Exogenous melatonin reduces somatic cell count of milk in Holstein cows

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High somatic cell counts in milk caused by mastitis significantly influence the quality of milk and result in substantial annual economic loss. This study evaluated the beneficial effects of melatonin (MT) on milk somatic cell count (SCC) in cows. To examine the effects of melatonin on SCC, one hundred twenty cows were divided into four groups based on milk SCC. In each group, half of the cows were treated with melatonin (S.C.). Melatonin treatment significantly reduced milk SCC. To explore the potential mechanism, 20 cows with relatively high SCC were selected to evaluate the biochemical and immunological profiles of their blood after melatonin treatment. Treatment with MT significantly reduced SCC in milk, lowered serum cortisol concentrations and increased the levels of albumin, alanine transaminase and lactate dehydrogenase. Following treatment with MT, the concentration of IgG and IgM rose transiently then decreased significantly, similar to changes observed for white blood cells and lymphocytes. In conclusion, MT treatment improved the quality of milk by reducing SCC. This may be due to melatonin improving immune activity in cows.

Mastitis is the most common disease in dairy cows1. There are two major forms of this disease, including clinical and subclinical mastitis2. Clinical mastitis is easily identified by swelling and redness in the teat and udder or increased temperature in the infected mammary gland2. However, the subclinical form is difficult to detect because there are no apparent symptoms. It is mainly characterized by an increase in the somatic cell count (SCC) of milk and a decrease in milk production3. Animals are considered to have a subclinical mammary gland infection if milk contains SCC > 1 × 10^6 cells/mL4, although some studies have used a threshold of 2 × 10^6 cells/mL5. The extremely high prevalence of subclinical mastitis causes great economic loss in the dairy industry. In China, 40–80% of milking cows have subclinical mammary gland infections, and the annual loss is estimated at 135 million Yuan6. Although antibiotic therapy has a direct and rapid effect on pathogenic bacteria, the frequent use of antibiotics leads to drug-resistant bacteria. In addition, drug residue in milk has a negative impact on human health7. Therefore, alternative therapies for bovine mastitis are necessary.

Reactive oxygen species (ROS) are continuously formed because of biochemical reactions and external stimulations during the substantial metabolic and physiological adaptations cows undergo between pregnancy and lactation7. Excessive ROS production causes oxidative stress in organisms, which is a significant underlying factor in the dysfunction of host immune and inflammatory responses. Thus, oxidative stress in dairy cows inevitably leads to increased susceptibility to a variety of health disorders, particularly during the high-temperature period8–14. Melatonin (MT), a hormone mainly synthesized in the pineal gland, affects a variety of physiological processes. It is a signalling molecule in circadian rhythms, regulates reproduction for photoperiodic breeders, modulates the neuroendocrine system and provides cardiovascular protection15–18. MT is a potent free radical scavenger and antioxidant19–21. In addition to its antioxidant function, MT balances immune responses and can be an immunostimulatory or immunosuppressive agent depending on conditions. For example, during acute inflammation, it exhibits anti-inflammatory effects and suppresses exacerbated immune responses22. MT implants in goats improved milk quality and reduced SCC in milk by decreasing oxidative stress in the udder. Reports have linked high nocturnal levels of MT in cows with low milk SCC23.

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This study investigated the effect of MT administration (S.C.) on the quality of the milk produced by cows with subclinical mastitis. Analyses included SCC in milk and cellular and humoural immune responsive parameters in blood.

Results

Effects of melatonin treatment on SCC in milk. Treatment with MT (4.64 mg/cow/day for 4 days) reduced the milk SCC in all treated groups (Fig. 1), including significant differences between the relatively high SCC groups (groups 2–4) compared with their untreated counterparts. This effect lasted for a minimum of 24 days after the termination of melatonin treatment.

Effects of melatonin on plasma cortisol levels. The results of MT treatment on cortisol concentrations are listed in Table 1. Plasma cortisol levels of Holstein cows were positively associated with their milk SCC. In cows with subclinical mastitis, cortisol levels were significantly higher (5.93 ± 1.70 to 6.12 ± 1.68 ng/mL) than in healthy cows, (3.42 ± 0.33 ng/mL). After MT treatment, the relatively high cortisol levels in cows with subclinical mastitis were reduced to normal ranges, as observed in the control group.

