Identification and immunolocalisation of melatonin MT₁ and MT₂ receptors in Rasa Aragonesa ram spermatozoa

Adriana Casao⁴, Margarita Gallego⁵, José Alfonso Abecia⁴, Fernando Forcada⁴, Rosaura Pérez-Pe⁵, Teresa Muiño-Blanco⁴ and José Álvaro Cebrían-Pérez⁴

⁴Grupo Biología y Fisiología de la Reproducción, Instituto de Investigación de Ciencias Ambientales de Aragón (IUCA), Facultad de Veterinaria, Universidad de Zaragoza, C/Miguel Servet 177, 500013, Zaragoza, Spain.
⁵Departamento de Patología Animal, Facultad de Veterinaria, Universidad de Zaragoza, C/Miguel Servet 177, 500013, Zaragoza, Spain.

Abstract. The reproductive seasonality of sheep suggests that melatonin receptors may be present in ram spermatozoa. The present study confirms the presence of melatonin MT₁ and MT₂ receptors. The MT₁ receptor was detected using immunocytochemistry, with four sperm subpopulations identified based on the following labelling patterns: (1) one small subpopulation with labelling over the entire head and tail; (2) one of two main subpopulations that exhibited reactivity at the equatorial, post-acrosomal, neck and tail regions; (3) another main subpopulation with equatorial and tail labelling only; and (4) a subpopulation in which staining was detected only in the tail. Immunocytochemistry revealed the presence of the melatonin MT₂ receptor, with intense staining on the acrosome, post-acrosomal region and neck and tail regions of all cells, but not in the equatorial region. Western blot identification of ram protein extracts revealed a 39-kDa band compatible with both MT₁ and MT₂ receptors, a 75-kDa band compatible with MT₁/MT₂ heterodimerisation, a 32-kDa band compatible with MT₁ receptor activation and a double band of 45–55 kDa that is compatible with MT₂ receptor homodimerisation or heterodimerisation with other G-proteins. In conclusion, we provide evidence of the presence of MT₁ and MT₂ receptors in ram spermatozoa, although the biochemical pathway triggered by these receptors and their function in terms of fertility remains to be elucidated.

Additional keywords: immunocytochemistry, immunofluorescence, western blot.

Received 21 September 2011, accepted 26 January 2012, published online 20 March 2012

Introduction

Sheep are short-day breeders and their seasonality is regulated by melatonin secretion. Although seasonality in the male is less marked than in the female, differences have been observed in both the quality of spermatozoa and fertility between reproductive and non-reproductive seasons (Mandiki et al. 1998; Rosa and Bryant 2003). Similarly, changes have been reported in sperm quality, testicular size and the biochemical composition of the seminal plasma following the use of melatonin implants during the non-reproductive season (Coyan et al. 1998; Kaya et al. 2000; Casao et al. 2010c).

Variations in in vivo sperm quality mediated by endogenous or exogenous melatonin can be explained by modulation of the hypothalamic–pituitary–gonadal axis (Webster et al. 1991; Lincoln 1996; Kokolis et al. 2000). However, we have recently shown that melatonin prevents in vitro ram sperm capacitation and apoptotic-like changes (Casao et al. 2010b), which can only be explained by a direct action of this hormone on spermatozoa. The effect of melatonin in preventing apoptotic-like changes may be related to its antioxidant and free radical scavenging activities, but whether this effect is mediated by receptor binding remains contentious. There is evidence that melatonin exerts its anti-apoptotic action via MT₁ and/or MT₂ receptors in monocytic cells (Radogna et al. 2007) and MT₁ receptors in human spermatozoa (Espino et al. 2011). Other studies in stallion spermatozoa have reported that in vitro incubation of spermatozoa with melatonin inhibits membrane lipid peroxidation and protects the mitochondria, although no melatonin receptors were found in these cells (Balao da Silva et al. 2011), suggesting that the effect of melatonin may also be related to its ability to cross the plasma membrane.

Melatonin MT₁ and MT₂ receptors, formerly known as Mel₁α and Mel₁β receptors, respectively (Dubocovich et al. 2009), are members of the G-protein-coupled receptor (GPCR) superfamily.
In mammals, activation of these receptors by melatonin binding modulates circadian, reproductive and endocrine functions (Dubocovich et al. 2003).

