Identification of serotoninergic system components in stallion sperm

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Summary. Assisted reproductive techniques have been used on several domestic animals to preserve desirable traits in strains of high genetic and commercial value; however in equines its efficiency rate is relatively low. To increase the conception ratio in stallions, some research groups have used pharmacological treatments which promote sperm hyperactivation in order to increase male's fertility rates. In this way, our previous work suggests that serotonin (5-HT) could be a good pharmacological candidate that facilitates conception rate in domestic horses. 5-HT is a neurohormone involved in several reproductive processes, i.e., it enhances hyperactivation, motility, and promotes the acrosome reaction in mammalian sperm, but it has not been described in the stallion sperm yet. Therefore, using both immunofluorescence and western blot techniques, we searched for and found some serotonin markers such as 5-HT, $5-HT_{1B}$, $5-HT_{2A}$, $5-HT_3$ receptors, both TPH1 and MAO_A enzymes, and serotonin transporter ($5-HT_T$) in stallion sperm. In addition, we found a non-neuroendocrine cell, $V-MAT_1$ transporter, which has not been previously reported in mammalian sperm. Our results suggest that serotoninergic system is present in stallion sperm, which could be a pharmacological target to increase the conception rates in domestic horses.

Key words: Stallion sperm, Serotonin, Serotonin Receptors, TPH, $VMAT_1$

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Introduction

One of the biomolecules which in recent years has generated more interest among researchers in reproductive physiology is serotonin (5-hydroxy-tryptamine; 5-HT), an indolamine which is present in both plants and animals, including humans. 5-HT is a monoamine which plays different key roles in the physiology of living beings (Gaspar et al., 2003; Adayev et al., 2005; Alavi et al., 2014). Its functions as a neurotransmitter in the central nervous system (CNS) and peripheral nervous system (PNS) are the most studied so far (Whalter and Bader, 2003; Pytliak et al., 2011).

In reproductive system of mammals, its pharmacological properties have made it a good candidate to efficiently treat several current disorders in reproductive health such as male infertility (Fujinoki, 2011; Jiménez-Trejo et al., 2012), mainly because of its influence in both CNS and PNS but also because 5-HT

participates in the regulation of testicular blood flow and in the secretion of both corticotrophin releasing factor and testosterone from Leydig cells (Dufau et al., 1993; Tinajero et al., 1993; Frungieri et al., 1999, 2002). In previous studies we have demonstrated that 5-HT plays an important role in reproductive tissues and some imbalances in its metabolism could negatively affect the reproductive status of mammals (Jiménez-Trejo et al., 2007, 2012, 2013; Pichardo et al., 2011), resulting in an infertility condition for some cases (Gonzales et al., 1992). 5-HT is synthesized and contained in cells of different sexual organs such as testes, epididymis, deferent ducts, prostate, and even human sperm (Anderson et al., 1979; Hay and Wadsworth, 1982; Young and Laing, 1989; Campos et al., 1990; Tinajero et al., 1993; Frungieri et al., 2002; Jiménez-Trejo et al., 2007, 2012, 2013).

Some 5-HT receptors have been identified in mammalian sperm and its actions within the cell have been described (Young and Laing, 1989; Jiménez-Trejo et al., 2007, 2012). Previous studies have postulated that these actions could be pharmacologically modulated to facilitate capacitation and sperm hyperactivity in mammals and invertebrates through positive regulation of acrosomal reaction and fertilization involving both dynein and tyrosine phosphorylation (Meizel and Turner, 1983; Bandivdekar et al., 1992; Stephens and Prior, 1992; Meizel, 2004; Fujinoki, 2011; Hinrichs and Loux, 2012; Jiménez-Trejo et al., 2012; Alavi et al., 2014; Ramírez-Reveco et al., 2017).

