Original research article

The *in vitro* effect of lipopolysaccharide on proliferation, inflammatory factors and antioxidant enzyme activity in bovine mammary epithelial cells

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ABSTRACT

Lipopolysaccharide (LPS) was selected as a stimulus to investigate its effect on cell viability and oxidative stress in bovine mammary epithelial cells (BMEC) by detecting the cell relative growth rate (RGR), antioxidant indicators and inflammatory factors. This information was used to provide the theoretical basis for the establishment of a LPS-induced oxidative damage model. The experiment was divided into two parts. The first part used a two-factor experimental design to determine the appropriate incubation time of LPS by detecting the RGR. The third-passage BMEC were divided into 24 groups with six replicates in each group. The first factor was LPS concentration, which was 0 (control), 0.1, 1.0 and 10.0 μg/mL; the second factor was LPS incubation time (2, 4, 6, 8, 12 and 24 h). The optimum LPS incubation time was 6 h according to the results of the first part of the experiment. The second part of the experiment was conducted using a single-factor experimental design, and the third-passage cells were divided into four groups with six replicates in each group. The cells were incubated with culture medium containing different concentrations of LPS (0 [control], 0.1, 1.0 and 10.0 μg/mL) for 6 h to select the appropriate concentration of LPS to measure the antioxidant indicators and inflammatory factors. The results showed the RGR was significantly reduced as the concentration of LPS and the incubation time increased; the interaction between concentration and incubation time was also significant. The cells treated with 0.1 μg/mL of LPS for 6 h had no significant difference in the activities of glutathione peroxidase (GPx) and superoxide dismutase (SOD) ($P > 0.05$) compared with the cells in the control group. On the contrary, catalase (CAT) activity and malondialdehyde (MDA) content were markedly lower and higher, respectively, in the 0.1 μg/mL LPS-treated group for 6 h compared with the control group ($P < 0.05$). The activities of GPx, CAT and SOD in the BMEC treated with 1.0 or 10.0 μg/mL of LPS were significantly lower compared with the cells treated with 0.1 μg/mL of LPS and cells in the control group after 6 h of incubation; however, the opposite trend was detected in MDA content. There was no significant ($P > 0.05$) difference between the 10.0 and 1.0 μg/mL LPS-treated groups. Compared with the control group, interleukin-1, interleukin-6 and nitric oxide concentrations and the activity of inducible nitric oxide synthase in the 0.1 μg/mL LPS-treated group significantly increased ($P < 0.0001$), but the levels of tumour necrosis factor did not significantly change ($P > 0.05$). All of observed indicators were higher in the 1.0 and 10.0 μg/mL LPS-treated groups ($P < 0.0001$) compared with the other groups, but there was no significant ($P > 0.05$) difference between the 1.0 and 10.0 μg/mL LPS-treated groups. The results indicated that a concentration of 1.0 μg/mL of LPS and an incubation time of 6 h were the optimum conditions necessary to induce oxidative stress in the BMEC and establish a model for oxidative damage.

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1. Introduction

Oxidative damage of the bovine mammary gland increases the risk of immune dysfunction and inflammatory disease associated with udder health, milk production and milk quality. Studies have

shown high levels of reactive oxygen species (ROS), malondialde-hyde (MDA), nitric oxide (NO) and inducible nitric oxide synthase (iNOS) in serum are the main factors contributing to udder oxida-tive damage and mastitis (Yin et al., 2011; Liu, 2007). In bovines, the mammary gland tissue is an area with complex biochemical re-actions and vigorous metabolism due to its high burden of milk production. The bovine mammary epithelial cells (BMEC) are important for synthesis and secretion of milk fat and milk protein and are the first cells affected by oxidative stress (Hu et al., 2011; Wang, 2011). Therefore, BMEC are an ideal model to study oxidative stress *in vitro* and can provide information to reduce mastitis, improve the health of the udder and produce healthy and high quality milk, as well as provide the basis for future studies on the mechanisms of oxidative stress.

