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Expression of the aromatase cytochrome p450 enzyme, α and β estrogen receptors, and androgens in cells of the spermatogenic lineage, Leydig and Sertoli of Hydrochoerus hydrochaeris (Linnaeus, 1766)

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Abstract: Capybaras (*Hydrochoerus hydrochaeris*) are the biggest rodents in the world and are of great importance in conservation. The species can be a rodent model in reproduction and have a massive role in the food chain in South America. Molecular studies of the reproduction of this animal are essential for all animal class conservation, mainly rodents. In this way, hormone signaling can help understand reproduction's external and internal mechanisms. In this work, estrogen receptors α and β, aromatase cytochrome P450 enzyme, and androgens were detected by immunohistochemistry on Leydig cells, Sertoli cells, myoid cells, and seminiferous epithelium *H. hydrochaeris* testis. Nine animals were sacrificed and collected the testicles, and for each staining (hematoxylin and eosin, Mallory, and immunohistochemistry for four antibodies) it was made five slides per animal; after that, a qualitative analysis was performed. The presence of estrogen receptors α and β in germinal epithelial cells, with more robust expression in spermatogonia and spermatocytes, may indicate the action of these hormones in the initial process of sperm cell development, needing more studies to investigate this first evidence. In other animals is believed that the aromatase cytochrome P450 enzyme can promote the transformation of androgens into estrogen, and in *H. hydrochaeris* is observed in those cells and androgen receptors. Sertoli cells exhibited moderate immunostaining for androgen and estrogen receptors α and β in the cytoplasm, with poor labeling for the aromatase cytochrome P450 enzyme in the nucleus in *H. hydrochaeris*. It is suggested that Sertoli cells may be responsible for producing estrogen in adult animals.
Keywords: Capybara; Reproduction; Spermatogenesis; Androgen; Estrogen.

1. Introduction

The capybara (*Hydrochoerus hydrochaeris*) is the largest rodent mammal in the world and has an important ecological influence in some Brazilian and South American biomes, such as Cerrado and the Amazon Forest (Alvarez, 2011). Because of their great adaptability and prolificacy, capybaras have currently been used in commercial breeding as an alternative for meat production in different biomes; besides, little is known about their reproductive physiology, with scarce data for implementing biotechniques in captivity. This data could help in understanding its reproduction in the wild (Nogueira and Nogueira, 2000).

Spermatogenesis is fundamental for the perpetuation of mammals. Its molecular events are complex. Therefore, the cells and regulatory mechanisms must be characterized to understand all events. Estrogen is one of the hormones that is involved in the processes of proliferation, apoptosis, maturation, and survival of cells in the germinal epithelium, and the regulation of these mechanisms encompasses membrane receptors, genes, receptors (ERα, β, γ), and their isoforms (Carreau et al., 2011; Hess at al. 2021; Dewael et al., 2022). It is converted from androgen hormones into estrogen by the enzyme cytochrome P450, a biological protein belonging to the group of active aromatases, being present in several species of mammals (Simpson et al., 1994).

Androgen hormones can regulate the microenvironment of the seminiferous epithelium, influencing a broad spectrum of genetic alterations in Sertoli cells in mice, and the loss of androgen receptors (AR) may affect, specifically in these cells. The Sertoli cells can lose the support function for germ cell movement and intercellular junctions, impairing the functional integrity of the blood-testis barrier without the AR. Also, the AR is important in the production and/or secretion of cell-specific proteases, transport proteins, and paracrine factors, which lead to their functions in developing germ cells (Wang et al., 2006). Furthermore, the lack of these receptors considerably affects Sertoli cell meiosis (Cooke and Walker, 2021). On rats that have the action of androgen hormones blocked, a 70% reduction in the area occupied by Leydig cells was observed. Consequently, lower concentration of testosterone levels, reduction of seminiferous tubules, and despite all seminiferous tubule cells being present. Deformation is observed in the elongated spermatids, showing the importance of this hormone in the formation of the sperm cell (Kaminska et al., 2020).

