

Low-Level Laser Therapy Attenuates LPS-Induced Rats Mastitis by Inhibiting Polymorphonuclear Neutrophil Adhesion

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ABSTRACT. The aim of this study was to investigate the effects of low-level laser therapy (LLLT) on a rat model of lipopolysaccharide (LPS)-induced mastitis and its underlying molecular mechanisms. The rat model of mastitis was induced by inoculation of LPS through the canals of the mammary gland. The results showed that LPS-induced secretion of IL-1 β and IL-8 significantly decreased after LLLT (650 nm, 2.5 mW, 30 mW/cm²). LLLT also inhibited intercellular adhesion molecule-1 (ICAM-1) expression and attenuated the LPS-induced decrease of the expression of CD62L and increase of the expression of CD11b. Moreover, LLLT also suppressed LPS-induced polymorphonuclear neutrophils (PMNs) entering the alveoli of the mammary gland. The number of PMNs in the mammary alveolus and the myeloperoxidase (MPO) activity were decreased after LLLT. These results suggested that LLLT therapy is beneficial in decreasing the somatic cell count and improving milk nutritional quality in cows with an intramammary infection.

KEY WORDS: low-level laser therapy, mastitis, polymorphonuclear neutrophil, transmigration

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Mastitis is one of the most common diseases in dairy herds and results in great economic losses in the dairy industry [16]. This disease is generally caused by bacterial infections of the mammary gland [32]. A key response of the cow to infection by pathogens is neutrophil recruitment in response to inflammatory mediators, which include tumor necrosis factor alpha (TNF- α), interleukin (IL)-8, IL-1 β and IL-6 [22, 30]. The major leukocyte cell type that enters the gland during inflammation is the polymorphonuclear neutrophil (PMN). During inflammation, the major increase in somatic cells is due to the influx of PMNs into the milk to fight infection. However, PMNs can promote tissue injury and disturb mammary function, via reactive oxygen metabolite generation and granular enzyme release [27]. Therefore, inhibiting the superabundant PMN adhesion and migration is essential for treatment mastitis. CD62L and CD11b/CD18 are important classes of adhesion molecules expressed in bovine PMNs, which mediate the migration of activated circulating PMNs through the blood-milk barrier [26]. Intercellular adhesion molecule-1 (ICAM-1)-mediated firm adhesion of leukocytes to endothelial cells is a requisite step in PMN transmigration [3, 35]. Platelet/endothelial cell adhesion molecule 1 (PECAM-1) expressed in the endothelial cell plasma mem-

brane is also critically involved in the mechanism of PMN transmigration [23]. Thus, PMN extravasation into alveolar spaces is a multistep process that is regulated by these adhesion molecules.

The development of noninvasive, nontoxic and non-pollutant methods for the treatment of mastitis represents a constant concern of veterinarians worldwide. Low-level laser therapy (LLLT), which fits in this category, has been promoted for its beneficial effects on tissue healing and pain relief [3]. The use of LLLT in treating inflammations has been the focus of various researchers around the world. Zilaitis *et al.* found that it is advisable to treat increases in somatic cell count (SCC) in environmental pathogens induced mastitis with low-intensity laser rays [35]. Fenyő *et al.* found that a regular linearly polarized light treatment of the udders of cows can significantly reduce the SCC of milk and significantly increase the milk yield [14]. Beneduci *et al.* revealed that a near-infrared low-intensity laser radiation treatment caused an effective beneficial response in cows against mastitis [4]. However, the detailed mechanisms by which LLLT decreases SCC remains unclear. We hypothesized that low-level laser radiation of the mammary gland would change the PMN adhesion and migration ability, which would result in SCC reduction. Therefore, the aim of this study was to investigate the effects of LLLT on a rat model of LPS-induced mastitis and its underlying molecular mechanisms.

MATERIALS AND METHODS

Experiments were carried out in accordance with the guiding principles in the use of animals, adopted by the Chinese Association for Laboratory Animal Sciences. The study protocol was approved by the Ethics Committee on the Use and

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Care of Animals, Heilongjiang Bayi Agricultural University (Daqing, P.R. China).

