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RESEARCH ARTICLE

The Effect of Exogenous Melatonin on Milk Somatic Cell Count in Buffalo

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ABSTRACT

High somatic cell counts (SCCs) in milk significantly influence the quality of milk and give rise to substantial economic loss. The present study was aimed to investigate the effect of extreme heat and cold compared to other season and melatonin (MLT) on milk SCCs in Chinese crossbred (Nili-Ravi×Murrah) buffaloes. We collected the 1948 milk SCCs data records from 2012 to 2017 to explore the effect of different month in China on milk SCCs. Meanwhile, twenty buffaloes with relatively high milk SCCs were employed and randomly divided into two groups (T1 and T2, n=10 each group) to evaluate the effect of MLT treatment on milk SCCs, blood antioxidant activities and immune levels of buffaloes during summer in China. Results showed that the milk SCCs in high temperature seasons (July and August) and low temperature seasons (December, January and February) were significantly higher compared with other months ($P<0.05$). In summer, MLT treatment significantly reduced milk SCCs and increased the IgM and superoxide dismutase (SOD) levels in plasma on day 1 after MLT treatment, and then both IgM and SOD levels were decreased significantly. In conclusion, our study demonstrated that environmental temperature stress (heat and cold) caused the higher milk SCCs and MLT treatment improved the quality of milk by reducing SCCs suggesting that MLT could improve immune activity in buffaloes.

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INTRODUCTION

The world population of buffaloes (*Bubalus bubalis*) is estimated to be 206 million today and more than 13% of the buffalo's population is located in China (http://faostat.fao.org/). Although the buffalo breeding industry is the main constitution of the agricultural economy in many countries and regions, the low productivity of buffalo limits its economic importance as the provider of milk, meat, horns, hide and draught power. The low milk production performance of buffalo due to elevated somatic cell counts (SCCs) has long been one of the main challenges in buffalo industry (Akhtar *et al.*, 2012). The causes of increased SCCs mainly include the bacterial infection of the mammary gland (Harmon, 1994) and nonbacterial factors, like age, season, management, and various stress (Rolde *et al.*, 2007; Alhussien and Dang, 2018; Bombade *et al.*, 2018). Although the nonbacterial factors are regard as less vital than bacterial infection status (Harmon, 1994), the various stresses, especially environment stress (heat and cold stress), give rise to the elevated SCCs (Hammami *et al.*, 2013; Bombade *et al.*, 2018; Gantner *et al.*, 2019; Kucevic *et al.*, 2019). It has been reported that environment could induce oxidative stress by increasing the concentration of cellular reactive oxygen species (ROS) (Alemu *et al.*, 2018), which would result in depressive immune function (Hammami *et al.*, 2013).

Melatonin (N-acetyl-5-methoxytryptamine, MLT) is synthesized in the pineal gland during the dark phase of photoperiod and plays a vital role in improving immune activity (Pierpaoli and Yi, 1990; Nabavi *et al.*, 2019). In addition, MLT can increase both the expression and activity of various antioxidant enzymes, such as

superoxide dismutase, catalase, and glutathione peroxidase which prevent the production of ROS (Guérin *et al.*, 2001; Rodriguez *et al.*, 2004). Therefore, we hypothesize that MLT can improve the immune function and then result in the decline of high milk SCCs owing to stress in buffaloes. Numerous studies have reported that MLT treatment can successfully improve milk quality by reducing milk SCCs in cows (Yang *et al.*, 2017; Yao *et al.*, 2020) and goats (Jiménez *et al.*, 2009). However, little information is available about the role of MLT in buffalo milk. Therefore, the aim of the present study was to investigate the efficacy of subcutaneous MLT injection on milk SCCs in buffaloes.

MATERIALS AND METHODS

Ethics statement: The experimental design and animal treatment protocols were approved by the Ethical Animal Care and Use Committee of Henan Agricultural University.

