

Nighttime or daytime? How milk melatonin levels relate to differential and somatic cell count in dairy cow milk

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Abstract

This study investigates the impact of winter nighttime versus daytime milking on melatonin concentration in milk and explores its relationship with somatic cell count (SCC) and differential somatic cell count (DSCC). Milk samples were collected from 40 Holstein cows at 12-hour intervals (04:00 AM Night samples and 04:00 PM Day samples) during the winter season (11 h of light and 13 h of darkness). The relationship between melatonin content, SCC and DSCC was assessed. Night milk had significantly higher melatonin levels (15.63 ± 1.90 pg/mL) compared to day milk (6.80 ± 0.75 pg/mL). SCC was significantly lower in night milk compared to day milk. The percentage of polymorphonuclear leukocytes (PMN) + lymphocytes (DSCC) was 66.54% in night milk and 63.36% in Day milk. The percentage of macrophages + epithelial cells was 33.46% in Night milk and 36.64% in Day milk. It has been shown that SCC are lower in Night milk compared to Day milk. This observation confirms a potential beneficial effect of melatonin on cow udder health and indicates that this effect occurs without modify the proportion of PMN + lymphocytes and macrophages + epithelial cells during Night milk and Day milk sampling.

Keywords: dairy cows, differential somatic cell count, melatonin, somatic cell count

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Introduction

Melatonin is the primary hormone whose synthesis is regulated by photoperiodism and known for its role in regulating the sleep-wake cycle. In mammals, its release into the bloodstream follows a circadian rhythm, with peak production occurring during the dark phase. Melatonin is an amphiphilic molecule, allowing its actions to be either receptor-mediated or to occur through passive diffusion across biological membranes. In mammals, this property enables melatonin to readily diffuse from the bloodstream into milk (Yu *et al.*, 2016; Romanini *et al.*, 2019). Melatonin is a potent antioxidant and modulates the immune system, exerting either immunostimulatory or immunosuppressive effects (Asher *et al.*, 2015; Yang *et al.*, 2017). In case of acute inflammation, melatonin acts as an anti-inflammatory agent by reducing excessive immune response activation (Carrillo-Vico *et al.*, 2013). In addition, the administration of melatonin in dairy cows, particularly during the dry-off period or in the weeks preceding parturition, has been shown to reduce embryo losses under heat stress conditions, while also contributing to a more efficient onset of subsequent lactation (Garcia-Isprierto *et al.*, 2013; De Rensis *et al.*, 2017; Morini *et al.*, 2018).

The biosynthesis and the role of melatonin in cow milk have been recently well reviewed (Andrani *et al.*, 2024). Melatonin concentration in milk is not solely dependent on milking time but is also influenced by factors such as breed, nutrition, stress levels, environmental temperature/humidity, and milk yield (Milagres *et al.*, 2014; Asher *et al.*, 2015). Melatonin concentration in milk follows its circadian rhythm, resulting in higher concentrations in milk collected during the early morning hours (Castro *et al.*, 2011; Teng *et al.*, 2021). Milking frequency and barn lighting conditions influence melatonin levels in milk: increased nighttime milking can enhance melatonin levels in milk, while barn light may suppress its synthesis. The exposure to light with an intensity above 10 lux and short wavelengths effectively suppresses melatonin synthesis (Asher *et al.*, 2015; Romanini *et al.*, 2019). Therefore, the control of lighting intensity in dairy farms can modulate melatonin secretion and its effect on cow health.

The physiological rhythm of melatonin synthesis leads to the production of naturally melatonin rich milk. It has been demonstrated the melatonin milk levels in dairy cows are correlated with a reduction of somatic cell count (SCC) and the risk of mastitis (Asher *et al.*, 2015; Yang *et al.*, 2017; Yao *et al.*, 2020; Wu *et al.*, 2021). SCC is the primary indicator of the inflammatory status of the bovine mammary gland. Somatic cells consist of polymorphonuclear leukocytes (PMN), lymphocytes, monocytes/macrophages and epithelial cells whose proportions vary depending on the animal's physiological or pathological state (Zecconi *et al.*, 2019, 2023; Dall'Olio *et al.*, 2025). Mastitis, a leading cause of economic losses in dairy herds and a major factor impairing cow welfare, and SCC is the primary indicator of this disease (Zecconi *et al.*, 2019; Cheng and Han, 2020). A high SCC is typically indicative of mammary gland inflammation (Sharun *et al.*, 2021).