Animal experimental groups and treatment: In total, 270 specific-pathogen-free Wistar rats, including 180 nonpregnant female rats (weighing 180–220 g) and 90 male rats (weighing 230–270 g), were procured from the Jilin University Animal Center. Two female rats and one male rat were housed per cage. Rodent chow and water were supplied *ad libitum*, and the rats were acclimated for 1 week prior to experimentation. When pregnancy was confirmed, the female rats were divided into 4 groups of 45 animals each. Seventy-two hr after parturition, the rats of groups A and B were infused 40 μg (shown to be the optimal dose of LPS for induction of stable mastitis in our previous studies) LPS into the inguinal mammary glands (left and right fourth glands) as described by Barham *et al.* [3]. Groups C and D were infused an equivalent sterile pyrogen-free physiological saline. LLLT was performed in groups A and C after perfusion for 1 hr and 15 min each time, twice each day, for 7 consecutive days of irradiation. An adjustable semiconductor laser with a beam diameter of 2 cm, wavelength of 650 nm, specified output power of 2.5 mW and power density of 30 mW/cm² (Harbin Institute of Technology, Harbin, P.R. China) was used as the laser source. Groups B and D were not irradiated; rats were anesthetized quickly after injection of LPS at 6 hr, 12 hr, 24 hr, 48 hr and 7 d, respectively, and the fourth pair of mammary glands were clipped as described by Plante *et al.* [28]. Finally, the mammary gland tissues were separated into 3 groups to determine ICAM-1 and PECAM-1 gene expressions and PMN number and to perform the myeloperoxidase (MPO) assay.

Cytokine assays: Blood samples were obtained from the abdominal aorta into 10 ml serum vacuum blood collection tubes (BD Biosciences, San Diego, CA, U.S.A.) for the cytokine assays. Blood samples were centrifuged at $3,000 \times g$ for 10 min to obtain serum. TNF- α , IL-1 β and IL-8 in the serum were evaluated with microplate readers (Bio-Rad, Hercules, CA, U.S.A.) and the corresponding ELISA kits according to the instructions of the manufacturer (R&D Systems, Minneapolis, MN, U.S.A.).

Determination of the percentages of CD62L- and CD11b-positive cells in blood PMNs: Blood samples were collected from the abdominal aorta into 10 ml vacuum blood collection tubes containing EDTA-2Na (BD Biosciences) for flow cytometric analysis. Purified blood PMNs were collected using Ficoll-Conray solution (Tianjin Haoyang Biological Manufacture Co., Ltd., Tianjin, P.R. China) and hypotonic red blood cell lysis solution according to the manufacturer's instructions. Triple immunofluorescent labeling was performed as described by Ozawa *et al.* [24]. Anti-rat CD62L (PE) and fluorescein isothiocyanate (FITC)-conjugated anti-rat CD11b were purchased from BioLegend (San Diego, CA, U.S.A.). Flow cytometry (BD Biosciences) experiments were performed as described by Li *et al.* [19].

Determination of ICAM-1 and PECAM-1 gene expressions in the mammary gland: Total RNA of the mammary gland was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, U.S.A.). First strand of cDNA was synthesized

with a reverse transcription kit (Takara, Dalian, China). The primer pairs for beta-actin (BACT) were 5'- TCAC-CAACTGGGACG-3' and 5'- GCATACAGGGACAACA-3', which yielded a 205-bp fragment. The primer pairs for ICAM-1 were 5'-TGTCAAACGGGAG ATGAATGGT-3' and 5'- CCTCTGGCGGTAATAGGTGTAAT-3', which yielded a 189-bp fragment. The primer pairs for PECAM-1 were 5'- ATTGCCGCCTTGA TAGTTG-3' and 5'- GTCG-TAATGGCTGTTGGT-3', which yielded a 155-bp fragment. The ICAM-1 and PECAM-1 gene expressions in the mammary gland were detected by quantitative RT-PCR (Hangzhou Bioer Technology, Hangzhou, P.R. China) analysis using a SYBR Premix Ex TaqTM II Kit (Takara), and this was performed in triplicate for each sample. The qRT-PCR experiment was set up according to the MIQE guidelines [6]. The relative expression levels for ICAM-1 and PECAM-1 were calculated relative to BACT (the normalizer) using the comparative cycle threshold method. The PCR products were submitted to a sequencing company (Huada Genetic Research Institute, Beijing, P.R. China) for sequence analysis. The sequencing results showed that the sequenced amplified fragments were identical to the sequences generated by the designed primers.

