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MELATONIN RECEPTORS MT_1 AND MT_2 ARE EXPRESED IN SPERMATOZOA FROM SEVERAL SEASONAL AND NON-SEASONAL BREEDER SPECIES

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ABSTRACT

Melatonin is a ubiquitous and multipurpose molecule, and one of its roles is to regulate reproduction in some seasonal mammals. Our group has previously shown the variation in the melatonin levels in ram seminal plasma along the year, and identified MT_1 and MT_2 receptors in ram spermatozoa. The objective of this study was to elucidate whether the presence of melatonin receptors (MT_1 and MT_2) in the sperm plasma membrane and melatonin in the seminal plasma is related to seasonal breeding. For this purpose, the presence of melatonin receptors and the levels of melatonin in seminal plasma have been examined in several species: donkey and stallion as long day breeders; red deer as a wild, short day, highly seasonal breeder (epididymal spermatozoa); bull as a conventional non-seasonal breeder; boar as a seasonal breeder under management techniques and dog as possible a seasonal breeder not regulated by melatonin. We have detected measurable levels of melatonin in the seminal plasma of all ejaculated semen samples (from donkey, stallion, boar, bull and dog). Also, and for the first time, we have demonstrated the presence of MT_1 and MT_2 melatonin receptors in the spermatozoa of all these species, regardless their type of reproduction or sperm source (ejaculated or epididymal), by using indirect immunofluorescence techniques and Western Blotting., Our findings suggest that melatonin and melatonin receptors may be universally distributed in the reproductive system of mammals, and that the sperm melatonin receptors cells may not be necessarily related with seasonal reproduction. Furthermore, the presence of MT_1 at the cytoplasmic droplet in immature ejaculated stallion spermatozoa found in one sample, and epididymal red deer spermatozoa suggests that melatonin may be involved in specific functions during spermatogenesis and sperm maturation, like protecting spermatozoa from oxidative damage, this activity being mediated through these receptors.

Keywords: donkey, stallion, boar, bull, deer, dog, melatonin

1. INTRODUCTION

Melatonin is a ubiquitous molecule, widely distributed in nature. It has been hypothesized that melatonin originally evolved as a free-radical scavenger [1], still one of its biological roles, and later on it acquired receptor-mediated important biological functions such as the chemical expression of darkness, immunomodulation and anti-inflammatory activity [2].

This hormone is also the main regulator of reproduction in photoperiodic animals. The melatonin signal works both as an inhibitor in long day breeders such as the Syrian hamster [3] and horse [4], and as a stimulator in short-day breeders such as sheep, goat [5] and deer [6]. Seminal plasma is a putative biological source of melatonin for mammal ejaculated spermatozoa, as this pineal hormone has been found in human [7] and ram [8] seminal plasma.

Seasonality is one of the most significant factors constraining reproduction in certain domestic animals, including sheep and horse. Despite that sperm production in these species is continuous throughout the year and that the seasonality is less marked in the male than in the female, the sexual behavior and sperm quality vary throughout the year in the ram and stallion, and they decrease during the non-breeding season [9-11]. In other temperate seasonal species like the red deer, sperm production is very low and even null during the non-reproductive season, with the reproductive organs undergoing dramatic changes at the beginning of the reproductive season (including testicular recrudescence), achieving a peak of sperm and glandular production for a short time in cervids [12].

In certain domestic species such as dairy cattle, this seasonality has been lost during its domestication process [13], or decreased by management techniques as in swine [14]. Dog seems to have seasonal reproduction, given that bitches tend to be in estrus in winter and summer [15], but this seasonal rhythm seems to be independent from short/long days or melatonin [16]. Nevertheless, male dogs constantly produce sperm and are fertile throughout the year [17].

Regardless seasonality, *in vitro* studies have shown a direct beneficial action of melatonin on sperm cells irrespective of the species being non-seasonal [18,19], long-day [20] or short-day breeders [21,22], which suggests a separate action of melatonin on spermatozoa from different species to the seasonal control of fertility. In general, the incubation of spermatozoa species with melatonin decreased the oxidative damage, improved their motility and increased their viability [23].

The direct action of melatonin on spermatozoa has been related with the free radical scavenging properties of this molecule [24] and its ability to cross the plasma membrane. However, in somatic cells, melatonin exerts most of its physiological actions by interacting MT_1 and MT_2 receptors. Both of them are involved in the circadian rhythm and play important roles in reproductive and endocrine functions in mammals [25].

We have previously shown the presence of both MT_1 and MT_2 melatonin receptors on the plasma membrane of ram spermatozoa using immuno-detection techniques [26]. Likewise, MT_1 and MT_2 activity has been reported in human spermatozoa by 2--[^{125}I]-iodomelatonin binding [27] and in hamster and human spermatozoa by using competitive antagonists [28,29]. Conversely, previous attempts to detect the MT_1 and/or MT_2 receptors in stallion, dog and boar spermatozoa have been unsuccessful [20]. These previous observations lead us to hypothesise that the presence of melatonin receptors in the sperm plasma membrane and the presence of melatonin in the seminal plasma might be related to seasonal breeding. Therefore, the aims of the present study were to determine the presence of i) melatonin in seminal plasma and ii) melatonin MT_1 and MT_2 receptors in spermatozoa of different types of breeders: donkey and stallion as long day breeders, red deer as a wild, short day, highly seasonal breeder, bull as a conventional non-seasonal breeder, boar as a seasonal breeder subjected to management techniques and dog as a seasonal but melatonin independent breeder.

2. MATERIAL AND METHODS

2.1. Animals and semen collection

Most experiments were performed using ejaculated spermatozoa. Semen was individually collected from five donkeys (Guara Catalá, ages 4 – 10 years), five stallions (Purebred Spanish Horse, ages 7 – 10 years) and seven dogs (four breeds, ages 2 – 6 years) from the Faculty of Veterinary Medicine of Barcelona (Spain). Donkey and stallion semen was obtained during the breeding season (March-June) by means of artificial vagina, and diluted in a commercial extender for transport. Dog semen was obtained between March and June by masturbation. Boar semen was obtained from six boars (Pietrain x Landrace, ages 18 – 24 months) belonging to the Porcine Producers Association of Aragon and EbroValley (APPAVE, Zaragoza, Spain) and the AI centre of AIM Ibérica (Calasparra, Murcia, Spain) by artificial vagina in spring, summer and autumn. Bull semen was obtained from three Frisian and three Limousine bulls (ages 1-3 years) by means of artificial vagina in May/June. Red deer spermatozoa were obtained from the cauda epididymis of adult males harvested in regulated hunting activities in September (Picos de Europa hunting reserve, León, Spain).

2.2. Melatonin concentration in seminal plasma

Seminal plasma in all the studied species but the red deer was extracted by semen centrifugation at 10000 x g for 10 min in a microfuge at 4 ºC. The supernatant was centrifuged again in the same conditions, and seminal plasma was recovered, filtered through a 0.22 µm Millipore membrane (Millipore Ibérica, Madrid, Spain) and stored at -20 ºC in darkness until analysed. Melatonin concentration was determined in several samples (between two and five) of each male, obtained in different days.

Melatonin concentration in seminal plasma was measured by means of a commercial competitive immunoassay (Direct saliva melatonin ELISA kit, Bühlmann Laboratories AG, Schönenbuch, Switzerland, sensitivity: 0.5 pg/ml, intro-assay variability: 5.2%), following the manufacturer's instructions. Briefly, 100 µL of each sample (in duplicate), control and calibrator were loaded in duplicate in a microtiter plate coated with an anti-melatonin antibody, and incubated for 16-20 h at 2-8 °C. After incubation, 50 µL of biotinylated melatonin were added to each well and incubated for 3 h at 2-8 °C. After three washes, 100 µL of streptavidin conjugated to horseradish peroxidase (HRP) were loaded to the wells and incubated for a further 60 min in a plate rotator set at 600 rpm at 18-28 °C. After incubation, the wells were washed three times, and 100 µL of tetramethylbenzidine substrate (TMB) were added to each well and incubated protected from direct light during 30 min on a plate rotator at 600 rpm and 18-28 °C. After incubation, 100 µL of 0.25 M H_2SO_4 solution were added and absorbance was measured on a microtiter plate reader (TECAN Spectrafluor plus, Männedorf, Switzerland) at 450 nm.

2.3. Immunolocalization of MT_1 and MT_2 melatonin receptors

The localization and distribution of melatonin receptors MT_1 and MT_2 was investigated by different methods: imaging of single-cell-flow using an imaging flow cytometer (AMNIS ImageStreamX, Amnis, Seattle, Washington, USA), confocal microscopy (Leica TCS SP2, Leica Microsystems, Wetzlar, Germany) and epi-fluorescence microscopy (Nikon DXM1200, Tokio, Japan). Otherwise stated, all reactives were purchased in Sigma-Aldrich (St. Louis, MO, USA).

