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Expression of Prolactin Receptor mRNA after Melatonin Manipulated in Cashmere Goats Skin during Cashmere Growth*

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ABSTRACT : The aim of this research was to investigate the dynamic changes of the level of total prolactin receptor (PRLR) mRNA and the short form prolactin receptor (S-PRLR) mRNA in skin of cashmere goats from the initiation of cashmere fibre growth to active growth. Eighteen half-sib wethers were allocated randomly to two groups. Melatonin implants were used in order to initiate growth of cashmere fibre before the normal time and reduce blood plasma prolactin (PRL) concentration. Real-time reverse transcription quantitative polymerase chain reaction (real-time PCR) was used to determine PRLR mRNA expression levels of skin from June to November. The results showed that, in Chinese Inner Mongolia cashmere goats, there were seasonal variations in expression of total PRLR mRNA in skin with levels decreasing from June to October. Synchronously, the cashmere fibre growth rate gradually increased during this period, but the expression levels of S-PRLR mRNA did not decrease along with seasonal variation from initiation to active growth of cashmere fibre. These results suggest that expression levels of S- PRLR mRNA might be involved in the process of cashmere growth. It was also possible that the change of alternative splicing of PRLR occurred in the skin of cashmere goats from proanagen to anagen. (**Key Words :** Prolactin Receptor, Real-time RT-PCR, Melatonin, Prolactin, Cashmere Fibre, Cashmere Goats)

INTRODUCTION

Studies have already indicated that changes in the concentration of several hormones are related to hair follicle activity, cashmere fibre growth and moulting (Klören et al., 1993; Dicks et al., 1994; Santiago-Moreno et al., 2005). Prolactin (PRL) is believed to mediate seasonal cues entraining reproductive and hair follicle growth cycles (Choy et al., 1997). Research has shown that peak levels of plasma PRL are associated with the initiation of cashmere growth and active growth which occurs as levels decline (Klören et al., 1993). Previous studies showed that PRL acts directly on growth-controlling cells in the wool follicles of Wiltshire sheep (Nixon et al., 1998). In isolated hair follicles, PRL supplementation of follicle cultures did not significantly affect wool production rate (Winder et al., 1995), but acted directly on the secondary hair follicle to stimulate elongation of the cashmere fibre *in vitro* (Ibraheem et al., 1994). However, when the skin of Angora goats was infused with PRL synthesis of mohair fiber decreased (Puchala et al., 2003).

The actions of PRL are initiated through an interaction with specific cell surface high-affinity PRL receptors (PRLR) (Shin and Friesen, 1974; Huang et al., 2006; Omelka et al., 2008). The PRLR is a single membrane-bound protein that belongs to class 1 of the cytokine receptor superfamily (Marc et al., 2000). Bignon et al. (1997) reported the full-length coding sequences for short and long ovine PRL receptors (long form PRL receptor, L-PRLR; short form PRL receptor, S-PRLR), and via PCR experiments on ovine genomic DNA showed that the 39 bp insert was directly linked to the downstream exon. The same result was found in bovine and caprine genomes (Bignon et al., 1997). PRLR immunoreactivity has been identified in ovine skin, and western blotting analysis showed major bands corresponding to molecular weights of 87 and 71 kDa in New Zealand Wiltshire ewes (Choy et al., 1997) and in Inner Mongolia cashmere goats (Sun et al., 2003).

Although there are no differences in hormone binding activity between L-PRLR and S-PRLR, the two forms of

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the PRLR may have distinct signaling pathways (Nixon et al., 2002). As circulating PRL began to rise from low levels, PRLR mRNA in the skin initially fell in ovine skin (Nixon et al., 2002), but has not been investigated in cashmere goats during fibre growth. Exogenous melatonin can initiate growth of cashmere fibre (Nixon et al., 1993; Wuliji et al., 2003; Yue et al., 2007) and reduce plasma PRL concentration (Emesih et al., 1993; Dicks et al., 1995; Santiago-Moreno et al., 2004).