In capybaras, many receptors for cytochrome P450 were identified in Leydig cells in the testis through immunohistochemistry (Miranda, 2003). Histologically, capybara testicles, when compared to other mammals, have very particular volumetric proportions of seminiferous tubules and Leydig cells. A low proportion of seminiferous tubules and a considerably high proportion of Leydig cells are observed. The proportion of Leydig cells per gram of testis is three times higher than the maximum range already described for other species as Cutia (*Dasyprocta leporina*), Paca (Cuniculus paca), which present this proportion in 16,2 +/- 5,25; 35,8+/- 2,8 respectively while this index in capybara is 126,44 +/- 28,93 (Carreta, 2012). The intertubular space comprises about 47.9% of the testicular parenchyma volume, 32.95% comprising Leydig cells, and 11.4% of tunica albuginea and mediastinum (Paula et al., 2006).

A few data about capybaras reproduction, especially in molecular aspects, are available; considering this animal's importance in wildlife and rodents' conservation, the need to further the studies is unquestionable. These would involve spermatogenesis and

hormonal dynamics to facilitate the implementation of biotechniques and improve their production in captivity, in addition to understanding the reproduction rate. This provides a basis for reproductive studies to conserve this species and all other related species, such as many endangered species of rodents. The present study aimed to characterize the expression of cytochrome P450 enzyme receptors, estrogen receptors α and β (ERα and ERβ), as well as androgen receptors in Leydig and Sertoli cells in seminiferous tubular cells.

2. Materials and Methods

2.1. Paraffin blocks

The experiment was carried out with paraffinized blocks from the University archive of adult capybaras (*H. hydrochaeris*) testis, kept in commercial breeding facilities in the state of São Paulo, and slaughtered in a slaughterhouse located in Iguape, State of Sao Paulo, accredited by the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA), and the Brazilian Federal Inspection System of the Ministry of Agriculture, Livestock and Food Supply (SIF/Mapa). At the time, testicles from nine animals were collected and fixed in Bouin, with subsequent inclusion in paraffin, and kept on file at the Laboratory of Wild Animals of the Preventive Veterinary Medicine sector of the School of Veterinary and Animal Science of the Federal University of Goiás. Cuts were used after thinning the blocks to make histological and immunohistochemical slides. These were stained with the HE solution to ensure the integrity of the tissue fragment to be used for the identification of estrogen receptors a and b, cytochrome P450, and androgens by immunohistochemistry, and it was made five slides per animal for each staining.

2.2. Histological processing of testis

Sections measuring five µm thick of paraffinized blocks of testis adhered to the histological slides, deparaffinized in an oven at 56 ºC in xylene and alcohol, and then hydrated. The slides were immersed in distilled water for 8 minutes and stained with hematoxylin (Sigma-Aldrich, HHS16, Saint Louis, USA) and subsequently stained with eosin (Sigma-Aldrich, E4009, Saint Louis, USA) for 40 seconds. The same sections were performed for Mallory staining. After deparaffinization and hydration, the slides were stained with acid fuchsin solution A (Sigma-Aldrich, F8129, Saint Louis, USA) for two minutes and passed directly to solution B (Aniline Blue - Sigma-Aldrich, 415049) Orange G and phosphotungstic acid.

2.3. 2.3 Immunohistochemistry

Sections measuring five µm thick were adhered to positive slides (Starfrost Sakura-Knittel), deparaffinized in an oven at 56 ºC for 30 minutes and in xylene (two steps of 15 minutes each), hydrated in three baths in absolute alcohol (5 minutes each), a 90% alcohol bath (5 minutes) and an 80% alcohol bath (5 minutes). The sample was transferred to a vat with SDS, where it remained for 5 minutes until it was washed with PBS and transferred to another vat, also containing PBS, where it remained for 5 minutes.

