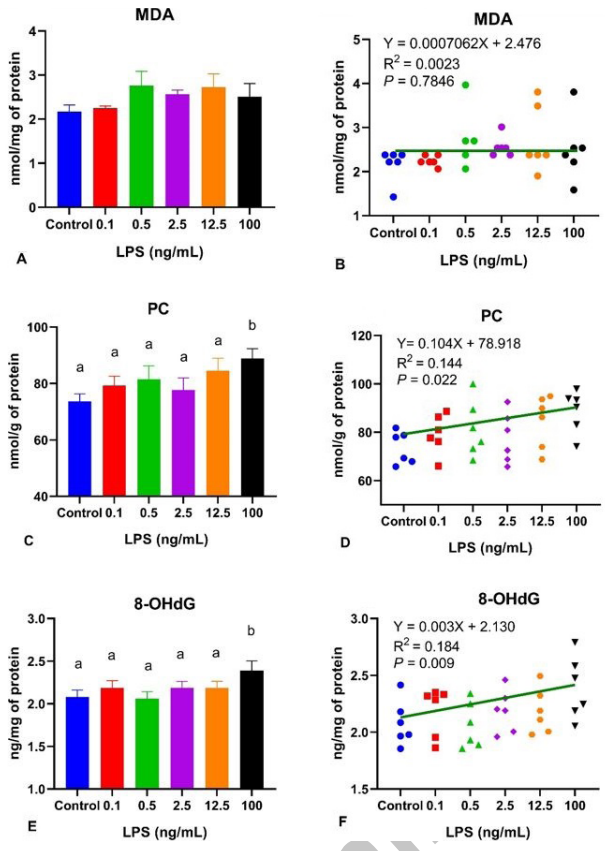

Fig. 3. Effects of different LPS levels on oxidative damage markers' concentrations (A, C, and E), and the correlation between LPS and markers' levels (B, D, and F) in MAC-T cells. Columns without a common lowercase letter (a, b) indicate significant difference among treatments ($P < 0.05$). MDA, malondialdehyde; PC, protein carbonyl; 8-OHdG, 8-hydroxy-2'-deoxyguanosine.

DNA damage marker protein expression

In MAC-T cells, γH2AX protein expression was upregulated with increasing concentrations of LPS from 0 to 12.5 ng/mL, but declined when LPS level reached 100 ng/mL (Fig. 7).

DISCUSSION

In order to evaluate the LPS induced oxidative stress and damage to major biomolecules in bMECs, we measured the cell viability, ROS production and oxidative damage markers in the present study. Although cell apoptosis plays an important role in maintaining homeostasis in organisms, excessive apoptosis stimulated by extracellular agents results in increased cell death and reduced cell viability. ROS can elicit protein misfolding and the sustained oxidative stress can initiate apoptotic cascades by activating plasma membrane death receptors (Li *et al.*, 2019). Previous studies had investigated cell apoptosis and viability in bMECs with different prooxidants (Jin *et al.*, 2016; Li *et al.*, 2019). With regard to LPS, cells treated with relatively high levels (1000 ng/mL or higher) exhibited reduction in viability and upregulated cell apoptosis-associated regulatory proteins, whereas relatively low concentrations showed no significant effects. In our study, the correlation analysis demonstrated negatively linear relationship between LPS concentrations and cell viability. Together with multi-fold increases in ROS production with increasing LPS levels, it can be seen that ROS induced cell apoptosis in a dose-depend manner, and the declined cell viability further attested to the occurrence of oxidative stress. Moreover, LPS treatments increased the yield of PC and 8-OHdG, which are common products of protein peroxidation and DNA damage (Simm and Brömme, 2005), indicating protein and DNA damage occurred during the LPS-induced oxidative stress.