Melatonin implants were used in this research which aimed to investigate dynamic changes in the level of total PRLR mRNA and S-PRLR mRNA in skin of cashmere goats during the period from initiation of fibre growth to active growth (from the summer solstice to November).

MATERIALS AND METHODS

Animals and treatment

The study was performed at the white cashmere goat stock farm of Inner Mongolia in China (latitude 39°06'N, longitude 107°59'E and at altitude of 1,500 m). Eighteen half-sib wethers (mean (±SD) age of 15±2.5 months and an initial body weight of 33.1±2.3 kg) were allocated randomly to two groups (n = 9/group). One group (M) was subcutaneously implanted with melatonin (2 mg/kg BW; Northeast Forest University, China) at the base of the ear on three occasions at 2-monthly intervals commencing in June (the summer solstice). The other group (C) served as the control. During the experimental period, all goats were maintained under natural photoperiodic conditions and were grazed on natural pasture. The composition of vegetation in this area has been described by Zhou et al. (2003).

Sample collections, recording and measurement

Blood samples were collected into EDTA anticoagulant at 10:00 a.m. from all experimental goats by jugular venepuncture at one-monthly intervals throughout the study. The blood was immediately centrifuged, and the plasma frozen at -20°C until assayed.

The fibre on the mid-side of each goat was dyed using human hair dye (CCP-N6, Jingxi Chemica Company, China) at the beginning of the study. The crimping frequency and the crimp amplitude are the same in the same cashmere goat variety, at the same age and in the same determination spot. Therefore, we used the relaxed length to replace the straight length according to the method of Rhind et al. (1995). The relaxed length of undyed cashmere fibre was recorded monthly on the right mid-side region. Cashmere fibre growth rate was calculated according to the method of Rhind et al. (1995).

Skin samples (approximately 1 cm^2) were cut monthly from the left mid-side region under local anaesthesia (Procaine hydrochloride, Huabei Medicine Company, China), snap frozen in liquid nitrogen and then stored at -70°C for subsequent extraction of total RNA.

Hormone assays

Plasma melatonin concentration was determined using the Melatonin ELISA kit (RE 54021, Immuno-Biological Laboratories GmbH of Hamburg, Germany) according to the instructions for use. Intra-assay and inter-assay coefficient of variation was 7.2% and 12.9%, respectively. Plasma PRL concentrations were determined by radioimmunoassay (RIA) as described by McNeilly and Andrews (1974). Sensitivity was 0.1 ng/ml; intra-assay and inter-assay coefficient of variation was 8.6% and 11.3%, respectively.

Extraction of total RNA and reverse transcription

Each sample of skin was first disrupted using a freezer mill (6750-230 Freezer/mill, Spex Co., NJ, USA) and total RNA was extracted from the fine powder using an RNAultra Kit (Tiangen Biotech Co., Beijing, China) according to the manufacturer's instructions. RNA was purified from the total RNA using a Purification Kit (TaKaRa Biotechnology Co., Ltd., Dalian China) according to the manufacturer's instructions. RNA concentration was measured by spectrophotometry at 260 nm and RNA integrity was verified by agarose gel electrophoresis.

Reverse transcription was carried out in a 50 μl final volume containing the total RNA (approximately 3 μg) solution, 200 U M-MLV reverse transcriptase (Promega Co., Madison, Wisconsin, USA), 25 U recombinant ribonuclease inhibitor (RNasin) (TaKaRa Biotechnology Co., Ltd., Dalian China), 5 ng oligo (dT) 18 primer (Promega Co., Madison, Wisconsin, USA) and 2 μl dNTP mix (10 mM) according to the manufacturer's instructions. The transcribed cDNA was inspected by PCR using paired primers of housekeeping gene (glycerolaldehyd-3-phosphate-dehydrogenase gene, GAPDH). Reaction cycles consisted of an initial denaturing step at 95°C for 3 min, followed by 33 cycles of denaturing at 95°C for 15 s, annealing at 62.7°C for 30 s and extension at 72°C for 30 s, followed by extension at 72°C for 7 min and 4°C for ever. The PCR products were inspected by gel electrophoresis.