Animals and management: The milk SCCs data from 2012-2017, totaling 1948 records, were collected to detect the SCCs variation in different month. To measure the effect of MLT treatment on milk SCCs, the healthy crossbred female buffaloes (Nili-Ravi×Murrah, n=20) were used in the present study. These animals (between 3 and 4 years of age and weighing 516±18.6 kg on average) were housed at the JINNIU Buffalo Farm located in the south of China. Buffaloes were milked daily at 05:00 am and 05:00 pm, separately. Roughage, concentrate supplement, and clean water were provided free of access.

Experimental design: Crystalline melatonin powder (Sigma-Aldrich Chemical Co. St. Louis, MO, USA.) was dissolved in ethanol and diluted with normal saline in a darkroom. Once dissolved, the suspension was used on the same day. Based on the three consecutive days of milk SCCs record, buffaloes with milk SCCs higher than 5×10^5 cells per millilitre were randomly allocated to treatment groups T1 (n=10) and T2 (n=10). The animals in T1 and T2 received a subcutaneous injection of MLT for 4 consecutive days at 4.68 mg and 20 mg per day, respectively.

Blood sampling: Blood samples were collected via jugular venipuncture on the day before and the day 1, 4, 7 and 10 after melatonin injection at 8:00 am. Blood samples were centrifuged at 3000 rpm for 15 min to separate the plasma, which were used for measuring the MLT (Cat. No. ml245196T, Shanghai, China), IgM (Cat. No. ml213696T, Shanghai, China) and SOD (Cat. No. ml213196T, Shanghai, China) levels using bovine ELISA test kits, as per the manufacturers' instructions.

Milk sampling: Milk samples were collected daily for three days before melatonin treatment and ten days after the last melatonin injection. Milk samples were analyzed using a fluorescence optical system (Fossomatic TM Minor; FossElectric; Hillerod; Denmark).

Statistical analysis: Owing to SCCs showed skewed distribution, we changed SCCs into somatic cell score (SCS) according to the formula for the subsequent analysis. $SCS = Log_2 (SCC/100) + 3$; ($SCC = 1\times10^3$ cells/mL).

ANOVA of repeated measurement data method was used to analyse the SCS changes at the whole MLT treatment period. The levels of plasma MLT, SOD and IgM, and the milk SCS variation among months were analysed by one-way ANOVA using the general linear model (GLM) procedure of SAS 9.4 (SAS Inst., Inc., Cary, NC, USA) and expressed as the means ± standard error mean (SEM). Differences were considered statistically significant at $P<0.05$.

RESULTS

Effect of month on SCS in milk: The milk SCS in high temperature months (July and August) were significantly higher than those in January, March, April, May, September, October, November, and December, respectively ($P<0.05$). The SCS in low temperature months (December, January, and February) were significantly higher than those in May, October and November, respectively ($P<0.05$) (Table 1). The percentages of individual with SCCs (more than 4×10^5 cells/mL) in summer season (June, July and August) and winter season (December, January and February) were slightly higher than other months (Fig. 1).

Effect of melatonin on milk SCCs in buffaloes: Treatment with MLT significantly reduced the milk SCCs in treated groups compared with the day before treatment ($P<0.05$), and the milk SCCs in T1 and T2 groups after MLT treatment were both less than 5×10^5 cells/mL. There were no differences in milk SCCs after MLT treatment between T1 and T2 groups (Fig. 2).

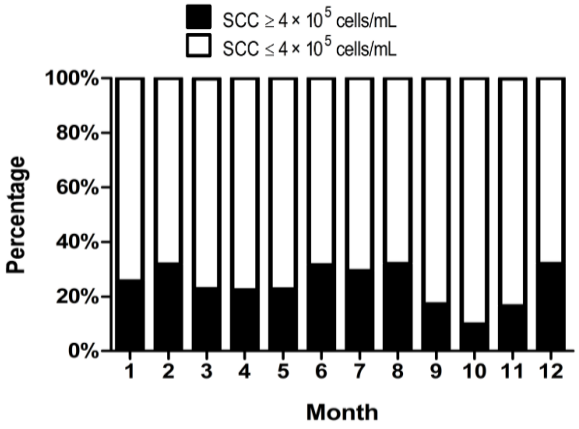

Fig. 1: The total proportion of samples with milk SCC higher than 4×10^5 cells/mL in different months.

