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Cloning, Sequencing and Analysis of Melatonin Receptor Subtype MT_1 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_1 expression. The length of MT_1 gene was 452 bp. RT-PCR assaying revealed the presence of the mt1 (Mel1a) 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_1 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_1 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_1, hypothalamus-pituitary-gonadal axis, bactrian camel, China

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-hypophysial and gonadal axis (Karsch *et al.*, 1988). This regulates the pulsatile secretion of Gonadotropin-Releasing Hormone (GnRH) from the hypothalamus (Malpaux *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 (Malpaux *et al.*, 1999).

Three specific melatonin receptor subtypes have been cloned and characterized in vertebrates: MT_1, MT_2 (previously known as Mel1a and Mel1b, respectively also known as MTNR1A and MTNR1B, respectively) and Mel1c (Reppert *et al.*, 1994, 1995a, b). There are three MT receptors found in lower vertebrates thus, so far only two MT receptors, MT_1 and MT_2 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_1 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

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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_1 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°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 μg) to get a volume of 5.0 μL. After this oligo (dt) primer (0.1 μg) was added and the mixture was heated to +70°C for 5 min then cooled immediately on ice. A ribonuclease inhibitor (10 U), 5×reaction buffer (2 μL), 1.0 μL of 10 mM dNTP mix (dATP, dGTP, dCTP anp dTTP) and a MuLV-reverse transcriptase (20 U) were added to make the final volume 10 μL. The reaction mixture was preincubated for 10 min at room temperature (+23°C) before cDNA synthesis. Reverse Transcription (RT) reactions were carried out for 60 min at +37°C and then samples were heated to +70°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_1 primer set were as follows: Sense primer, 5'-TTGCTACATCTGCCACAGTC-3' and antisense primer 5'-CAAACAGCCACTCTGGGAT-3'. A total volume of 50 μL was used for all PCR reactions, containing 0.5 μL of template DNA, 5 μL of 10×Ex Taq Buffer, 4 μL of dNTP Mixture (2.5 mM each), 0.5 μL of TaKaRa Ex Taq polymerase (5 units μL^{-1}), 1 μL each primer. PCR amplification was performed in a Gene Cycler (Bio-Rad, USA) under the following conditions: denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1min, annealing at 5°C for 1 min for MT_1. 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_1 gene: The PCR amplified product of the MT_1 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°C overnight using T_4 DNA ligase. Competent *E. coli* DH5α 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^{-1} 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 MT1 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_1 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_1 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), 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_1 comparing with other mammal MT_1 known in GenBank.

Comparison of the nucleotide sequences of MT_1 melatonin receptors revealed that the Bactrian camel anterior pituitary MT_1 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

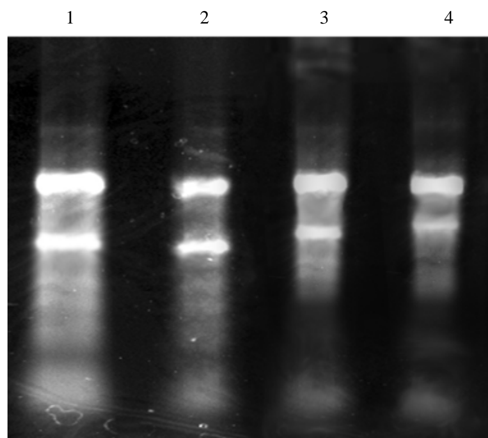

Fig. 1: Agarose gel electrophoresis of total RNA extracted from 1) pineal, 2) ovary, 3) hypothalamus and 4) pituitary

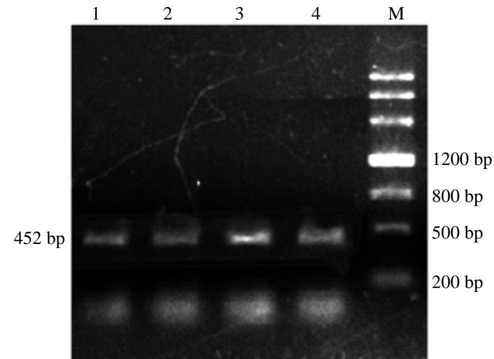

Fig. 2: Agarose gel electrophoresis of RT-PCR, 1) pineal, 2) ovary, 3) hypothalamus and 4) pituitary. M: Marker III