Mammary gland histopathology and PMN quantification: PMN quantification was performed by hematoxylin and eosin (HE) staining. The mammary gland samples were fixed with 4% formalin solution for 48 hr, dehydrated in ethanol and embedded in paraffin wax. Then, paraffin blocks were cut into several mammary tissues slices (5 μm thickness) using a microtome (Leica, Wetzlar, Germany) and mounted onto slides. The slices were then rehydrated with xylene, 100%, 95%, 90%, 80% and 70% alcohol and distilled water. Then the slices were stained in hematoxylin solution for 30 sec, distilled water for 1 min, eosin solution for 10 sec and distilled water for 1 min, respectively. The slices were then dehydrated with 70%, 80%, 90%, 95% and 100% alcohol and xylene. They were then mounted in gum for permanent cover slips. The pathological changes in the mammary glands were observed under a light microscope. The PMN stains neutral pink and contains a nucleus divided into 2–5 lobes.

MPO assay: MPO activity indirectly represents the parenchymal infiltration of PMNs. The quantitative supernatant from the tissue homogenates was used for the MPO assay, according to the manufacturer's protocols for the MPO kit (Jiancheng Bioengineering Institute of Nanjing, Nanjing, P.R. China). Briefly, the mammary tissues were weighed and then homogenized in PBS according to a previous study [12]. Then, the samples were sonicated 3 times for 15 sec on ice and centrifuged at 16,000 g for 30 min at 4°C. Protein concentrations were determined with a BCA protein assay (Beyotime Co., Nantong, P.R. China). MPO activity was determined in 100 μl of supernatants in duplicate using development reagent. Activity was measured over 25 sec at 450 nm. Development reagent without a sample was used as a control. MPO activity was expressed as the change in absorbance per milligram of protein.

Statistical analyses: Results were expressed as means \pm

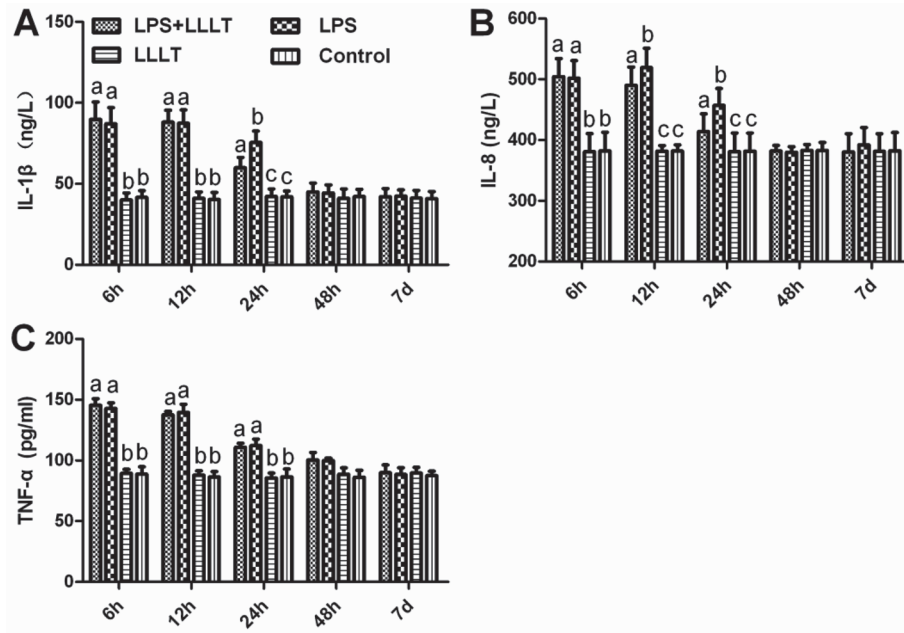


Fig. 1. Effect of LLLT on cytokine concentration. The levels of IL-1 β , IL-8 and TNF- α in serum (n=8 in each group) were detected by ELISA. A: effect of LLLT on the IL-1 β concentration. B: effect of LLLT on the IL-8 concentration. C: effect of LLLT on the TNF- α concentration. The values are presented as means \pm SEM (n=8 in each group). Values with different superscript letters at the same time point are significantly different from one another: a versus b= P <0.05; a versus c= P <0.05; b versus c= P <0.01.