Several issues are present in the reproductive system of domestic horses resulting in important economic losses. These complications include 1) difficulties in the conservation and preservation of stallion sperm for artificial insemination, 2) difficulties to maintain adequate viability, 3) low success rates of *in vitro* fertilization procedures, 4) delays in the conception of the offspring and 5) the intrinsic seasonality in the stallions (Khlifaoui et al., 2004; Squires et al., 2004; Alvarenga et al., 2005; Glazar et al., 2009; Hinrichs and Loux, 2012; Oldenhof et al., 2013). We postulate that the pharmacologic use of 5-HT could help to resolve some of these problems, but currently there are no reports in the literature describing 5-HT or serotoninergic component in the reproductive tract of this species. With this idea in mind, in the present study we search for the presence of serotoninergic markers in domestic horse sperm. We used both immunofluorescence and western blot techniques, and found the presence of 5-HT, some 5-HT receptors ($5-HT_{1B}$, $5-HT_{2A}$ and $5-HT_3$); tryptophan hydroxylase isoform 1 (TPH1) and monoamine oxidase A (MAO_A) enzymes, and both membrane and vesicular 5-HT transporters ($5-HT_T$; $VMAT_1$) in the horse sperm. We have previously described the localization and physiological effects of 5-HT components in reproductive tissues of other mammal species (Jiménez-Trejo et al., 2007, 2012, 2013), so we postulate that the reproductive role of 5-HT is highly conserved across species. In horses, it is a possibility

that modulation of this pathway could promote hypermotility in stallion sperm, enhancing the conception ratio and diminishing economic losses.

Materials and methods

Stallion sperm samples

Sperm samples were obtained from four healthy horses of thorough bred race and different ages between 5 and 10 years old, with a body weight between 300-500 kg. Horses were housed in stables of the San Francisco Ranch property of UNAM patronage, located in Valle de Chalco, Mexico State. The sperm was collected during three time frames, first from August to December 2014, second from August to December 2015 and third from August to December 2016. The handling of the animals was realized under strict agreement of the guidelines established by the ethic committee of the Faculty of Veterinary Medicine of UNAM (Facultad de Medicina Veterinaria y Zootecnia, FMVZ, UNAM).

Samples of semen were diluted with a kenney extender, stored at room temperature and transported to the Faculty of Chemistry of UNAM (Province et al., 1985). Then we followed the procedure described by Schuh et al. (2006) to separate the sperm; briefly, sperm samples were divided into 0.25 mL aliquots, then were diluted 4 fold with Na7.4 medium (in mM:135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 20 Hepes, 5 glucose, 10 lactic acid, 1 pyruvic acid, adjusted to pH 7.4 with NaOH), and sedimented at 333 g for 7 min. Separated sperm were re-suspended in 0.75 mL of Na7.4 medium, sediment for 3 min, and then dispersed in 0.5 mL of Na7.4 medium to achieve a final concentration of $1x10^8$ cells/mL or greater. At the end, they were exanimated and conserved in optimal conditions of temperature until use (González-Martínez et al., 2002; Jiménez-Trejo et al., 2012).

Immunofluorescence of serotoninergic system components in stallion sperm

Once the equine sperm was separated from seminal liquid, smears of sperm were mounted over slides, fixed in 4% paraformaldehyde during 20 minutes and then left drying; after this the slides were desiccated in a vacuum chamber during 20 minutes. Then smears were incubated with specific antibodies for detecting several serotoninergic system components (see below).

Immunofluorescence procedures were performed using the following antibodies: 1) mouse anti-5-HT (1:100, Genetex, Irvine, CA, USA), 2) goat anti-TPH1 (1:100), 3) rabbit anti-MAOA (1:200), 4) goat anti-5-HT_T (1:200), 5) rabbit anti-5-HT V-MAT_1 (1:100), 6) goat anti 5-HT1B receptor (1:100), 7) goat anti 5-HT2A receptor (1:100) and 8) goat anti 5-HT_3 receptor (1:100); (all from Santa Cruz Biotechnology, Inc). Control slides were incubated with pre-immune serum; all slides were incubated with a preparation of different second

antibodies, as described by Jiménez-Trejo et al., (2012), and Vela-Hinojosa et al., (2013). We used adult rat brainstem tissue as positive control for serotoninergic components immunostaining.