Divergence	1	2	3	4	5	6	7		
1		85.8	87.2	85.8	86.5	86.3	85.4	1	pMD 18-T-RA
2	15.8		86.6	100.0	85.1	85.3	84.2	2	Pig (U73326.1)
3	14.2	14.9		85.6	97.1	97.1	96.3	3	Cattle (EU716174)
4	15.8	0.0	14.9		85.1	85.3	84.2	4	Human (NM_005958)
5	15.0	16.9	2.9	16.9		99.3	98.7	5	Sheep (HQ658145.1)
6	15.3	16.6	2.9	16.6	0.7		98.9	6	Sheep MTNR1Aα(U14109.1)
7	16.1	18.0	3.8	18.0	1.3	1.1		7	Sheep MTNR1A:β (AF045219.1)
	1	2	3	4	5	6	7		

Fig. 3: Percent identity of melatonin receptor MT1 of different mammals using DNAStar's Megalign with Clustal W Method

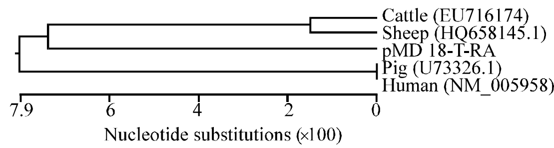

Fig. 4: Phylogenetic tree of the melatonin receptors MT_1 of different mammals using DNAStar's Megalign with Clustal W Method

analyzing homology of the melatonin receptors MT_1 (Fig. 4). There is evidence that melatonin ploys 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_1 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_1 in hypothalamus-pituitary-gondola 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 mt1 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_1 mRNA is expressed in hypothalamus-pituitary-gondola axis and pineal of female Bactrian camel and sequence has 87.2% identity to cattle MT_1 (EU716174) and 86.3% identity to sheep MT_1 (U14109.1). This shows unambiguously the existence of a functional MT_1 receptor in brain and ovary of Bactrian camel. At same time, the expression of melatonin receptor MT_1 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 codom change can affect melatonin regulation of seasonal breeding in Bactrian camel.

The melatonin receptor MT_2 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_2 subtype mRNA also express hypothalamus-pituitary-gondola 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_1 receptors on hypothalamus-pituitary-gondola axis, to influence reproductive philology of Bactrian camel.

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REFERENCES

Arendt, J., 1995a. Physiology of the Pineal: Role in Photoperiodic Seasonal Functions. In: Melatonin and the Mammalian Pineal Gland, Arendt, J. (Ed.). Chapman and Hall, London, UK., pp: 110-160.

Arendt, J., 1995b. Role of the Pineal Gland and Melatonin in Circadian Rhythms. In: Melatonin and the Mammalian Pineal Gland, Arendt, J. (Ed.). Chapman and Hall, London, pp: 161-200.

Audinot, V., A. Bonnaud, L. Grandcolas, M. Rodriguez and N. Nagel *et al*., 2008. Molecular cloning and pharmacological characterization of rat melatonin MT_1 and MT_2 receptors. Biochem. Pharmacol., 75: 2007-2019.

Bosaev, J., 1938. Sexual activity of camels and organisation of service. Konevodstvo, 26: 8-9.

Chen, B.X., Z.X. Yuen and G. W. Pan, 1985. Semen-induced ovulation in the bactrian camel (*Camelus bactrianus*). J. Reprod. Fertil., 74: 335-339.

Clemens, J.W., M.J. Jarzynka and P.A. Witt-Enderby, 2001. Down-regulation of mt1 melatonin receptors in rat ovary following estrogen exposure. Life Sci., 69: 27-35.

Coge, F., S.P. Guenin, I. Fery, M. Migaud and S. Devavry *et al*., 2009. The end of a myth: Cloning and characterization of the ovine melatonin MT_2 receptor. Br. J. Pharmacol., 158: 1248-1262.

Drew, J.E., P. Barrett, S. Conway, P. Delagrange and P.J. Morgan, 2002. Differential coupling of the extreme C-terminus of G protein alpha subunits to the G protein-coupled melatonin receptors. Biochim. Biophys. Acta, 1592: 185-192.

Dubocovich, M.L. and M. Markowska, 2005. Functional MT_1 and MT_2 melatonin receptors in mammals. Endocrine, 27: 101-110.

Dubocovich, M.L., 1995. Melatonin receptors: Are there multiple subtypes? Trends Pharmacol. Sci., 16: 50-56.

El-Allali, K., M.R. Achaaban, B. Vivien-Roels, B. Bothorel, N.S. Tligui and P. Pevet, 2005. Seasonal variations in the nycthemeral rhythm of plasma melatonin in the camel (*Camelus dromedarius*). J. Pineal Res., 39: 121-128.