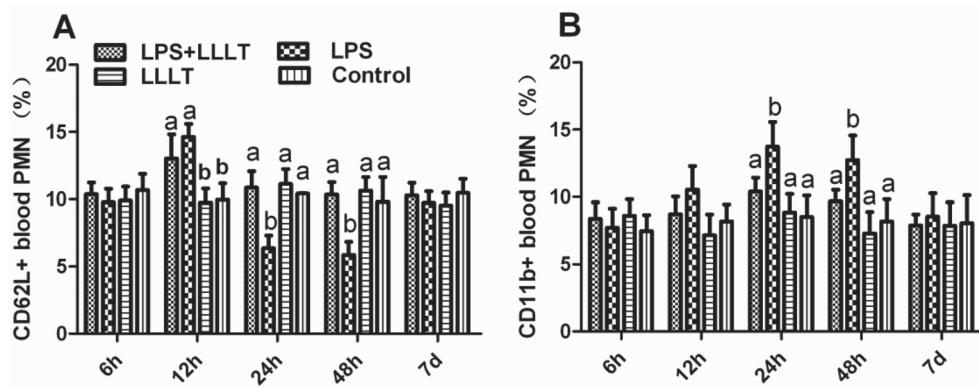


Fig. 2. Effect of LLLT on CD62L and CD11b positive cell percentages in blood PMNs. A: effect of LLLT on the CD62L positive cell percentage in blood PMNs; B: effect of LLLT on CD11b positive cell percentage in blood PMN. The values are presented as means \pm SEM (n=8 in each group). Values with different superscript letters at the same time point are significantly different from one another: a versus b= P <0.05.

SD. Data were analyzed by using the statistical software package SPSS 17.0 (SPSS Inc., Chicago, IL, U.S.A.). Groups were compared by one-way analysis of variance (ANOVA) followed by the least significant difference test. A P value of less than 0.05 was considered statistically significant, and values less than 0.01 were considered markedly significant.

RESULTS

Effect of LLLT on cytokine concentration: As shown in Fig. 1, the concentrations of IL-1 β , IL-8 and TNF- α increased significantly at 6, 12 and 24 hr after LPS infusion compared with the control group (P <0.05). LLLT decreased the IL-1 β concentration significantly at 24 hr after LPS infusion compared with group B (Fig. 1A). LLLT decreased the

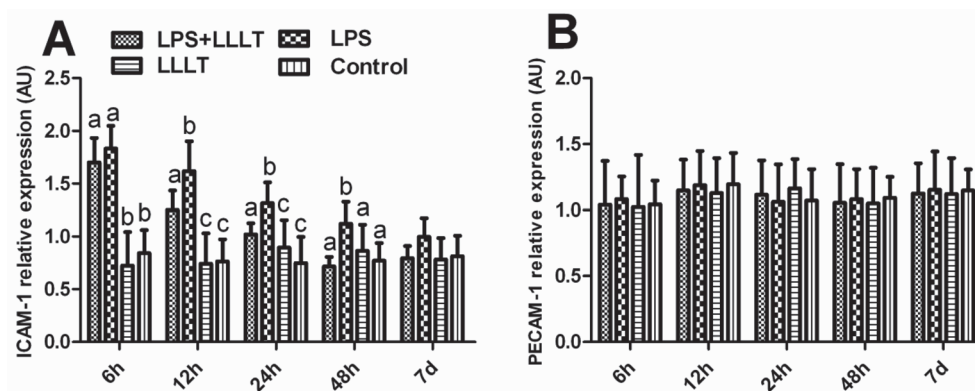


Fig. 3. Effect of LLLT on ICAM-1 and PECAM-1 gene expressions. A: effect of LLLT on ICAM-1 gene expression; B: effect of LLLT on PECAM-1 gene expression. The values are presented as means \pm SEM ($n=8$ in each group). Values with different superscript letters at the same time point are significantly different to one another: a versus b, and a versus c= $P<0.05$; b versus c= $P<0.01$.