2.3.1. Imaging Flow Cytometry

Due to the commercial extender in which donkey and stallion spermatozoa were preserved, microscope visualization was not possible and an imaging flow cytometer was used instead. Cell suspensions previous to flow cytometry imaging examination

were prepared as follows: aliquots of 8 x 10^6 spermatozoa/mL were fixed in 3.7% formaldehyde (v:v in Phosphate Buffered Saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.76 KH_2PO_4, pH 7.2), pH 7.2) for 20 min at room temperature. After that, samples were centrifuged 6 min at 500 x g, and the pellet resuspended and incubated in the blocking solution (5% BSA in PBS) for 2 h at room temperature. Following incubation, samples were washed three times by centrifugation at 500 x g for 5 min and resuspension of the pellet in PBS. Following the last centrifugation, the pellet was resuspended with the primary antibody (rabbit Mel 1A-R antibody; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA for MT_1 receptor, or rabbit melatonin receptor 1B antibody; Acris Antibodies GmbH, Herford, Germany, for MT_2 receptor, both diluted 1:50 in PBS with 1% BSA) and incubated overnight at 4 ºC. Finally, samples were washed by centrifugation in PBS three times, and incubated for 75 min at room temperature and in darkness with an anti-rabbit secondary antibody (Alexa Fluor 488 chicken anti-rabbit, Invitrogen, Carlsbad, California, USA) diluted 1:500 in PBS with 1% BSA. After that, cells were washed three times with PBS and evaluated by imaging flow cytometry (AMNIS ImageStreamX, Amnis, Seattle, Washington, USA).

2.3.2. Microscopy

Slides for microscopy examination were prepared as follows: aliquots of 2 x 10^6 spermatozoa/mL from dog, boar, bull and deer were fixed with 3.7% (v:v) formaldehyde diluted in PBS for 20 min at room temperature. Once fixed, the samples were centrifuged 6 min at 900 x g and the pellet resuspended in PBS. Forty µL of cell suspension were smeared onto poly-L-lysine-coated slides andonce the cells were properly adhered, slides were washed three times for 5 min with PBS, and non-specific binding sites were blocked with 5% BSA in PBS for 2 h at room temperature in a wet chamber. After three washes in PBS, spermatozoa were incubated with the primary antibody for melatonin receptor MT_1 (MTNR1A mouse polyclonal antibody, Abnova, Taipei, Taiwan) or melatonin receptor MT_2 (rabbit melatonin receptor 1B antibody, Acris Antibodies GmbH, Herford, Germany), both diluted 1:50 in PBS with 1% BSA overnight at 4 ºC in a wet chamber. Following the incubation with primary antibodies, the slides were washed in PBS three times and incubated for 75 min at room temperature in darkness with the secondary antibodies Alexa Fluor 594 chicken anti-mouse (Invitrogen, Carlsbad, California, USA) for melatonin receptor MT1 and Alexa Fluor 488 chicken anti-rabbit (Invitrogen, Carlsbad, California, USA) for melatonin

receptor MT_2, both diluted 1:800 in PBS containing 1% BSA. After three washes in PBS, 5 µL of 0.22 M triethylenediamine (DABCO) in glycerol:PBS (9:1) were added in order to enhance and preserve fluorescence. Boar and dog spermatozoa were visualized under confocal microscopy (Leica TCS SP2, Leica Microsystems, Wetzlar, Germany) and bull and deer spermatozoa under epi-fluorescence microscopy (Nikon DXM1200, Tokio, Japan).

2.4. Western Blotting

Sperm proteins were extracted by diluting samples in PBS (10^8 cells/mL) and centrifuging them in a microfuge at 900 x g for 6 min at room temperature. The supernatant was discarded and the pellet was resuspended in 100 µL extraction buffer (0.0626 M TRIS-HCl, 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 1% glycerol and 0.002% bromophenol blue). After incubation at 100 °C in a sand bath for 5 min, samples were centrifuged again at 13000 x g for 5 min at 4°C. The supernatant was recovered, 10% protease inhibitor cocktail was added and samples were stored at -20 °C.

For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), 5×10^6 cells were loaded on 12% and 10% (w/v) SDS-PAGE gels for MT_1 and MT_2 receptors respectively. Proteins were separated by standard SDS-PAGE and transferred onto apolyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, California, USA) using a wet transfer unit (Mini Trans Blot Electrophoretic Transfer Cell Unit, Bio-Rad, Hercules, California, USA). After the blocking of non-specific sites on the membrane with 5% BSA in 0.5% Tween-20–PBS for 4 h at RT, the proteins were immunodetected by incubating overnight at 4 °C with the primary antibody, namely Mel-1A-R rabbit polyclonal antibody against the MT_1 receptor (GeneTex, Irvine, California, USA) or rabbit melatonin receptor 1B antibody (Acris Antibodies GmbH, Herford, Germany) for MT_2 receptor, diluted both 1:1000 in 0.1% Tween-20–PBS containing 1% BSA. Following incubation with the primary antibodies, membranes were washed three times for 15 min each time in 0.1% Tween-20–PBS and then incubated with a secondary donkey anti-rabbit IRDye 800RD antibody in all the studied species but the donkey and stallion (LI-COR Biosciences, Lincoln, Nebraska, USA), or a secondary goat anti-rabbit DyLight 680 Conjugated (Thermo Scientific, Waltham, Massachusetts, USA) for donkey and stallion samples, both of them diluted 1:15000 in 0.1% Tween-20 PBS containing 1% BSA for 1 h and 15 min at room temperature and in darkness. Finally,

fluorescent detection was performed, after extensive washing in darkness, in an Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, Nebraska, USA). Ram sperm protein extracts were used as a positive control [26].

2.5. Statistical analysis

Normality of seminal plasma melatonin values were first evaluated by the Kolmogorov-Smirnov test. After normality of data was established, differences between either species, individuals or breeds within each specie were analysed by the Kruskal-Wallis test, and, when this test revealed significant differences, analyses by pairs were performed with the Mann-Whitney test. All statistical analysis was performed using SPSS (v.15.0, IBM Software, Armonk, New York, U.S.A.).

3. RESULTS

Measurable melatonin levels were detected in all studied species (Table 1). The mean concentration value in seminal plasma of donkey and stallion was similar, although the variation range was broader in donkey than in stallion. However, no statistical differences between individual males in these species were found. A broad range of melatonin values was also detected in seminal plasma of dog and boar, although average values were no significantly different from those in donkey or horse. Male variability was high, due to the fact that the melatonin concentration in one dog was several times higher than in the others (29.50 ± 8.01 pg/mL, $P<0.05$ compared to each other). However, no age or breed influence was detected in that males. Likewise, the melatonin concentration in two boars was double than in the others ($P<0.05$). The highest melatonin mean concentration was found in bull (19.10 ± 7.37 pg/mL), being significantly different ($P<0.05$) to the other species. The maximun melatonin concentration in seminal plasma was found in the Frisian bulls (26.88 ± 9.96 vs. 2.00 ± 0.51 pg/mL for Frisian and Limousine bulls, respectively, $P<0.05$).

Likewise, indirect immunofluorescence assays against melatonin receptors revealed the presence of both types, MT_1 and MT_2, in all the studied species. However, their distribution within the spermatozoa appears to be species-specific and, in some cases, differences between cells within the same ejaculate were also detected.

MT_1 receptor was located at the acrosomal region in almost all donkey spermatozoa (Fig. 1 a-c), some of them also showing a brighter band at the equatorial or postacrosomal region (Fig. 1 d-f). In stallion, the reactivity was found at the head and

tail of all spermatozoa (Fig. 2 a-c). Furthermore, the immature spermatozoa found in one sample showed a very intense immunoreactivity in the cytoplasmic droplet (Fig. 2 d-f). In boar spermatozoa, the MT_1 receptor distribution was identical in all the cells of the studied ejaculates, showing an intense band at the equatorial region, and immunoreactivity at the neck and midpiece of the flagellum (Fig. 3 a-c). Dog spermatozoa presented a very characteristic MT_1 "banded" pattern in the head, with up to three bands, located at the edge of the acrosome, equatorial band and/or postacrosome, plus some staining at the neck and midpiece (Fig. 3 d-f). In bull spermatozoa, MT_1 receptor was located at the postacrosome and flagellum; most cells also showed an intense staining on the equatorial band and neck, and only a few of them presented an additional signal on the acrosomal ridge (Figure 4 a-c). MT_1 location was very similar in deer spermatozoa, with some cells showing immunoreactivity at the postacrosomal region and flagellum, while other cells showed an intense band of staining at the equatorial region and flagellum (Figure 4 d-f). Unlike the stallion immature spermatozoa, the immunoreactivity intensity at the cytoplasmic droplet of red deer was not higher than in the rest of the flagellum.