Recently, it has been shown a dose of vitamin A (VA) and organic selenium exceeding above the current recommended level can improve immune function and antioxidant function in dairy cows; however, the reason for this effect is still unclear (Gong et al., 2014; Jin et al., 2014a,b). Shi et al. (2015) suggested VA regulates NO production, which improves levels of antioxidants, removes spare free radicals, and prevents inflammation and oxidative stress. Vitamin A is thought to down-regulate the expression and activity of the *iNOS* gene by inhibiting tumour necrosis factor alpha (TNF-α) and cytokine interleukin (IL), which suppresses the pro-duction of NO. Shin et al. (2009) found diaryl diselenide com-pounds have strong inhibitory effects on lipopolysaccharide (LPS)-induced NO production and prostaglandin E2 (PGE2) production in RAW 264.7 macrophages; diaryl diselenide compounds also significantly reduced the mRNA levels of *iNOS*, *TNF-α*, *IL-1β*, and *IL-6*. Kim et al. (2004) demonstrated selenium attenuated LPS-induced ROS and NO production in murine macrophage cultures *in vitro* through modulation of the p38 mitogen-activated protein kinase (p38 MAPK) and nuclear factor-kappaB (NF-κB) signalling pathways. These findings suggest LPS can be used as a stimulus to induce oxidative stress damage through increased NO generation associated with the secretion of IL and TNF-α. Therefore, estab-lishing an LPS-induced oxidative stress model is necessary to un-derstand the mechanism of LPS-induced oxidative damage. Presently, studies of animal models of LPS-induced oxidative damage are widely used to study inflammation and bacterial infection (Wang and Xu, 2008); however, these studies mainly focus on rats or humans, and the results have been inconsistent. Moreover, little research is available on LPS-induced oxidative damage of BMEC.

The aim of the present study was to determine the *in vitro* ef-fects of LPS concentration and incubation time on proliferation, inflammatory factors and antioxidant enzyme activity of BMEC and to establish a LPS-induced oxidative stress model in cells to provide the theoretical basis for discussing the mechanism of cell oxidative stress.

2. Materials and methods

The experimental protocol was approved by the Animal Care and Use Committee at the Inner Mongolia Agricultural University, Hohhot, China.

2.1. Cell culture and treatments

Primary cells were isolated at a local abattoir from the mam-mary glands of 5-year-old late lactating Holstein dairy cows with a parity of 3, a body weight (BW) of 650 ± 50 kg, and a milk/d of 20.75 ± 0.50 kg according to a modified procedure described by Wellnitz and Kerr (2004) and Qi et al. (2014). Briefly, several sam-ples (approximately 1 cm^3) of the mammary gland tissue were

removed aseptically and washed with cold phosphate buffered solution (PBS) (HyClone, NWJ0467, China) containing 100 U/mL of penicillin and 100 μg/mL of streptomycin. The mammary tissue fragments were minced with sterile scissors and then digested by collagenase type II (GIBCO, 17101-015, US) at 37°C and 5% CO$_2$ for 1 h with shaking every 20 min. The digests were filtered through a 200 μm nylon mesh to remove the large tissue fragments, and the filtered liquid was centrifuged at 179 × g at room temperature for 5 min and the supernatant liquid was removed. The cell pellet was resuspended in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) (GIBCO, 12400-024) supplemented with 10% foetal bovine serum (FBS) (GIBCO, 10099-141), 0.5% insulin (GIBCO, 51500-056), 4 μg/mL of prolactin (Sigma, L6520), 1 μg/mL of hydrocortisone (Sigma, H0135), 100 U/mL of penicillin and 100 μg/mL of strepto-mycin (GIBCO) at 37°C and 5% CO$_2$. The cells were passaged twice and subsequently cryopreserved in the DMEM/F12 medium con-taining 10% FBS and 10% dimethyl sulfoxide (DMSO). The third-generation cells were used in this experiment and identified by immunofluorescence staining (Hu et al., 2009).