Table 1: The milk SCS in different month during 2012 to 2017

Month	Sample (N)	SCS (Mean±SEM)
1	113	3.313 ± 0.243^{b}
2	110	3.765 ± 0.238^{a}
3	219	3.401 ± 0.171^{b}
4	197	3.160 ± 0.176^{bc}
5	238	2.554 ± 0.161^{c}
6	187	3.541 ± 0.178^{ab}
7	177	3.700 ± 0.182^{a}
8	203	4.028 ± 0.176^{a}
9	174	2.956 ± 0.181^{bc}
10	142	2.508 ± 0.221^{c}
11	91	2.627 ± 0.282^{c}
12	97	3.107 ± 0.266^{b}

a, b and c: The different superscripts between months with same column show significant difference ($P<0.05$).

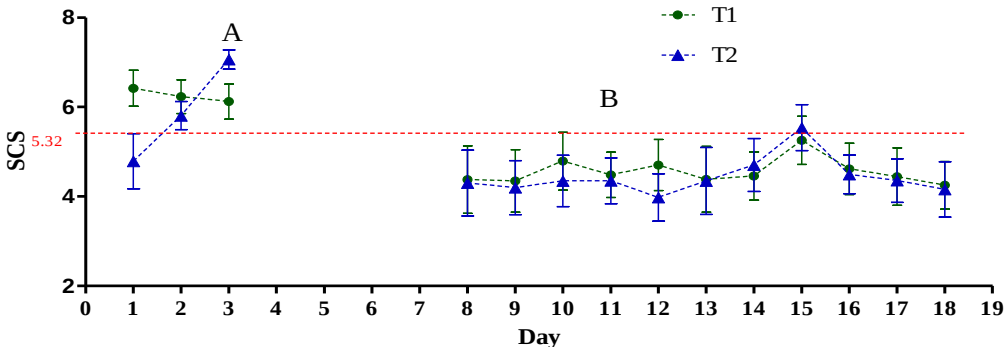

Fig. 2: Changes in milk SCS following the subcutaneous injection of melatonin into buffaloes for four consecutive days. A and B: The different superscript in T1 and T2 groups show significant difference (P<0.05).

Table 2: Effect of melatonin treatment on plasma melatonin (MLT), superoxide dismutase (SOD) and immunoglobulin (IgM) levels in buffaloes (Mean±SEM)

Index	Group	Before injection	After injection			
			1d	4d	7d	10d
SOD (U/mL)	T1	169.86±44.56^{b}	335.42±29.61^{a}	279.17±51.41^{ab}	188.33±15.90^{b}	265.83±58.37^{ab}
	T2	235.27±39.03^{b}	629.86±73.71^{a}	353.33±51.65^{b}	356.94±62.24^{b}	307.07±49.38^{b}
IgM (ug/mL)	T1	238.34±57.13^{b}	412.75±31.16^{a}	293.37±57.78^{ab}	258.39±48.33^{b}	293.45±23.46^{b}
	T2	289.61±33.33^{b}	509.74±27.37^{a}	394.71±57.36^{ab}	352.30±40.60^{b}	309.13±30.49^{b}
MLT(pg/mL)	T1	177.24±11.64^{b}	376.06±66.70^{a}	166.93±15.83^{b}	179.73±18.70^{b}	158.17±10.09^{b}
	T2	166.23±31.24^{b}	575.55±42.76^{a}	233.33±11.11^{b}	212.39±43.25^{b}	227.09±18.50^{b}

Within a row and column, means with different superscript letters (a and b) differ (P<0.05).

Effect of melatonin on plasma melatonin (MLT), superoxide dismutase (SOD) and immunoglobulin (IgM) levels in buffaloes: The results of MLT treatment on blood parameters, including plasma IgM, SOD and MLT are listed in Table 2. Treatment with MLT in T1 and T2 groups significantly increased MLT, IgM, and SOD levels on the day 1 after treatment and then decreased significantly (P<0.05). There were no significant differences in SOD, MLT, and IgM levels between groups T1 and T2 before or after MLT injection.