The MT_2 receptor distribution differed from that of MT_1 in all the studied species. In donkey, spermatozoa were stained all over the head and tail (Fig. 1 g-i), although some of them showed more intensity at either the acrosome (Fig. 1 j-l) or post-acrosome (Fig. 1 m-o). Stallion spermatozoa showed an intense staining at the acrosome of all cells in the ejaculates (Fig. 2 g-i), plus a fainter staining at the postacrosome (Fig. 2 m-o) and tail in some of them (Fig. 2 j-l); the cytoplasmic droplet present in immature spermatozoa was not stained at all (Fig. 2 m-o). All the spermatozoa observed in boar (Fig. 3 g-ci), bull (Fig. 4 g-i) and deer (Figure 4 j-l) samples showed an intense staining in the neck, while in dog spermatozoa the reactivity was found at the acrosome, with a faint signal at the midpiece of the flagellum (Fig. 3 j-l). A summary of both melatonin receptors distribution is shown in Table 2.

In order to confirm these results, Western-blot analysis of the extracted proteins from all sperm samples was carried out. The results obtained for the MT_1 receptor revealed a 39 kDa band, compatible with this receptor [30], in donkey, stallion (Fig. 9a, lanes 1 and 2), boar, bull and deer sperm extract, but not in dog (Fig. 9a, lanes 3-6). Another band of 32 kDa was also found in boar, bull, deer and dog protein extracts. This 32 kDa band, along with another one of 26 kDa, was also visible in donkey, but not in horse. Ram

298 sperm proteins, used as a positive control [26] also showed the 39 and 32 kDa bands
299 (Fig. 9a, lane 7).
300 Western-blot analyses against MT_2 receptor revealed several small bands between 15
301 and 28 kDa in donkey sperm extract (Fig. 9b, lane 1). In stallion, a faint 42 kDa band
302 along with another one of 32 kDa was detected (Fig. 9b, lane 2). These bands were also
303 found in dog (32 kDa, Fig. 9b, lane 6), bull and deer (42 kDa, Fig. 9b, lanes 4 and 5,
304 respectively) sperm extracts. Likewise, faint bands of 37 kDa and 39 kDa, compatible
305 with the MT_2 receptor molecular weight [31], were identified in bull (Fig. 9b, lane 4)
306 and dog (Fig. 9b, lane 6) sperm, respectively. Bands of 45 kDa were also found in boar
307 (Fig. 9b, lane 3) and bull (Fig. 9b, lane 4) protein extracts, along with another 65 kDa
308 band in the former. Finally a strong band of 75 kDa was detected in bull (Fig. 9b, lane
309 4) and deer (Fig. 9b, lane 5). Ram sperm proteins, the positive control, showed the 39
310 kDa, the double 45-50 kDa and the 75 kDa bands (Fig. 9b, lane 7).
311
312
313 **4. DISCUSSION**
314 We have previously detected the presence of melatonin in ram seminal plasma [8], and
315 its relationship with testosterone, estradiol and antioxidant enzymes [32]. In this study,
316 we have found measurable levels of melatonin in seminal plasma of donkey, stallion,
317 boar, bull and dog. The mean values detected in the bull seminal plasma were
318 statistically higher than those in the other analyzed species, with the lowest
319 concentrations found in donkey and stallion. Furthermore, we observed a great intra-
320 species individual variation, being the dog and boar the species with a higher deviation.
321 An age-effect on melatonin concentration has been previously reported in the human
322 nocturnal pineal melatonin secretion [33]. However, no age-effect was found in the
323 individual variation observed in dog samples. In bulls, individual variation in the
324 seminal plasma melatonin concentration seems to be breed-related, with the Frisian
325 bulls showing the higher values.
326 In a previous study, we demonstrated the presence of melatonin receptors MT_1 and MT_2
327 in ram spermatozoa [26]. In this study, we have confirmed, for the first time, that
328 melatonin receptors MT_1 and MT_2 are present in ejaculated spermatozoa of donkey,
329 stallion, boar, bull, dog and in epididymal spermatozoa from red deer.
330 The presence of melatonin receptors in spermatozoa had been initially hypothesized in
331 human by the detection of melatonin binding sites [27] and the use of antagonists

against these receptors [28]. Later on, the presence of the melatonin receptor MT_1 was confirmed by immunofluorescence and RT-PCR in human spermatozoa [34], but not MT_2. Regarding the presence of melatonin receptors in domestic mammalian spermatozoa other than the ram, a previous study using western blotting failed to detect the presence of melatonin receptors in stallion, boar and dog spermatozoa [20]. However, in the present study, we have verified the presence of both melatonin receptors MT_1 and MT_2 in all the tested species. This result suggests that their presence may be universal in mammalian spermatozoa and their role might be other than seasonal control. The differences in the receptor distribution were corroborated by the band pattern obtained by western-blot, and can be related to receptor activation [35,36] and/or dimerization [37,38], which may vary among species.

However, the unequal distribution of these receptors on the sperm plasma membrane of the studied species, even those closely related such as stallion and donkey, together with the presence of different immunotypes in the same ejaculate suggest that the function of these receptors in spermatozoa may vary. They could be related to the fertilization process by improving their motility or extending their viability [23], or they could be involved in the antioxidant defense of the gametes by scavenging excessive ROS and RNS (Reactive Oxigen/Nitrogen Species) as already reported for human spermatozoa [39], rather than in seasonal control. These functions are compatible, and melatonin could be helping to preserve and regulate sperm functionality both by having a direct antioxidant effect and through receptor binding. However, the fact that melatonin is present in the seminal plasma of all studied species, and melatonin receptors in all spermatozoa even in the non-seasonal ones, lead us to suggest that its function might be other than seasonal control.

Several results have reported that melatonin may exert its antioxidant and antiapotoptic effect, via MT_1 and/or MT_2 receptors, after ejaculation. It has been demonstrated that exogenous melatonin can prevent oxidative damage in boar [40], stallion [20] and human [41] spermatozoa. Due to its antioxidant properties, melatonin has also been used as an extender additive in sperm refrigeration and cryopreservation of boar [19], ram [42], and bull [18], and its addition increased the post-thawing sperm quality on red deer [21]. However, melatonin did not seem to exert any beneficial effects on dog sperm cryopreservation [43].

A species-specific effect has already been shown for the modulation of sperm motility through melatonin receptors. Thus, exogenous melatonin enhanced hyperactivation of

hamster sperm through the MT_1 receptor [29], whereas it increased progressive motility and other kinematics parameters in ram [42,44], bull [18], human [45] and even Iberian ibex spermatozoa [46], but not in the stallion [20]. In addition, several studies have reported contradictory results on the melatonin effects on boar sperm motility [19,40]. Our results show that although the distribution of MT_1 and MT_2 melatonin receptors is unequal in the sperm head of the studied species, all of them have one or both receptors in the neck and midpiece of the flagellum, which might be related to the modulation of sperm kinematics and hyperactivation, potentially contributing to increase the sperm fertilizing capacity. Furthermore, melatonin can exert its antiapoptotic effects in human spermatozoa via the MT_1 receptor [28]. Therefore, the presence of melatonin receptor MT_1 in the cytoplasmic droplet of stallion immature spermatozoa and epididymal red deer spermatozoa suggests that melatonin may protect the future spermatozoon from oxidative damage during spermatogenesis and sperm maturation [47-49] through these receptors.

Melatonin receptors could also be involved in the fertilization process by modulating the capacitation process or the acrosome reaction. In fact, incubation of ram spermatozoa with different physiological doses of melatonin not only prevents apoptotic-like changes, but also modulates sperm capacitation and increases *in vitro* fertilization [22]. Moreover, we have recently shown that the melatonin effect on ram sperm capacitation is modulated through MT_2 receptors [50].

5. CONCLUSIONS

In conclusion, our study shows the presence of melatonin receptors MT_1 and MT_2 in spermatozoa of several domestic species and a wild, highly seasonal species, regardless their seasonality. It also shows the existence of measurable levels of melatonin in the seminal plasma of ejaculated semen, despite a great intra-species individual variation. These results open new interesting perspectives of research to explore the exact role of melatonin and melatonin receptors in the fertility of domestic animals. A wider study is currently in progress to establish the cause of the wide inter-individual variation and the high variability found in seminal plasma samples, using a broader range of individuals and breeds in each species, and a higher number of samples.

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Author contribution: Dr. Cebrián-Perez designed the experimental study, Marta Gonzalez-Arto and Alejandro Vicente-Carrillo performed indirect immunofluorescence analyses and Western blot in dog, boar, donkey and stallion, Dr. Martinez-Pastor and Estela Fernández-Alegre analyzed bull and deer spermatozoa, Dr. Casao analyzed seminal plasma, Drs. Roca, Miró, Rigau and Rodriguez-Gil provided dog, boar, donkey and stallion samples and revised the parts concerning to these species, Dr. Casao drafted the manuscript, whereas critical revision of the manuscript and approval of the article was completed by Drs. Pérez-Pé, Muiño-Blanco and Cebrián-Pérez.