This experiment was divided into two parts. The first part used a two-factor experimental design in which the third generation BMEC were randomly divided into 24 treatment groups in two independent experiments of six replicates. The first factor was LPS concentration, which were 0 (control), 0.1, 1.0 and 10.0 μg/mL; the second factor LPS incubation time (2, 4, 6, 8, 12 and 24 h). The second part of the experiment was conducted using a single-factor experimental design. The cells were randomly divided into 4 groups in two independent experiments of six replicates. The first group was used as a control, and groups 2, 3 and 4 were treated with 0.1, 1.0, and 10.0 μg/mL of LPS, respectively. The control group was treated without LPS for 6 h; LPS-treated groups were treated with different concentrations of LPS for 6 h. The cells from the different treatment groups were lysed on ice for 30 min in lysis buffer (NO.80807A, Beyotime, China). The lysates were centrifuged at 1,200 × g at 4°C for 10 min to remove cell debris, and the su-pernatant was used to analyse the activity of glutathione peroxi-dase (GPx) and the concentration of malondialdehyde (MDA). The cell-free supernatant was collected for analysis of other parame-ters, including iNOS activity and the levels of NO, IL-1, IL-6 and TNF-α, to select the suitable dose of LPS for oxidative damage. The LPS incubation time (6 h) used in the second part of the experiment was chosen based on the first part of the experiment.

2.2. Working solution preparation

The LPS working solution was prepared as follows: 1 mg of LPS (sigma, L4391) was dissolved in DMEM/F12 at room temperature to a final concentration of 1 mg/mL. The resulting solution was added to the cell culture medium to obtain the desired treatment con-centrations of 0.1, 1 and 10 μg/mL and then filter sterilized before the experiments. The working solutions were stored at −4°C before use.

2.3. Cell proliferation assay

Cell proliferation was determined by methyl thiazolyl tetrazo-lium (MTT) assay. The data were obtained from three independent experiments of six replicates. The cells were distributed into 96-well plates at 1 × 10^4 cells/well. The LPS was added to the cells according to the experimental design. Briefly, after 6 h of incuba-tion, 20 μL of MTT (5 mg/mL in 1 × PBS) was added to each well and incubated at 37°C for 4 h. Next, the formazan crystals in each well were dissolved in 100 μL DMSO for 10 min with shaking. The absorbance at 490 nm in each well was recorded immediately using an ELISA microplate reader (Bio Tek, USA). The higher absorbance

values indicated more cell proliferation. The cell proliferation was expressed as a cell relative growth rate (RGR); RGR (%) = [OD_{490} (treatment group)/OD_{490} (control group)] × 100.

2.4. Inflammatory factors and antioxidant parameter analysis

The activity of GPx in cells was measured using a commercial colorimetric assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The concentrations of CAT, SOD and MDA in cells were measured using a colorimetric method, xanthine oxidase method and thiobarbituric acid method, respectively, according to the manufacturer's instruction for the three different commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Inducible nitric oxide synthase and NO were measured using an ELISA kit according to the manufacturer's instructions (R&D Systems, USA). The concentrations of IL-1, IL-6 and TNF-α were measured using an ELISA kit according to the manufacturer's instructions (R&D Systems, USA). The absorbance values were read using the STAT FAX 2100 automatic enzyme standard instrument (Awareness Technology, Inc. USA).

2.5. Statistical analysis

Data were analysed using an analysis of variance (ANOVA) with SAS software (SAS Version 9.0) to test the significance followed by Duncan's test for multiple comparisons. A value of $P < 0.05$ was considered to be significant, whereas the differences were considered to be a statistical trend when $0.05 < P < 0.10$.