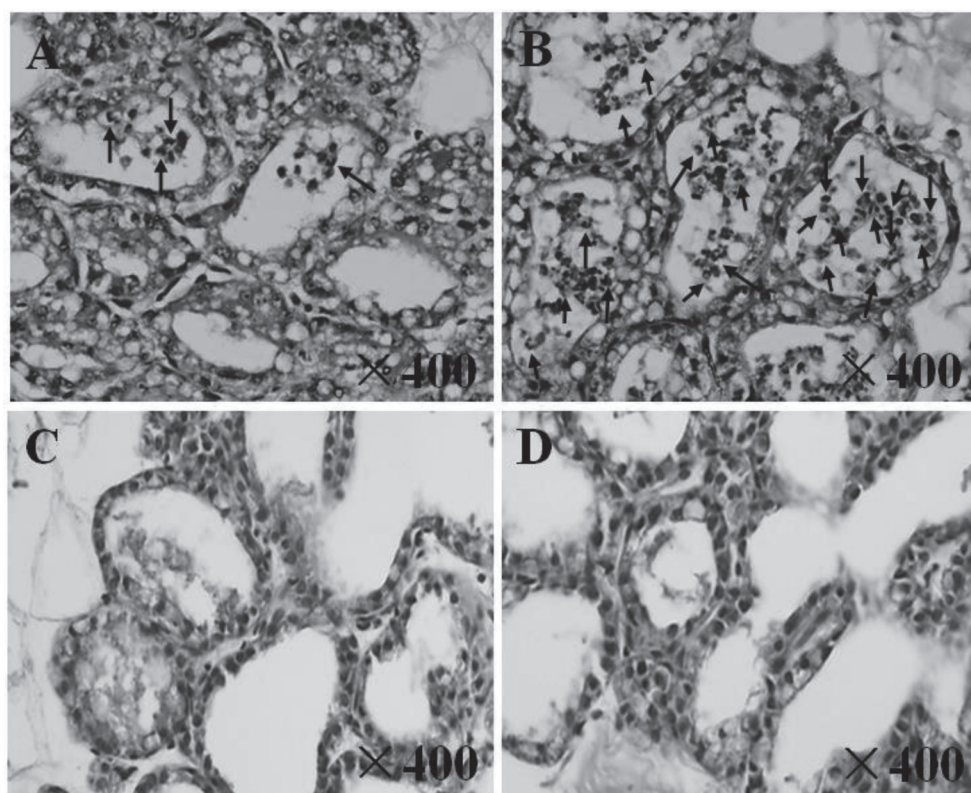


Fig. 4. Histological changes in the mammary tissues from each group (HE staining, $\times 400$). Mammary tissues ($n=8$ in each group) from each group were processed for histological evaluation at 24 hr after LPS infusion. Representative histological changes of mammary tissues from each group: control group A, LPS+LLLTT group; B, LPS group; C, LLLT group; D, control group. The black arrow indicates a representative PMN.

IL-8 concentration significantly at 12 and 24 hr after LPS infusion compared with group B ($P<0.05$, Fig. 1B). However, LLLT did not affect the LPS-induced TNF- α increase at any sampling points ($P>0.05$, Fig. 1C).