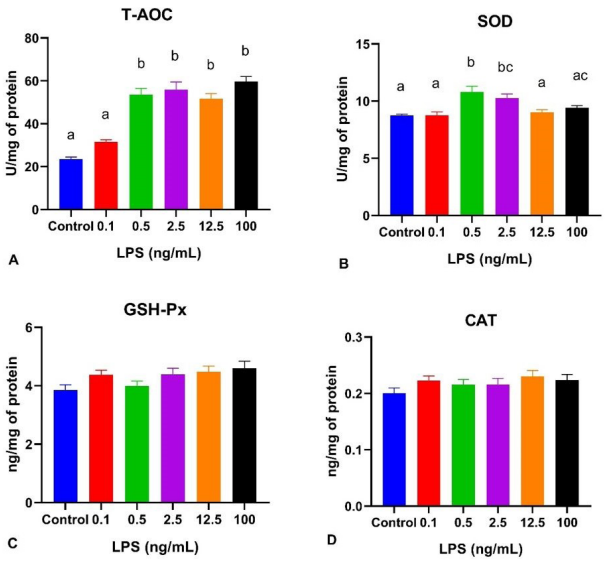

Fig. 4. Effects of different LPS levels on the activities of antioxidants in MAC-T cells. Columns without a common lowercase letter (a, b, c) indicate significant difference among treatments ($P < 0.05$). T-AOC, total antioxidant capacity; GSH-Px, glutathione peroxidase; CAT, catalase; SOD, superoxide dismutase.

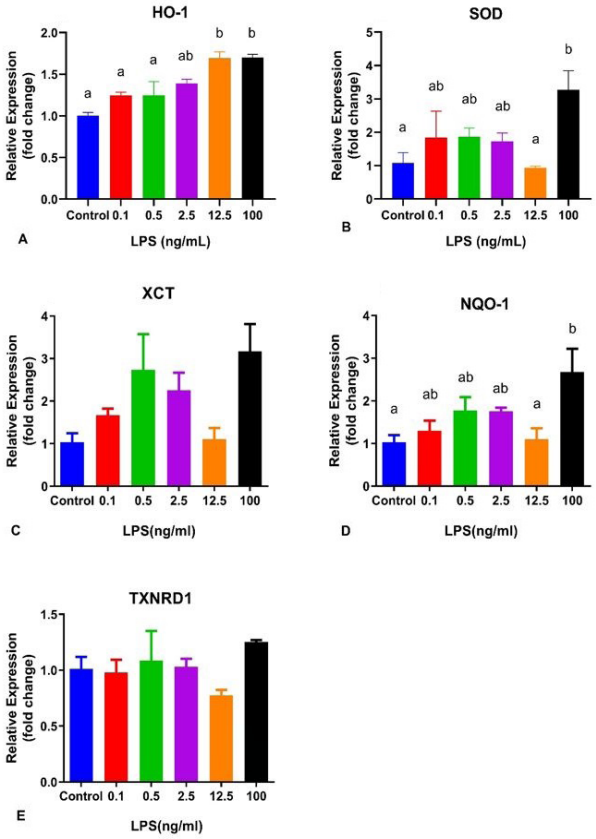

Fig. 5. Effects of different LPS levels on the relatively expression of antioxidative genes in MAC-T cells. Columns without a common lowercase letter (a, b) indicate significant difference among treatments ($P < 0.05$). HO-1, hemeoxygenase 1; SOD, superoxide dismutase; XCT, cysteine uptake transporter; NQO-1, NADPH-quinone oxidoreductase; TXNRD1, thioredoxin reductase 1.

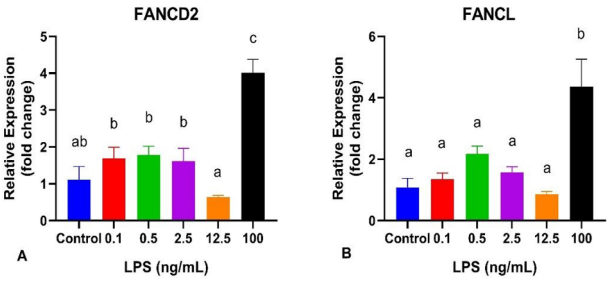

Fig. 6. Effects of LPS on the relatively expression of DNA repair genes in MAC-T cells. Columns without a common lowercase letter (a, b, c) indicate significant difference among treatments ($P < 0.05$). FANCD2, Fanconi Anemia complementation group D2 protein; FANCL, Fanconi Anemia complementation group L.