Oligonucleotide primers were designed using Primer Premier 5.00 software (Premier Co., Santa Cruz, CA) for PRLR, S-PRLR and GAPDH. The primers were synthesised by a professional company (Sangon Biotech Co., Shanghai, China). The difference of cDNA sequences between S-PRLR and L-PRLR are shown in Figure 1 together with the positions of designed real-time quantitative PCR primers.

Real-time quantitative PCR

Fluorescent real-time quantitative PCR was used to

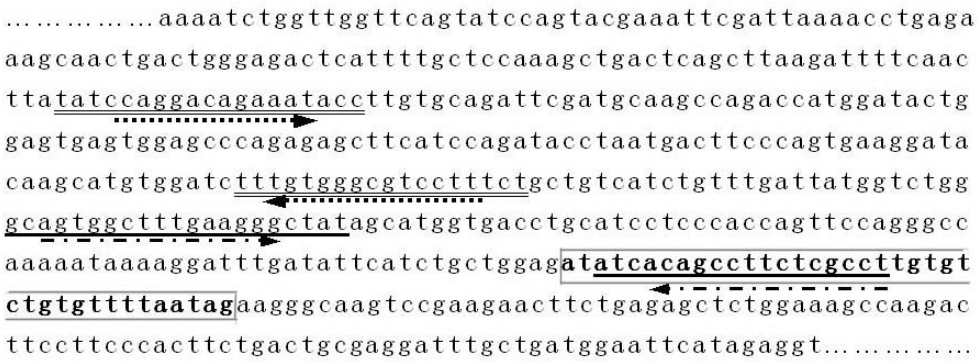

Figure 1. Partial sequence for the difference between L-PRLR and S-PRLR. The only difference between S-PRLR and L-PRLR coding sequences was, respectively, the presence or absence of a 39 bp insert (in the framed box), and the other sequences were common for L-PRLR and S-PRLR. The real-time quantitative PCR primers were designed at the positions with subscript "_____" for S-PRLR in order to determine S-PRLR mRNA levels, and at the positions with subscript "_____" for PRLR in order to determine total PRLR mRNA levels.

determine mRNA expression levels of skin for total PRLR and S-PRLR from the initiation of cashmere fibre growth to active growth.

Primer concentrations were optimized and absence of primer-dimers was verified before actual mRNA quantification. PCR reactions for each gene were conducted in a final volume of 20 μl that contained 10 μl of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), optimal primer concentrations (0.4 μl each, 200 nM) and 2 μl cDNA using a Prism 7000 Real time PCR system (ABI Co., Foster City, California, USA). Real-time PCR procedure was performed according to the manufacturer's instructions. The real-time PCR primer sequences are shown in Table 1. Relative efficiencies of amplification of PRLR, S-PRLR and GAPDH were validated as approximately equal by running a serial dilution of highly concentration cDNA. Calibrated and non-template controls were include in each assay. Each sample was run in duplicate. SYBR Green dye intensity was analysed using the ABI prism 7000 SDS software (ABI Co., Foster City, California, USA). All results were normalized to the GAPDH (an unaffected housekeeping gene).

Statistical analysis

Fibre growth rate was analyzed using the t-test and plasma hormone concentration and mRNA expression levels were analyzed using the ANOVA procedure of SAS (SAS, 1999). Significant means were separated using Duncan's multiple range test.

RESULTS

Hormone concentrations

Plasma melatonin concentration showed no significant difference between both groups before administration of melatonin in June ($p>0.05$) but there were significant differences after administration (Figure 2a). There were significant effects in July and August ($p<0.01$), and in September ($p<0.05$) on plasma PRL concentration after administration (Figure 2b). There was also marked seasonal variation and a trend towards decreasing plasma PRL concentration from the summer solstice to November for both M and C groups.

Cashmere fibre growth rate

The cashmere fibre growth rate of M group was markedly greater than that of C group in July ($p<0.01$) (C group: three of the nine goats cashmere fibre outgrowth; M group: the entire nine goats cashmere fibre outgrowth), August ($p<0.001$) and September ($p<0.05$). There was no significant difference between M and C groups in October, November and December ($p>0.05$) (Figure 3). There was a trend towards increasing cashmere fibre growth rate from July to October in C group ($p<0.05$).

