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THE MELATONIN RECEPTOR IN THE BACTRIAN CAMEL PINEAL: CLONING EXPERIMENTS AND DISTRIBUTION STUDIES

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ABSTRACT

The aim of this study was to identify the high-affinity melatonin-receptor subtypes expressed in pineal of camel. To achieve this, reverse transcription-polymerase chain reaction (RT-PCR), sequencing and immunohistochemistry were used. By using RT-PCR, mRNA of melatonin receptors MT1/MT2 were detected in the pineal of bactrian camel. The highest identity was 87.2% and 97.6%, respectivly for MT1 and MT2 compatively with white yak. The pridict protein of the deduce amino acid sequence showed a tendency towards forming the 7-TM motif profile of G-protein. The immunohistochemistry studies showed that melatonin receptors are expressed in pinealocytes and glial cells of pineal. Overall, these results showed that melatonin receptors are expressed in the bactrian camel pinealocytes, and melatonin, possible as intracrine or autocrine, can influence pinealocytes and pineal physiological function.

Key words: Bactrian camel, expression, melatonin receptor, pineal

The pineal gland is a small endocrine gland and unpaired organ located near the geometric centre of the vertebrate brain. It produces the serotonin derivative melatonin, a hormone that affects on behavioral rhythmicity, sleep, reproduction, thermoregulation, immune function, intracellular antioxidative processes, aging, tumor growth, and certain psychiatric disorders. These effects of melatonin are produced via at least two high-affinity G protein-coupled receptors, the MT1 and MT2 expressed in various areas of the central nervous system and in peripheral tissues in mammals (Dubocovich and Markowska, 2005; Hardeland *et al,* 2011).

In mammals central nervous system, melatonin receptors (MT1 and MT2) are located in hypothalamus (Anhe *et al*, 2004; Wu *et al,* 2006), pituitary (Nonno *et al,* 1995). Through the receptors, melatonin regulate the secretion of gonadotropins from pituitary to drive their activity in accordance with the season of the year in seasonal breeding mammals. At the same time, melatonin receptor MT1 was cloned in sheep pineal by performing reverse transcription polymerase chain reaction (RT-PCR) (Zhang *et al,* 2009). However, whether the MT2 are also expressed in pineal remains to be determined. The lack of distribution and location of melatonin receptors makes the identification of the receptors subtype function involved difficult. So, it is very much necessary to study melatonin receptors distribution and expression in pineal.

The female bactrian camel is a seasonal polyestrous animal in which oestrus usually appear from December to April (Chen *et al,* 1985). Seasonal variations in the nycthemeral rhythm of plasma melatonin in dromedary camel had been investigated. The pattern of melatonin secretion in the camel showed 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 has 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 24h ($p<0.01$), 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 just like the other seasonal breeding animals. The purpose of this study was, therefore, to identify melatonin receptor subtype(s) expressed with RT-PCR and IHC in the bactrian camel pineal.

Materials and Methods

Tissue collection

Pineals of adult female bactrian camels were collected from the slaughter house of the Ningxia

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Laoheqiao Muslim Meat Food Company in the Ningxia Autonomous Region, China in mid-November within 30 minutes. Some tissues were immediately frozen in liquid nitrogen, and stored at -70°C until RNA isolation was performed.

These were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 24 h. After fixation, pineal tissue blocks were dehydrated in a graded series of alcohols, cleared in xylene and embedded in paraffin. Series of semithin sections of 4 µm were cut and mounted on 0.1% (W/V) poly-l-lysine solution (Sigma Chemical Co., Poole, UK) coated slides for immunohistochemistry staining. At least one section from each case was directly mounted on clean slide to inspect the integrity of tissue structure through haematoxylin and eosin (H&E) staining.

