

## Cloning, Sequencing and Analysis of Melatonin Receptor Subtype MT<sub>1</sub> in Hypothalamus-Pituitary-Gonadal Axis and Pineal of Female Bactrian Camel

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**Abstract:** It is well known that melatonin is a coordinating signal for mammalian reproduction. In order to confirm the presence of melatonin receptors in hypothalamus-pituitary-gonadal axis and pineal of female Bactrian camel, the researchers used a Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) procedure to examine receptor MT<sub>1</sub> expression. The length of MT<sub>1</sub> gene was 452 bp. RT-PCR assaying revealed the presence of the mtl (Mella) melatonin receptor subtype in reproduction axis and pineal which were obtained from the slaughterhouse in the Ningxia Autonomous Region, China. Sequence has been confirmed a high identity (above 85%) with melatonin receptor MT<sub>1</sub> of other mammal known in GenBank. Comparing with other tissue's sequences, one base substitution changed the 108th TTC codon (encoding Phenylalanine) to TAC (Tyrosine) on hypothalamus. Although, there are base substitutions in pineal's gene, encoded amino acid are coincident with pituitary and ovary. The current results, the expression of MT<sub>1</sub> receptor mRNA in brain and ovary, suggest that melatonin regulate reproduction function through not only neuroendocrine but also directly acting on the ovary in Bactrian camel.

**Key words:** Clone, sequence, MT<sub>1</sub>, hypothalamus-pituitary-gonadal axis, bactrian camel, China

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### INTRODUCTION

Many mammalian species from temperate latitudes exhibit seasonal variations in breeding activity which is controlled by the annual photoperiodic cycle (Hafez, 1952). Photoperiodic information is conveyed through several neural relays from the retina to the pineal gland where the light signal is translated into a daily cycle of melatonin secretion: high at night, low in the day. This duration of melatonin secretion is then processed to regulate the activity of the hypothalamo-hypophyseal and gonadal axis (Karsch *et al.*, 1988). This regulates the pulsatile secretion of Gonadotropin-Releasing Hormone (GnRH) from the hypothalamus (Malpoux *et al.*, 1999). Changes in GnRH release induce corresponding changes in Luteinising Hormone (LH) secretion which are responsible for the alternating presence or absence of ovulation in the female and varying sperm production in the male (Malpoux *et al.*, 1999).

Three specific melatonin receptor subtypes have been cloned and characterized in vertebrates: MT<sub>1</sub>, MT<sub>2</sub> (previously known as Mella and Mell b, respectively also known as MTNR1A and MTNR1B, respectively) and

Mell c (Reppert *et al.*, 1994, 1995a, b). There are three MT receptors found in lower vertebrates thus, so far only two MT receptors, MT<sub>1</sub> and MT<sub>2</sub> found in mammalian species. All these subtypes display similar high binding affinity for melatonin and the same rank of order for the binding of common ligands (Dubocovich, 1995; Dubocovich and Markowska, 2005).

Structurally, high-affinity melatonin receptor subtypes define a distinct receptor family within the superfamily of G Protein-Coupled Receptors (GPCRs) as they have been shown to be functionally coupled to both Pertussis toxin-sensitive and Pertussis toxin-insensitive G proteins (Morgan *et al.*, 1990; Drew *et al.*, 2002). Previously, melatonin MT<sub>1</sub> receptor has been pay more attention to melatonin regulation of seasonal breeding in mammal (Migaud *et al.*, 2005).

Numerous experimental data point that melatonin receptors localized to the suprachiasmatic nuclei of the anterior hypothalamus and pars tuberalis of the pituitary as putative target sites for chronobiological actions of melatonin on circadian rhythms and seasonal changes, respectively (Morgan 1991; Morgan *et al.*, 1994; Arendt, 1995a, b). It was also reported that melatonin receptors