Table 1. Real-time PCR primer sequences and PCR product size for GAPDH, PRLR and S-PRLR

Gene	Primers (5'→3')	Product (bp)	Genbank
GAPDH	Forward: GCAAGTTCCACGGCACAG Reverse: GGTTCACGCCCATCACAA	249	AJ431207
PRLR	Forward: TATCCAGGACAGAAATACC Reverse: AGAAAGGACGCCCACAAA	147	AF041257
S-PRLR	Forward: GCAGTGGCTTTGAAGGGCTAT Reverse: AGGCGAGAAGGCTGTGAT	113	AF041979

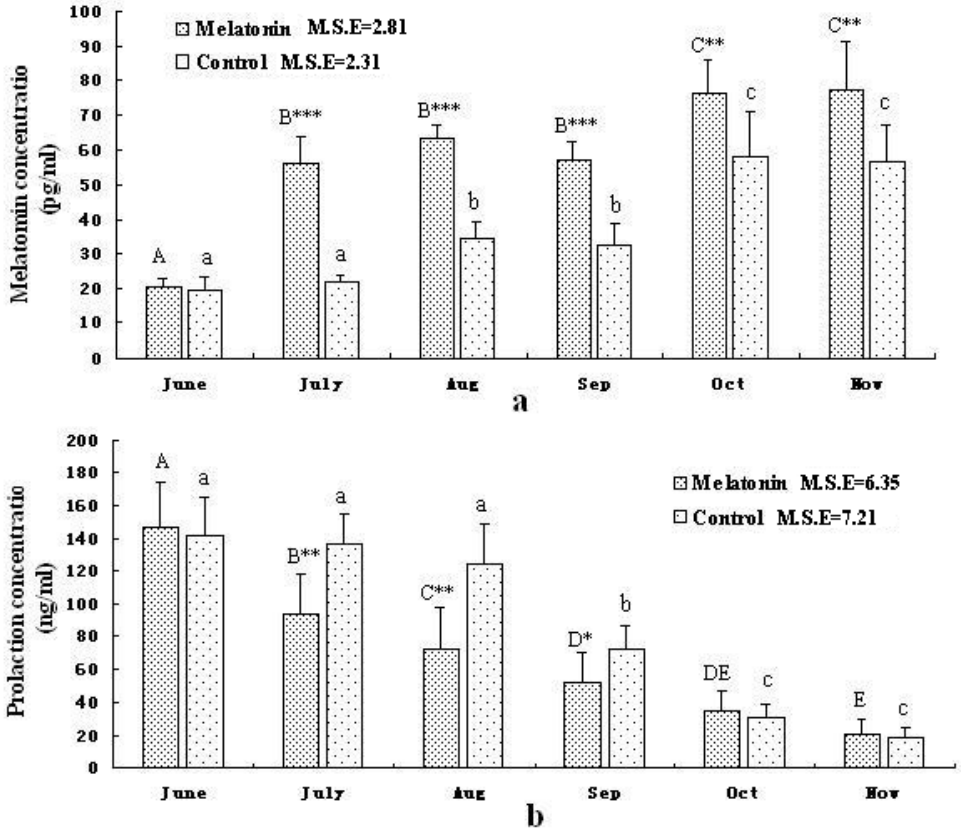

Figure 2. Melatonin implant (M) and control (C) group mean plasma melatonin concentration (pg/ml) (a) and prolactin (PRL) concentration (ng/ml) (b) at monthly intervals (samples for June were taken before implantation of melatonin) from June to November. Values with different capital letters show significant differences ($p<0.05$) in M group; values with different small letters show significant differences ($p<0.05$) in C group. The M group values superscribed with * are significantly different ($p<0.05$) to C group (** $p<0.01$; *** $p<0.001$).