3. Results

3.1. The effect of LPS on cell morphology

The results showed the cells had a uniform size and the characteristic cobblestone morphology or slender shape in the control group (Fig. 1A). Compared with the control group, the BMEC morphology had no significant changes in the 0.1 μg/mL LPS-treated group (Fig. 1B). As shown in Fig. 1C, in the 1.0 μg/mL LPS-treated group the cell–cell contact region was bigger and the cells had irregular cell edges with sharp edges or even rupture in some of the cells. In addition, the number of exfoliated cells increased. When the LPS concentration reached 10.0 μg/mL, the cell number decreased significantly and the number of dead and exfoliated cells increased significantly (Fig. 1D). These results show LPS damages the cellular morphology in a dose-dependent manner.

3.2. The effect of LPS on cell proliferation

As shown in Table 1, there was a negative effect on RGR with the different concentrations of LPS. The RGR in the 1.0 and 10.0 μg/mL LPS-treated groups was significantly lower (77.98% and 70.35%, respectively) compared with the 0 and 0.1 μg/mL LPS-treated groups ($P < 0.05$). The MTT assay revealed the lower concentrations of LPS (0 and 0.1 μg/mL) had a lower percentage of cell death (77.98% and 70.35%, respectively) compared with the 1.0 and 10.0 μg/mL LPS-treated groups ($P < 0.05$). The effect of LPS incubation time (2, 4, 6, 8, 12 and 24 h) on RGR showed a time-dependent significant negative effect (Table 1). Specifically, incubation for 12 or 24 h showed a RGR of 79.70% and 74.67%, respectively, which was significantly lower compared with the other incubation times ($P < 0.05$). We observed a significant interaction between the LPS dose and incubation time ($P < 0.05$). The addition of 0.1 μg/mL of LPS for 24 h, 1 μg/mL of LPS for 6 or 8 h, and 10 μg/mL of LPS for 6 h reduced RGR between 70% and 80% (73.31%, 78.33%,
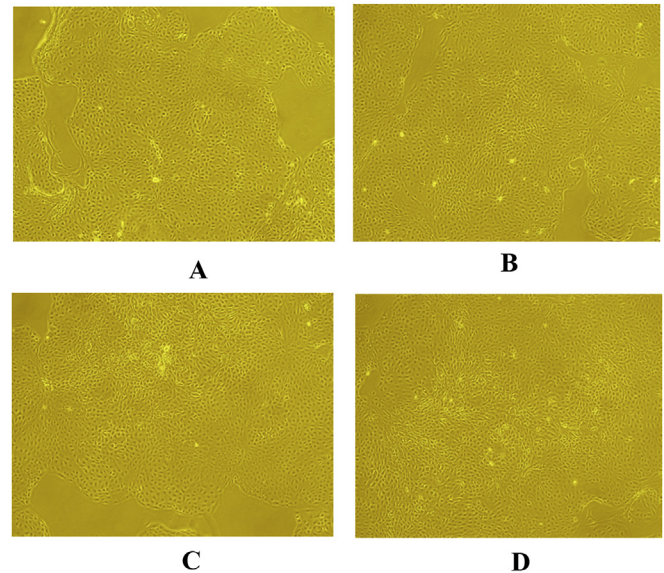
A：0 μg/mL，6 h;
B：0.1 μg/mL，6 h;
C：1.0 μg/mL，6 h;
D：10.0 μg/mL，6 h

Fig. 1. Morphological observation of bovine mammary epithelial cells (BMEC) stimulated by different concentrations of lipopolysaccharide (LPS) (×100).

70.94% and 72.06%, respectively). The cell RGR in other combinations, including 1.0 μg/mL of LPS for 12 or 24 h, 10.0 μg/mL of LPS for 8, 12 or 24 h were notably lower compared with the groups mentioned above ($P < 0.05$).

