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RESEARCH ARTICLE

Effect of Melatonin on Casein Expression in Buffalo Mammary Epithelial Cells

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ABSTRACT

The objectives of this study are to find out the effect of melatonin treatments (43µM & 430µM) on mRNA and protein expression of casein, and melatonin receptors in buffalo mammary epithelial cells (BuMEC) using reverse transcriptional polymerase chain reaction (qRT-PCR) and Western blotting. Furthermore, by using specific inhibitors against mTOR (Rapamycin) and Jak2-Stat5 pathway (AG490), effect of melatonin treatment on translational level of casein was measured. The results showed that melatonin treatment (430 µM) significantly up-regulated casein level both at transcriptional ($P<0.001$) and translational ($P<0.05$) levels. Melatonin treatment significantly up-regulated mRNA expression of ($P<0.05$) melatonin receptor 1a (MT1) but melatonin receptor 1b (MT2) did not significantly varied. In the presence of mTOR inhibitor, rapamycin, melatonin failed to up regulate ($P<0.05$) protein expression of casein but no significant difference was found in the level of casein in the presence of Jak2-Stat5 inhibitor (AG490). These results indicate the significance of melatonin in altering milk casein expression in mammary epithelial cells in buffalo. Keeping in view the seasonality of buffalo in tropical and subtropical region, this study is important in the establishment of melatonin as the key modulator in changing concentration of casein in milk.

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INTRODUCTION

Milk is mainly constituted by fat, protein and lactose, and is a source of nutrition for human consumption. It is well established that regulation of milk protein in under variety of hormonal control such as insulin, prolactin, growth hormone, thyroid stimulating hormone and corticosteroids (Neville *et al.*, 2002). The mammalian target of rapamycin (mTOR) signaling pathway offers a pivotal function in the production of milk protein in mammary epithelial cells (Burgos *et al.*, 2010). In response to insulin signaling mTOR complex phosphorylates 4EBP1 and allows the release of eiF4E which forms as an initiation complex, binds to 40S ribosomal subunit and initiates the process of translation. Similarly, transcriptional activation of protein synthesis has also been reported by prolactin induced Jak2-Stat5 pathway (Bionaz and Loor, 2011). In dairy animals, due to lower prolactin expression in lactation, co activator of Stat5, E74-like facor 5 (ELF5) plays important role in controlling milk protein synthesis (Bionaz *et al.*, 2012).

Melatonin (MT), a hormone synthesized by the pineal gland, has multiple physiological roles in regulating circadian rhythms, reproduction and neuroendocrine system. Its biosynthesis in all animals is dependent of the amount of lighting conditions with highest concentration at night and that's why it is known as "hormone of darkness". In mammals, it works through two cell membrane bound receptors, melatonin receptor type 1a (MT1) and 1b (MT2) (Emet *et al.*, 2016). Recent studies have shown its protective effect on viability of LPS-stimulated bovine mammary epithelial cells and mastitis, and improved quality of milk by reducing somatic cells count (Shao *et al.*, 2015; Yang *et al.*, 2017; Yu *et al.*, 2017). Studies have shown that administration of exogenous melatonin during long days or artificial

stimulation of short days affect milk protein and casein contents in sheep and cows (Dahl *et al.,* 2012; Molik *et al.,* 2012). However, no reports are available regarding protein contents in buffalo.

The reproductive efficiency in buffaloes is limited by poor expression of estrus, silent heat and lower conception during summer, and that's why this animal behaves as a seasonal breeder in tropical and subtropical part of the world (Phogat *et al.,* 2016). The concentration of melatonin significantly increased 2 h after sunset and difference between day and night melatonin concentration is >28 times higher in adult buffaloes. Although this confined seasonal pattern severely disturbs the milking pattern in buffaloes, no direct report is available on effect of melatonin on milk production, milk protein or casein contents. So in the present study we tried to find out the effect of melatonin treatment on casein level in buffalo mammary epithelial cells. Furthermore, effect of melatonin treatment on its receptors (MT1 and MT2) and protein pathways (mTOR, Jak2-Stat5) was also measured.

MATERIALS AND METHODS

Chemicals and reagents: The following chemicals were purchased from HyClone laboratories Inc., USA, Dulbecco's Modified Eagle Medium (DMEM)-F12, Hank's balance salt solution (HBSS), phosphate buffer saline (PBS), fetal bovine serum (FBS), and penicillin and streptomycin. Melatonin, Collegenase, Hyaluronidase, Dispase, DNAseI, Trypsin EDTA, Rapamycin, AG490 were obtained from Sigma–Aldrich Chemical Co., USA.