References

[1] Tan D-X, Manchester LC, Terron MP, Flores LJ, Reiter RJ. One molecule, many derivatives: A never-ending interaction of melatonin with reactive oxygen and nitrogen species? J Pineal Res 2007;42:28-42.

[2] Tan DX, Hardeland R, Manchester LC, Paredes SD, Korkmaz A, Sainz RM, et al. The changing biological roles of melatonin during evolution: from an antioxidant to signals of darkness, sexual selection and fitness. Biol Rev Camb Philos Soc 2010;85:607-23.

[3] Turek FW, Desjardins C, Menaker M. Melatonin-induced inhibition of testicular function in adult golden hamsters. Proc Soc Exp Biol Med 1976;151:502-6.

[4] Argo CM, Cox JE, Gray JL. Effect of oral melatonin treatment on the seasonal physiology of pony stallions. J Reprod Fertil Suppl 1991;44:115-25.

[5] Chemineau P, Malpaux B, Delgadillo JA, Guerin Y, Ravault JP, Thimonier J, et al. Control of sheep and goat reproduction: Use of light and melatonin. Anim Reprod Sci 1992;30:157-84.

[6] Adam CL, Moir CE, Atkinson T. Induction of early breeding in red deer (Cervus elaphus) by melatonin. J Reprod Fertil 1986;76:569-73.

[7] Luboshitzky R, Shen-Orr Z, Herer P. Seminal plasma melatonin and gonadal steroids concentrations in normal men. Arch Androl 2002;48:225 - 32.

[8] Casao A, Cebrian I, Asumpcao M, Perez-Pe R, Abecia J, Forcada F, et al. Seasonal variations of melatonin in ram seminal plasma are correlated to those of testosterone and antioxidant enzymes. Reprod Biol Endocrinol 2010;8:59.
[9] D'Alessandro AG, Martemucci G. Evaluation of seasonal variations of semen freezability in Leccese ram. Anim Reprod Sci 2003;79:93-102.
[10] Mandiki SNM, Derycke G, Bister JL, Paquay R. Influence of season and age on sexual maturation parameters of Texel, Suffolk and Ile-de-France rams: 1. Testicular size, semen quality and reproductive capacity. Small Rumin Res 1998;28:67-79.
[11] Hoffmann B, Landeck A. Testicular endocrine function, seasonality and semen quality of the stallion. Anim Reprod Sci 1999;57:89-98.
[12] Martinez-Pastor F, Guerra C, Kaabi M, Garcia-Macias V, de Paz P, Alvarez M, et al. Season effect on genitalia and epididymal sperm from Iberian red deer, roe deer and Cantabrian chamois. Theriogenology 2005;63:1857-75.
[13] Berthelot X, Neuhart L, Gary F. Photoperiodicity, melatonin and reproduction in cows. Rec Med Vet 1991;167:219-25.
[14] Peltoniemi OA, Virolainen JV. Seasonality of reproduction in gilts and sows. Soc Reprod Fertil Suppl 2006;62:205-18.
[15] Bouchard G, Youngquist RS, Vaillancourt D, Krause GF, Guay P, Paradis M. Seasonality and varibility of the interestrous interval in the bitch. Theriogenology 1991;36:41-50.
[16] Sen Majumder S, Bhadra A. When Love Is in the Air: Understanding Why Dogs Tend to Mate when It Rains. PLoS ONE 2015;10:e0143501.
[17] Ortega-Pacheco A, Segura-Correa JC, Bolio-Gonzalez ME, Jimenez-Coello M, Forsberg CL. Reproductive patterns of stray male dogs in the tropics. Theriogenology 2006;66:2084-90.
[18] Ashrafi I, Kohram H, Ardabili FF. Antioxidative effects of melatonin on kinetics, microscopic and oxidative parameters of cryopreserved bull spermatozoa. Anim Reprod Sci 2013;139:25-30.
[19] Martín-Hidalgo D, Barón FJ, Bragado MJ, Carmona P, Robina A, García-Marín LJ, et al. The effect of melatonin on the quality of extended boar semen after long-term storage at 17 °C. Theriogenology 2011;75:1550-60.
[20] Balao da Silva CM, Macías-García B, Miró-Morán A, González-Fernández L, Morillo-Rodriguez A, Ortega-Ferrusola C, et al. Melatonin reduces lipid

peroxidation and apoptotic-like changes in stallion spermatozoa. J Pineal Res 2011;51:172-9.

[21] Domínguez-Rebolledo ÁE, Fernández-Santos MR, Bisbal A, Ros-Santaella JL, Ramón M, Carmona M, et al. Improving the effect of incubation and oxidative stress on thawed spermatozoa from red deer by using different antioxidant treatments. Reprod Fertil Dev 2010;22:856–70.

[22] Casao A, Mendoza N, Pérez-Pé R, Grasa A, Abecia JA, Forcada F, et al. Melatonin prevents capacitation and apoptotic-like changes of ram spermatozoa and increases fertility rate. J Pineal Res 2010;48:39-46.

[23] Reiter RJ, Rosales-Corral SA, Manchester LC, Tan DX. Peripheral Reproductive Organ Health and Melatonin: Ready for Prime Time. Int J Mol Sci 2013;14:7231-72.

[24] Reiter RJ, Tan DX, Osuna C, Gitto E. Actions of melatonin in the reduction of oxidative stress - A review. J Biomed Sci 2000;7:444-58.

[25] Dubocovich ML, Rivera-Bermudez MA, Gerdin MJ, Masana MI. Molecular pharmacology, regulation and function of mammalian melatonin receptors. Front Biosci 2003;8:D1093-D108.

[26] Casao A, Gallego M, Abecia JA, Forcada F, Pérez-Pé R, Muiño-Blanco T, et al. Identification and immunolocalisation of melatonin MT1 and MT2 receptors in Rasa Aragonesa ram spermatozoa. Reprod Fertil Dev 2012;24:953-61.

[27] van Vuuren RJ, Pitout MJ, van Aswegen CH, Theron JJ. Putative melatonin receptor in human spermatozoa. Clin Biochem 1992;25:125-7.

[28] Espino J, Ortiz Á, Bejarano I, Lozano GM, Monllor F, García JF, et al. Melatonin protects human spermatozoa from apoptosis via melatonin receptor- and extracellular signal-regulated kinase-mediated pathways. Fertil Steril 2011;95:2290-6.

[29] Fujinoki M. Melatonin-enhanced hyperactivation of hamster sperm. Reproduction 2008;136:533-41.

[30] Reppert SM, Weaver DR, Ebisawa T. Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. Neuron 1994;13:1177-85.

[31] Coge F, Guenin SP, Fery I, Migaud M, Devavry S, Slugocki C, et al. The end of a myth: cloning and characterization of the ovine melatonin MT2 receptor. Br J Pharmacol 2009;158:1248-62.

500 [32] Casao A, Pérez-Pé R, Abecia JA, Forcada F, Muiño-Blanco T, Cebrián-Pérez
501 JA. The effect of exogenous melatonin during the non-reproductive season on
502 the seminal plasma hormonal profile and the antioxidant defence system of Rasa
503 Aragonesa rams. Anim Reprod Sci 2013;138:168-74.
504 [33] Bubenik GA, Konturek SJ. Melatonin and aging: prospects for human treatment.
505 J Physiol Pharmacol 2011;62:13-9.
506 [34] Dimitrov R, Georgiev G, Todorov P, Dimitrov Y, Konakchieva R. Membrane
507 melatonin receptor type MT1 expression in human ejaculated spermatozoa. C R
508 Acad Bulg Sci 2012;65:947-52.
509 [35] Dubocovich ML, Markowska M. Functional MT1 and MT2 melatonin receptors
510 in mammals. Endocrine 2005;27:101-10.
511 [36] Gilman AG. G-Proteins and regulation of adenylyl-cyclase. Biosci Rep
512 1995;15:65-97.
513 [37] Ayoub MA, Levoye A, Delagrange P, Jockers R. Preferential formation of
514 MT1/MT2 melatonin receptor heterodimers with distinct ligand interaction
515 properties compared with MT2 homodimers. Mol Pharmacol 2004;66:312-21.
516 [38] Levoye A, Dam J, Ayoub MA, Guilllaume JL, Couturier C, Delagrange P, et al.
517 The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor
518 function through heterodimerization. Embo J 2006;25:3012-23.
519 [39] Du Plessis SS, Hagenaar K, Lampiao F. The in vitro effects of melatonin on
520 human sperm function and its scavenging activities on NO and ROS. Andrologia
521 2010;42:112-6.
522 [40] Jang HY, Kim YH, Kim BW, Park IC, Cheong HT, Kim JT, et al. Ameliorative
523 Effects of Melatonin against Hydrogen Peroxide-Induced Oxidative Stress on
524 Boar Sperm Characteristics and Subsequent In Vitro Embryo Development.
525 Reprod Domest Anim 2010;45:943-50.
526 [41] Bejarano I, Monllor F, Marchena AM, Ortiz A, Lozano G, Jimenez MI, et al.
527 Exogenous melatonin supplementation prevents oxidative stress-evoked DNA
528 damage in human spermatozoa. J Pineal Res 2014;57:333-9.
529 [42] Succu S, Berlinguer F, Pasciu V, Satta V, Leoni GG, Naitana S. Melatonin
530 protects ram spermatozoa from cryopreservation injuries in a dose-dependent
531 manner. J Pineal Res 2011;50:310-8.
532 [43] Varesi S, Vernocchi V, Morselli MG, Luvoni GC. DNA integrity of fresh and
533 frozen canine epididymal spermatozoa. Reprod Biol 2014;14:257-61.