3.3. The effect of LPS on antioxidant indicators and MDA

The results from Table 2 shown there was no significant difference in the enzyme activity of GPx or SOD between the 0.1 μg/mL LPS-treated group and the control group ($P > 0.05$). In contrast, the enzyme activity of CAT was significantly lower and the level of MDA was significantly higher compared with the control group ($P < 0.05$). As compared with the 0.1 μg/mL LPS-treated group and the control group, the enzyme activities of GPx, CAT and SOD were significantly lower in 1.0 and 10.0 μg/mL LPS-treated groups. However, the opposite results were observed for the MDA levels. There was no significant ($P > 0.05$) difference in these measures between the 10.0 and 1.0 μg/mL LPS-treated groups.

3.4. The effect of LPS on inflammatory factors

Table 3 shows 0.1 μg/mL of LPS significantly increased the levels of IL-1, IL-6 and NO, and the activity of iNOS compared with the control group ($P < 0.0001$); however, there was no significant difference in the level of TNF-α ($P > 0.05$). After 6 h of incubation, the production of IL-1, IL-6 and NO, and the enzyme activity of iNOS in the 1.0 or 10.0 μg/mL LPS-treated groups were significantly higher compared with the control group and the 0.1 μg/mL LPS-treated group ($P < 0.01$). However, there were no significant differences in these measures between the 1.0 and 10.0 μg/mL LPS-treated groups.

4. Discussion

The mammary gland has complex biochemical reactions and a vigorous metabolism. Due to their high lactation, high producing

Table 1
Effects of concentration and action times of lipopolysaccharide (LPS) on relative growth rate (RGR) (%) of bovine mammary epithelial cells.

Item	LPS concentrations, μg/mL				Action time effect
	0	0.1	1.0	10	
Action time, h					
2	100[a]	98.13[ab]	95.51[abc]	90.99[cde]	96.53[A]
4	100[a]	93.64[bcd]	88.32[ef]	84.07[fg]	92.57[B]
6	100[a]	90.70[de]	78.33[h]	72.06[ij]	86.91[C]
8	100[a]	86.53[ef]	70.94[ij]	65.16[k]	82.40[D]
12	100[a]	80.19[gh]	68.08[jk]	60.39[l]	79.70[E]
24	100[a]	73.31[i]	59.44[l]	49.45[m]	74.67[F]
SEM	0.013				
Concentration effect	100[A]	87.55[B]	77.98[C]	70.35[D]	
P-value					
Action time	0.0001				
Concentration	0.0001				
Action time × Concentration	0.0001				

SEM = standard error of the average.
[a–m] The values in rows or columns with the same lowercase letters mean no significant interaction ($P > 0.05$), and the different lowercase letters mean significant interaction ($P < 0.05$).
[A–F] Values in the same column within different action times with the different capital letters mean significant difference ($P < 0.05$). Values in the same row within concentrations of LPS with different capital letters mean significant difference ($P < 0.05$).

Table 2
Effect of lipopolysaccharide (LPS) concentrations on antioxidant enzyme activity and superoxide dismutase (MDA) content.

LPS concentrations, μg/mL	GPx, IU/mg prot	CAT, IU/mL	SOD, IU/mL	MDA, nmol/mg prot
0	151.80[a]	1.94[a]	17.96[a]	0.69[c]
0.1	139.57[a]	1.51[b]	16.02[a]	1.53[b]
1.0	111.39[b]	0.93[c]	10.77[b]	2.37[a]
10.0	100.54[b]	0.89[c]	9.33[b]	2.82[a]
SEM	5.193	0.119	0.753	0.159
P-value	<0.0001	<0.0001	<0.0001	<0.0001

GPx = glutathione peroxidase; CAT = catalase; SOD = thioredoxinreductase1; SEM = standard error of the average.
[a,b,c] The values in the same column with the same superscript letter mean no significant difference ($P > 0.05$), while with different superscript letters mean significant difference ($P < 0.05$).