Slides were observed and photographed in a Nikon Eclipse E600 epifluorescence microscope (Nikon Instruments, Melville, NY), equipped with a Digital Camera System for Microscopy (Digital Sight Series), or in a confocal Nikon A1R$^+$. Images were digitized and figures elaborated using Adobe Photoshop Software 5.1 (Adobe Systems Incorporated, San Jose, CA, USA).

Western blotting

Stallion sperm samples stored at -75°C were thawed and homogenized in a buffer containing Trizma hydrochloride (Tris-HCl; 0.05 M, pH 7.4), dithiothreitol (1 mM), [ethylenebis (oxyethylenenitrilo)]tetraacetic acid and ethylene glycol bis (2-aminoethyl ether),N,N9,N9-tetraacetic acid (EGTA, 1 mM), supplemented with a mixture of protease inhibitors (Complete, EDTA-free, Roche-Mannheim, Germany). After the buffer addition, the cells were subjected to a lysis procedure to obtain the proteins with a special drill (Polytron PT-MR 2100, Kinematica AG, Luzern, Switzerland); all these procedures were performed in low temperature conditions to avoid protein denaturing. Once the proteins were obtained by the lysis, samples were centrifuged at 14,049 gravitational force (x g), during 20 minutes. The supernatant with proteins of each aliquot was collected to be quantified by Bradford assay using Epoch Microplate Spectrophotometer software and GEN 5.1. Software (BYOTEK, Instruments, Inc.). Samples (30 μg of protein/well), diluted in Laemmli solution were electrophored under reducing conditions (5% β-mercaptoethanol) through 12% sodium dodecyl sulphate-polyacrylamide gels at 140–150V for 2 hours.

Pre-stained molecular weight markers (BIO-RAD Laboratories Inc. Hercules CA, USA), were used to determine the relative mobility of proteins. Following electrophoresis, gels were equilibrated in a buffer containing 25 mM Tris, 192 mM glycine and 20% methanol. The proteins were then transferred to nitrocellulose sheets (BIO-RAD) at 300 mA for 1 hour at 4°C. Membranes were blocked with non-fat milk (5%) dissolved in Tris (20 mM), -sodium chloride (500 mM) buffer (TBS) for 2 hours at room temperature. They were then washed with TBS containing Tween-20
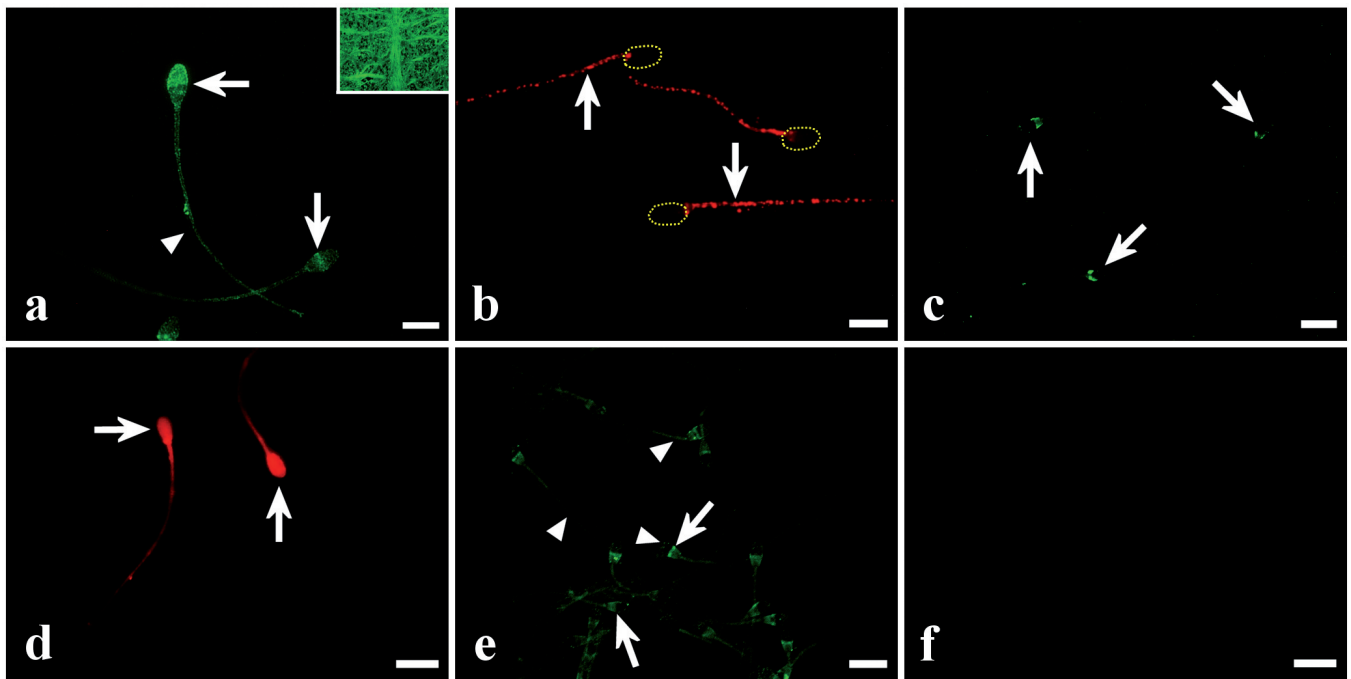