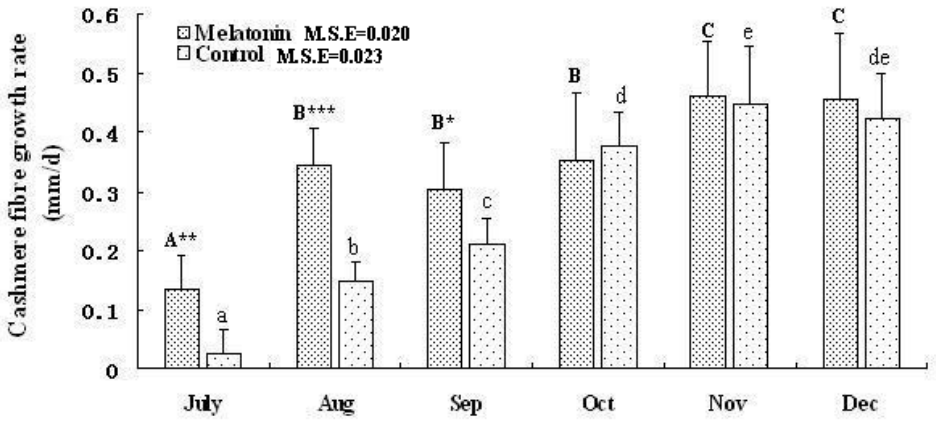

Figure 3. Melatonin implant and control group mean cashmere fibre growth rate (mm/d) from July to December. Values with different capital letters are significantly different ($p<0.05$) in M group; values with different small letter are significantly differenct ($p<0.05$) in C group. The M group values with * are significantly different ($p<0.05$) to C group (** $p<0.01$; *** $p<0.001$).

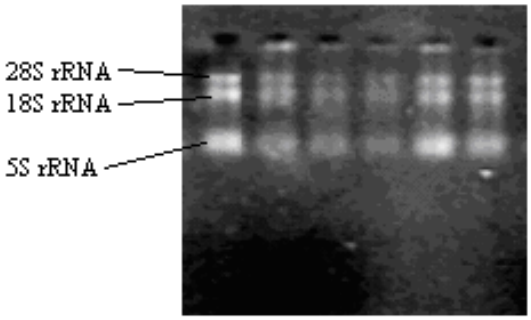

Figure 4. Result of agarose gel electrophoresis of total RNA from skin of cashmere goats.

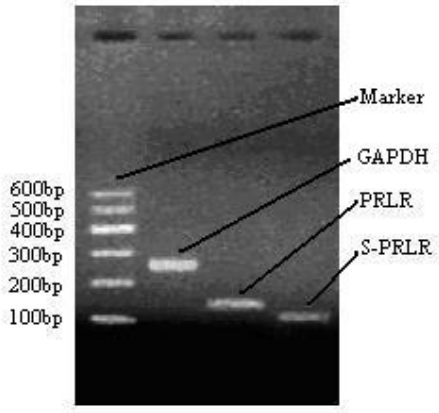

Figure 5. Identification of real-time PCR products. Sizes of PCR products for GADPH (249 bp), PRLR (147 bp) and S-PRLR (113 bp) were validated using agarose gel electrophoresis.

Expression levels of PRLR mRNA

The result of total RNA integrity verified by agarose gel electrophoresis is indicated in Figure 4. All RNA samples had A260/A280 ratios between 1.7 and 1.9, indicating pure and clean RNA isolates. Primer specificity was analyzed by a comparison of PCR products (Figure 5) using agarose gel electrophoresis. The results showed that the primers of GADPH, PRLR and S-PRLR were practicable and optimum for real-time quantitative PCR; sizes of PCR products matched the theoretically designed length. Dissociation curve plots (melting temperature analysis) showed only one peak for each product, thus indicating that specific amplification formation had occurred during the PCR. Linearity and efficiency of PCR amplification were deduced from the given standard curves ($R^2 \geq 0.995$) by ABI Prism 7000 SDS software (ABI, USA).