cows are more sensitive to oxidative stress and the resulting excess metabolites, including oxygen free radical and lipid peroxidation. An excess exposure to these negative metabolites may result in cell apoptosis, inflammation of the udder (Djordjevic, 2004; Valavanidis et al., 2006) and inhibition of the detoxifying capacity of antioxidants. Therefore, the susceptibility of the udder to immune dysfunction and inflammatory disease affects the health of the udder, milk production and milk quality (Sordillo et al., 2009). It is reported at least a third of dairy cows in the world suffer from mastitis and the rate of recessive mastitis is even higher; together, this incurs an economic loss as high as $35 billion a year (Giraudo et al., 1997; Wu, 2011). The incidence of recessive mastitis is 40% to 80% in China (Wu, 2011). Mastitis is listed as one of the four main diseases in cows, however, there is still no suitable solution to prevent and treat mastitis. Therefore, it is necessary to establish a model of oxidative stress in the bovine

mammary gland to study the mechanism of oxidative stress of the udder and identify antioxidant protection measures to guarantee the healthy development of the dairy industry and to improve the quality of milk production.

Lipopolysaccharide is the major constituent of the outer membrane of Gram-negative bacteria associated with pathogenic bacteria substance composition. Studies have shown LPS, a type of toxin, induces oxidative damage and has been widely used to establish animal models of inflammation and bacterial infection (Wang and Xu, 2008; Gu et al., 2008; Wang et al., 2012; Wu et al., 2012). However, these studies have been limited to experimental animals such as the murine macrophage or the rat peritoneal mesothelium cell (Kim et al., 2004; Li et al., 2011). Few models of oxidative stress have used LPS in BMEC *in vitro*. Therefore, the current experiment investigated the optimum concentration and incubation time of LPS with regards to cell RGR, antioxidant

Table 3
Effect of concentrations of lipopolysaccharide (LPS) on the inflammatory factors.

LPS concentrations, μg/mL	IL-1, ng/L	IL-6, ng/L	TNF-α, ng/L	iNOS, μmol/L	NO, μmol/L
0	86.39[c]	21.46[c]	141.75[b]	11.12[c]	63.94[c]
0.1	100.00[b]	24.03[b]	147.29[b]	12.05[b]	65.53[b]
1.0	109.67[a]	28.68[a]	155.42[a]	12.73[a]	67.12[a]
10.0	113.00[a]	29.17[a]	160.83[a]	13.07[a]	68.27[a]
SEM	2.694	0.809	1.918	0.211	0.422
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

IL-1 = interleukin-1; IL-6 = interleukin-6; TNF-α = tumour necrosis factor-alpha; iNOS = inducible nitric oxide synthase; NO = nitric oxide; SEM = standard error of the average.
[a,b,c] The values in the same column with the same superscript letter mean no significant difference ($P > 0.05$), while with different superscript letters mean significant difference ($P < 0.05$).

indicators and inflammatory factors to establish a model of oxidative stress of BMEC.

The RGR is the main indicator of cell viability and damage. It has been reported an appropriate degree of damage is crucial for establishing an oxidative stress cell model. A low survival rate of cells indicates irreversible damage and a high proliferation rate indicates less oxidative damage and a poor oxidative stress cell model (Farombi et al., 2004). Several studies suggest a cell survival rate between 70% and 80% is optimal to establish an oxidative damage cell model (Zhou, 2011). In a previous study from our lab, we established a H_2O_2-induced oxidative stress model in BMEC using the same RGR range of 70% to 80% (Jin et al., 2014a,b). The present study has shown LPS concentration and its incubation time, as well as the interaction between the two, have a significant effect on cell proliferation. The bovine mammary epithelial cells showed a trend towards a decrease in cell proliferation as the concentration of LPS and incubation time increased. We observed significant interactions between the dose and incubation time and found when cells were treated with 0.1 μg/mL of LPS for 24 h, 1.0 μg/mL of LPS for 6 h, 8 h and 10.0 μg/mL of LPS for 6 h, the RGR were 73.31%, 78.33%, 70.94% and 73.31%, respectively. Therefore, we selected 6 h as the optimum time to induce the oxidative stress model in BMECs.