Fig. 1. 5-HT, both TPH and MAOA enzymes and 5-HT transporters are present in stallion sperm. **a.** Intense 5-HT immunoreactivity was found in the stallion sperm head (arrows) and a more scarce signal through the rest of sperm body (arrowhead); adult rat brainstem neurons and fibers immunoreactive for 5-HT are shown in inset as positive control. **b.** TPH was found strongly stained in a vesicle-like manner along the sperm tail (arrows), yellow dashed line indicates the area of the sperm head. **c.** MAOA was located in the post-acrosomal zone of the head (arrows); serotonin transporter [5-HTT; **(d)**] was observed strongly stained through the whole sperm, while vesicular monoamine transporter [V-MAT1, **(e)**] was differentially detected; in some sperm an intense signal in post-acrosomal zone was found (arrow) but all showed a moderate signal in the midpiece, a weaker one through the flagella and some small vesicles scarcely distributed in the sperm head (arrowheads). No detectable signal was found when stallion sperm was incubated with pre-immune serum **(f)**. Scale bars: 10 μm.

(0.05%; Tween-Tris sodium chloride (TTBS), and the immunoblots were performed using the following primary antibodies: 1) goat anti-TPH1 (1:3500), 2) rabbit anti-MAO$_A$ (1:5000), 3) rabbit anti-V-MAT$_1$ (1:3500), 4) goat anti 5-HT$_{1B}$ (1:3500), 5) goat anti 5-HT$_{2A}$ (1:5000), and 6) goat anti 5-HT$_3$ (1:5000). The immunoblots were incubated with the following secondary antibodies: 1) donkey anti-goat IgG- HRP (1:7500) and 2) goat anti-rabbit IgG-HRP (1:7500); all provided by Santa Cruz Biotechnology, Inc. Adult rat brainstem samples were homogenized and used as positive control for serotoninergic system markers.

Membranes were exposed to film sheets for 15 seconds at room temperature and the films were developed (Dektol-19, Kodak, Rochester, NY) and fixed (Rapid Fixer, Kodak). Images of these films were captured using Epson perfection 4490 photo scanner and digitized with Photoshop 5.1 software (n=5 for each treatment).