Effects of melatonin on blood biochemical indices. The results of MT treatment on blood biochemical parameters, including IgG, IgM, albumin, alanine aminotransferase (ALT) and serum lactate dehydrogenase (S-LDH) are listed in Table 2. IgG and IgM levels in cows with subclinical mastitis (10.26 ± 1.31 to 10.86 ± 1.82 g/L) were significantly higher than in control groups (2.63 ± 0.21 g/L to 2.74 ± 0.63 g/L) (p < 0.05). In contrast, the blood levels of ALB, ALT and LDH were significantly lower in cows with subclinical mastitis than in healthy cows. There were no significant differences in blood glucose levels among these groups (p > 0.05). There was a transient increase in blood IgG and IgM levels in cows with subclinical mastitis one day after the termination of melatonin treatment followed by a significant decrease three days after termination. Following treatment, IgM concentrations were reduced to levels observed in the healthy group. Blood ALB, ALT and LDH levels in cows with subclinical mastitis were significantly increased after melatonin treatment compared with their untreated counterparts (p < 0.05). There were no significant differences in the blood levels of LDH among groups before or after melatonin injection.

Effects of melatonin on blood cells. The effects of MT treatment on blood cells are listed in Table 3. WBC, LYM and GRN were significantly higher in cows with subclinical mastitis compared with healthy cows (p < 0.05). There were no significant differences in RBC between groups (p > 0.05). In cows with subclinical mastitis treated with melatonin, the number of WBC and LYM changed in a pattern similar to that described for IgG and IgM, there was a transient increase then a decrease compared with untreated counterparts. However, levels were not reduced to the concentrations observed in the healthy group.

Discussion

SCC in milk is an important index of breast health in cows. To some degree, the quality of milk is determined by its SCC. A significant increase of SCC in milk indicates a poor quality product, with high SCC accompanied by relatively low levels of butterfat, milk protein, calcium and relatively high levels of sodium and chloride. All these factors lower the nutritional value of milk and, in some cases, necessitate discarding the milk. A high SCC in milk is also an important factor for determining whether to cull a cow. Approximately 30% of cows are culled due to high SCC in their milk. When SCC in milk is higher than 7 × 10⁵ cells/mL, the culling rate is 3–4 times higher than in cows with milk SCC lower than 2.5 × 10⁵ cells/mL. In this study, cows with milk SCC greater than 3 × 10⁵ cells/mL were classified as having subclinical mastitis.

ROS, caused by high environmental temperatures, cause immunosuppression in dairy cows, aggravate dairy mastitis and improve SCC. Dairy cow mastitis is connected with the activity of reactive oxygen species, cytokines, and other classic inflammatory mediators. The conventional treatment for mastitis in cows is antibiotics, due to their rapid therapeutic effect on mastitis. However, the frequent use of antibiotics causes drug resistance, and drug residues in milk directly jeopardize human health, especially in children. Therefore, it is crucial to find suitable alternative therapies for dairy cow mastitis. Naturally occurring antioxidants have antiviral effects, scavenge reactive oxygen species and improve immune response. They also improve reproductive performance in cows, lower SCC in raw milk and reduce the incidence of mastitis. Dietary supplementation with tea polyphenol effectively reduced the SCC in raw milk. We observed that melatonin, a naturally occurring antioxidant, effectively eliminates ROS and enhances immunity. We also found that a short period (four days) of administration (S.C.) significantly reduced the SCC of milk in Holstein cows with subclinical mastitis. Cortisol plays a pivotal role in inflammatory responses, such as mastitis. It regulates glucose metabolism, which is a blood immune inhibitor, and inhibits immune-active Serum cortisol levels increase in stress states and promote protein decomposition and glycogen dysplasia. When cows are stressed, cortisol secretion increases and immune function is repressed, including the atrophy of immune organs and a decrease in number of lymphocytes and acidophilic leukocytes in blood. All of these factors lead to the inhibition of cellular immunity and resistance to disease. This is consistent with our observation that blood cortisol levels in cows with subclinical mastitis (milk SCC > 3 × 10⁵ cells/mL) were significantly higher in healthy cows (milk SCC < 1 × 10⁵ cells/mL). Melatonin injection significantly reduced cortisol levels in cows with subclinical mastitis, possibly via melatonin-mediated suppression of adrenal cell corticotropin releasing hormone and adrenocorticotropic hormone, which leads to a reduction in butyrylcholinesterase cAMP-induced cortisol production. In addition, the rate-limiting step in cortisol synthesis is the conversion of cholesterol to pregnenolone. Melatonin reduces the synthesis of cortisol by inhibiting prostaglandin, which reduces the concentration of intracellular cAMP in lymphocytes.