Somatic cell count is a robust quantitative measurement, but does not provide the proportion of lymphocytes, macrophages, and PMN (Kehrl and Shuster, 1994; Rivas *et al.*, 2001). While the lymphocytes regulate the induction and suppression of immune responses (Sordillo and Nickerson, 1988; Nickerson, 1989), macrophages initiate the immune response (Paape *et al.*, 2002; Oviedo-Boyso *et al.*, 2007). Neutrophils play a central role in the inflammatory process, acting not only as first responders of the innate immune system against infections and tissue damage, but also by modulating inflammation through the production of cytokines, chemokines, and the release of granular components, exhibiting remarkable functional heterogeneity (Rosales, 2018). Due to the specific functions of the individual cell populations, the distribution of PMN differs between normal milk and mastitic milk (Nickerson, 1989). Specifically, proportions of PMN can reach up to 95% in milk from cows with mastitis (Paape *et al.*, 1979; Kehrl and Shuster, 1994) while in the uninfected mammary gland, are predominantly macrophages and lymphocytes (Schwarz *et al.*, 2011; Pilla *et al.*, 2013; Zecconi *et al.*, 2019).

Recently, it has become possible to improve the accuracy of SCC by introducing a new parameter: the differential somatic cell count (DSCC), which represents the combined proportion of PMN and lymphocytes, expressed as a percentage (Damm *et al.*, 2017; Halasa and Kirkeby, 2020). This provides a better assessment of the immune system compared to SCC alone. This study aimed to determine the melatonin content in cow milk from either daytime or nighttime winter milking in a commercial dairy farm and give a better understanding of the mechanism by which melatonin has a beneficial effect on the udder immune system by determining the DSCC.

Materials and Methods

Ethical Statement: This study involved dairy cows raised on a commercial farm located in northern Italy (44°48'05.3"N 10°19'40.8"E). Milk samples for analysis were obtained during routine milking carried out from ARAER (Regional Breeders Association of Emilia-Romagna), as part of functional checks, and processed following the established guidelines for dairy cattle milk recording and analysis.

Animals and housing conditions: The study was conducted on 40 Holstein cows, with an average parity of 2.43 ± 1.50 , days in milk (DIM) of 174 ± 140 , and a mean milk yield of 26.09 ± 9.21 kg/day. The cows were housed in pen barns and were milked twice per day in milking parlors. Sampling was performed on February 2024, under environmental conditions characterized by an average temperature of 11 °C (maximum: 13 °C, minimum: 9 °C), relative humidity of 95%, and rainfall. The natural photoperiod was 11 h of light and 13 h of darkness. The cows were fed in accordance with the Parmigiano Reggiano Consortium's production guidelines (Consorzio del Parmigiano Reggiano, 2018), ensuring compliance with the specific requirements for Parmigiano Reggiano cheese production.

Milk sampling and analysis: From each cow milk samples were collected at 4:00 AM (Night milk), while those from daytime milking were collected at 4:00 PM (Day milk), resulting in a total of 80 samples. One liter of milk per cow were sampled from each milking session and stored immediately after collection. The samples were immediately analysed for composition for protein, lactose, urea, and casein content using the MilkoScan FT3 (Foss Electric A/S, Hillerød, Denmark), while SCC and DSCC was determined with the Fossomatic 7DC (Foss Electric A/S, Hillerød, Denmark). Thereafter, milk samples were aliquoted into 15 mL Falcon tubes and immediately stored at -20 °C for subsequent melatonin quantification.

Melatonin quantification: Melatonin concentrations were determined employing the Melatonin ELISA kit (REF54021; Tecan, Hamburg, Germany), strictly adhering to the manufacturer's instructions, as briefly described below.

Prior to quantification, milk samples were skimmed by centrifugation (15 mL at 5000 × g for 10 minutes, repeated three times), removing the lipid layer after each step. Skimmed samples were then analysed for melatonin content using the ELISA kit, with a standard range of 0.0–300 pg/mL and a standard curve efficiency (R^2) of 0.99.

For extraction, standards, controls, and undiluted samples (500 µL each, in triplicate) were loaded onto methanol-conditioned columns and centrifuged at 120 × g for 5 minutes. After two washes with 10% methanol, melatonin was eluted with 1 mL of methanol, centrifuged, dried under nitrogen, and reconstituted in 150 µL of bi-distilled water.