Isolation and culture of buffalo mammary epithelial cells: All the experiments in the current study were approved by the Buffalo Care Committee of Guangxi Buffalo Research Institute (Nanning, China). To culture the buffalo mammary epithelial cells, a previously described protocol was followed with brief modifications (Anand *et al.,* 2012). Buffalo parenchymal tissues were retrieved from buffalo and transported to the laboratory in icy antibiotic supplemented sterile HBSS. After cleaning from fat and other connective tissues, tissues were minced and digested with 0.05% collagenase, 0.05% Hyaluronidase for 3 h at 37°C. Later, these mammary tissues were soaked in trypsin EDTA, 1% Dispase at 37°C and filtered through 40 µm cell strainer. After brief centrifugation at 80g and washing with DMEM/F12, the cells were seeded in 60mm dish in a culture medium having DMEM/F12, 10% FBS, 5 µg/ml bovine insulin (Sigma, USA), 1 µg/ml hydrocortisone (Sigma, USA), 1 µg/ml transferrin (Sigma, USA), 10 ng/ml EGF (Sigma, USA), 5 µg/ml Prolactin in an incubator at 37°C under 5% CO_2.

For melatonin treatments, BuMECs were cultured and treated with or without respective melatonin concentrations (43 and 430 µM) for 12 h in serum free medium. The concentration of melatonin selected was based on previous study in which similar dilutions were used to study antioxidant effect of melatonin in bovine mammary epithelial cells (Yu *et al.,* 2017). In some experiments, melatonin was co-treated with rapamycin (0.1nM) or AG490 (50µM) respectively for 12 h.

RNA isolation and quantitative real-time PCR (RT-PCR): For amplification of respective genes, total RNA was isolated from BuMECs by extraction in TRIzol reagent. Complementary DNA (cDNA) was synthesized using ReverTra Ace-α-first strand cDNA synthesis kit (Toyobo Co, Osaka, Japan). Quantitative PCR was performed by using Bio-Rad iQ5 Real Time PCR system in 96 wells (LightCycler 480 Multiwell plate 96, Roche, IN, USA). The relative quantification between target and control was measured by comparative threshold cycle method. The expression values were normalized against values of GAPDH. The primer pairs are listed in Table 1.

Table 1: Primers used for qRT-PCR

Gene	Primer sequence	Tm (°C)	Accession number
MT1	F: CCGTGGTGGTGTTCCATTTC R: GGGGCTTCAGTTTCCGTTTG	58.0	XM_006076173
MT2	F: AGCCTTGTACCCCTACCCG R: GACCACGCTCAGACCCATCA	58.0	XM_006053939
GAPDH	F: ATGCTGGTGCTGAGTATGTG R: CTTCTGGGTGGCAGTGAT	58.0	XM_006065800.1

Western blotting: After respective treatments, BuMEC were washed with ice cold PBS and total protein was extracted by using RIPA buffer (Santa Cruz Biotechnology, CA, USA). The protein samples were separated on polyacrylamide gel and later processed to transfer to PVDF membrane (Millipore, Bedford). The PVDF membranes were soaked by overnight incubation at 4°C with primary antibodies (anti-casein, 1:1000, abcam, USA, ab166596; anti-GAPDH, Proteintech Group, IL, USA, 60004-1). Next morning, after washing with PBS containing 0.1% Tween-20 three times, membranes were saturated with respective secondary antibodies (1:2000, HRP labeled rabbit anti-goat or goat anti-rabbit, Beyotime, Beijing, China) for 1 h at room temperature. After washing, chemiluminescence detection was performed using BeyoECL plus (Beyotime, China).

Statistical analysis: All the data were presented as the mean ± SEM of at least three independent experiments. The data statistics was analyzed using a *t*-test in the SAS 8.2 software (SAS Institute Inc., Cary, NC, USA). As for the qRT-PCR results, the individual difference were measured from three pairs of CT values corresponding to three pairs of technical replicate reactions.

RESULTS

Melatonin treatment increased casein expression in BuMEC: To find out the casein expression, we performed qRT-PCR and Western blotting of casein in BuMEC cultured in the presence of respective melatonin treatments (43µM and 430 µM). The results showed that mRNA expression of beta casein was significantly up-regulated (>10 times) after melatonin treatment (430 µM) compared to that of control (Fig 1A). Similarly, after respective melatonin treatment, protein expression was also increased (~2.0 times) in BuMEC (Fig 1B).