Melatonin receptors have been located along the hypothalamic–pituitary–testis axis (Dubocovich and Markowska 2005). Despite the fact that the receptors are yet to be immunolocated, MT₁ and MT₂ receptor activity has been demonstrated in hamster and human spermatozoa by the use of 2-[125I]-iodomelatonin binding (van Vuuren et al. 1992) and competitive antagonists (van Vuuren et al. 1992; Fujinoki 2008; Espino et al. 2011).

The high reproductive seasonality of sheep (Rosa and Bryant 2003) and the stimulatory effect of melatonin implants during the non-reproductive season on semen quality (Coyan et al. 1998; Kaya et al. 2000; Casao et al. 2010c) suggest that melatonin receptors may be present in ram spermatozoa. Furthermore, receptors for other reproductive hormones have recently been immunolocated in human spermatozoa (Solakidi et al. 2005). Based on these observations, the aim of the present study was to confirm the presence of melatonin MT₁ and/or MT₂ receptors in ram spermatozoa using immunocytochemical and immunofluorescence localisation, as well as western blot identification.

**Materials and methods**

**Animal and semen collection**

Semen was collected from Rasa Aragonesa rams kept under uniform nutritional conditions at the Experimental Farm of the University of Zaragoza, Spain (latitude 41°41′N), in compliance with the European Union Directive for Scientific Procedures (European Community Commission 1986). All experimental procedures were performed under the supervision of the Ethics Committee of the University of Zaragoza.

All rams belonged to the National Association of Rasa Aragonesa Sheep Breeders (ANGRA) and were 4–6 years of age. Rams were kept under natural photoperiod conditions and all experiments were performed during the reproductive season (September–February).

Second ejaculates from nine rams, divided in two groups of four and five sires, were collected separately using an artificial vagina. To eliminate individual differences, daily ejaculates from rams in each group were pooled and processed together (Ollero et al. 1996). The ejaculates were kept at 37°C until laboratory analysis.

A seminal plasma-free sperm population was obtained using a dextran swim-up procedure (García-López et al. 1996) performed in medium with the following composition: 200 mM sucrose, 50 mM NaCl, 18.6 mM sodium lactate, 21 mM HEPES, 10 mM KCl, 4 mM NaHCO₃, 3 mM CaCl₂, 2.8 mM glucose, 0.4 mM MgSO₄, 0.3 mM sodium pyruvate, 0.3 mM K₃HPO₄, 5 mg mL⁻¹ bovine serum albumin (BSA), 1.5 IU mL⁻¹ penicillin and 1.5 mg mL⁻¹ streptomycin (pH adjusted to 7.2 with NaOH). The quality of the spermatozoa was assessed by computer-assisted motility analysis (ISAAS 1.0.4; Proiser, Valencia, Spain), whereas viability was assessed by fluorescent staining with 6-carboxyfluorescein diacetate (6-CFDA) and propidium iodide (Sigma Chemical, Madrid, Spain). The cells were then examined under a Labo-phot-2 fluorescence microscope (Nikon Corporation, Tokyo, Japan) with a B–2A filter (excitation 450–490 nm) and a G–2A filter (excitation 510–560 nm) at ×400 magnification. The number of fluorescein-positive (intact plasma and acrosomal membranes) and propidium iodide-positive (damaged plasma and acrosomal membranes) spermatozoa per 100 cells was counted and recorded. At least 200 cells were counted in duplicate for each sample.

**Sample preparation for immunocytochemical assays**

Aliquots of 4 × 10⁶ cells were fixed with 3.7% formaldehyde (v/v) in phosphate-buffered saline (PBS); 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.76 mM KH₂PO₄, pH 7.2) for 20 min at room temperature. The cells were then centrifuged for 6 min at 800g at room temperature and the pellet resuspended in PBS. The final concentration of resuspended cells was 4 × 10⁶ cells in 1 mL PBS. After fixation, 40 μL cell suspension was smeared onto poly-i-lysine-coated slides and maintained at room temperature for 3 h to ensure good adhesion onto the slide.