Results

Identification and localization of serotoninergic markers in stallion sperm

In Fig. 1a we show 5-HT immunopositive stallion sperm intensely stained on its head zone (arrows; Fig. 1a) and scarce through flagella (arrowhead); in inset we show 5-HT immunoreactivity in neurons of adult rat brainstem as positive control. In addition, we detected
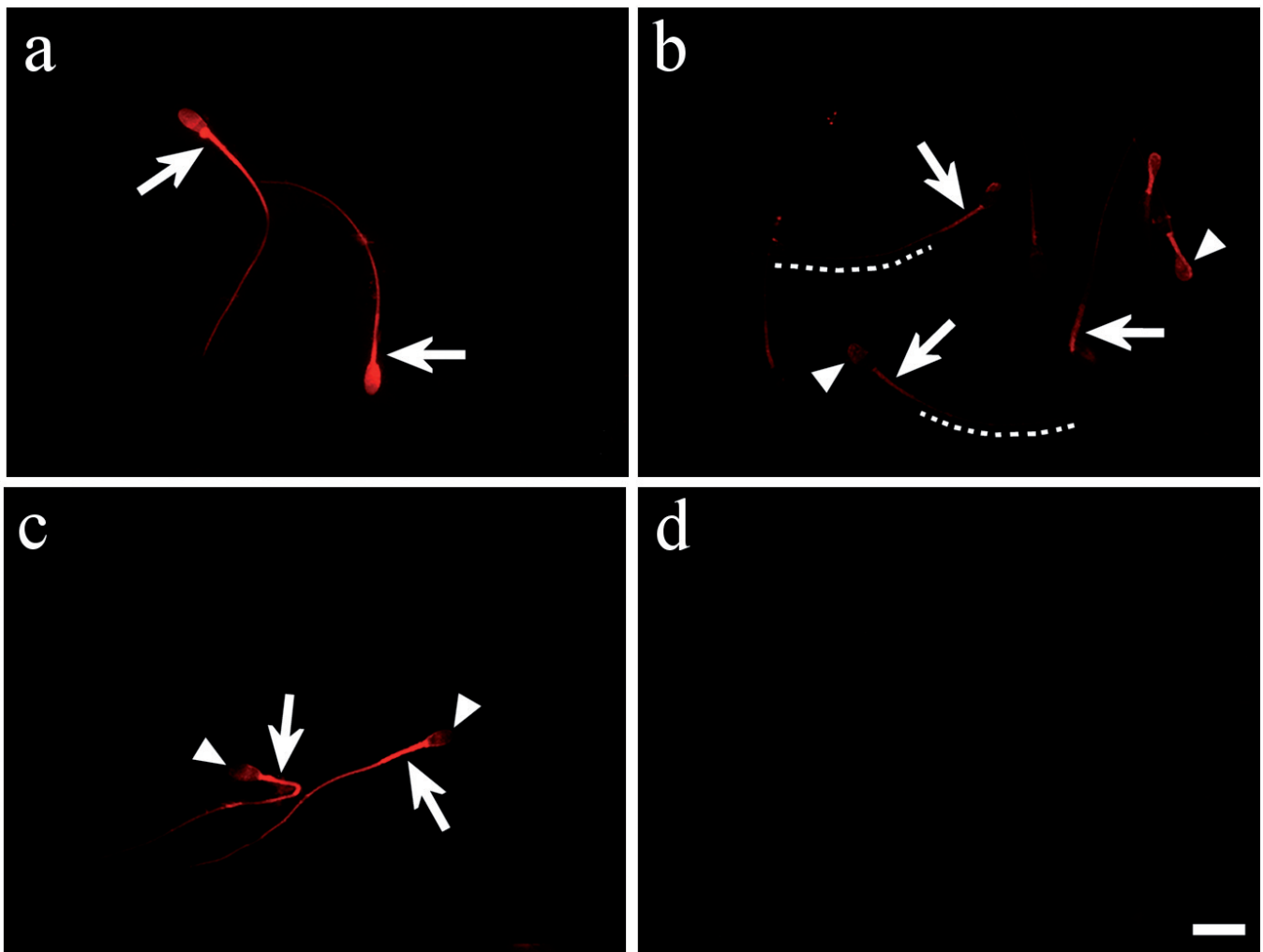