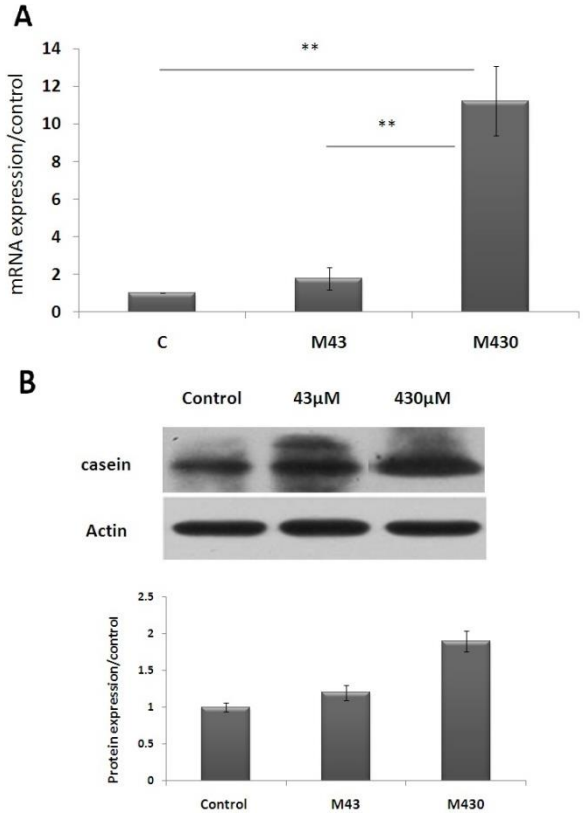

Fig. 1: Expression of casein level after different melatonin treatments in BuMEC. A: The mRNA expression of beta casein was measured in BuMEC after respective melatonin treatments for 12 h as measured by qRT-PCR (C: Control, M43=43µM and M430=430µM). The experiment was repeated three times with three independent replicates and values are presented as mean±SEM. ** indicates P<0.01. **B:** Protein expression of casein was measured in BuMEC after respective melatonin treatments as detected by Western blotting. The protein ratio was measured with actin as having control. The experiment was independently repeated two times and values are presented as mean±SD.

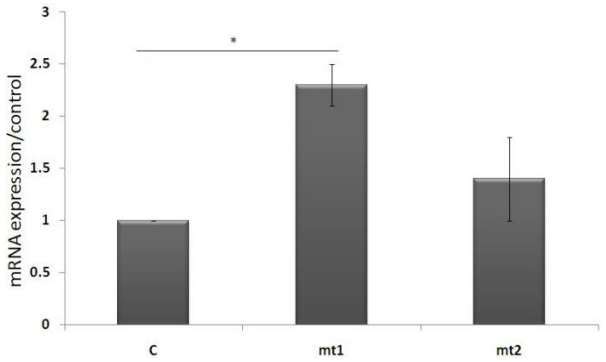

Fig. 2: Effect of treatment of melatonin on mRNA level of melatonin receptor 1a (MT1) and melatonin receptor 1b (MT2) gene in BuMEC. BuMECs were cultured and treated with melatonin, 430µM, for 12 h. After extraction, total RNA was reverse-transcribed into cDNA, and mRNA level of MT1 and MT2 was measured by quantitative real-time PCR and normalized to GAPDH. All experiments were independently replicated three times* indicates P<0.05.

Treatment of melatonin increased its own receptors: As treatment with higher concentration of melatonin increased casein expression, we checked the expression of its receptors in BuMECs (Fig 2). The results showed that treatment of melatonin significantly increased mRNA expression of MT1 but not that of MT2, indicating the involvement of MT1 in facilitating melatonin functioning.

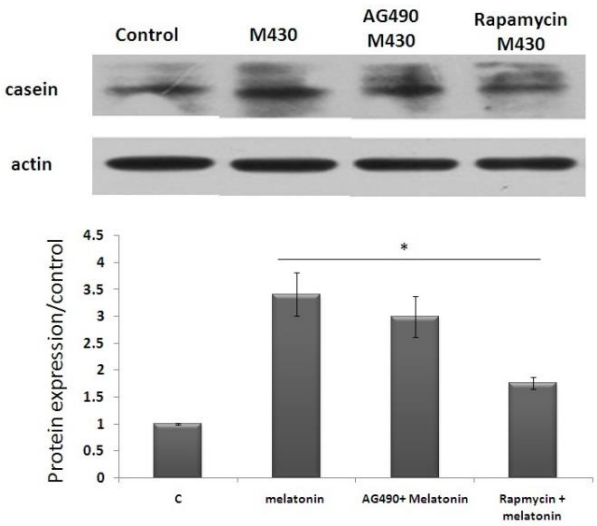

Fig. 3: Melatonin regulates casein expression by activating mTOR pathway. a,b,c BuMEC were cultured with melatonin along with specific signal pathway inhibitors AG490 (Jak2-Stat5), Rapamycin (mTOR), and protein expression of casein, was determined by Western blotting.. Actin served as internal control while data is presented as mean ±SD of at least two independent experiments.