Endogenous peroxidase activity was blocked with the addition of hydrogen peroxide (30 mL) diluted in methyl alcohol (70 mL) and incubated in a humid chamber for 30 minutes (in three 10-minute steps). After this period, the slides were bathed for 5 minutes on PBS. For the antigenic recovery of estrogen receptors α and estrogen β, citrate solution added with Tween 0.05%, pH 6.0, heated in a microwave for 20 minutes was used; for cytochrome P450 aromatase enzyme and androgens, antigen retrieval was performed in a water bath at 96 ºC for 20 minutes, followed by washing in PBS for 5 minutes. Nonspecific labeling was blocked with 3% BSA in a humid chamber at room temperature for one hour and then again washed with PBS.

Primary rabbit polyclonal antibodies, anti-androgen (1:1000, AB74272, Abcam, Cambridge-UK), anti-cytochrome P450 (1:1000, AB28146, Abcam, Cambridge-UK), anti-estrogen receptor α (1: 1000, AB75635, Abcam, Cambridge-UK) and the anti-estrogen receptor β monoclonal antibody (1:1000, AB133467, Abcam, Cambridge-UK), were diluted in 1.5% BSA, instilled on the fragment and incubated in a chamber wet, at 4°C, overnight. After washing with PBS, biotinylated anti-rabbit secondary antibody (1:2000, ZyMAX, Rockford, IL, USA) was added and incubated at room temperature for one hour, washed with PBS, and added with the ABC Vectastain Elite kit (PK-6200, Burlingame, CA-USA), for 30 minutes, and washed again with PBS. Diaminobenzidine peroxidase was added to the antibody-labeled fragments, and after washing with PBS (5 minutes), the slide was counterstained with methyl green and hematoxylin. Slides were mounted with Entellan (Merck, 1079600500, Billerica, MA-USA) and histological coverslips.

2.4. Evaluations of immunohistochemical reactions

Ten photomicrographs per animal were taken in random fields, at magnifications of 50x and 100x, in the DM 4500 Leica microscope installed in the Animal Pathology sector of the School of Veterinary and Animal Science of the Federal University of Goiás. These were evaluated for the morphology of Leydig and Sertoli cells and cellular structures with immunostaining for the tested antibodies (cell membrane, cytoplasm, and nucleus). The staining intensity scores were as follows: negative (-), weak staining (+), moderate staining (++), and Strong staining (+++).

2.5. Statistical analysis

The results were submitted to descriptive statistical analysis.

3. Results

3.1. Seminiferous epithelium

The seminiferous tubules in the capybara presented seminiferous epithelium surrounded by a basal lamina, connective tissue sheath, and, more internally, myoid cells. The seminiferous epithelium, composed of cells of the spermatogenic lineage arranged in 4 to 8 layers and Sertoli cells was intact and with histological characteristics of normal tissue (Carreta, 2012) (Junqueira and Carneiro 2008) (Paula et al., 2006) (Figure 1).

Testicle fragments stained with HE and Mallory solutions were viable for immunohistochemistry with the preservation of the original histological characteristics, such as lobes filled with seminiferous tubules and surrounded by loose connective tissue, with blood vessels and Leydig cells (Figure 1A and 1B) (Junqueira and Carneiro, 2008). A marked presence of Leydig cells was noted, occupying about 1/3 of the testicle. This agrees with the literature (Carreta, 2012; Paula et al., 2006).