Effect of LLLT on the percentage of CD62L and CD11b positive cells in blood PMNs: As shown in Fig. 2, the percentage of CD62L⁺ blood PMNs showed an increasing trend at less than 12 hr after LPS administration and then showed a

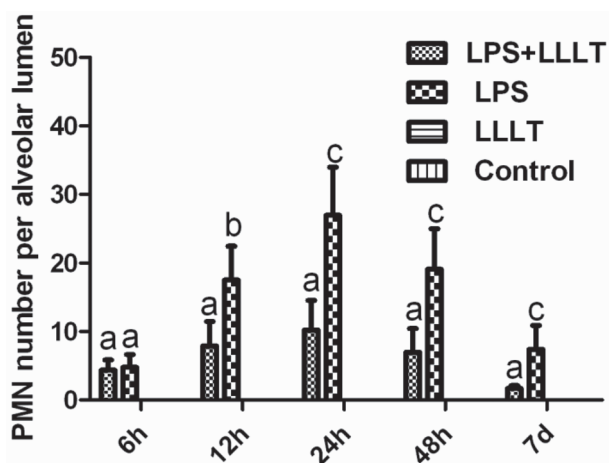


Fig. 5. Effect of LLLT on the number of PMNs in the mammary gland. The values are presented as means \pm SEM ($n=8$ in each group). Values with different superscript letters at the same time point are significantly different from one another: a versus b= $P<0.05$; a versus c= $P<0.01$.

decreasing trend later. At 7 d after LPS infusion, the percentage of CD62L⁺ blood PMNs recovered to normal (Fig. 2A). The percentage of CD62L⁺ blood PMNs showed a significant increase at 24 and 48 hr after LLLT ($P<0.05$). The percentage of CD11b⁺ blood PMNs showed an increasing trend at 24 and 48 hr after endotoxin administration ($P<0.05$) and then showed a decreasing trend after endotoxin administration 7d (Fig. 2B). LLLT decreased the percentage of CD11b⁺ blood PMNs significantly at 24 and 48 hr after endotoxin administration ($P<0.05$). The percentages of CD62L⁺ and CD11b⁺ blood PMNs remained unchanged at all sampling points in groups C and D ($P>0.05$, Fig. 2A and 2B).

Effect of LLLT on ICAM-1 and PECAM-1 gene expressions: Only the RNA samples with an A260/A280 ratio of 1.8–2.0 were chosen for analysis. Agarose gel electrophoresis revealed that the 28S:18S ratio was approximately 2:1, which indicated that the sample was high-quality RNA. Agarose gel electrophoresis revealed that all primer pairs amplified a single PCR product with the expected size. As shown in Fig. 3, the expression of ICAM-1 increased significantly at 6, 12, 24 and 48 hr after LPS infusion ($P<0.05$, Fig. 3A). The expression of PECAM-1 remained unchanged at all sampling points ($P>0.05$, Fig. 3B). LLLT decreased the expression of ICAM-1 significantly at 12, 24 and 48 hr after LPS infusion compared with group B ($P<0.05$, Fig. 3A).

Effect of LLLT on mammary gland PMN number: As shown in Fig. 4, the blank control group (group D) of mammary glands displayed no abnormal histopathological changes (Fig. 4D). LLLT had no influence on alveolar structure (Fig. 4C). After LPS infusion, the alveolar structure was severely damaged and was characterized by alveolar epithelium vacuolization and thickening of the alveolar walls (Fig. 4B). In addition, the alveolar spaces were infiltrated with inflammatory cells (Fig. 4B). However, PMN influx into the alveolar spaces was decreased after LLLT (Fig. 4A,

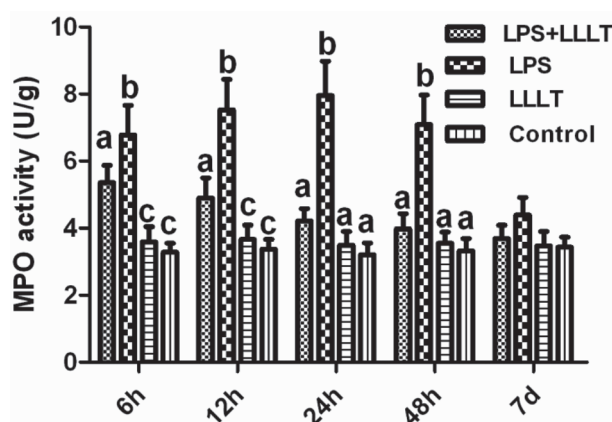


Fig. 6. Effect of LLLT on MPO activity.

black arrow), and the histopathological changes of alveolar epithelium vacuolization were also ameliorated after LLLT. The PMN number per alveolar lumen was counted. As shown in Fig. 5, there were no PMNs in the alveolar lumen in the control and LLLT treatment groups. The number of PMNs increased markedly and significantly after LPS infusion compared with the control group. LLLT decreased the number of PMNs significantly at 12 hr after LPS infusion ($P<0.05$). LLLT decreased the number of PMNs significantly at 24 hr, 48 hr and 7 d after LPS infusion compared with group B ($P<0.01$).