localized to ovary in human (Niles *et al.*, 1999), rat (Witt-Enderby and Dubocovich, 1996; Soares *et al.*, 2003; Clemens *et al.*, 2001) and sheep (Zhang *et al.*, 2009; Coge *et al.*, 2009). This means that the sites of action of melatonin are hypothalamus-pituitary-gonadal axis and reproduction is effected by melatonin regulating reproductive hormone secreting through melatonin receptors of this sites (Malpaux *et al.*, 1996). So, it is very necessary to study melatonin receptors distribution and expression in reproduction axis. The female Bactrian camel is a seasonal polyestrous animal which oestrus usually appear December to April (Chen *et al.*, 1985). The length of the oestrus cycle is normally 2-3 weeks, sometimes the period can extend to 30-40 days (Bosaev, 1938). Seasonal variations in the nycthemeral rhythm of plasma melatonin in dromedary camel had been investigated: the pattern of melatonin secretion in the camel show a significant seasonal variation parallel to the photoperiodic changes of the year (El-Allali *et al.*, 2005). In the Bactrian camel, the concentration of plasma melatonin is extremely significant difference between day and night in breeding season. There was a markedly significant positive correlation between plasma melatonin and prolactin concentration of Bactrian camels during 24 h ( $p < 0.01$ ) and a significant positive correlation between melatonin and FSH concentration in the Bactrian camels ( $p < 0.01$ ) (Yong, 2000). This implies that melatonin regulate camel reproduction and breeding just like the other seasonal breeding animals. The objectives of the present study are to clone and to analyze the hypothalamus-pituitary-gonadal axis and pineal melatonin receptor *MT<sub>1</sub>* gene with RT-PCR in breeding season of Bactrian camel. This achievement provides a basis for further research on the relationship of melatonin and seasonal breeding and reproduction of the camel.

## MATERIALS AND METHODS

**Tissue samples:** The pineal, hypothalamus, anterior pituitary and ovary of adult female Bactrian camels were collected during winter (mid-November) from the slaughterhouse of the Ningxia Laoheqiao Muslim Meat Food Company in the Ningxia Autonomous Region, China. These tissues were immediately frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until RNA isolation was performed. All experimental procedures were performed according to authorization granted by the Chinese Ministry of Agriculture.

**Total RNA extraction and reverse transcription:** Total RNA was extracted from the tissue samples using trizol reagent (Takara, Dalian, China) according to the

manufacturer's instructions. Total RNA was converted into cDNA by oligo (dt) priming using a First Strand cDNA Synthesis kit (#K1612, MBI Fermentas, Lithuania). Deionized, nuclear free water was added to the total RNA (0.5  $\mu\text{g}$ ) to get a volume of 5.0  $\mu\text{L}$ . After this oligo (dt) primer (0.1  $\mu\text{g}$ ) was added and the mixture was heated to  $+70^{\circ}\text{C}$  for 5 min then cooled immediately on ice. A ribonuclease inhibitor (10 U), 5 $\times$ reaction buffer (2  $\mu\text{L}$ ), 1.0  $\mu\text{L}$  of 10 mM dNTP mix (dATP, dGTP, dCTP and dTTP) and a MuLV-reverse transcriptase (20 U) were added to make the final volume 10  $\mu\text{L}$ . The reaction mixture was preincubated for 10 min at room temperature ( $+23^{\circ}\text{C}$ ) before cDNA synthesis. Reverse Transcription (RT) reactions were carried out for 60 min at  $+37^{\circ}\text{C}$  and then samples were heated to  $+70^{\circ}\text{C}$  for 10 min to terminate the RT reaction.

**Polymerase chain reaction:** Since, the melatonin receptor gene sequences of camel has not been reported in GenBank, the primers design was based on an alignment of the sequences of melatonin receptor cDNAs of sheep, cattle, pig and human which have been reported in Genbank. Oligonucleotids primers were designed using the Primer Premier Version 5.0 (Premier Biosoft International, Palo Alto, CA, USA) and the Oligo 6 program (Molecular Biology Insights Inc., Cascade, CO, USA). The sequences of the primers of the *MT<sub>1</sub>* primer set were as follows: Sense primer, 5'-TTGCTACATCTGCCACAGTC-3' and antisense primer 5'-CAAACAGCCACTCTGGGAT-3'. A total volume of 50  $\mu\text{L}$  was used for all PCR reactions, containing 0.5  $\mu\text{L}$  of template DNA, 5  $\mu\text{L}$  of 10 $\times$ Ex Taq Buffer, 4  $\mu\text{L}$  of dNTP Mixture (2.5 mM each), 0.5  $\mu\text{L}$  of TaKaRa Ex Taq polymerase (5 units  $\mu\text{L}^{-1}$ ), 1  $\mu\text{L}$  each primer. PCR amplification was performed in a Gene Cyclor (Bio-Rad, USA) under the following conditions: denaturation at  $94^{\circ}\text{C}$  for 5 min followed by 35 cycles of denaturation at  $94^{\circ}\text{C}$  for 1min, annealing at  $5^{\circ}\text{C}$  for 1 min for *MT<sub>1</sub>*. The PCR products were separated by electrophoresis on 1.5% agarose gels (Promega, Madison, WI, USA) in parallel with a DNA marker III. The amplified product was verified by 1% agarose gel electrophoresis and analyzed using gel imaging system (Bio-Rad, USA).