Hafez, E.S.E., 1952. Studies on the breeding season and reproduction of the ewe Part I. The breeding season in different environments Part II. The breeding season in one locality. J. Agric. Sci., 42: 189-231.

Karsch, F.J., B. Malpaux, N.L. Wayne and J.E. Robinson, 1988. Characteristics of the melatonin signal that provide the photoperiodic code for timing seasonal reproduction in the ewe. Reprod. Nutr. Dev., 28: 459-472.

Malpaux, B., C. Viguie, D.C. Skinner, J.C. Thiery, J. Pelletier and P. Chemineau, 1996. Seasonal breeding in sheep: Mechanism of action of melatonin. Anim. Reprod. Sci., 42: 109-117.

Malpaux, B., J.C. Thiery and P. Chemineau, 1999. Melatonin and the seasonal control of reproduction. Reprod. Nutr. Dev., 39: 355-366.

Migaud, M., A. Daveau and B. Malpaux, 2005. MTNR1A melatonin receptors in the ovine premammillary hypothalamus: Day-night variation in the expression of the transcripts. Biol. Reprod., 72: 393-398.

Morgan, P.J., 1991. The Pars Tuberalis as a Target Tissue for Melatonin Action. In: Advances in Pineal Research, Foldes, A. and R.J. Reiter (Eds.). Vol. 6. John Libbey, London, UK., pp: 149-158.

Morgan, P.J., G. Davidson, W. Lawson and P. Barrett, 1990. Both pertussis toxin-sensitive and insensitive G-proteins link melatonin receptor to inhibition of adenylate cyclase in the ovine pars tuberalis. J. Neuroendocrinol., 2: 773-776.

Morgan, P.J., P. Barrett, H.E. Howell and R. Helliwell, 1994. Melatonin receptors: Localization, molecular pharmacology and physiological significance. Neurochem. Int., 24: 101-146.

Niles, L.P., J. Wang, L. Shen, D.K. Lobb and E.V. Younglai, 1999. Melatonin receptor mRNA expression in human granulosa cells. Mol. Cell. Endocrinol., 156: 107-110.

Nishiyama, K., Y. Shintani, K. Hirai and S.I. Yoshikubo, 2009. Molecular cloning and pharmacological characterization of monkey MT_1 and MT_2 melatonin receptors showing high affinity for the agonist ramelteon. J. Pharmacol. Exp. Ther., 330: 855-863.

Reiter, R.J., 1991. Pineal melatonin: Cell biology of its synthesis and of its physiological interactions. Endocr. Rev., 12: 151-180.

Reppert, S.M., C. Godson, C.D. Mahle, D.R. Weaver, S.A. Slaugenhaupt and J.F. Gusella, 1995b. Molecular characterization of a second melatonin receptor expressed in human retina and brain: The Mel1b melatonin receptor. Proc. Natl. Acad. Sci. USA., 92: 8734-8738.

Reppert, S.M., D.R. Weaver and T. Ebisawa, 1994. Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. Neuron, 13: 1177-1185.

Reppert, S.M., D.R. Weaver, V.M. Cassone, C. Godson and L.F. Kolakowski Jr., 1995a. Melatonin receptors are for the birds: Molecular analysis of two receptor subtypes differentially expressed in chick brain. Neuron, 15: 1003-1015.

Soares Jr., J.M., M.I. Masana, C. Ersahin and M.L. Dubocovich, 2003. Functional melatonin receptors in rat ovaries at various stages of the estrous cycle. J. Pharmacol. Exp. Ther., 306: 694-702.

Witt-Enderby, P.A. and M.L. Dubocovich, 1996. Characterization and regulation of the human ML1A melatonin receptor stably expressed in Chinese hamster ovary cells. Mol. Pharmacol., 50: 166-174.

Xiao, C.T., M.X. Chu, Y. Fu, L. Fang and S.C. Ye, 2007. Analysis of polymorphism, structure and function of exon 2 of ovine melatonin receptor 1b gene: A clue as to why it lacks expression in sheep. J. Pineal Res., 42: 97-104.

Yong, Z., 2000. The relationship between melatonin and gonadotropins in the breeding season of camel (*Camelus bactrianus*) and Yak (*Bos grunniens*). Ph.D. Thesis, Gansu Agricultural University, China.

Zhang, Y., J.Q. Zhu, F.D. Li, Y.J. Ma and X.X. Zhao, 2009. Cloning of melatonin receptor subtype MT_1 in ovary of ewe. Chin. J. Vet. Sci., 29: 1495-1499.