Fig. 2. 5-HT1B, 5-HT2A and 5-HT3 receptors show different distribution in stallion sperm. 5-HT1B receptor was observed along the whole spermatozoa, being more intensely stained in both head and midpiece (arrows in **a**); 5-HT2A immunoreactivity was higher in the midpiece (arrows in **b**) and in some regions of the sperm head (arrowheads in **b**) but a discrete staining along the sperm flagella (dashed lines in **b**) was also found; 5-HT3 immunoreactivity was higher in both midpiece and flagella (arrows in **c**) but a discrete staining in postacrosomal zone of the head (arrowheads) was also observed. We found no detectable signal when stallion sperm were incubated with pre-immune serum **(d)**. Scale bars: 10 μm.

the presence of two main enzymes of the 5-HT metabolic pathway: TPH1, which was localized along in the flagella in stallion sperm (arrows; Fig. 1b), and MAO_A, which was evident in the post acrosomal zone (arrows; Fig. 1c). 5-HTT was observed strongly stained along the whole stallion sperm (arrows; Fig. 1d). Interestingly, we found the vesicular monoamine transporter (V-MAT$_1$) differentially expressed in stallion sperm; in some of them we found an intense signal in post acrosomal zone (Fig 1e, arrows); but in all we found a moderate signal in the midpiece and weaker across the flagella plus some small vesicles that were scarcely present in the sperm head (arrowheads, Fig. 1e). No staining for any of the antigens analyzed was observed in control cells (Fig. 1f) when they were incubated with pre-immune serum.

We detected 5-HT$_{1B}$ receptor along the whole spermatozoa (Fig. 2a); its signal was relatively higher in both head and midpiece; 5-HT$_{2A}$ was found intensely stained in the midpiece (arrows; Fig. 2b) and in some regions of the sperm head (arrowheads, Fig. 2b), but also showed a discrete staining along the flagella (dashed lines, Fig 2b); 5-HT$_3$ was intensely stained in both midpiece and flagella (arrows; Fig. 2c) and showed a discrete staining in the sperm head (arrowheads; Fig. 2c). Fig. 2d shows a negative control of stallion sperm incubated with pre-immune serum.

Detection of serotonin markers in homogenates of stallion sperm

Fig. 3a shows a control gel with protein homogenates of adult rat brainstem loaded in well 1 and used as control of 5-HT system markers, and protein extracts of stallion sperm loaded in wells 2 and 3. The presence of serotoninergic components in stallion sperm was confirmed by immunoblots. Fig. 3b shows positive bands of both MAO_A (~61kDa) and TPH1 (~51kDa) enzymes; V-MAT$_1$ transporter (~65kDa); and 5-HT$_{1B}$ (~47 kDa), 5-HT$_{2A}$ (~53kDa) and 5-HT$_3$ (~48kDa) receptors. We did not detect 5-HT$_T$ using this method. Experiments were performed in triplicate.

Discussion

In the present study we explore the presence of some serotonergic system components in stallion sperm; our results have demonstrated the presence of 5-HT itself, 5-HT receptors (5-HT$_{1B}$, 5-HT$_{2A}$ and 5-HT$_3$), both MAO_A and TPH1 enzymes, vesicular monoamine transporter (V-MAT$_1$) and confirmed the presence of 5-HT transporter (5-HTT).