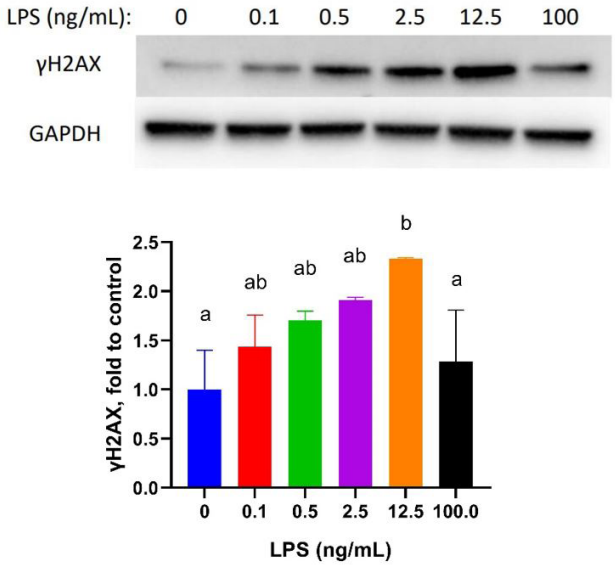

Fig. 7. Effects of different LPS levels on γH2AX protein expression in MAC-T cells. Columns without a common lowercase letter (a, b) indicate significant difference among treatments ($P < 0.05$). γH2AX, phosphorylated histone 2AX.

we also analyzed the activity and gene expression of representative antioxidants under the LPS treatments. The activity of SOD, GSH-Px and CAT, and T-AOC is important indicator of the antioxidant capacity in organisms. SOD and CAT play crucial roles in reducing high level of intracellular superoxide radicals by catalyzing their catabolism into hydrogen peroxide and water, while GSH-Px degrades reactive oxygen intermediates to prevent them from interacting with critical cellular components, such as the phospholipids of bio-membranes, nucleic acids, and proteins (Hsu *et al*., 2006; Moriya *et al*., 2000). Under SARA, increased SOD activity and reduced T-AOC, GSH-Px, and CAT activity were found in both the liver and plasma of dairy cows (Abaker *et al*., 2017). When bMECs were treated with 1000 ng/mL LPS, cellular levels of SOD, T-AOC, GSH-Px, and CAT significantly declined (Li *et al*., 2019; Zhang *et al*., 2019). HO-1, NQO-1, XCT and TXNRD1 are genes mediated by nuclear factor erythroid 2-related factor 2 (Nrf2), which is a crucial transcription factor in regulating the expression of antioxidant/detoxifying enzymes and protecting cells against oxidative-induced cytotoxicity (Cho *et al*., 2005; Jin *et al*., 2016). HO-1 and NQO-1 are major enzymes with the abilities to enhance antioxidant activity (Liang *et al*., 2013). As an important antioxidant selenoprotein in bovine, Txnrd1 can reduce oxidized cysteine groups on proteins using the redox-active center together with

NADPH (Sordillo and Aitken, 2009). XCT has the capability to regulate cell defense in maintaining redox homeostasis (Ma, 2013). Previous research evaluated HO-1 expression in different organs of mice after 4 and 24 h LPS administration, and found LPS markedly induced HO-1 mRNA expression in the liver, kidneys, and lungs (Wiesel *et al*., 2000). On the other hand, an *in vitro* study using MECs detected decreased HO-1 and Nrf2 gene expression after 12 h LPS stimulation (Yu *et al*., 2017). When continuous monitoring the gene expression in MAC-T cells within 24 h, upregulated gene expression of HO-1, XCT and Txnrd1 were observed in the first 4 h and then declined with persistent H_2O_2 inducement (Jin *et al*., 2016). In our current study, the antioxidative capability of MAC-T cell was higher under LPS treatments with increased activity of T-AOC and SOD and mRNA expression of antioxidants genes including HO-1, NQO-1 and SOD. In contrast with previous results, we speculated that the activation of antioxidative defense and oxidative damage of cellular biomolecular synchronized during the first stage of oxidative stress. However, as LPS levels increased, excessive oxidative damage to cellular biomolecules resulted in impaired antioxidative defense.