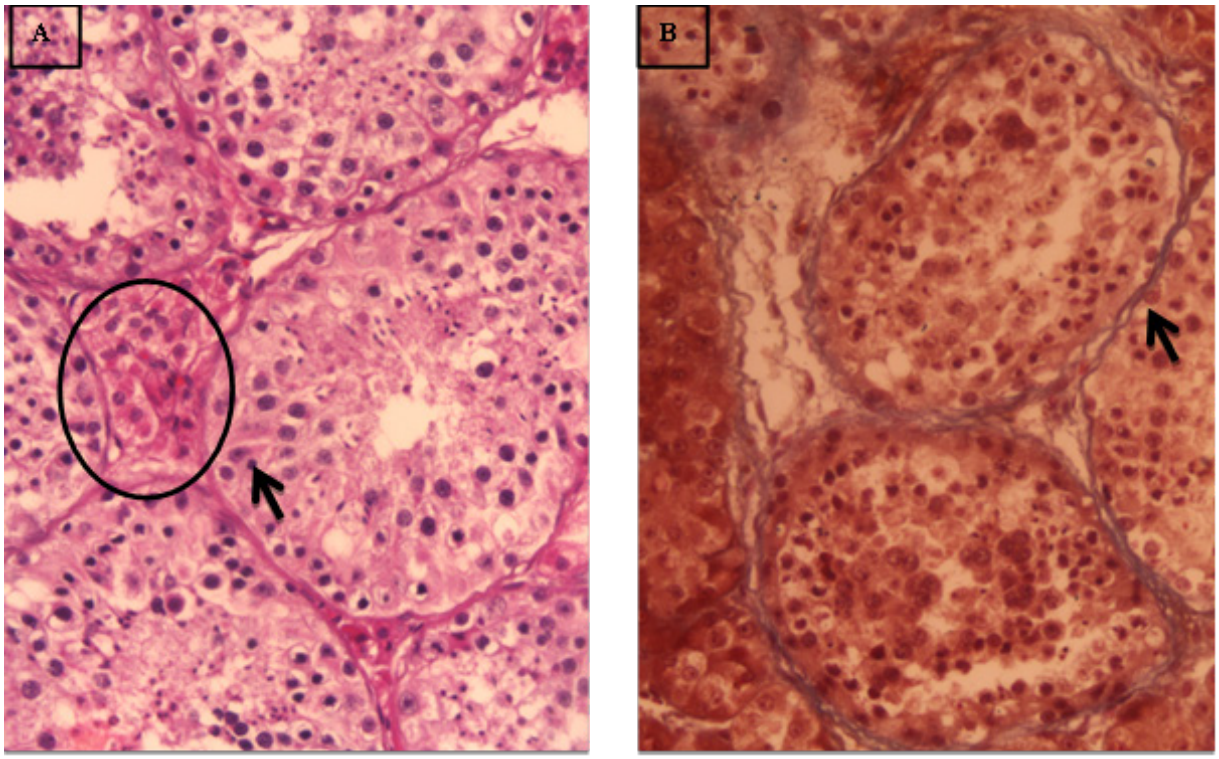

Figure 1 – A – Intact Sertoli cells (black arrow) and intertubular space full of Leydig cells (black circle) in capybara testis (*H. hydrochaeris*). Hematoxylin and eosin. 40x increase. B – Intact loose connective tissue surrounding the seminiferous tubules (black arrow) in a capybara testicle (*H. hydrochaeris*). Mallory. 400x magnification.

Immunolocalization of the aromatase cytochrome p_{450} enzyme, α, and β estrogen receptors, and androgens in cells of the spermatogenic lineage, Leydig and Sertoli of *H. hydrochaeris* (Linnaeus, 1766)

Immunostaining for cytochrome P450 aromatase enzyme receptors, estrogen receptors α and β, and androgen receptors varied in terms of cell location (membrane, cytoplasm, or nucleus) and intensity, as shown in Tables 1 and 2.

	SERTOLI CELLS			LEYDIG CELLS		
	Membrane	Cytoplasm	Nucleus	Membrane	Cytoplasm	Nucleus
Eα	++	++	-	+	+	-
E β	-	++	-	-	-	++
P_{450}	-	-	+	+++	+++	-
Androgens	-	++	++	-	+++	+++

Table 1 – Location and intensity of immunostaining of receptors for estrogen α (Eα), estrogen β (Eβ), androgens (AR), and cytochrome P450 aromatase enzyme (P450) in Leydig cells and Sertoli cells from capybara testis (*H. hydrochaeris*).