DISCUSSION

According to the European Union (EU) standard that milk and milk products with the SCCs more than 400,000 cells/mL is regards as to be unfit for human consumption (http://www.adhis.com.au/). In the present study, the percentages of individual with SCCs (more than 4×10^5 cells/mL) in summer season (June, July and August) and winter season (December, January and February) were slightly higher than other months. Meanwhile, the SCS in high temperature months (July and August) and low temperature months (December, January and February) were significantly higher than that in other months, respectively (P<0.05), which were consistent with the previous studies that heat and cold temperature could lead to the elevated SCCs (Hammami *et al.*, 2013; Testa *et al.*, 2017; Bombade *et al.*, 2018). The reason for the difference between different months could be attribute to the environment stress which may depress immune function via inducing oxidative stress (Hammami *et al.*, 2013). Our data indicated that the proper management should be taken to reduce the milk SCCs in the environment stress condition.

Subcutaneous MLT injection resulted in the increase (P<0.05) of SOD and MLT levels in plasma, which was consistent with previous findings that serum SOD (Ramadan *et al.*, 2016) and MLT levels (TA *et al.*, 2014) were upregulated following subcutaneous MLT implantation in buffalo. MLT is a mitochondrial-targeted antioxidant and protects mitochondrial by scavenging ROS (Tan *et al.*, 2016). MLT also protects bovine mammary epithelial cells from oxidant stress damage via up-regulating the expression of Nrf2 and heme oxygenase-1 in the Nrf2 antioxidant defense pathway (Yu *et al.*, 2017). In our study, the antioxidant stress effect was indicated by the increased plasma SOD level after MLT treatment. Meanwhile, it has been reported that SOD responds positively to MLT both *in vitro* (Aydogan *et al.*, 2004) and *in vivo* (Abdel-Wahhab *et al.*, 2004; Hua *et al.*, 2005). SOD has also been demonstrated to play an essential role in reducing milk SCCs in goats (Jiménez *et al.*, 2009). Therefore, the increased SOD levels observed in buffaloes receiving subcutaneous MLT treatment might be attributed to the lower milk SCCs. Reiter (1995) has reported that MLT is more efficiency than glutathione and vitamin E in scavenging highly toxic hydroxyl and neutralizing peroxyl radicals, separately. In addition, MLT, a free radical scavenger and antioxidant, is much more potent than that of vitamin E and vitamin C (Pieri *et al.*, 1994). Similarly, MLT has been shown to have the protective effect by reducing oxidative stress and elevating antioxidant levels, which resulting in the higher oestrus induction rates in anoestrous buffaloes (Kumar *et al.*, 2014). IgM plays a vital role in anti-infection and immune response. Our findings showed that MLT treatment significantly improved the levels of plasma IgM, which was consistent with previous studies that in mouse treated with inactivated Venezuelan equine encephalomyelitis virus (Negrette *et al.*, 2001) and in 28-month-old Wistar rats (Akbulut *et al.*, 2001). The immune response effect was confirmed by the increased plasma IgM level after MLT treatment in our study. IgM has also been demonstrated, at least in part, to play an essential role in reducing milk SCCs in cows (Yang *et al.*, 2017). Therefore, the higher IgM levels might enhance the immune response, which affected milk SCCs in buffaloes. However, there were no significant differences in SCCs and plasma SOD, IgM, and MLT levels after MLT treatment between the two treatment doses (4.68 mg and

20 mg). This result showed that effect of MLT may not be depend on the treatment concentration and low dose of MLT seems to be effective to alter the parameters. The further experiment will be required to identify the optimum dose.

Conclusions: Our study demonstrated that temperature stress (heat and cold) caused higher milk SCCs. Subcutaneous MLT injection improved the quality of milk by reducing SCCs in buffaloes. Importantly, the beneficial effects of the approach can last for quite a long period after treatment. The speculated mechanisms are that MLT improves antioxidant and immune activity via enhancement of plasma SOD and IgM levels in buffaloes.

Authors contribution: LSH conceived and designed the review/project/study. LSH, LZP, LF, MJA and CYX executed the experiment and analyzed the sera and tissue samples. LZP, LM, GTY and WZC analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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