Furthermore, we explored the relationship between DNA damage and DNA repair function under LPS treatments. Fanconi anemia (FA) is a recessively inherited genetic disorder associated with severe bone marrow failure and increasing cancer susceptibility, characterized by chromosomal abnormalities and hypersensitivity to DNA crosslinking agents (Sarren *et al*., 2012). Among fifteen identified FA genes whose protein products shared a common pathway (the FA pathway), FANCD2 and FANCL are the downstream targets when the FA pathway is activated upon DNA damage (Kitao and Takata, 2011; Moldovan and D'Andrea, 2009). FANCD2 and FANCL were proved to guard genome stability by cooperating with many known repair proteins and pathways including ataxia telangiectasia mutated (ATM) pathway in nearly all phases of DNA damage response, including damage-sensing, signal transduction, and execution of DNA repair. In stressed cells, FANCD2 can also facilitate the initiation of ATM signaling upstream via its involvement in the phosphorylation of H2AX which is a substrate of ATM (Nepal *et al*., 2017). As a histone protein variant, phosphorylation of H2AX is one of the first processes initiated by DNA damage, especially in double strand breaks (Redon *et al*., 2002). Previous studies detected increased protein expression of γH2AX under short-term oxidative stress induced by H_2O_2 or cortisol (Chen *et al*., 2005; Flaherty *et al*., 2017). In our current study, γH2AX protein expression gradually increased as LPS levels increased from 0 to 12.5 ng/mL, but significantly declined when LPS level reached 100 ng/mL. The gene expression of both FANCD2 and FANCL was upregulated about 4 folds at 100 ng/mL LPS, whereas LPS at lower doses had minor effects on their gene expression. These results suggest that as a sensitive biomarker of DNA damage, phosphorylation of H2AX occurred at the early stage of oxidative stress. The rise of γH2AX due to increased DNA damage initiated DNA repair pathways as a feedback mechanism, resulting in alleviation of DNA damage.

CONCLUSION

Overall, LPS induced oxidative stress in a dose-dependent manner in bMECs. Meanwhile, LPS stimulated the antioxidative defense which was enhanced with increasing LPS levels. However, the enhanced antioxidative defense could not offset the LPS induced damage to cellular biomolecules including DNA in bMECs.

ACKNOWLEDGEMENTS

This study was sponsored by natural science foundation of Chongqing, China (Grant number: cstc2018jcyjA0834), and the National Natural Science Foundation of China (Grant number: 31672448).

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Abaker, J.A., Xu, T.L., Jin, D., Chang, G.J., Zhang, K. and Shen, X.Z., 2017. Lipopolysaccharide derived from the digestive tract provokes oxidative stress in the liver of dairy cows fed a high-grain diet. *J. Dairy Sci*., **100**: 666-678. https://doi.org/10.3168/jds.2016-10871

Altieri, F., Grillo, C., Maceroni, M. and Chichiarelli, S., 2008. DNA damage and repair: from molecular mechanisms to health implications. *Antioxid. Redox Signa.*, **10**: 891-937. https://doi.org/10.1089/ars.2007.1830

Bilal, M.S., Abaker, J.A., Aabdin, Z., Xu, T.L., Dai, H.Y., Zhang, K., Liu, XX. and Shen, X.Z., 2016. Lipopolysaccharide derived from the digestive tract triggers an inflammatory response in the uterus of mid-lactating dairy cows during SARA. *BMC Vet. Res*., **12**: 284-290. https://doi.org/10.1186/s12917-016-0907-1

Chen, J.H., Ozanne, S.E. and Hales, C.N., 2005. Heterogeneity in premature senescence by oxidative stress correlates with differential DNA

damage during the cell cycle. *DNA Repair*, **4**: 1140-1148. https://doi.org/10.1016/j.dnarep.2005.06.003

Chen, J., Wu, Y., Sun, Y., Dong X., Wang, Z., Zhang, Z., Xiao, Y. and Dong, Z., 2019. Bacterial lipopolysaccharide induced alterations of genome-wide DNA methylation and promoter methylation of lactation-related genes in bovine mammary epithelial cells. *Toxins*, **11**: 298-312. https://doi.org/10.3390/toxins11050298

Cho, H.Y., Reddy, S.P., Debiase, A., Yamamoto, M. and Kleeberger, S.R., 2005. Gene expression profiling of Nrf2-mediated protection against oxidative injury. *Free Radic. Biol. Med*., **38**: 325-343. https://doi.org/10.1016/j.freeradbiomed.2004.10.013

Dong, G., Qiu, M., Ao, C., Zhou, J., Erdene, K., Wang, X., Zhang, Z. and Yang, Y., 2014. Feeding a high-concentrate corn straw diet induced epigenetic alterations in the mammary tissue of dairy cows. *PLoS One*, **9**: e107659. https://doi.org/10.1371/journal.pone.0107659

Dröge, W., 2002. Free radicals in the radicals in the physiological control of cell function. *Physiol. Rev*., **82**: 47-95. https://doi.org/10.1152/physrev.00018.2001

Eckel, E.F. and Ametaj, B.N., 2016. Invited review: role of bacterial endotoxins in the etiopathogenesis of periparturient diseases of transition dairy cows. *J. Dairy Sci*., **99**: 5967–5990. https://doi.org/10.3168/jds.2015-10727