Cloning of MT1, MT2 cDNAs

Bactrian camels pineal gland tissues were used to extract total RNA using Trizol®, based on the protocol of the manufacturer (Takara, Dalian, China). First Strand cDNA Synthesis Kit was used to transcribe total RNA into cDNA, following manufacturer's protocol (#K1612, MBI Fermentas, Lithuania). A portion of the cDNA synthesis reaction was amplified using a thermocycler and the following cycling protocol: a 5 min denaturation at 94°C followed by 34 cycles of a 1 min hold at 94°C, a 1 min primer annealing step at 54°C for MT1 and 60 for MT2, and a 1 min extension step at 72°C. 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: MT1 forward 5'-TTGCTACATCTGCCACAGTC-3', reverse 5'- CAAACAGCCACTCTGGGAT-3'. MT2 forward 5'- CTGGCG TTCGCTGACCTG-3', reverse 5'- CCTGGCTGCCCTTGGAAG-3'. The amplified products were subcloned into pMD18-T Easy vector (Dalian TAKARA Biotechnology Co. China), and transformed into *E. coli* DH5α cells according to established methods. Nucleotide sequence analysis of positive clones was performed on both strands by automated DNA sequencing (Dalian TAKARA Biotechnology Co. China).

Immunohistochemistry

Immunohistochemical staining for MT1 and MT2 was performed on 4 µm paraformaldehyde fixed paraffin embedded sections. The primary antibody that was used for MT1 detection was a 1:100 diluted rabbit polyclonal antibody to human (Cat. No. bs-0027R, Beijing Boisynthsis Biotechnology Co. Ltd). Rabbit polyclonal antibody to human MT2 (Cat. No. bs-0963R, Beijing Boisynthsis Biotechnology Co.Ltd) was diluted 1:50 in phosphate buffered saline (PBS) when it was used to detect the MT2. Localisation of the primary antibody was performed using the histostain-plus kits (Invitrogen Zymed Laboratories, California, USA). In short, paraffin-embedded sections were dewaxed, rehydrated and incubated for 5 min with 3% hydrogen peroxide in distilled water to block endogenous peroxidase activity. For pretreatment, sections were heated for 10 min in citrate buffer, pH 6.0 applying 350 W microwave irradiation. After a thorough wash with PBS, sections were incubated for 15 min with normal goat blocking serum followed by incubation with anti-MT1 or anti-MT2 antibody for 120 min at 37°C. Then sections were incubated for 15 min with biotinylated goat anti-rabbit IgG working solution (Invitrogen Zymed Laboratories, California, USA) and washed again in PBS before avidin–biotin peroxidase was applied. After further washing in PBS, peroxidase was displayed with 3,3-diaminobenzidine tetra hydrochloride substrate kit (DAB; Invitrogen Zymed Laboratories, California,USA) as the substrate to give a brown reaction product. Sections were lightly counterstained with haematoxylin, dehydrated, cleared and mounted with resin. Negative controls were performed by substituting the primary antibody with the isotype matched non-immune IgG.

Result

Cloning and sequencing of MT1 and MT2 mRNA in the camel pineal gland

As expected, a nucleotide sequence production has been amplificated with the specific primers which designed on the basis of other mamamals melatonin receptors gene in GenBank. The bactrian camel MT1 has been sequencing 452 bp, and there are GCA codon (Serine) deletion at 423bp compared with other mammal. The MT1 nucleotide sequence of the cloned fragment encoded a 150 amino acid protein. With predictprotein (www.predictprotein.org), predicted protein show a G-protein structure, which include 40% Helix, 20.67% Extended, 35.33% Loop, and 3 transmembrane domain, 2 intracellular loop and 2 extracelluar loop. Comparing with full MT1 protein structure of *Ovis aries* (NM_001009725.1), the cloned camel part MT1 protein was located inside 3 to 7 transmembrane domain of the 7-TM motifs profile of G-protein. The Serine (GCA codon) deletion was positioned in outside region after TM-6.

The cloned MT2 nucleotide sequence was 411bp cDNA. It encoded a 137 amino acid protein. With predictprotein, predicted protein show a G-protein structure, which include 40.88% Helix, 31.39% Extended, 27.74% Loop, and 4 transmembrae domain, 2 intracellular loop and 1 extracelluar loop. Comparing with full MT2 protein structure of *Ovis aries* (NM_001130938.1), the cloned camel part MT2 protein is located inside 3 to 6 transmembrane domain of the 7-TM motifs profile of G-protein.