All three 5-HT receptors evaluated were found in the stallion sperm but their pattern of distribution was quite different between them; 5-HT$_{1B}$ receptor was found equally distributed in the whole sperm; 5-HT$_{2A}$ was found intensely stained in the midpiece and in some regions of the sperm head but with a discrete staining along the flagella, and 5-HT$_3$ was intensely stained in both midpiece and flagella and showed a discrete staining in the sperm head. This differential distribution for 5-HT receptors suggest that they have different roles in the stallion sperm as has been suggested for human sperm (Jiménez-Trejo et al., 2012); both 5-HT$_{1B}$ and 5-
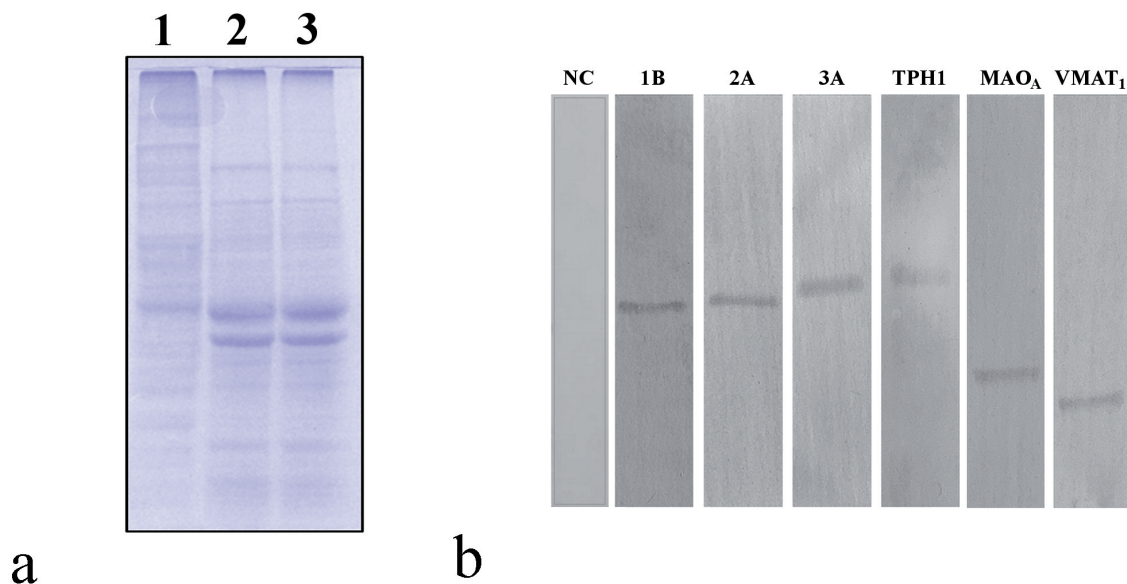

a

b

Fig. 3. Immunodetection of serotoninergic system components in stallion sperm. **a.** Control gel showing the presence of equine sperm protein (a2 and a3), adult rat brainstem homogenates were used as control (a1). **b.** positive bands of 5-HT1B (~47kDa, 1B), 5-HT2A (~53kDa, 2A), and 5-HT3 (~48kDa, 3A) receptors; TPH1 (~51kDa) and MAOA (~61kDa) enzymes, and V-MAT1 transporter (~65kDa) from stallion sperm homogenates. A representative negative control (NC) incubated with pre-immune serum is shown.

HT_{2A} are metabotropic receptors which could induce second messenger cascades (Gaspar et al., 2003; Adayev et al., 2005), while $5\text{-}HT_3$ is a ionotropic receptor which could induce fast ionic responses to 5-HT (Gaspar et al., 2003; Jiménez-Trejo et al., 2012). Interestingly, we found $5\text{-}HT_3$ intensely stained in midpiece, the region where mitochondria are highly concentrated and where sperm motility is generated. The presence of these receptors suggests that 5-HT could promote sperm hyperactivation and participate in the acrosomal reaction (Meizel and Turner, 1983; Meizel, 2004; Fujinoki, 2011; Jiménez-Trejo et al., 2012), but more functional studies are necessary to demonstrate these statements.

We also found the expression of both MAO_A and TPH1 enzymes and 5-HT in the stallion sperm, in a similar way to what we had previously found in human sperm. Because 5-HT receptors, $5\text{-}HT_T$ and MAO_A are all present in the stallion sperm we could suppose that the role of 5-HT in humans and horses is similar; one exciting idea we have is that serotoninergic system acts like a sensing mechanism for the sperm on its way to oocyte along the female reproductive tract, promoting the hypermotility of sperm through it (see below).