Flaherty, R.L., Owen, M., Fagan-Murphy, A., Intabli, H., Healy, D., Patel, A., Allen, M.C., Patel, B.A., Flint, M.S., 2017. Glucocorticoids induced production of reactive oxygen species/reactive nitrogen species and DNA damage through an iNOS mediated pathway in breast cancer. *Breast Cancer Res*., **19**: 35-47. https://doi.org/10.1186/s13058-017-0823-8

Gressley, T.F., Hall, M.B. and Aremtano, L.E., 2011. Ruminant nutrition symposium: Productivity, digestion, and health responses to hindgut acidosis in ruminants. *J. Anim. Sci*., **89**: 1120-1130. https://doi.org/10.2527/jas.2010-3460

Hsu, D.Z., Li, Y.H., Chu, P.Y., Chien, S.P., Chuang, Y.C. and Li, M.Y., 2006. Attenuation of endotoxin-induced oxidative stress and multiple organ injury by 3,4-methylenedioxyphenol in rats. *Shock*, **25**: 300-305. https://doi.org/10.1097/01.shk.0000194719.82845.39

Iwasaki, A. and Medzhitov, R., 2004. Toll-like receptor control of the adaptive immune responses. *Nat. Immunol*., **5**: 987-995. https://doi.org/10.1038/ni1112

Jin, D., Chang, G., Zhang, K., Guo, J., Xu, T. and Shen, X., 2016. Rumen-derived lipopolysaccharide enhances the expression of lingual antimicrobial peptide in mammary glands of dairy cows fed a high-concentrate diet. *BMC Vet. Res*., **12**: 128-137. https://doi.org/10.1186/s12917-016-0755-z

Jin, X., Wang, K., Liu, H., Hu, F., Zhao, F. and Liu, J., 2016. Protection of bovine mammary epithelial cells from hydrogen peroxide-induced oxidative cell damage by resveratrol. *Oxid. Med. Cell Longev*., **2016**: 2572175. https://doi.org/10.1155/2016/2572175

Khafipour, E., Krause, D.O. and Plaizier, J.C., 2009. A grain-based subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation. *J. Dairy Sci*., **92**: 1060-1070. https://doi.org/10.3168/jds.2008-1389

Khovidhunkit, W., Kim, MS., Memon, R.A., Shigenaga, J.K., Moser, A.H., Feingold, K.R. and Grunfeld, C., 2004. Effects of infection and inflammation on lipid and lipoprotein metabolism: Mechanisms and consequences to the host. *J. Lipid Res*., **45**: 1169-1196. https://doi.org/10.1194/jlr.R300019-JLR200

Kitao, H., and Takata, M., 2011. Fanconi anemia: a disorder defective in the DNA damage response. *Int. J. Hematol*., **93**: 417-424. https://doi.org/10.1007/s12185-011-0777-z

Li, L., Wang, H., Nie, X., Jiang, W. and Zhang, Y., 2019. Sodium butyrate ameliorates lipopolysaccharide-induced cow mammary epithelial cells from oxidative stress damage and apoptosis. *J. Cell Biochem*., **120**: 2370-2381. https://doi.org/10.1002/jcb.27565

Liang, L., Gao, C., Luo, M., Wang, W., Zhao, C., Zu, Y., Efferth, T. and Fu, Y., 2013. Dihydroquercetin (DHQ) induced HO-1 and NQO-1 expression against oxidative stress through the Nrf2-dependent antioxidant pathway. *J. Agric. Fd. Chem*., **61**: 2755-2761. https://doi.org/10.1021/jf304768p

Ma, Q., 2013. Role of Nrf2 in oxidative stress and toxicity. *Annu. Rev. Pharmacol*., **53**: 401-426. https://doi.org/10.1146/annurev-pharmtox-011112-140320

Magata, F., Morino, I., Teramura, M., Tsunoda, E., Kawashima, C., Haneda, S., Miyamoto, A., Kida, K. and Shimizu, Y., 2017. Impact of metritis on the generation of reactive oxygen species by circulation phagocytes and plasma lipopolysaccharide concentration in peripartum dairy cows. *Anim. Sci. J*., **88**: 248-253. https://doi.org/10.1111/asj.12642

Moldovan, G.L. and D'Andrea, A.D., 2009. How the Fanconi anemia pathway guards the genome. *Annu. Rev. Genet*., **43**: 223-249. https://doi.org/10.1146/annurev-genet-102108-134222