Effect of LLLT on MPO activity: As shown in Fig. 6, the MPO activity increased markedly and significantly at 6, 12, 24 and 48 hr after LPS infusion compared with the control group ($P<0.01$). LLLT decreased the MPO activity significantly at 6 and 12 hr after LPS infusion compared with group B ($P<0.05$). The MPO activity showed a markedly significant decreasing trend at 24 and 48 hr after endotoxin administration ($P<0.01$).

DISCUSSION

Mastitis, an inflammatory response of the mammary tissue, is a common disease in dairy herds in many countries. It can be very detrimental for dairy farm profitability, because of lost production and treatment costs. Mastitis is caused by bacterial invasion and colonization of the teat canal, and mammary pathogenic *Escherichia coli* is a leading cause of the disease in dairy cows [25]. Following detection of pathogen invasion into the mammary gland, alveolar macrophages and epithelial cells release inflammatory mediators and modulators. These agents trigger the migration of PMNs from the blood toward the alveolar lumen [29]. Activated blood PMNs were found to be cytotoxic for mammary epithelial cells, possibly via the release of extracellular reactive oxygen species, such as hydroxyl radicals [18]. Thus, inhibiting the superabundant PMN adhesion and migration is essential for treatment of mastitis.

In veterinary medicine, LLLT has been used since the mid 1980s [7]. LLLT induces mainly photochemical effects in

biological tissues. The use of LLLT in treating inflammations has been the focus of various researchers around the world. Alves *et al.* reported that LLLT decreased the expression of IL-1 β and IL-6 and neutrophils and macrophages in joint inflammation in rats induced by papain [2]. Yamaura *et al.* reported that LLLT decreased the level of pro-inflammatory cytokines/chemokines produced by rheumatoid arthritis synoviocytes [34]. Aimbire *et al.* reported that LLLT decreased pulmonary microvascular leakage, neutrophil influx and IL-1 β levels in the airway and lung of rats subjected to LPS-induced inflammation [1]. In the present study, we showed that LLLT delivered at a dose of 30 mW/cm² and wavelength of 650 nm was able to relieve LPS-induced mastitis through of a mechanism that involves reduction of the level of the pro-inflammatory cytokines IL-1 β and IL-8. Elazar reported that neutrophil recruitment into the milk spaces in LPS-induced murine mastitis was dependent on IL-1 β and IL-8 signaling [11]. Thus, it represents an important effect of LLLT on neutrophil recruitment, since it is well known that neutrophil recruitment across the alveolar epithelium is IL-1 β and IL-8 dependent.

Many studies indicate that LLLT could inhibit the expression and secretion of inflammatory cytokines, such as IL-1 β , TNF- α , prostaglandin E₂ (PGE₂) and cyclooxygenase-2 (COX-2), in the initial stage of inflammation [5]. LLLT involves a complexity of biophysical, photobiological, pathological and clinical aspects, and treatment success may be compromised by a lack of understanding of the mechanisms. However, the TNF- α , IL-1 β and IL-8 genes are regulated by nuclear factor- κ B (NF- κ B), which plays a central role in the inflammatory responses [12]. Mafra *et al.* also reported that LLLT attenuated TNF- α mRNA expression in rat bronchi segments in *E. coli* LPS-induced airway inflammation caused by a NF- κ B-dependent mechanism [21]. LPS is a major component of the outer membrane of Gram-negative bacteria, and it stimulates a host immune response upon interaction with the pattern recognition receptor toll-like receptor (TLR) expressed on host cells [19]. Thus, we hypothesize that LLLT may inhibit inflammatory cytokine gene transcription via the TLR/NF- κ B pathway. Further studies are needed to understand the exact mechanisms of LLLT inhibition of inflammatory cytokines.