Melatonin failed to induce casein expression in the presence of rapamycin: To further explore the role of melatonin in casein, we used inhibitors of mTOR (Rapmycin) and Jak-Stat (AG490) pathways in the presence of melatonin. The results showed that melatonin did not induce casein expression in the presence of rapamycin, while no significant difference was found while using AG490 indicating that melatonin helps in the casein synthesis by the mammalian target of rapamycin (mTOR) signaling pathway in BuMEC (Fig. 3).

DISCUSSION

Among all livestock species, buffalo milk represents >6% protein contents and along with sheep, it represents highest concentration of casein contents (~4 g/100 ml of milk, Zicarelli 2004; Dario *et al.*, 2008) compared to cow (Zicarelli 2004), goat (Leitner *et al.*, 2004) and camel (all av. ~2) (Khaskheli *et al.*, 2005). A previous study has shown seasonal variation in protein contents in buffaloes where highest crude protein contents were observed in winter and vice versa (Ahmad *et al.*, 2013). This could be due to lower concentration of prolactin in summer and shorter daylight, both of which leads to higher melatonin level (Ramadan, 2017). In our study, we also found higher casein level in BuMEC after melatonin treatment. Since mammary epithelial cells in the udder are responsible for all the synthesis and secretion of milk proteins, studies regarding seasonal variations can be more accurately conducted by using buffalo mammary epithelial cell line. Similar reports were accounted in sheep, where introduction of melatonin implants or changes in the day length both increased casein contents in milk (Molik *et al.*, 2012).

In the present study, we found that melatonin treatment significantly up-regulated MT1 mRNA whereas increase in the MT2 receptor was non-significant. The regulation of melatonin is attributed with its receptors and different cells exhibit participation of its single or both

receptors in performing cellular functions. For example, in bovine GCs, melatonin treatment significantly increased MT1 receptor expression while MT2 expressions were only up-regulated in time and dose dependent manners (Wang *et al.,* 2012). Similarly, Melatonin preferred MT1 rather than MT2 to inhibit cholangiocyte hyperplasia in cholestatic rats (Renzi *et al.,* 2011). It is pertinent to state that many recent studies have described the role of melatonin in mammary epithelial cells as receptor independent free radical scavenger and broad spectrum antioxidant in reducing somatic cell count, improving immune activity, and curing mastitis by preventing LPS-induced inflammatory and oxidative stress damage (Yang *et al.,* 2017; Yu *et al.,* 2017)

A significant role of milk protein synthesis through mTOR pathway has been previously described (Wang *et al.,* 2006). Similarly, regulation in the expression of milk protein has also been associated by Jak2-Stat5 pathways, where both insulin and prolactin has been described to phosphorylate Stat5. The phosphorylation of Stat5 induces transcription of many factors including ELF5 (a co activator of Stat5) ultimately increasing the transcription of milk protein genes. Our study showed that melatonin failed to induce casein expression in the presence of mTOR inhibitor, rapamycin while no significant effect was observed in the presence of AG490 (Jak2-Stat5 inhibitor) (Bionaz *et al.,* 2012). One possibility of this insignificance is that comparing to rodents, the role of Stat5 in controlling milk protein expression is weak in dairy animals suggesting species difference in controlling milk protein synthesis (Wheeler *et al.,* 2001). In bovines, major growth factors including insulin, prolactin, leptin, growth hormone and insulin-like growth factor increase protein synthesis through mTOR pathway. Recently, the role of melatonin in protecting the primodial follicles to oxidative stress via mTOR pathway has been described in rat ovaries (Behram-Kandemir *et al.,* 2017). Similarly, melatonin induced cell death via the PI3K/Akt/mTOR pathway is accounted in melanoma cells (Kim *et al.,* 2014). It appears that this kind of strong association between melatonin and mTOR pathway is important in physiological variations in casein contents in buffalo milk and future studies must be planned on effect of melatonin on an *in-vivo* model in buffaloes.

Conclusions: we found out that treatment of melatonin significantly enhance casein mRNA and protein level in BuMEC. Melatonin treatment significantly increased MT1 expression but not MT2 furthermore, melatonin failed to induce casein expression in the presence of rapamycin indicating its involvement in protein synthesis through mTOR pathway.

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Authors contribution: Conceived the idea: HR, executed by: HR, LSS, MXY, AQD, LXR wrote the paper: HR, Revised by: DXT, MRY, CYP, LXW.

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