**Purifying, ligating, transforming and sequencing of the *MT<sub>1</sub>* gene:** The PCR amplified product of the *MT<sub>1</sub>* gene was purified by the Gel Extraction kit according to the manufacturer's instructions. The purified product was ligated into pMD18-T vector which was a TA cloning vector at  $16^{\circ}\text{C}$  overnight using *T<sub>4</sub>* DNA ligase. Competent *E. coli* DH5 $\alpha$  cells were transformed with the ligation mixture by the Heat Shock Method. The cells were

cultured at 37°C on Luria-Bertani broth plates containing 100 mg mL<sup>-1</sup> ampicillin for 16 h. Then the recombinant plasmid was confirmed by PCR. The correct recombinant plasmid was sent to Dalian TAKARA Biotechnology Co. (China) for sequencing. Pair distance and phylogenetic tree of MT<sub>1</sub> sequence of Bactrian camel, cattle, sheep, swine and human are analyzed using DNASTar soft(Demonstration System DNASTAR, Inc.).

## RESULTS AND DISCUSSION

The total RNA of the Bactrian Camel was extracted by 1% agarose gel electrophoresis. The extracted RNA from ovaries had the normal ultraviolet absorption, the value of OD260/OD280 varied from 1.8-2.0 which indicated that the RNA was not contaminated by proteins or phenol. The average yields of the total RNA from 100 mg fresh tissues samples were 80.2 µg. Two bright bands, 28S rRNA and 18S rRNA were very clear and the brightness value of these two bands was close to 2:1 while the band of 5S rRNA was very weak which proved that the totle RNA had no DNA contamination and had a good integrity and high purity without obvious degradation (Fig. 1).

A single PCR product of the expected size (452 bp) was detected on the amplification with specific mt1 melatonin receptor (Fig. 2). The researchers initially cloned the Bactrian camel MT<sub>1</sub> melatonin receptors by PCR amplification of camel pineal, hypothalamus, anterior pituitary and ovary mRNA-derived cDNAs with the primer based on an alignment of sheep, cattle, pig and human MT<sub>1</sub> sequences. The major PCR products of 452 bp were gel purified, recombined to plasmid and sequenced. Recombination plasmids are marked ,respectively with pMD18-T-RP (Pineal), pMD18-T-RH (Hypothalamus),

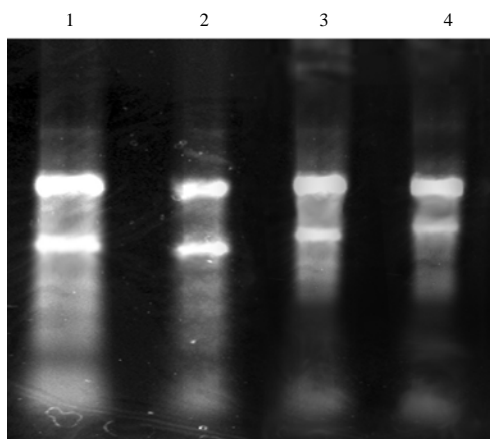


Fig. 1: Agarose gel electrophoresis of total RNA extracted from 1) pineal, 2) ovary, 3) hypothalamus and 4) pituitary

pMD18-T-RA (Anterior pituitary) and pMD18-T-RO (Ovary). Alignment of sequence of four recombination plasmids shows that pMD18-T-RA and pMD18-T-RO have identical sequence. Comparing with the pMD18-T-RA, there are two bases substitution of degenerate codon on pMD18-T-RP (Pineal) and one base substitution which changed the 108th TTC codon (encoding Phenylalanine) to TAC (Tyrosine) on pMD18-T-RH. Sequencing of the camel product confirmed that there are a lots of similarities (6.5%) between pMD18-T-RA and 190-644 bp of exon 2 and partial cds of Ovis aries isolate K13 melatonin receptor type 1A (*MTNR1A*) gene (GenBank: HQ658146.1) and there are GCA base deletion at 423 bp in all of the Bactrian camel MT<sub>1</sub> comparing with other mammal MT<sub>1</sub> known in GenBank.

Comparison of the nucleotide sequences of MT<sub>1</sub> melatonin receptors revealed that the Bactrian camel anterior pituitary MT<sub>1</sub> melatonin receptor has 87.2% identity to cattle MTNR1A (EU716174.1), 85.8% identity to human (NM\_005958.1) and pig sequence (U73326.1), 86.3% identity to sheep MTNR1Aα (U14109.1) and 85.4% identity to sheep MTNR1Aβ (AF045219.1) (Fig. 3). A phylogenetic tree was constructed using DNASTar's MegAlign with Clustal W Method and support for