Subsequently, 50 µL of each sample, standard, and control were pipetted into the microplate wells along

with 50 µL of Melatonin Biotin and Melatonin Antiserum. Following a 16-hour incubation at 4°C, the plate was washed, incubated with Enzyme Conjugate for 2 hours at room temperature, then developed with Substrate Solution for 40 minutes. The reaction was stopped, and absorbance was measured at 405 nm using a VICTOR® Nivo™ Multimode Microplate Reader (PerkinElmer, Waltham, MA, USA), with melatonin levels calculated based on the standard curve.

Statistical analysis: Differences for each parameter (melatonin, SCC, DSCC) between Night milk and Day milk samples were assessed using a paired t-test. The differences in DSCC percentages, expressed as the proportion of PMN + lymphocytes and macrophages + epithelial cells, were analysed using an ordinary one-way ANOVA with the milking time (day vs. night) as the fixed factor. Since two groups (Day and Night milk) were compared for DSCC, Tukey's post hoc test was applied to adjust for multiple pairwise comparisons between these groups. Data normality was assessed for all datasets using the Shapiro-Wilk normality test. Statistical analysis was performed using IBM SPSS Statistics software (v.29.0.1.0, IBM Corp., Armonk, NY, USA) and statistical significance was set at $P < 0.05$.

Results

Night and day milk composition: Table 1 presents the mean values ± SEM of composition for Night milk and Day milk. No significant differences were observed for protein, lactose, casein, and urea content ($P > 0.05$) (Table 1).

Table 1 Mean values ± SEM of Night and Day milk composition and milk yield.

	Night milk	Day milk	P-value
Protein (%)	3.28 ± 0.06	3.31 ± 0.07	0.08
Lactose (%)	4.56 ± 0.05	4.59 ± 0.05	0.22
Casein (%)	2.59 ± 0.07	2.62 ± 0.07	0.12
Urea (mg/dL)	36.73 ± 0.85	36.06 ± 1.05	0.16

Melatonin, SCC, and DSCC: Melatonin concentrations were significantly higher in Night milk than in Day milk samples ($P < 0.001$), with 15.63 ± 1.9 pg/mL and 6.80 ± 0.75 pg/mL, respectively (Fig. 1A). SCC was significantly lower in Night milk compared to Day milk ($P < 0.001$), with $129.71 \pm 25.01 \times 10^3$ cell/mL and $196.39 \pm 33.80 \times 10^3$ cell/mL, respectively (Fig. 1B).

DSCC was not different between Night and Day milk ($P = 0.78$). Figure 2 describes the proportion of PMN + lymphocytes and macrophages + epithelial cells. In Night milk, PMN + lymphocytes were 66.54%, and in Day milk, was 63.36%. In night milk, the percentage of macrophages + epithelial cells was 33.46%, and in Day milk was 36.64%.