Moriya, S., Yokoyama, H., Fukuda, M., Okamura, Y., Kamegaya, Y., Mizukami, T., Ohgo, H. and Ishii, H., 2000. Glutathione depletion enhances the formation of superoxide anion released into hepatic sinusoids after lipopolysaccharide challenge. *Alcohol. clin. exp. Res*., **24**: 59S-63S. https://doi.org/10.1111/j.1530-0277.2000.tb00014.x

Nepal, M., Che, R., Ma, C., Zhang, J. and Fei, P., 2017. FANCD2 and DNA damage. *Int. J. mol. Sci*., **18**: 1804-1812. https://doi.org/10.3390/ijms18081804

Porcherie, A., Cunha, P., Trotereau, A., Roussel, P., Gilbert, F.B., Rainard, P. and Germon, P., 2012. Repertoire of *Escherichia coli* agonists sensed by innate immunity receptors of the bovine udder and mammary epithelial cells. *Vet. Res*., **43**: 14-21. https://doi.org/10.1186/1297-9716-43-14

Redon, C., Pilch, D., Rogakou, E., Sedelnikova, O., Newrock, K. and Bonner, W., 2002. Histone H2A variants H2AX and H2AZ. *Curr. Opin. Genet. Dev*., **12**: 162-169. https://doi.org/10.1016/S0959-437X(02)00282-4

Sareen, A., Chaudhury, I., Adams, N. and Sobeck, A., 2012. Fanconi anemia proteins FANCD2 and FANCL exhibit different DNA damage responses during S-phase. *Nucl. Acids Res*., **40**: 8425-8439. https://doi.org/10.1093/nar/gks638

Shukla, P.C., Singh, K.K., Yanagawa, B., Teoh, H. and Verma, S., 2010. DNA damage repair and vardiovascular diseases. *Can. J. Cardiol*., **26**: 13A-16A. https://doi.org/10.1016/S0828-282X(10)71055-2

Simm, A. and Brömme, H.J., 2005. Reactive oxygen species (ROS) and aging: Do we need them, can we measure them, should we block them? *Signal Transduct*., **5**: 115-125. https://doi.org/10.1002/sita.200400053

Sordillo, L.M. and Aitken, S.L., 2009. Impact of oxidative stress on the health and immune function of dairy cattle. *Vet. Immunol. Immunop*., **128**:104-109. https://doi.org/10.1016/j.vetimm.2008.10.305

Steele, M.A., Croom, J., Kahler, M., Alzahal, O. and Mcbride, B.W., 2010. Bovine rumen epithelium undergoes rapid structural adaptations during grain-induced subacute ruminal acidosis. *Am. J. Physiol-Reg. I*., **300**: R1515-1523. https://doi.org/10.1152/ajpregu.00120.2010

Wiesel, P., Patel, A.P., Difonzo, N., Marria, P.B., Sim, C.U. Pellacani, A., Maenura, K., LeBlanc, B.W., Marino, K., Doerschuk, C.M., Yet, S.F., Lee, M.E. and Perrella, M.A., 2000. Endotoxin-induced mortality is related to increased oxidative stress and end-organ dysfunction, not refractory hypotension, in heme oxygenase-1-deficient mice. *Circulation*, **102**: 3015-3022. https://doi.org/10.1161/01.CIR.102.24.3015

Yu, G.M., Kubota, H., Okita, M. and Maeda, T., 2017. The anti-inflammatory and antioxidant effects of melatonin on LPS-stimulated bovine mammary epithelial cells. *PLoS One*, **12**: e0178525. https://doi.org/10.1371/journal.pone.0178525

Zhang, B., Guo, Y., Yan, S., Guo, X., Zhao, Y. and Shi, B., 2019. The protective effect of selenium on the lipopolysaccharide-induced oxidative stress and depressed gene expression related to milk protein synthesis in bovine mammary epithelial cells. *Biol. Trace Elem. Res*., https://doi.org/10.1007/s12011-019-01961-7

Zhou, J., Dong, G., Ao, C., Zhang, S., Qiu, M., Wang, X., Wu, Y., Erdene, K., Jin, L. Lei, C. and Zhang, Z., 2014. Feeding a high-concentrate corn straw diet increased the release of endotoxin in the rumen and pro-inflammatory cytokines in the mammary gland of dairy cows. *BMC Vet. Res*., **10**: 172-180. https://doi.org/10.1186/s12917-014-0172-0