The results indicated that the expression levels of total PRLR mRNA decreased gradually in skin of cashmere goats from the summer solstice (June) to September (Figure 6a) for both groups. The total PRLR mRNA level of M group was significantly different ($p<0.05$) to C group in July (one month after melatonin implant). The S-PRLR mRNA in skin did not gradually decrease along with seasonal variation (Figure 6b), but it was higher in October than in July and August in C group. S-PRLR mRNA levels of M group were significantly higher ($p<0.05$) than those of C group in July, August and September (Figure 6b). However, the S-PRLR/total PRLR ratio (Figure 6c) gradually increased from June to October for M group and from August to October for C group. In M group, the ratios of S-PRLR/total PRLR were significantly higher than C group in July ($p<0.05$) and August ($p<0.01$).

DISCUSSION

PRLR was abundant in the dermal papilla, outer root sheath, inter root sheath and hair fibre matrix, and also existed to a small extent in the sebaceous glands and epidermis of sheep (Nixon et al., 1998) and cashmere goats (Sun et al., 2003). These authors suggested that the hair follicle activity was regulated and partially dependent on the pattern of expression of PRLR. Full-length sequences of ovine L-PRLR and S-PRLR have been cloned, and results showed that the 39 bp insert was directly linked to the downstream exon, and separated from the upstream exon by an 800 bp intron on genomic ovine, bovine and caprine DNA (Bignon et al., 1997). The only difference between S-PRLR and L-PRLR coding sequences was, respectively, the presence or absence of a 39 bp insert at the beginning of the cytoplasmic domain with two contiguous inframe stop codons at its 3' end (Bignon et al., 1997).

Real-time quantitative PCR is a sensitive method that allowed us to use low amounts of starting copy DNA (cDNA) and amplify it to quantitative levels (Bustin, 2000). In this research, it was used to determine mRNA expression levels which showed that, in Chinese Inner Mongolia cashmere goats, there were seasonal variations in expression levels of total PRLR mRNA, decreasing in skin from long daylight to short daylight (from June to October). At the same time, there was a trend towards decreasing plasma PRL concentration. This trend is consistent with previous reports in ovine skin (Nixon et al., 2002; Soboleva et al., 2005). We demonstrated that expression of the total mRNA in skin of cashmere goats gradually decreased from the summer solstice to October. Synchronously, the cashmere fibre growth rate gradually increased. This result is consistent with a previous report that the level of PRLR expression peaked during proanagen and returned to a low level during anagen in sheep (Nixon et al., 2002).

Our data also show that the expression of the S-PRLR mRNA was comparatively low in the total PRLR mRNA in cashmere goats skin. This result is similar to previous reports in ovine skin (Nixon et al., 2002) and in murine skin (Foitzik et al., 2003). However, the expression levels of S-PRLR mRNA did not decrease along with seasonal variation from the initiation of cashmere fibre growth to