Analysing homology of the melatonin receptors MT1 and MT2

The degree of identity, about nucleotide sequence of melatonin receptors MT1 and MT2, is shown in table 1. Compared with the homologous sequences from other species (*Bos grunniens, Bos taurus, Ovis aries, Sus scrofa, Homo sapiens, Bubalus bubalis, Rattus norvegicus, Mus musculus*), the nucleotide sequence is a part of camel MT1. At nucleotide sequence level, Bactrian camel MT1 exhibited highest and least identity with that of *Bos grunniens* (87.2%) and *Mus musculus* (80.8%), respectively. At the same time, bactrian camel MT2 exhibited highest and least identity with that of *Bos grunniens* (97.2%) and *Mus musculus* (80.6%), respectively. The results showed that the homology of both MT1 and MT2 of bactrian camels were most similar to *Bos grunniens*.

Table 1. Identity (%) at nucleotide of the bactrian camel (MT1 and MT2) with eight mammal species[a]).

Species	MT1(%)	MT2(%)
Bos grunniens	87.2	97.6
Bos taurus	87.0	81.8
Ovis aries	86.3	84.2
Sus scrofa	86.1	91.3
Homo sapiens	86.1	87.6
Bubalus bubalis	85.2	82.5
Mus musculus	81.9	80.6
Rattus norvegicus	80.8	80.6

a) the Genbank accession numbers sequence used in sequence comparison were as follows: melatonin receptors MT1 are *Bos grunniens* (KF569810.1), *Bos taurus* (EU716174.1), *Ovis aries* (HQ658145.1), *Sus scrofa* (U73326.1), *Homo sapiens* (NM_005958.1), *Bubalus bubalis* (GU817415.1), *Mus musculus* (NM_008639.2), *Rattus norvegicus* (NM_053676.1) respectively. Melatonin receptors MT2 are *Bos taurus* (NM_001206907.1), *Ovis aries* (NM_001130938.1), *Sus scrofa* (AJ276454.1), *Homo sapiens* (NM_005959.3), *Bubalus bubalis* (XM_006053939.1), *Rattus norvegicus* (NM_001100641.1), Mus musculus(NM_145712.2), respectively, and *Bos grunniens* had been cloned by our lab.

Distribution of melatonin receptor MT1 and MT2 in camel pineal

Using immunohistochemistry, the expression and localisation of melatonin receptors MT1 and MT2 were demonstrated in pineal of adult females bactrian