IgG and IgM are important factors in immune response and anti-infection. They lyse and activate complements to promote phagocytosis. Research shows that melatonin enhances humoral immunity. In mice treated...
with inactivated Venezuelan equine encephalomyelitis virus, subcutaneous injection of melatonin significantly increased serum globulin and IgM levels. Melatonin administration also enhanced the levels of IgG and IgM in 28-month-old Wistar rats. We observed that melatonin treatment transiently increased IgG and IgM levels in
cows with subclinical mastitis before levels were reduced toward normal. This implies that melatonin treatment decreases symptoms of mastitis while it decreases IgG and IgM levels, consistent with our observation that melatonin treatment decreases SCC in milk. Lymphocytes and neutrophils are also important factors in inflammatory and immune responses. Studies show that extending the illumination time at night decreases the percentage of peripheral lymphocytes, which also suppresses the secretion of melatonin in animals. However, treatment with melatonin significantly increased the percentage of lymphocytes. In pinealectomized animals, thymus and spleen function were suppressed, as indicated by a decrease in the number of lymphocytes and neutrophils. Treatment with melatonin led to a full recovery. These studies are consistent with our observation that melatonin injection significantly enhanced the number of lymphocytes and neutrophils. Melatonin is a naturally occurring antioxidant with low to no toxicity that is inexpensive and widely available. SCC in milk directly reflects the health of consumers. Treatment with melatonin may provide an effective, safe, and quick method to lower SCC in milk and replace the use of the antibiotics, which have many potential adverse effects for cows and human health. To understand the exact mechanisms of melatonin’s beneficial effects on cows with high levels of SCC in their milk, additional studies are necessary.

### Table 1. Effects of subcutaneous injection of melatonin on plasma cortisol in Holstein cows (ng/mL).

| Group            | Index     | Before injection | 1 d after injection | 3 d after injection |
|------------------|-----------|------------------|---------------------|---------------------|
| MT (SCC:3 × 10⁵–1 × 10⁶ cells/mL) | IgG (g/L) | 10.26 ± 1.31 ⁹   | 12.18 ± 1.26 ⁹   | 8.92 ± 0.63 ⁹   |
| NON-MT (SCC:3 × 10⁵–1 × 10⁶ cells/mL) | IgM (g/L) | 2.63 ± 0.21 ⁹   | 3.39 ± 0.23 ⁹   | 2.14 ± 0.20 ⁹   |
| Healthy control (SCC < 1 × 10⁵ cells/mL) | ALB (g/L) | 29.92 ± 1.44 ⁹   | 33.58 ± 1.31 ⁹   | 33.92 ± 1.44 ⁹   |
| MT (SCC:3 × 10⁵–1 × 10⁶ cells/mL) | ALT (U/L) | 30.08 ± 1.78 ⁹   | 31.75 ± 1.60 ⁹   | 28.25 ± 1.22 ⁹   |
| NON-MT (SCC:3 × 10⁵–1 × 10⁶ cells/mL) | LDH (U/L) | 25.42 ± 4.03 ⁹   | 34.17 ± 4.04 ⁹   | 35.50 ± 4.38 ⁹   |
| Healthy control (SCC < 1 × 10⁵ cells/mL) | GLU (mmol/L) | 24.83 ± 3.93 ⁹   | 30.00 ± 4.81 ⁹   | 31.42 ± 5.95 ⁹   |
| MT (SCC:3 × 10⁵–1 × 10⁶ cells/mL) |   | 35.5 ± 6.42 ⁹   | —   | —   |
| NON-MT (SCC:3 × 10⁵–1 × 10⁶ cells/mL) |   | 28.91 ± 8.56 ⁹   | —   | —   |
| Healthy control (SCC < 1 × 10⁵ cells/mL) |   | 801.33 ± 66.48 ⁹ | 940.67 ± 103.13 ⁹| 904.67 ± 96.38 ⁹|
| MT (SCC:3 × 10⁵–1 × 10⁶ cells/mL) |   | 847.92 ± 85.03 ⁹ | 844.83 ± 86.80 ⁹| 842.83 ± 93.57 ⁹|
| NON-MT (SCC:3 × 10⁵–1 × 10⁶ cells/mL) |   | 945.7 ± 87.28 ⁹ | —   | —   |
| Healthy control (SCC < 1 × 10⁵ cells/mL) |   | 4.06 ± 0.23 ⁹   | 4.32 ± 0.24 ⁹   | 4.21 ± 0.19 ⁹   |
| MT (SCC:3 × 10⁵–1 × 10⁶ cells/mL) |   | 3.96 ± 0.23 ⁹   | 4.16 ± 0.31 ⁹   | 4.14 ± 0.23 ⁹   |
| NON-MT (SCC:3 × 10⁵–1 × 10⁶ cells/mL) |   | 4.16 ± 0.22 ⁹   | —   | —   |
| Healthy control (SCC < 1 × 10⁵ cells/mL) |   | 4.16 ± 0.22 ⁹   | —   | —   |