	SEMINIFEROUS EPITHELIUM									Myoids Cells		
	Spermatogonia			Spermatocyte			Spermatid					
	M	C	N	M	C	N	M	C	N	M	C	N
Eα	+++	++	-	+++	++	-	-	+	-	-	-	-
E β	-	-	-	-	+	-	-	+	-	-	-	-
P_{450}	+++	+++	-	++	-	-	-	++	-	-	+	-
Androgens	++	++	-	+++	+	+++	+++	+	-	-	++	-

Table 2 – Location and intensity of immunostaining of receptors for estrogen α (Eα), estrogen β (Eβ), androgens (AR), and cytochrome P450 aromatase enzyme (P450) in myoid cells and seminiferous epithelium cells (spermatogonia, spermatocytes, and spermatids) in testicles of capybara (*H. hydrochaeris*). M – membrane; C – cytoplasm; N – nucleus.

Figure 2A shows that the immunostaining for androgen receptors can be observed, which appear expressively within the seminiferous tubules and Leydig cells. For α estrogen receptors, more evident marking was observed in spermatogonia and spermatocytes (Figure 2B). Marking for β estrogen receptors was more discreet and more evident in Leydig cells and Sertoli cells (Figure 3A). For P450, the aromatase enzyme antibody was more evident in Leydig cells but less evident in seminiferous tubules (Figure 3B).