PMN extravasation requires CD62L-mediated tethering, CD11b- and ICAM-1-dependent firm adhesion and PECAM-1-mediated transendothelial migration [20]. CD62L and CD11b are important classes of adhesion molecules expressed in bovine PMNs that mediate the migration of activated circulating PMNs through the blood-milk barrier. After PMN activation, CD62L molecules are rapidly shed from the cell membrane, whereas expression of CD11b is upregulated on the PMN membrane, facilitating firm contact of the PMN to the vascular endothelium followed by PMN extravasation [9]. Diez-Fraille *et al.* reported that the percentage of CD11b⁺ and CD62L⁺ blood PMNs decreased at 12 hr after intramammary administration of *E. coli* [10]. In that study, CD11b molecules were upregulated, and CD62L molecules were downregulated on blood PMNs at 24 hr after LPS infusion. Similar results were also reported by

Ozawa *et al.* [26]. Our results show that the percentage of CD62L⁺ blood PMNs showed an increasing trend at 12 hr after endotoxin administration and then showed a decreasing trend later and that the percentage of CD11b⁺ blood PMNs showed an increasing trend at 24 and 28 hr after endotoxin administration and then showed a decreasing trend later. These tendencies are consistent with the previous report. The percentage of CD11b⁺ blood PMNs showed a significant decrease after LLLT 24 and 48 hr. Activation-dependent attachment of CD11b on neutrophils to endothelial ICAM-1 is essential for firm or secondary cell adhesion to the vessel wall [15]. Therefore, these results indicate that LLLT can reduce PMN adhesion to endothelial cells. Blockade of the IL-1 β or IL-8 pathway could reduce CD11b integrin expression on leukocytes [31]. Thus, it is possible that the reduction of CD11b expression on PMNs after LLLT might be caused by the reduction of the levels of the pro-inflammatory cytokines IL-1 β and IL-8 indirectly.

The Ig superfamily cell adhesion molecules are calcium-independent transmembrane glycoproteins [17]. Five members of this family expressed on the endothelium are involved in leukocyte adhesion [8]. ICAM-1, which is expressed at a low level on unactivated endothelial cells, is upregulated in response to inflammatory mediators [33]. ICAM-1 is a counter-receptor for CD11b, and this receptor pair is responsible for the adhesion between stimulated neutrophils and stimulated endothelial cells. PMNs adhere to the endothelium via an interaction between CD11b (on the PMN) and ICAM-1 (on the endothelium) [13]. In the present study, we showed that LLLT decreased the percentage of CD11b⁺ blood PMNs significantly at 24 and 48 hr after LPS treatment and decreased the expression of ICAM-1 significantly at 12, 24 and 48 hr after LPS infusion. We hypothesize that LLLT may inhibit ICAM-1 gene transcription in the mammary gland via inhibition of the percentage of CD11b⁺ blood PMN in the smaller blood vessels in mammary gland. PECAM-1 expressed at endothelial cell junctions appears to be required for transmigration by binding homophilically to PECAM-1 expressed on leukocytes. However, the expression of PECAM-1 remained unchanged at all sampling points [24]. Thus, it is possible that the reduction in the amount of PMN influx into the alveolar spaces after LLLT is caused by the inhibition of PMN adhesion.

MPO is a marker of neutrophil function and activation that can be used as a surrogate for the level of neutrophilic inflammation and activation. In this study, LPS markedly increased the infiltration of PMNs and MPO activity. However, LLLT significantly reduced the infiltration of PMNs and MPO activity. In conclusion, LLLT significantly reduced the levels of IL-1 β and IL-8, inhibited the activation of the PMNs, reduced PMN adhesion to endothelial cells and attenuated the PMN infiltration in LPS-induced mouse mastitis. These results suggested that LLLT therapy is beneficial in decreasing the somatic cell count and improving milk nutritional quality in cows with an intramammary infection.

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