[44] Casao A, Vega S, Palacín I, Pérez-Pe R, Laviña A, Quintín FJ, et al. Effects of Melatonin Implants During Non-Breeding Season on Sperm Motility and Reproductive Parameters in Rasa Aragonesa Rams. Reprod Domest Anim 2010;45:425-32.

[45] Ortiz A, Espino J, Bejarano I, Lozano GM, Monllor F, García JF, et al. High endogenous melatonin concentrations enhance sperm quality and short-term in vitro exposure to melatonin improves aspects of sperm motility. J Pineal Res 2011;50:132-9.

[46] Santiago-Moreno J, Toledano-Diaz A, Castano C, Coloma MA, Esteso MC, Prieto MT, et al. Photoperiod and melatonin treatments for controlling sperm parameters, testicular and accessory sex glands size in male Iberian ibex: A model for captive mountain ruminants. Anim Reprod Sci 2013;139:45-52.

[47] Ateşşahin A, Şahna E, Türk G, Çeribaşi AO, Yılmaz S, Yüce A, et al. Chemoprotective effect of melatonin against cisplatin-induced testicular toxicity in rats. J Pineal Res 2006;41:21-7.

[48] Koksal M, Oguz E, Baba E, Eren MA, Ciftci H, Demir ME, et al. Effects of melatonin on testis histology, oxidative stress and spermatogenesis after experimental testis ischemia-reperfusion in rats. Eur Rev Med Pharmacol Sci 2012;16:582-8.

[49] Sarabia L, Maurer I, Bustos-Obregón E. Melatonin prevents damage elicited by the organophosphorous pesticide diazinon on mouse sperm DNA. Ecotoxicol Environ Saf 2009;72:663-8.

[50] Gonzalez-Arto M, Luna C, Perez-Pe R, Muino-Blanco T, Cebrian-Perez JA, Casao A. New evidence of melatonin receptor contribution to ram sperm functionality. Reprod Fertil Dev 2014.

Figure 1: Distribution of melatonin MT_1 (panels a-f) and MT_2 (panels g-o) receptor in donkey spermatozoa, evaluated by imaging flow cytometry. For MT_1 receptor, spermatozoa with staining at the acrosome (a-c), and with a brighter band at the postacrosomal region (d-f) are represented. For MT_2 receptor, spermatozoa with staining all over the head and tail (g-i), acrosome (j-l) and postacrosomal region (m-o) are shown. Bright field (a, d, g, j, m), MT_1 receptors (b, e), MT_2 receptors (h, k, n) and merged images (c, f, i, l, o) are shown.

Figure 2: Distribution of melatonin MT_1 (panels a-f) and MT_2 (panels g-o) receptor in horse spermatozoa, evaluated by imaging flow cytometry. For MT_1 receptor, spermatozoa with staining all over the head and tail (a-c), and cytoplasmic droplet (d-f) are represented. For MT_2 receptor, spermatozoa show staining at the acrosome (g-i), or acrosome and tail (j-l), but not cytoplasmic droplet (m-o). Bright field, (a, d, g, j, m) MT_1 receptors, (b, e), MT_2 receptors (h, k, n) and merged images (c, f, i, l, o) are shown.

Figure 3: Distribution of melatonin MT_1 (panels a- f) and MT_2 (panels g-l) receptor in boar (a-c, g-i), dog (d-f, j-l) spermatozoa. Magnification 400x. Differential Interference Contrast (a, d, g, j), melatonin receptors (b, e, h, k) and merged images (c, f, i, l) are shown.

Figure 4: Distribution of melatonin MT_1 (panels a- f) and MT_2 (panels g-l) receptor in bull (a-c, g-i) and red deer (d-f, j-l) spermatozoa. Magnification 1000x. Bright field (a, d, g, j), melatonin receptors (b, e, h, k) and merged images (c, f, i, l) are shown.

Figure 5: Western-blot images of the presence of MT_1 (a) and MT_2 (b) melatonin receptor in sperm protein extracts from donkey (1), horse (2), boar (3), bull (4), deer (5), dog (6) and ram (7, positive control).

Table 1: Concentration of melatonin (pg/mL) in donkey, stallion, boar, bull and dog seminal plasma. Values are shown as mean ± S.E.M. of different males (number of analyzed males (n), shown in brackets in each species). Range of values obtained in all samples from in each species is also displayed. Different letters account for significant differences between species ($P<0.05$).

	Mean ± SEM (pg/mL)	Range (pg/mL)
Donkey (n = 4)	2.82 ± 0.38^{b}	0.68 - 6.47
Stallion (n = 4)	2.48 ± 0.14^{b}	1.24 - 3.39
Boar (n = 6)	9.34 ± 1.38^{b}	1.07 - 26.71
Bull (n = 6)	19.10 ± 7.37^{a}	0.74 - 88.03
Dog (n = 7)	6.06 ± 2.28^{b}	0.74 - 46.22

Table 2: Summary of melatonin MT_1 (1) and MT_2 (2) receptor distribution in donkey, stallion, boar, bull, deer and dog spermatozoa, assessed by indirect immunofluorescence. Brackets ([]) indicate that in that location, the receptor was not detected in all the spermatozoa of the sperm sample. The absence of a number indicates that there was no immunostaining in that sperm region.

	Head			Flagellum		
	Acrosome	Equatorial band	Postacrosome	Neck	Midpiece	Tail
Donkey	1, 2	[1], 2	2	2	2	2
Stallion	1,2	1	1, [2]	1, [2]	1, [2]	1, [2]
Boar		1		1, 2	1	
Bull	[1]	[1]	[1]	[1], 2	1	1
Deer	[1]	[1]	[1]	[1], 2	1	1
Dog	[1], 2	[1]	[1]	1	1, 2	

Figure 1

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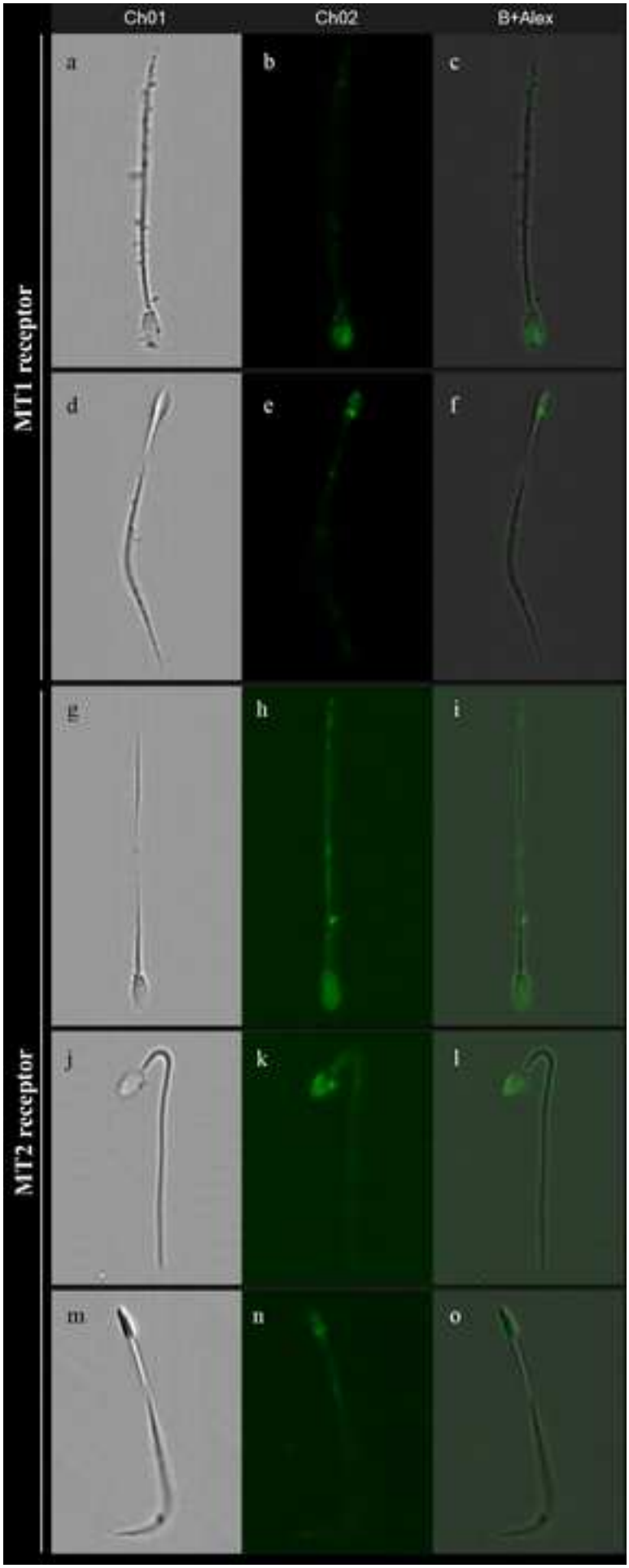