The second experiment was conducted to stimulate the cells at different concentrations of LPS for 6 h to the effect of LPS on the oxidative stress indicators. There are two systems to monitor the status of various antioxidant defence mechanisms against free radicals. The antioxidant defence system of living organisms can subdivided into enzymatic antioxidants, such as SOD, CAT, and GPx, which can terminate the chain reaction of free radicals and non-enzymatic antioxidants, including vitamin A, vitamin E, vitamin C, carotenoids, coenzyme Q, and thiol compounds, etc (Wang, 2005). The antioxidant enzymes, SOD, CAT and GPx, can remove a variety of active oxygen free radicals, lipid peroxides and the peroxide of aldehyde compounds, such as MDA, to reduce the degree of oxidative damage (Hu and Niu, 2005). The most widely used assay for lipid peroxidation is MDA formation (Juranek, 2005). Therefore, the RGR, the enzyme activities of GPx, SOD, CAT, and the levels of MDA are important indicators of oxidative damage from free radicals and lipid peroxidation (Gu et al., 2008; Zhang, 2013; Khodir et al., 2016), which can also indicate if oxidative stress has occurred or not (Wang and Xu, 2008; Farombi et al., 2004; Juranek and Bezek, 2005). Shin et al. (2009) indicated selenide inhibits NO and PGE2 production, and reduces the mRNA levels of *iNOS*, *TNF-α*, *IL-1β* and *IL-6* in LPS-induced macrophages. There are data suggesting the sensitivity of macrophage membranes to LPS regulates the release of cytokines and the generation of NO, which is associated with the inflammatory response (Watters et al., 2002). Kim et al. (2004) demonstrated selenium attenuated LPS-induced ROS and NO production and suppressed the activation of the MAPK signal transduction pathway and NF-κB transcription in murine macrophage cultures *in vitro*. These studies show the relationship between oxidative damage and LPS-induced IL overproduction. Therefore, the cytokines, IL and TNF-α, are also indicators cellular oxidative damage. The present study showed the enzyme activities of GPx, CAT and SOD were significantly lower in all of the LPS-treated groups compared with the control group, but the activity of iNOS, and the concentrations of IL-6, TNF-α, IL-1α, NO were significantly higher compared with the control group in a dose-dependent manner. These results indicate LPS induced oxidative cell damage in a dose-dependent manner. Because there were no significant differences between the 1.0 and 10.0 μg/mL LPS-treated groups, the present study demonstrated 1.0 μg/mL of LPS for 6 h could induce oxidative damage in BMEC *in vitro* and the cellular RGR was 78.63%.

Li et al. (2011) found rat peritoneal mesothelial cells stimulated with 10 mg/L of LPS for 6 h induced the expression of *IL-18* and *IL-6*, and the production of MDA, leading to increased inflammation. Wang and Ai (2012) discussed the protection of gastrodine on rat adrenal pheochromocytoma (PC12) cells and successfully established an LPS-induced model of oxidative stress in PC12 cells with 200 ng/mL of LPS for l h. Liang et al. (2013) investigated the effects of the isocoumarin compound on expression of LPS-induced inflammatory molecules in human umbilical vein endothelial cells at a dose and incubation time of 50 ng/mL and 6 h, respectively. Therefore, the optimal dose or incubation time of LPS-induced oxidative stress is different for various animal or cells. In the present experiment, we successfully established an oxidative stress model using 1.0 μg/mL of LPS for 6 h in BMEC *in vitro*. Furthermore, the data presented in this study of a LPS-induced oxidative stress model in BMEC *in vitro* does not fully represent the *in vivo* action of LPS, and the results of our study warrant further validation in other animal models.

5. Conclusions

This study indicates the oxidative stress measure of RGR, MDA content and antioxidant enzyme activity, NO and inflammatory factors status can be used as biomarkers for oxidative stress in BMEC. The optimum concentration was shown to be 1.0 μg/mL of LPS with an incubation time of 6 h to induce oxidative stress in BMEC.

Acknowledgements

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