**Avidin–biotin peroxidase assays**

Slides selected for avidin–biotin peroxidase assays were rehydrated in PBS, pH 7.2. Endogenous peroxidase was inactivated with 1.70% hydrogen peroxide in 100% ethanol for 30 min. The slides were then washed in PBS, pH 7.2, and incubated in normal horse serum as a blocking reagent (Vector Laboratories, Los Angeles, CA, USA) for 45 min, followed by overnight incubation at 4°C in a humidity chamber with Mel-1A-R rabbit antibody polyclonal antibody against the MT₁ receptor (GeneTex, San Antonio, TX, USA) or rabbit polyclonal antibody against the MT₂ receptor (Acris Antibodies GmbH, Herford, Germany), both at a dilution of 1:50. Subsequently, slides were incubated with a biotinylated anti-rabbit antibody for 40 min at room temperature. An avidin–biotin–peroxidase complex (Vector Laboratories) was then applied for 45 min at room temperature. The binding sites of the primary antibodies were visualised by diaminobenzidine (DAB) and hydrogen peroxide solution (20 μg DAB in 100 mL of 0.05 M TRIS-HCl buffer, pH 7.6, containing 0.005% H₂O₂ for 5 min. Counter-staining with Carazzi’s haematoxylin was followed by dehydration and mounting. As a negative control, samples were incubated with normal serum instead of the primary antibody; all other remaining procedures were the same.

Cells were examined using a Nikon Eclipse E-400 microscope under brightfield illumination at ×600 and ×1000 magnification. Microscopic images were captured and processed with Nikon image software.

**Indirect immunofluorescence assays**

Sperm samples were washed three times with PBS and non-specific binding sites were blocked with 5% BSA in PBS for 4 h at room temperature in a humidity chamber. After three washes in PBS, spermatozoa were incubated overnight at 4°C in a humidity chamber with a combination of two primary antibodies, namely MTNR1A mouse polyclonal antibody (Abnova, Taipei, Taiwan) for the MT₁ receptor and a rabbit polyclonal antibody against the MT₂ receptor (Acris Antibodies), both
Melatonin receptors in ram spermatozoa

Reproduction, Fertility and Development

Melatonin receptors in ram spermatozoa

Results

Sperm quality

Sperm samples freed from seminal plasma by a dextran swim-up procedure were used for the localisation of melatonin receptors. Mean (±s.e.m.) values of the different populations in the samples were 80.7 ± 2.3% motile spermatozoa, 48.8 ± 1.6% progressive motile spermatozoa and 82.1 ± 0.9% viable spermatozoa.

Immunocytochemical localisation of MT₁ and MT₂ receptors

The avidin–biotin peroxidase complex assays for detecting the melatonin MT₁ receptor revealed a variety of sperm staining. Specifically, four sperm subpopulations were detected: (1) a small subpopulation with labelling all over the head and tail (Fig. 1b); (2) one of two main subpopulations with reactivity at the equatorial, post-acrosomal, neck and tail regions (Fig. 1c); (3) another main subpopulation with equatorial and tail labelling only (Fig. 1d); and (4) a subpopulation in which staining was present only on the tail (Fig. 1e). However, these immunotypes were not well defined in all cells, so transitional forms were also seen (Fig. 1f, g).

Avidin–biotin peroxidase complex assays for detection of the MT₂ receptor revealed intense staining on the acrosome, the post-acrosomal region and the neck and tail of all cells, but not in the equatorial region. Although nearly all the spermatozoa seemed to share this staining pattern, the intensity of the staining varied between cells, mainly in the acrosomal region (Fig. 2).

These results were corroborated by double indirect immunofluorescence assays against the MT₁ and MT₂ receptors, the aim of which was to simultaneously evaluate the relative distribution of each receptor subtype in ram spermatozoa. Both receptor subtypes were present in all spermatozoa, although the intensity and labelling distribution varied between cells (Fig. 3). All four spermatozoa subtypes (i.e. those defined on the basis of the distribution of MT₁ receptors determined in the avidin–biotin peroxidase assays) were also identified using indirect immunofluorescence. Most of the spermatozoa showed a stronger MT₁ receptor staining pattern, whereas only a few showed a predominance of the MT₂ type receptor. In these spermatozoa, both MT₁ and MT₂ receptors were coincident in the post-acrosomal area, but only MT₁ receptors reached the equatorial region, with MT₂ receptors remaining below and above it. Spermatozoa with MT₁ receptor staining exclusively in the equatorial region also showed post-equatorial labelling of the MT₂ receptor. Both receptor subtypes were coincident in the neck and tail of most...
cells, although MT1 receptors seemed to predominate at the end of the flagellum in those cells that also exhibited strong post-acrosomal MT1 receptor labelling.