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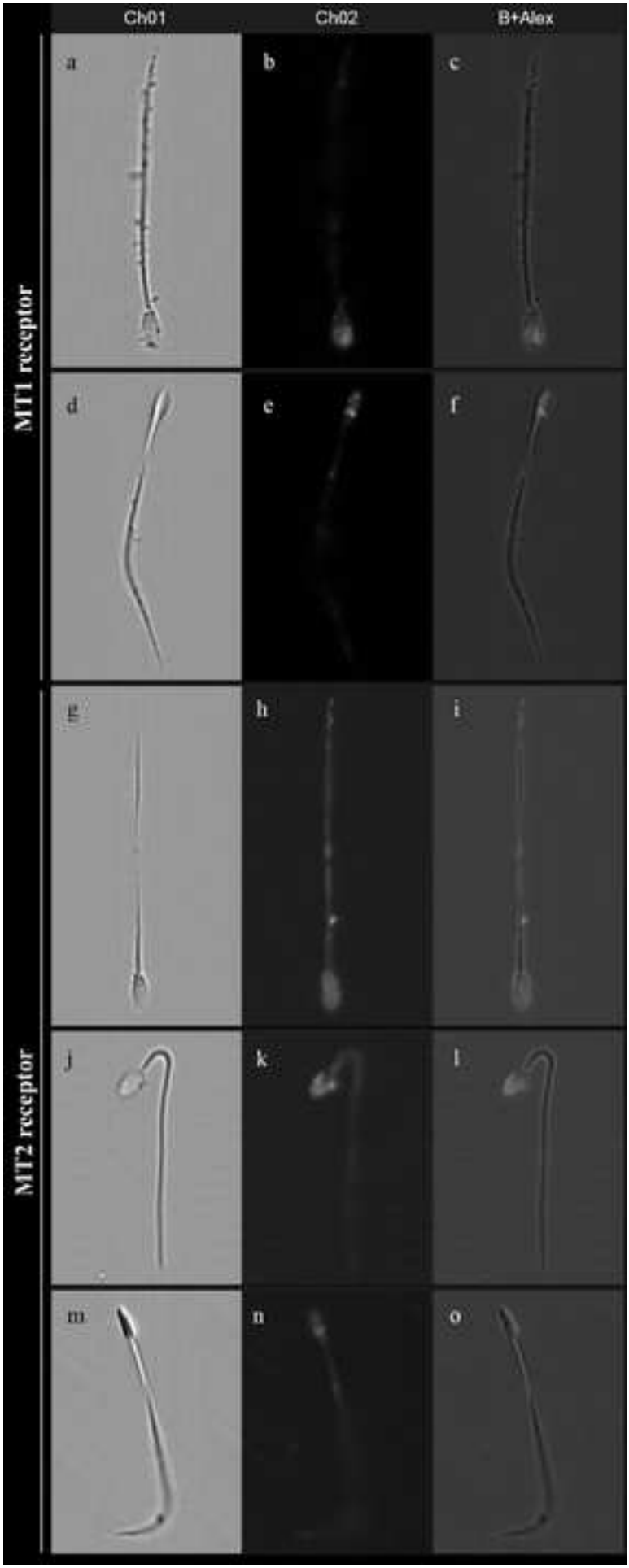

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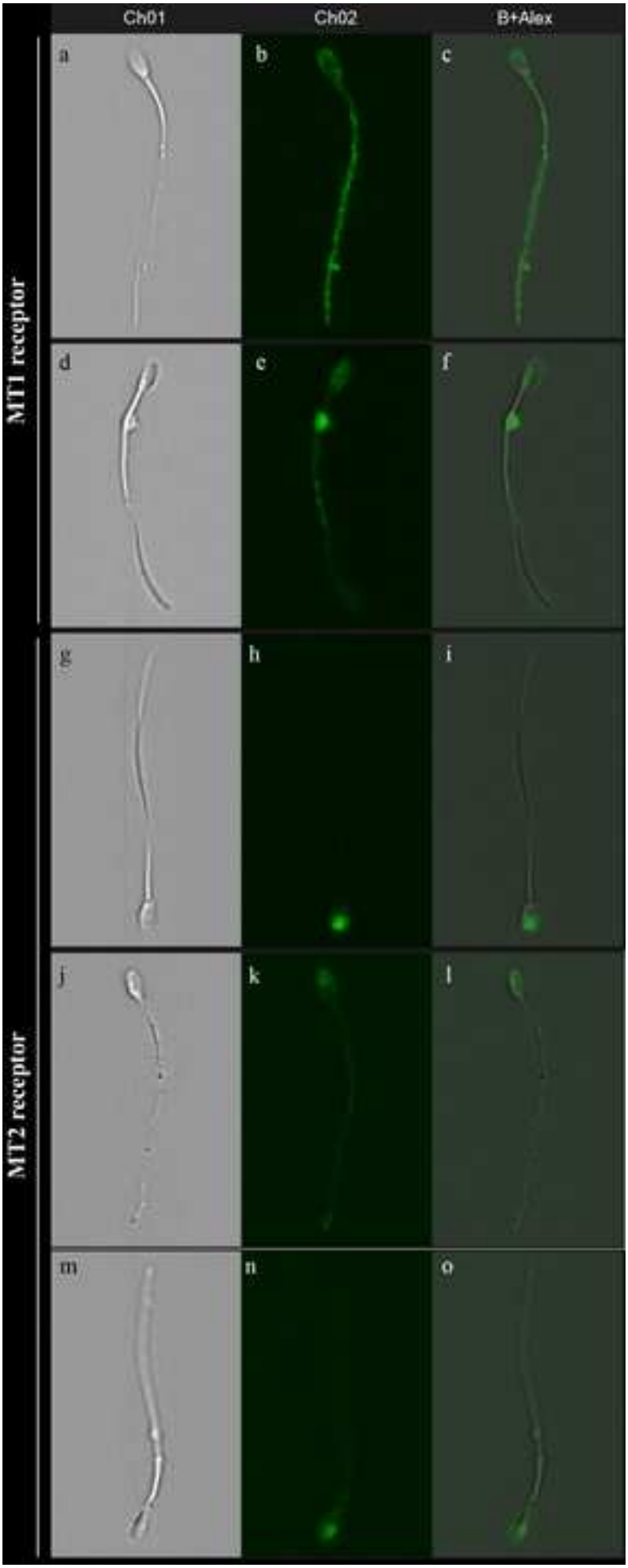