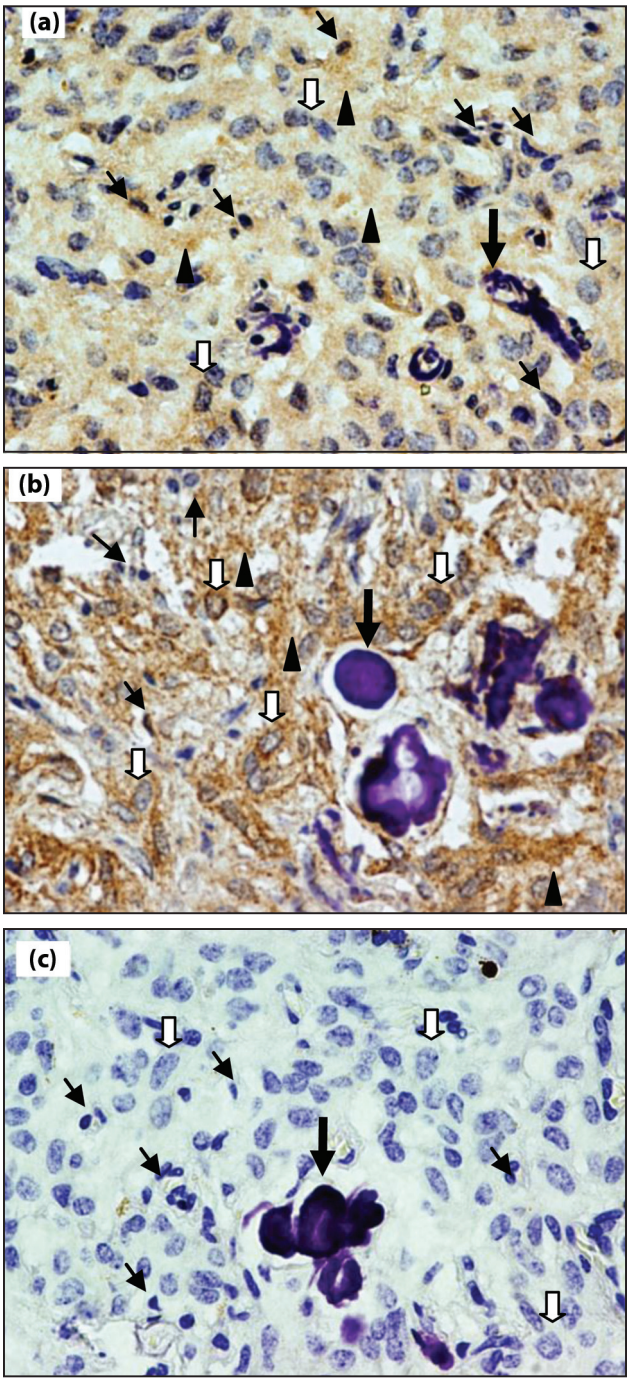

Fig 1. Section through the pineal. (a) expression of MT1 in pineal (×400). (b) expression of MT2 in pineal (×400). (c) control (×400). Melatonin receptors, which were stained brown, were extensive expressed in pinealocytes (white arrow), glial cells (black arrow) and nerve fibre (arrow head) of pineal in bactrian camel. Pineal calcification was stained blue with hematoxylin (thick black arrow)