In both types of assay, the omission of the primary antibody resulted in no fluorescent signal, which rules out the possibility of non-specific binding of the secondary antibodies (Fig. 4).

Western blotting detection of MT1 and MT2 receptors
Western blot analyses of proteins extracted from spermatozoa for investigation of melatonin MT1 receptors revealed two strong protein bands of approximately 39 and 32 kDa and a weaker band of 41 kDa. Another 75-kDa band, of intermediate intensity, was also found. In the positive control (rat brain cell extract), the strong 32-kDa protein band was also seen, in addition to a smaller band around 26 kDa and other weaker bands at 38, 50 and 80 kDa (Fig. 5).

Western blot analyses for the melatonin MT2 receptor identified a 39-kDa protein band, compatible with the MT2 receptor, as well as a very strong signal that appeared to be composed of a double band of 45–50 kDa and a weaker band at 75 kDa. Only the 38-kDa protein band was seen in the positive control (Fig. 6).
Discussion

Previous studies conducted by our group have identified the presence of melatonin in ram seminal plasma (Casao et al. 2010a) and have demonstrated the actions of melatonin on the apoptosis and capacitation of ram spermatozoa in vitro (Casao et al. 2010b). The results of the present study show, for the first time, the immunolocalisation of the melatonin MT₁ and MT₂ receptors in ram spermatozoa. These results were
The present study also revealed differences in the distribution of both receptor subtypes in ram spermatozoa. The MT1 and MT2 receptors are colocalised in the neck and tail region of the spermatozoa, as well as in the post-acrosomal region in some cells. However, MT2 receptors are more likely to be found in the acrosome than MT1 receptors, and MT1 receptors (but not MT2 receptors) are located at the equatorial region in certain cells. The distribution of the MT2 receptor is very similar in all cells, although the intensity of labelling, which could be related to the receptor density (Witt-Enderby et al. 2003), seems to be lower in some cells. The MT1 receptor, however, showed a wide variety of cell labelling, which allowed us to identify four immunotypes, as well as transitional forms between them. This variability in MT1 receptor distribution and MT2 receptor density could be related to the high heterogeneity of ejaculated semen. The physiological status of the spermatozoa, including maturation, capacitation, apoptosis and cell death processes, involves membrane changes that may affect the distribution and density of melatonin receptors.

Western blot analysis of sperm membrane proteins revealed two strong bands of 39 and 32 kDa for the MT1 receptor, and a weaker band of 75 kDa. The 39-kDa band is compatible with the MT1 receptor (Reppert et al. 1994), whereas the 32 kDa band may be related to receptor activation. This is because MT1 and MT2 receptors are GPCR and their activation promotes the dissociation of these proteins into α and βγ dimers (Gilman 1995; Dubocovich and Markowska 2005). Therefore, the smaller band (32 kDa) detected by western blot analysis could indicate the presence of active MT1 receptors in ram spermatozoa. Ejaculated spermatozoa are diluted in the seminal plasma, which contains melatonin (Casao et al. 2010a) that is able to bind to MT1 receptors on the spermatozoa (van Vuuren et al. 1992). Similarly, the lack of the smaller 32 kDa band in western blot analysis of MT2 receptors suggests that these receptors are not activated.

Western blot analysis of the MT2 receptor revealed a 39 kDa band, as described previously (Cogo et al. 2009), and another strong signal, which appeared to be formed by a double band of 45–50 kDa, together with a weaker band of 75 kDa. These bands may be related to the oligomerisation of the MT2 melatonin receptor, which, as a member of the GPCR family, can form dimers with itself (homodimers) or with other GPCR receptors (heterodimers; Pin et al. 2007). In HEK 293 transfected cells, MT1/MT2 receptor heterodimers are formed preferentially over MT2 homodimers (Ayoub et al. 2004). Therefore, the presence of the 75 kDa band western blot analysis of both MT1 and MT2 receptors suggests that MT1/MT2 heterodimers are formed in ram spermatozoa, which is consistent with their close localisation on the sperm plasma membrane. However, the presence of the strong 45–50 kDa band in the western blot analysis of the MT2 receptor and its absence in analyses of MT1 receptors could suggest that the MT2 receptor homodimers and/or heterodimers with another GPCR receptor (Levoye et al. 2006) are more prevalent than MT1/MT2 heterodimers in ram spermatozoa. The fact that both receptors appear in the neck of all the spermatozoa would suggest that MT1/MT2 heterodimer formation takes place in the sperm neck, but not in the head.