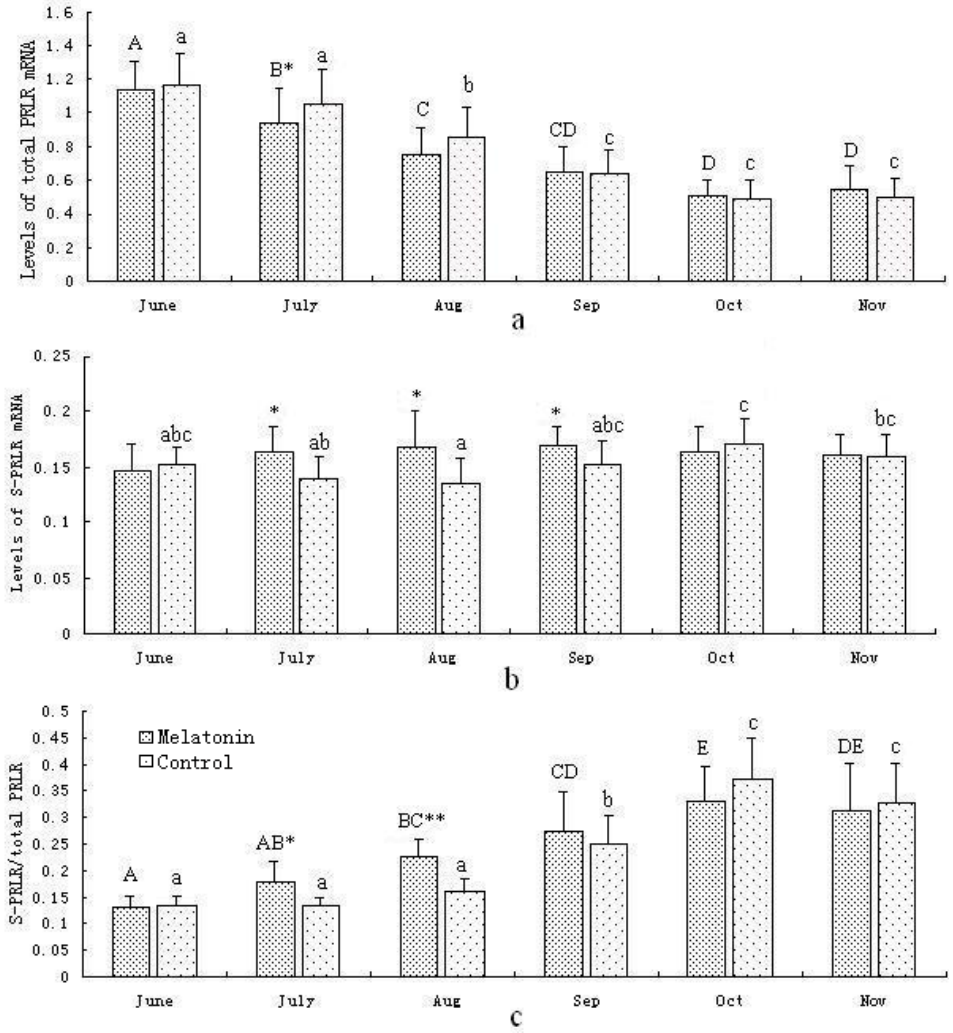

Figure 6. The expression levels of total PRLR mRNA (a) and S-PRLR mRNA (b) and the ratios of S-PRLR/total PRLR (c) in skin of cashmere goats at monthly intervals (samples for June were taken before implantation of melatonin) from June to November for melatonin implant (M) and control (C) groups. Values with different capital letters are significantly different ($p<0.05$) in M group; values with different small letter are significantly different ($p<0.05$) in C group. The M group values superscribed with * are significantly different ($p<0.05$) to C group (** $p<0.01$).

active growth. It is possible that S-PRLR has different signaling pathways compared with L-PRLR in skin of cashmere goats from proanagen to anagen.

The data show that there were significantly depressed plasma PRL concentrations as a consequence of the melatonin implants, especially during the three-month period after melatonin treatments. These results are similar to previous reports (Emesih et al., 1993; Santiago-Moreno et al., 2004). At the same time, cashmere fibre growth rate of M group was higher than that of C group. During this period, the S-PRLR mRNA levels of M group were significantly higher than those of C group in July, August and September. This result indicated that the expression levels of S- PRLR mRNA were involved in the process of cashmere growth.

Of importance is the ratio of S-PRLR/total PRLR which differed significantly between M and C groups in July and August. Synchronously, there was a gradual increase in each group from the initiation of cashmere fibre growth to active growth. Therefore, it is possible that the change of alternative splicing of PRLR occurred from proanagen to anagen in cashmere skin.

CONCLUSIONS

This research shows that the expression levels of total PRLR mRNA gradually decreased in skin of Chinese Inner Mongolia cashmere goats from the summer solstice (June) to October. This was consistent with decreasing plasma PRL concentration and increasing cashmere fibre growth rate occurring at the same time, but the expression levels of S-PRLR mRNA did not decrease along with seasonal variation from the initiation of cashmere fibre growth to active growth. However, the increasing S-PRLR/total PRLR

ratio was usually consistent with increasing cashmere growth rate at the same stage. These results suggest that the expression levels of S-PRLR mRNA may be involved in the process of cashmere growth, and the S-PRLR may have a specific signaling pathway in the skin of cashmere goats. Synchronously, it was also possible that the change of alternative splicing of PRLR occurred from proanagen to anagen in the cashmere skin.

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