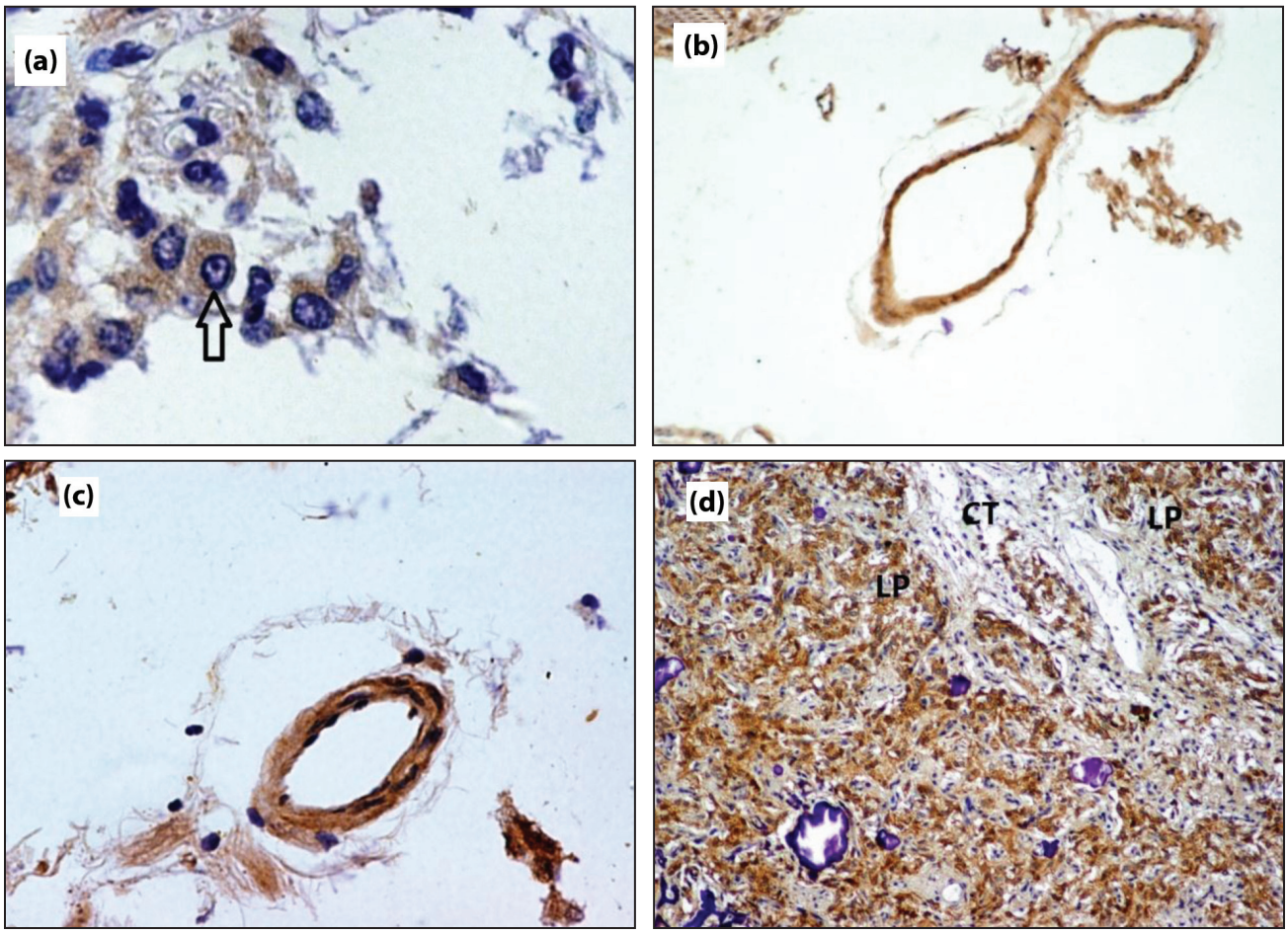

Fig 2. Section through the pineal. (a) distribution of MT1 in pinealocyte (×400). white arrow, expression of MT1 in cytoplasm and membrane of pinealocytes. (b) expression of MT1 in vein (×50). (c) expression of MT1 in artery (×200). (d) weak expression of MT2 in the connective tissue (CT) of lobular parenchyma (LP) of pinealocytes.

camel. Just like other mammals, the camel's pineal gland was composed of pinealocytes, occasional glial cells, nerve fibre and capillaries. The MT1 and MT2 expressed in pinealocytes, glial cells and nerve fiber (Fig 1a and b). Pineal calcification, which was stained blue with hematoxylin, were observed in camel's pineal (Fig 1). The melatonin receptor MT1 existed in cytoplasm and membrane of pinealocytes (Fig 2a). The receptors also expressed in subcapsular vein and artery of pineal (Fig 2b and c). The expression of MT2 was weak in the connective tissue of lobular parenchyma of pineal (Fig 2d).

Discussion

In the current study, the expression of melatonin receptors MT1/MT2 in the pineal of bactrian camel was investigated by using RT-PCR and immunohistochemistry for the first time.

The past 50 years have seen a proliferation of research on the pineal gland and its main product, melatonin. Melatonin is a pleiotropia, orchestrating regulator molecula to vertebrate (Hardeland *et al,* 2011). Melatonin receptors is a G protein-coupled receptors and expressed in various areas of the central nervous system and peripheral tissues. Through the membrane receptors, melatonin play important roles in circadian regulation, immune modulation, cell antioxidative protection, effecting on mitochondrial function, energy regulation and photoperiodically controlled reproduction. With good reason, melatonin is regarded as a chronobiotic and, also, as a chronobiological regulator molecule in the periphery (Zhang *et al,* 2009).

The present paper showed that melatonin is synthesised not only by the pineal gland or related structures, such as the retina, but also in quite a number of different organs or cells. These include gastrointestinal tract, testes, leukocytes, bone marrow, membranous cochlea, Harderian gland, ovary, thymus and skin (Raikhlin and Kvetnoi, 1974; Biesalski *et al,* 1988; Itoh *et al,* 1999; Conti *et al,* 2000; Djeridane and Touitou, 2001; Carrillo-Vico *et al,*

2005; Jimenez-Jorge *et al,* 2005; Slominski *et al,* 2008; Sakaguchi *et al,* 2013).

It is interesting that the membrane receptors are found in the organs and cell types. Melatonin synthesis and the receptors expression in human lymphocytes suggest that physiological significance of melatonin are possible role as intracrine, autocrine, and/or paracrine substance in the cell (Carrillo-Vico *et al,* 2004).

Axelrod demonstrated that the pinealocytes possess all the machinery necessary for the synthesis of melatonin (Axelrod, 1974). The biochemical cascade of melatonin has been clearly summarised in pinealocytes (Macchi and Bruce, 2004). However, we found that mRNA of melatonin receptors expressed in camel pineal with RT-PCR and the receptors located in pinealocytes in camel pineal with immunohistochemistry. Therefore, melatonin, possible as intracrine or autocrine, can influence pinealocytes and pineal physiological function.

Conclusion

In conclusion, MT1 and MT2 gene expression have been demonstrated by RT-PCR and sequencing in pineal of bactrian camels. The immunohistochemistry studies showed that pinealocytes are the mainly cells of melatonin receptors expression. The previous studies demonstrated that the pinealocytes are the mainly cells of melatonin synthesis. Therefore, melatonin, possible as intracrine or autocrine, can influence pinealocytes and pineal physiological function.

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