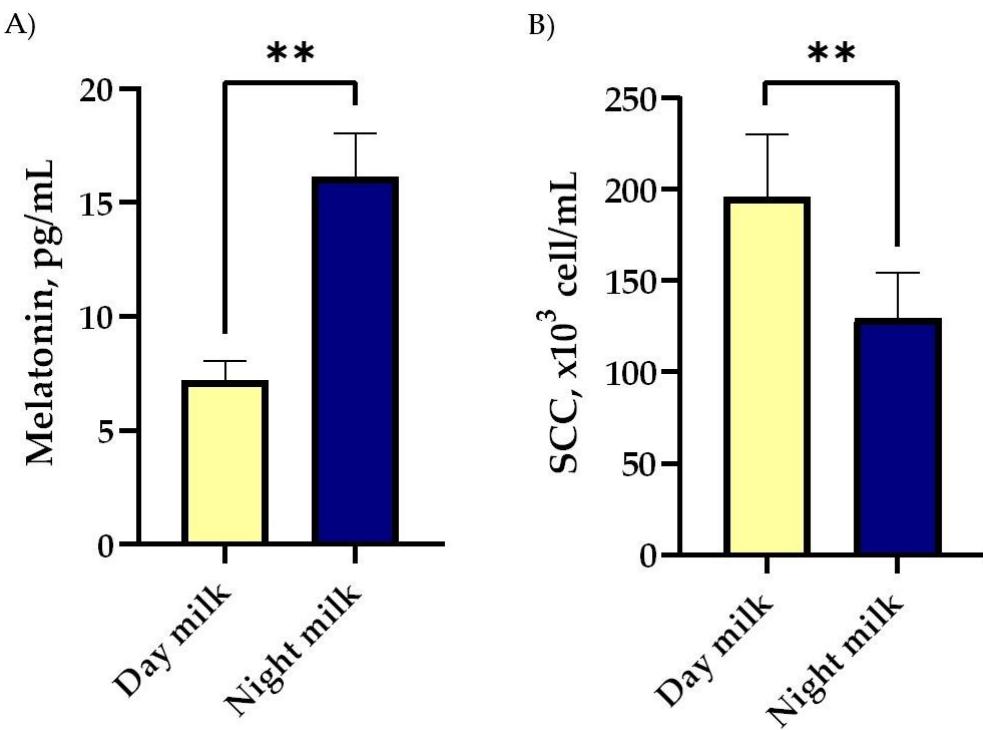


Figure 1 Mean \pm SEM values of A) melatonin concentration and B) SCC in milk from cows milked at night (04:00 AM; Night milk) or day (04:00 PM; Day milk). ** indicates a significant difference with P -value < 0.001 . SCC = somatic cell count.

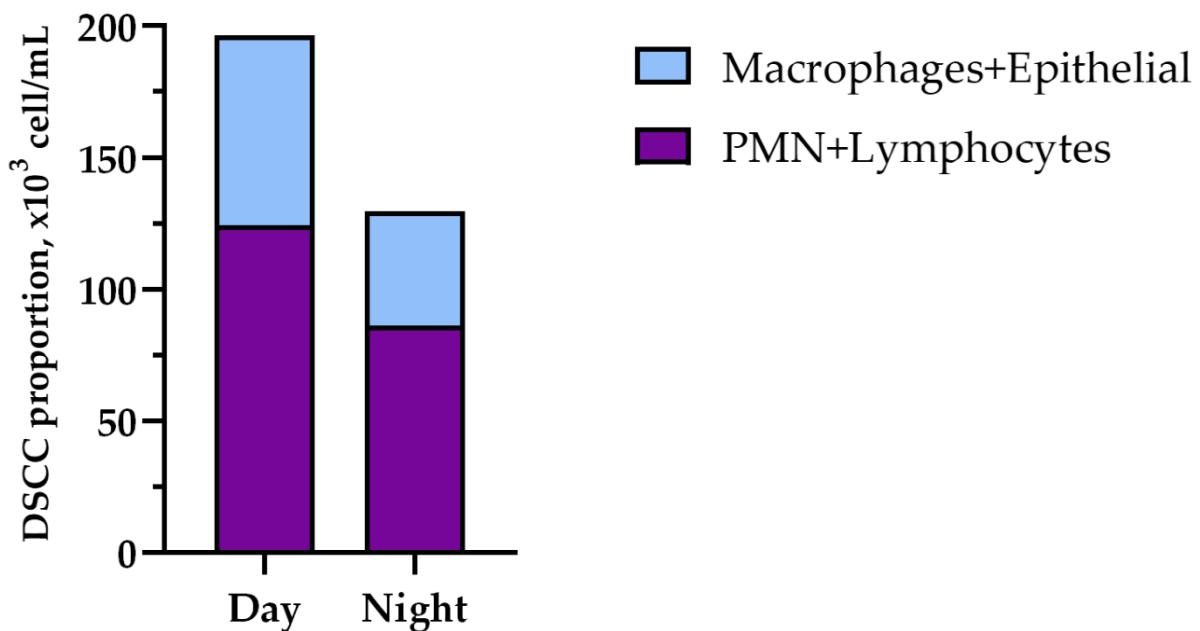


Figure 2 Proportion of PMN + lymphocytes and macrophages+epithelial cells in milk from cows milked at night (04:00 AM; Night milk) or day (04:00 PM; Day milk) in relation to the total somatic cell count (SCC). DS SCC = differential somatic cell count; PMN = polymorphonuclear leukocytes.