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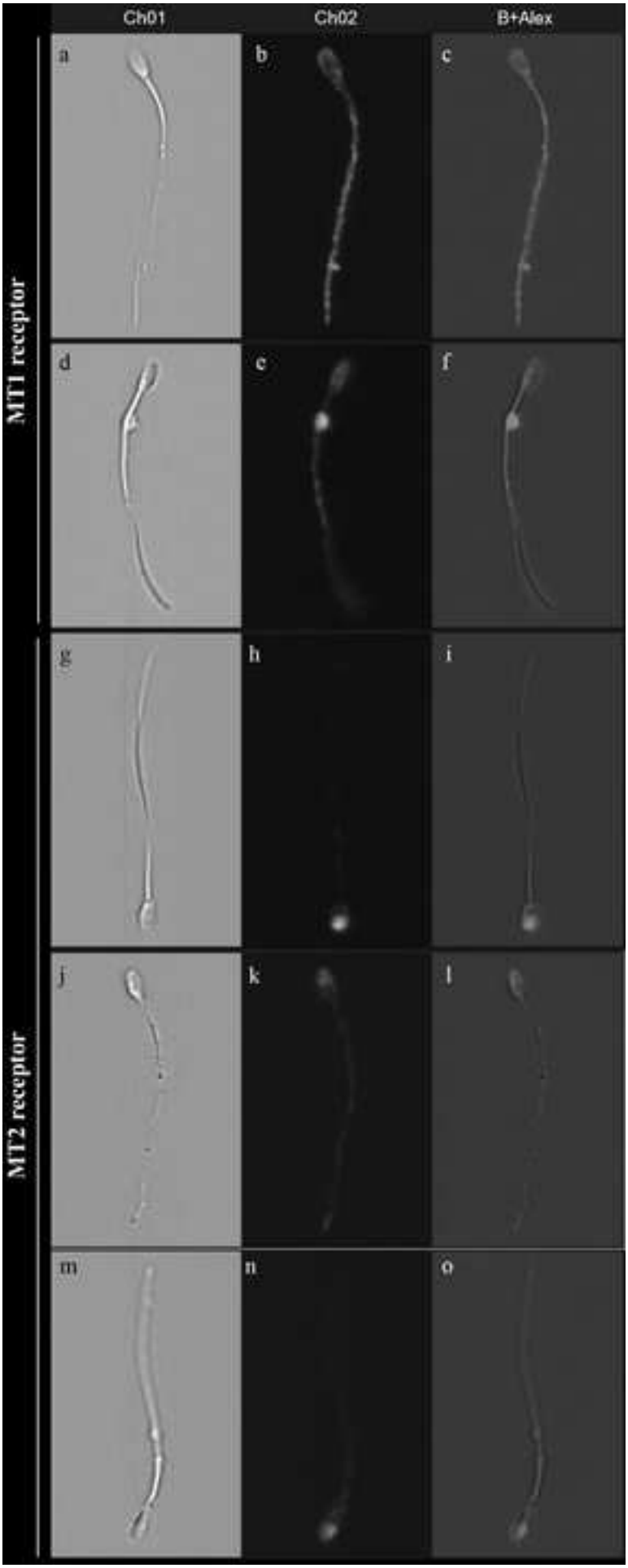

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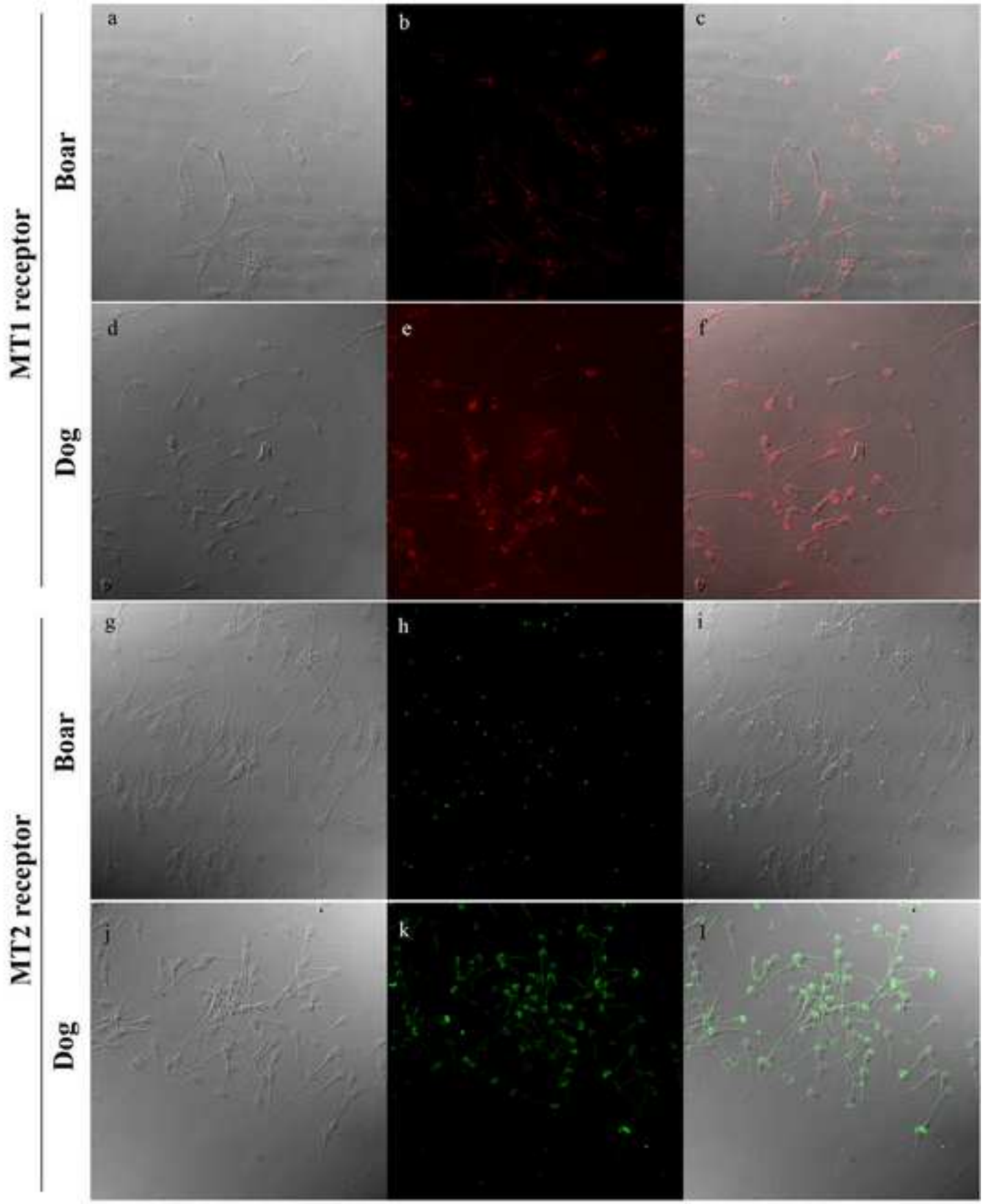

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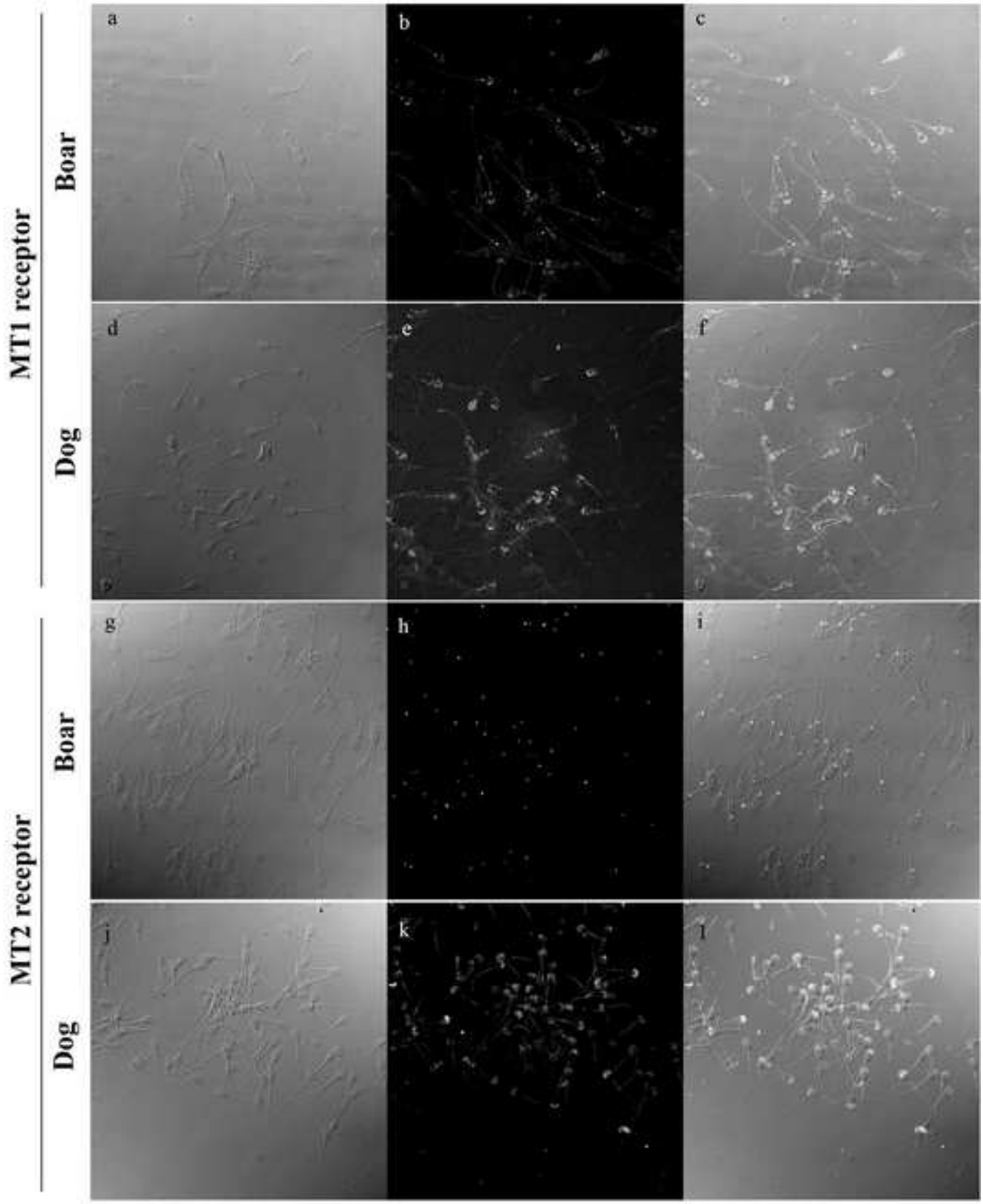