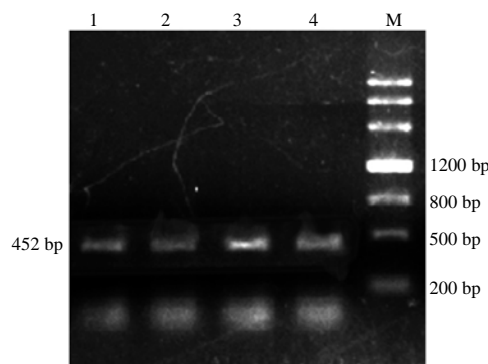


Fig. 2: Agarose gel electrophoresis of RT-PCR, 1) pineal, 2) ovary, 3) hypothalamus and 4) pituitary. M: Marker III

	1	2	3	4	5	6	7	
1		85.8	87.2	85.8	86.5	86.3	85.4	pMD 18-T-RA
2	15.8		86.6	100.0	85.1	85.3	84.2	Pig (U73326.1)
3	14.2	14.9		85.6	97.1	97.1	96.3	Cattle (EU716174)
4	15.8	0.0	14.9		85.1	85.3	84.2	Human (NM_005958)
5	15.0	16.9	2.9	16.9		99.3	98.7	Sheep (HQ658145.1)
6	15.3	16.6	2.9	16.6	0.7		98.9	Sheep MTNR1Aα(U14109.1)
7	16.1	18.0	3.8	18.0	1.3	1.1		Sheep MTNR1Aβ (AF045219.1)
	1	2	3	4	5	6	7	

Fig. 3: Percent identity of melatonin receptor MT<sub>1</sub> of different mammals using DNASTar's MegAlign with Clustal W Method

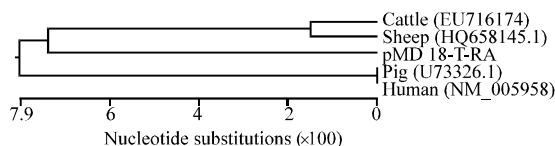


Fig. 4: Phylogenetic tree of the melatonin receptors MT<sub>1</sub> of different mammals using DNASTar's Megalign with Clustal W Method

analyzing homology of the melatonin receptors MT<sub>1</sub> (Fig. 4). There is evidence that melatonin plays an important role in the regulation of reproductive activity in seasonal breeders (Reiter, 1991). The effects of melatonin on reproductive function are thought to be mediated by G protein-coupled MT<sub>1</sub> receptors in the pars tuberalis of the sheep and other seasonal breeders (Reppert *et al.*, 1994). Therefore, it is very important to investigate distribution and expression of melatonin receptor MT<sub>1</sub> in hypothalamus-pituitary-gonadotropin axis and pineal for clarifying mechanism of melatonin regulating seasonal reproduction through light in seasonal breeding animals. It has been reported that melatonin regulate reproduction through melatonin receptors mtl which are distributing in hypothalamus, pituitary and ovary of mammals (Morgan, 1991; Niles *et al.*, 1999; Zhang *et al.*, 2009; Coge *et al.*, 2009). In the Bactrian camel, the concentration of plasma melatonin is relevance to prolactin and FSH concentration in breeding season (Yong, 2000). However, there were a little further studies on melatonin receptor in Bactrian camel in the past. The present research demonstrate that melatonin receptor MT<sub>1</sub> mRNA is expressed in hypothalamus-pituitary-gonadotropin axis and pineal of female Bactrian camel and sequence has 87.2% identity to cattle MT<sub>1</sub> (EU716174) and 86.3% identity to sheep MT<sub>1</sub> (U14109.1). This shows unambiguously the existence of a functional MT<sub>1</sub> receptor in brain and ovary of Bactrian camel. At same time, the expression of melatonin receptor MT<sub>1</sub> mRNA in ovary may suggest that ovary is also a direct target organ of melatonin and mediate reproduction in Bactrian camel.

There are two bases substitution of degenerate codon on pMD18-T-RP and one base substitution which changed the 108th TTC codon (encoding phenylalanine) to TAC (tyrosine) on pMD18-T-RH. It remains to determine whether this codon change can affect melatonin regulation of seasonal breeding in Bactrian camel.

The melatonin receptor MT<sub>2</sub> also express in reproduction axis of mammals (Niles *et al.*, 1999; Xiao *et al.*, 2007; Audinot *et al.*, 2008; Coge *et al.*, 2009;

Nishiyama *et al.*, 2009). Further studies are required to determine whether the MT<sub>2</sub> subtype mRNA also express hypothalamus-pituitary-gonadotropin axis and to learn more reproductive physiology of melatonin receptors in Bactrian camel.

## CONCLUSION

Taken together with earlier observations, the present findings suggest that melatonin acts directly on G protein-coupled MT<sub>1</sub> receptors on hypothalamus-pituitary-gonadotropin axis, to influence reproductive physiology of Bactrian camel.

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