Although the biological functions of ovine melatonin MT1 and MT2 receptors remain to be elucidated, this theoretically lower heterodimer formation suggests that melatonin MT1 and MT2 receptors could have different functions in spermatozoa, as is implied by the differences in the relative distribution of the receptors. In addition, coexpression of both melatonin receptor subtypes may be compatible with possible cross-regulation of both receptors, as well as with a complex regulation of seasonal breeding in sheep, in which MT2 receptors may modulate the...
action of MT$_1$ receptors, as already postulated for sheep brain tissues (Cogé et al. 2009). The results of the present study show that the distribution of MT$_2$ receptors is consistent on the sperm plasma membrane, with variations only in density, whereas labelling of the MT$_1$ receptor revealed considerable differences between cells. Because both receptors are related to photoperiod regulation (Morgan et al. 1994) and are expressed in mammalian testes (Frungieri et al. 2005; Izzo et al. 2010), the MT$_2$ receptor, alone or via modulation of the MT$_1$ receptor, may be involved in spermatogenesis in the testes, which can also be affected by the reproductive seasonality of rams (Chehimeau et al. 1992; Rosa and Bryant 2003). Furthermore, melatonin treatment during the non-reproductive season seems to increase sperm concentration and quality (Coyan et al. 1998; Kaya et al. 2000). Hence, it may be that MT$_2$ and/or MT$_1$ receptors exert their function in the testes during sperm maturation, increasing sperm concentration during the reproductive season. However, sperm quality may also be related to melatonin receptors in the spermatozoa, because melatonin levels in ram seminal plasma also exhibit seasonal variation (Casao et al. 2010a), with higher levels detected during the reproductive season.

The melatonin MT$_1$ and MT$_2$ receptors located in the sperm neck may be involved in sperm motility. High doses of melatonin have been shown to improve the percentage of motile and progressive motile human spermatozoa after short-term *in vitro* incubation (O’Flaherty 2008; Iwanaga et al.) may be that both processes are partially modulated by melatonin enzymes are related to sperm capacitation and the acrosome reaction of mammalian spermatozoa (Ortiz et al. 2002). However, only the binding of melatonin to MT$_2$ receptors is consistent on the sperm plasma membrane, whereas melatonin levels in ram seminal plasma also exhibit seasonal variation (Casao et al. 2010a), with higher levels detected during the reproductive season.

In conclusion, we have demonstrated in the present study, for the first time, the presence and distribution of melatonin MT$_1$ and MT$_2$ receptors in ram spermatozoa, although the biochemical pathways triggered by these receptors and their function in terms of fertility remains to be elucidated.

**Acknowledgements**

This work was supported by grants from the Ministerio de Ciencia e Innovación, Gobierno de España (AGL2010-18975) and Diputación General de Aragón (DGA-A26). The authors thank María Royo (Instituto Aragonés de Ciencias de la Salud, Zaragoza, Spain) for technical assistance with the confocal microscopy, ANGRA for supplying the rams and Santiago Morales (Departamento de Bioquímica y Biología Molecular y Celular, Facultad de Veterinaria, Universidad de Zaragoza, Zaragoza, Spain) for collecting the semen samples.

**References**


Cebrian-Perez, J., and Muino-Blanco, T. (2010c). Effects of melatonin implants during non-breeding season on sperm motility and reproductive parameters in Rasa
H Reproduction, Fertility and Development

A. Casao et al.


Morgan, P. J., Lawson, W., Davidson, G., and Howell, H. E. (1989). Melatonin inhibits cyclic-AMP production in cultured ovine par tuber-


H Reproduction, Fertility and Development