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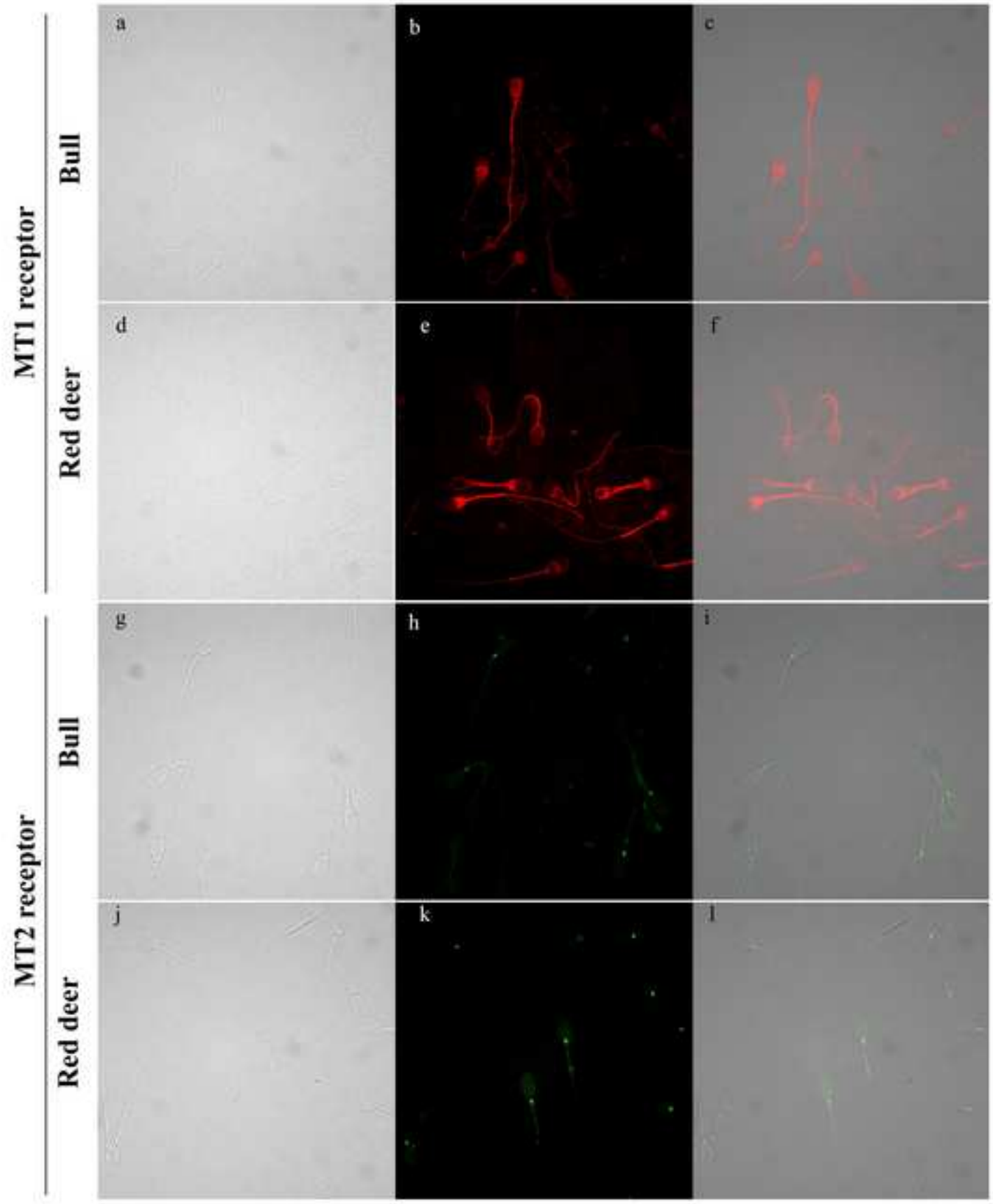

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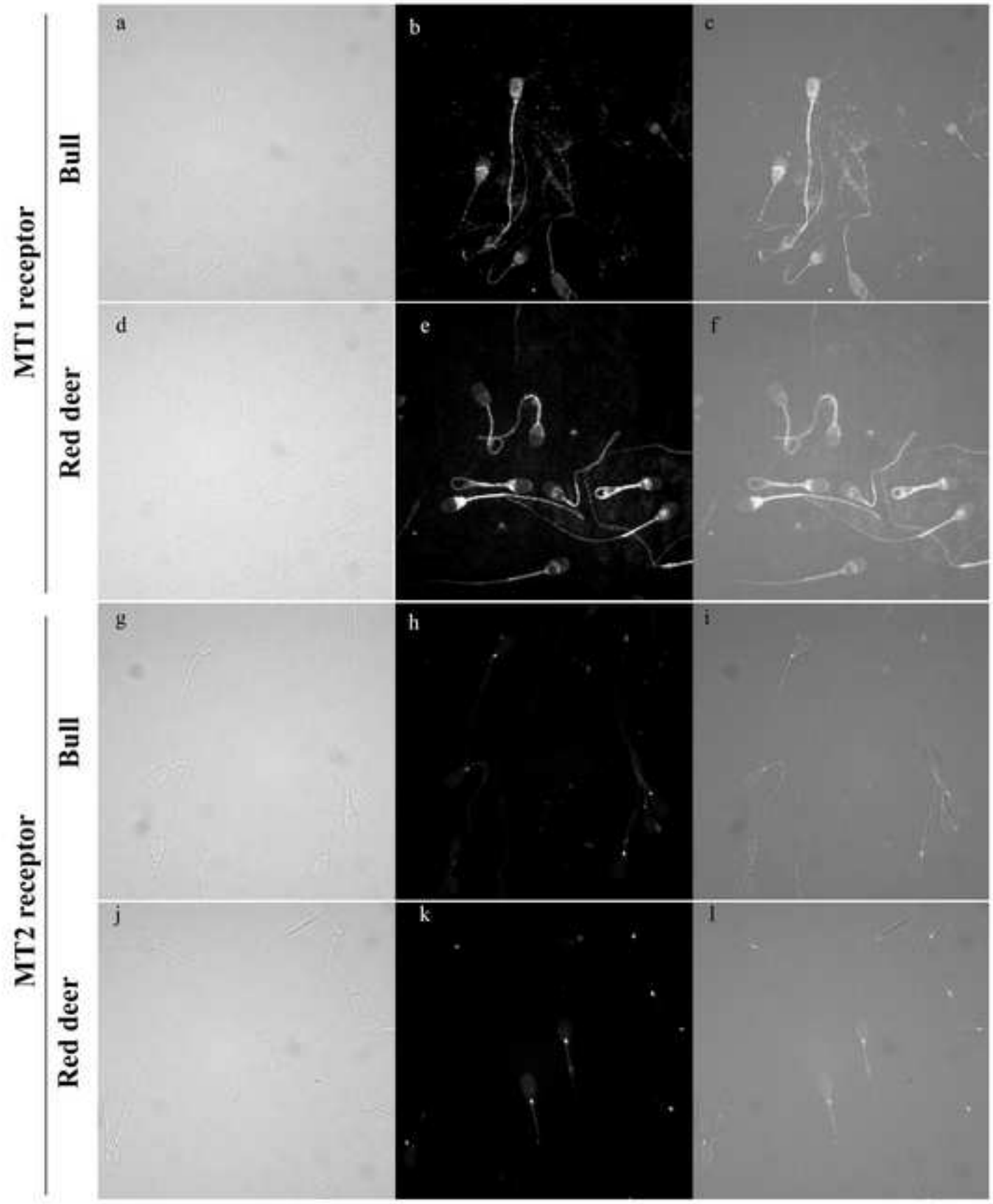

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