TPH, the rate-limiting enzyme in 5-HT synthesis is encoded by two different genes (Walther and Bader, 2003); TPH1, expressed predominantly in peripheral nervous tissues and TPH2, isoform expressed exclusively in CNS (Walther and Bader, 2003; Jiménez-Trejo et al., 2013). Concomitant with our findings in human sperm, TPH1 enzyme was detected in stallion sperm, which suggests that 5-HT could be directly synthesized in this species too. The effects of several neurotransmitters in sperm has been reported previously (for review see Ramírez-Reveco et al., 2017) but to our knowledge, 5-HT and GABA are the only synthetized directly in sperm (Persson et al., 1990; Jiménez-Trejo et al., 2012; Jan et al., 2017). Because of the apparently tight regulation of 5-HT in the non-capacitated sperm, we postulate that TPH1 is activated only in the female reproductive tract when the sperm requires a high 5-HT concentration outside to be able to continuously activate $5\text{-}HT_3$ receptors located in the midpiece, creating a positive feedback loop to achieve and sustain hypermotility. Future studies would have to be done to evaluate the functional role of TPH1 in this process.

In neural cells, MAOA participates in the degradation of 5-HT, epinephrine (E), norepinephrine (NE) and dopamine (DA) (Gaweska and Fitzpatrick, 2011), and we found this enzyme located in the post-acrosomal zone of the sperm head. Besides our characterization for the serotoninergic components, the presence of 5-HT, E/NE and DA transporters has been previously reported for stallion sperm (Urra et al., 2014). Interestingly, a biphasic, dose-dependent response for E/NE (Way and Killian, 2002), melatonin (Casao et al., 2010) and DA (Urra et al., 2014) in mammalian sperm has been reported and it has been postulated that the monoamine transporters are involved in such mechanisms (Ramírez-Reveco et al., 2017); although it

has not been tested for 5-HT, it is a possibility that a similar response would be obtained. From our results, it is tempting for us to postulate that MAO_A expressed directly in the sperm could terminate the actions that those different neurotransmitters exert on it.

While $5HT_T$ is located in the external membrane of the cell and intrudes 5-HT to the cytoplasm, $VMAT_1$ transports cytosolic monoamines such as 5-HT, DA, E/NE to the interior of synaptic vesicles or large dense core granules which could serve as a transitory storage location ready to be released to the extracellular space (Wimalasena, 2011; Lawal and Krantz, 2013).The presence of both transporters $5\text{-}HT_T$ and $VMAT_1$ we found along the presence of both NE/E and D transporters in stallion sperm previously reported strengthen the idea of a tight regulation of monoamine concentration in the external medium; this is the first time $VMAT_1$ has been reported in a non-neural cell and we think that is a possibility that in sperm $V\text{-}MAT_1$ could promote the packaging of monoamines in vesicles that could be released in the female reproductive tract to promote the fertilization process (Tinajero et al., 1993; Jiménez-Trejo et al., 2007, 2012; Bakst and Akuffo, 2008; Fujinoki, 2011; Ramírez-Reveco et al., 2017).

While this study was based only on the description of serotonin components in stallion sperm, the results obtained by our research group in previous studies may lead us to suggest that in mammalian sperm we have a functional serotonergic system that could participate in the acquisition of sperm hypermotility during its transit across the female reproductive tract; if this is true we think this pathway could be pharmacologically manipulated for enhancing or inhibiting sperm physiological function in fertilization procedures in mammals. Further functional experiments will be realized to try to demonstrate these ideas.

Finally, we think our results and others are slowly changing the idea that sperm is a limited, determined cell; now we know that sperm could synthesize several bioactive molecules (i.e. 5-HT) and have special protein synthesis mechanisms, such as reported recently in which a protein—not mRNA—determines tRNA recruitment and the tagging of nascent chains with carboxy-terminal Ala and Thr extensions ("CAT tails"), like both Rqc2p protein and 60S ribosomal subunits do on nascent chains of protein in mammal sperm (Shen et al., 2015). With no doubt, sperm physiology will continue to be an interesting research field for the years to come.

In conclusion, our results provide 1) further evidence of the existence of a serotonergic system in the reproductive system of mammals; 2) new knowledge and tools into equine reproduction that could lead to a better understanding of the stallion sperm to develop a study model and 3) 5-HT could be pharmacologically manipulated to achieve better, promising results in assisted reproduction on this animal species such as sperm freeze to cryopreservation and artificial insemination.

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