Discussion

In this study, under a photoperiod of 11 hours of light and 13 hours of darkness, melatonin concentrations were significantly higher in Night Milk (15.6 ± 1.9 pg/mL) than in Day Milk (6.8 ± 0.8 pg/mL), in agreement with previous studies (Milagres *et al.*, 2014; Asher *et al.*, 2015; Teng *et al.*, 2021; Sahin *et al.*, 2021). For instance, Romanini *et al.* (2019) found mean

melatonin concentrations of 14.9 ± 7.7 pg/mL in nighttime milk and 6.9 ± 3.1 pg/mL in daytime milk in Brazil, using samples collected during short (10.3 hours of light) and long photoperiodism (13.3 hours of light). Furthermore, during the winter, the average melatonin concentration in milk was 13.9 ± 8.4 pg/mL, 74.7% higher than in summer, where the average was 7.9 ± 3.4 pg/mL (Romanini *et al.*, 2019).

However, these values reported in our and Asher *et al.* (2015) and Romanini *et al.* (2019) studies are considerably lower than the concentrations observed in two other studies. In fact, Sahin *et al.* (2021), found melatonin concentrations of 163.1 ± 8.9 pg/mL in nighttime milk and 103.7 ± 6.6 pg/mL in daytime milk. This discrepancy may be attributed to several factors, including the different standard testing range of the ELISA kit used for quantification, as well as the fact that the cows were kept in darkness for an entire week prior to nighttime milking and all cows were in their first lactation (Sahin *et al.*, 2021) and not information on seasonality or the natural photoperiod was provided. Teng *et al.* (2021), during the summer season in China (13 hours of light and 11 hours of darkness), in cows that were entirely free from artificial lighting, reported melatonin concentrations of 120.1 pg/mL in nighttime milk and 90.2 pg/mL in daytime milk. Similarly, the differences with our study could be attributed to variations in quantification procedures.

In the present study, milk yield was reduced during nighttime milking (12.08 ± 0.96 kg/day) compared to daytime milking (13.34 ± 1.05 kg/day). This difference could be related to several factors, but it must be considered the melatonin action on prolactin. In fact, an increase in plasma melatonin concentration, achieved through the administration of slow-release melatonin implants for 12 weeks, can lead to a reduction in plasma prolactin levels and, consequently, a decrease in milk production in dairy cows, because melatonin has an inhibitory action on prolactin secretion (Auldist *et al.*, 2007). Furthermore, it is well-documented that extended photoperiod enhances milk production (Dahl *et al.*, 2000). However, because the percentage of melatonin that is filtered is constant in the milk composition, milk yield does not affect the concentration of melatonin (Kollmann *et al.*, 2008).

The main aim of this study was to investigate the potential effect of milk melatonin content on SCC and the proportion of PMN + lymphocytes and macrophages + epithelial cells relative to the total somatic cell population from milk samples collected in night or daytime. Concerning SCC, our results revealed a significantly lower SCC in Night milk ($129.71 \pm 25.01 \times 10^3$ cells/mL vs. $196.39 \pm 33.80 \times 10^3$ cells/mL in Day milk), confirming the findings of Asher *et al.* (2015) and other studies demonstrating a reduction in SCC following exogenous melatonin administration, via subcutaneous injection at doses of 9.3 mg/cow/day or 4.6 mg/cow/day for four consecutive days (Yang *et al.*, 2017; Wu *et al.*, 2021), or through rumen bypass melatonin feeding at doses of 40 or 80 mg/cow/day for 7 or 14 days (Yao *et al.*, 2020) or by edible glutinous rice capsules at 120 mg/cow/day (Li *et al.*, 2024).

We also analysed the percentages of PMN + lymphocytes and macrophages + epithelial cells in milk samples during night and daytime, on the basis of melatonin immunoregulatory properties (for review see Andrani *et al.*, 2024). However, in our study, we did not detect any significant difference (Fig. 2). Our results highlight that in night milk (melatonin-rich milk), the immune cells are lower compared with day milk, confirming that melatonin provides a natural

defence at the mammary level (Romanini *et al.* 2019), and our study showed that this effect is not related to differences in the proportion of PMN + lymphocytes and macrophages + epithelial cells.

In conclusion, our results show that Night Milk has lower somatic cell counts compared to Day Milk, suggesting a beneficial effect of melatonin on udder health which could play a role in mastitis prevention. This effect is not associated with differences in the proportion of immune cell populations, indicating that melatonin enhances local defence mechanisms without significantly altering immune cell distribution.

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