### Table 2. Effects of subcutaneous injection of melatonin on blood biochemical index of Holstein cows.

| Group            | Index     | Before injection | 1 d after injection | 3 d after injection |
|------------------|-----------|------------------|---------------------|---------------------|
| MT (melatonin-treated cows); NON-MT (non-melatonin-treated cows). Data expressed as the means ± SEM (n = 20). Values with different superscript letters, such as a⁹ represent a significant difference within the same group, (P < 0.05); values with different superscript letters b⁹ represent a significant difference between different groups (P < 0.05); The same letters represent no significant difference (P > 0.05).
Table 3. Effects of subcutaneous injection of melatonin on white and red blood cell counts of Holstein cows. MT (melatonin-treated cows); NON-MT (non-melatonin-treated cows); WBC: white blood cells; LYM: lymphocytes; GRN: granulocyte neutrophil; RBC: red blood cells. Data expressed as the means ± SEM (n = 10). Values with different superscript letters, such as a represent a significant difference within the same group, (P < 0.05); values with different superscript letters A represent a significant difference between different groups, (P < 0.05); the same letters represent no significant difference (P > 0.05).

Materials and Methods

Ethics Statement. This study was carried out in strict accordance with the guidelines for the care and use of animals of China Agricultural University. All animal experimental procedures were approved by the Animal Care Commission of the College of Animal Science and Technology, China Agricultural University. Every effort was made to minimize animal pain, suffering, and distress and to reduce the number of animals used.

Management of animals. Studies were performed in a commercial dairy herd in Beijing China. One hundred forty Holstein dairy cows were included in the experiment. The approximate annual milk production was 9,102 to 11,020 kg per cow for the herd during this study period. Cows were milked three times per day and fed with TR diet (NRC2001). The mean annual culling rate for the study period was 29.5%. Lactating cows were reared in cubicle and feeding areas. In the feeding area, water sprinklers facing the resting place of cows were made to minimize animal pain, suffering, and distress and to reduce the number of animals used.

Experimental design. Experiment I. One hundred twenty cows were divided into four groups (< 10 × 10⁶ cells/mL, 1 × 10⁷ to 3 × 10⁶ cells/mL, 3 × 10⁶ to 5 × 10⁶ cells/mL, and 5 × 10⁶ to 1 × 10⁷ cells/mL, respectively) depending on milk SCC levels. Each group was randomly subdivided into melatonin treated and untreated cows. Melatonin treated cows were given subcutaneous injections of MT (4.64 mg) for four consecutive days. Milk was sampled to examine the effects of melatonin on milk SCC.

Experiment II. Twenty cows with milk SCC in the range of 3 × 10⁵ to 1 × 10⁶ cells/mL were selected. Half were treated with MT (4.64 mg) for four consecutive days and the remainder served as untreated controls. This experiment was designed to find associations between melatonin treatment, milk SCC and alterations in the biochemical and immunoresponsive parameters in blood. Thus, both milk and blood were sampled from cows at different time points.

Melatonin injection. Melatonin (Sigma-Aldrich Chemical Co. St. Louis, MO, USA.) was dissolved in ethanol and diluted with normal saline while in a darkroom. Each animal was given 4.64 mg melatonin in a parenteral solution. Cows were injected subcutaneously in the neck with melatonin for four consecutive days at 8:00 am.

Blood sampling. Blood samples were drawn from the coccygeal blood vessel of each cow just before the first melatonin injection at 8:00 am. Thereafter, blood samples were collected at 8:00 am on days one and three after the last melatonin injection. Blood samples were collected in the vacutainers containing heparin or EDTA for analyses of cortisol, the number of red blood cells, lymphocytes, and neutrophils and measurement of physiologic and biochemical indices, such as IgG, IgM, albumin and alanine transaminase and lactate dehydrogenase. Samples were immediately placed on ice, and plasma was separated by centrifugation within 1 h of collection. Measurements of serum metabolites and routine blood tests were conducted at the laboratories of BJ. XinChuangYuan BIOTECH CO., LTD (Beijing, China).

Milk sampling. Milk was sampled each day for three days before melatonin treatment and then sampled for 18 days after the last melatonin injection. Milk samples were used for analysis of SCC. All samples were collected after morning milking. The milk samples were heated to 40–42 °C. Samples were well shaken, then analysed using a fluorescence optical system (Fossomatic TM Minor; FossElectric; Hillerød; Denmark).
Statistical analysis. Data expressed as the mean ± SEM, were analysed with univariate analysis of variance (ANOVA) followed by Duncan’s test using SPSS 19.0 statistical software. The significant difference between treatments was set at P < 0.05.

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Author Contributions
Jianmin Shi and Minghui Yang designed and participated in the entire experiment and wrote the manuscript. Minghui Yang, Yukun Song and Zhiyuan Xu participated in data collection and analyses. Jingli Tao, Jianhua Tian and Kuanfeng Zhu assisted with sample collection. Jing Wang performed the melatonin analysis. Pengyun Ji participated in different stages of the study. All experiments were directed by Guoshi Liu. All authors reviewed the manuscript.

Additional Information
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