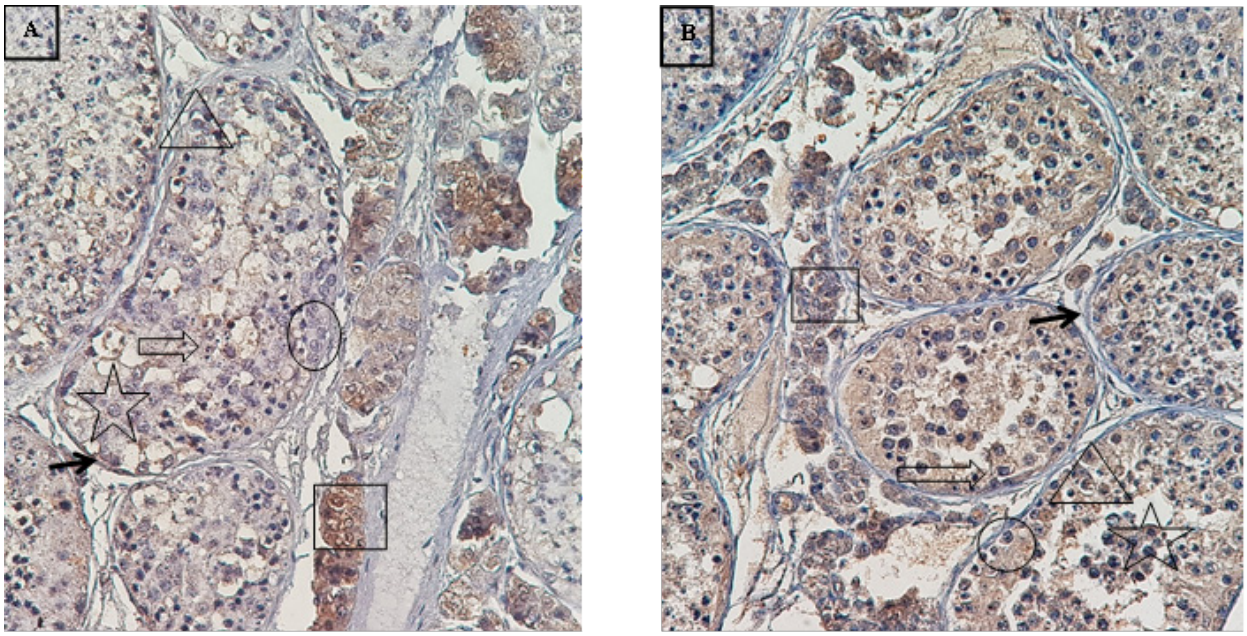

Figure 2 – A – Immunohistochemistry of capybara testis (*H. hydrochaeris*) for androgen receptor antibodies. Square: Leydig cell (Cytoplasm +++, nucleus +++), triangle: Sertoli cell (Cytoplasm ++, nucleus ++), arrow: Myoid cell (Cytoplasm +), circle: Spermatogonia (Membrane ++, cytoplasm ++), star: Spermatocyte (membrane +++, cytoplasm +, nucleus +++), empty arrow: Spermatid (Membrane +++, cytoplasm +). Hematoxylin. Magnification 400x. FIGURE B – Immunohistochemistry of capybara testis (*H. hydrochaeris*) for estrogen receptor α antibody. Square: Leydig cells (Membrane +, cytoplasm +), triangle: Sertoli cell (Membrane ++, cytoplasm ++), arrow: Myoid cell (no marking), arrow: Spermatogonia (Membrane +++, cytoplasm ++), star: Spermatocyte (Membrane +++, Cytoplasm ++), empty arrow: Spermatid (Cytoplasm +). Hematoxylin. 400x magnification.

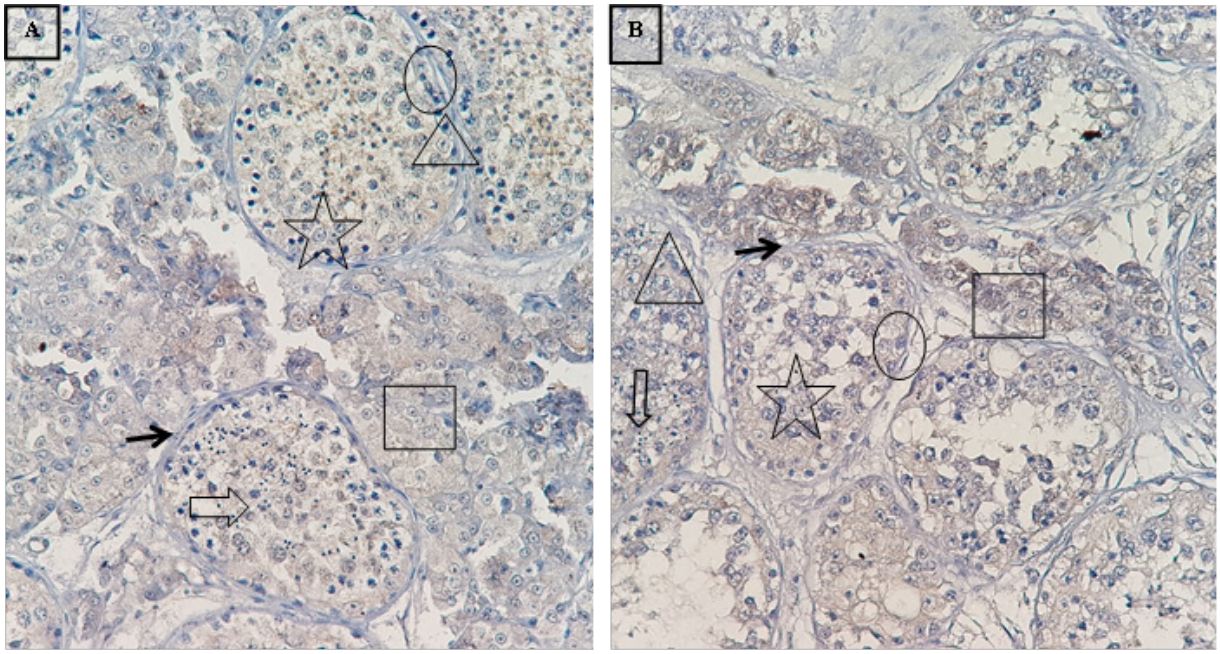

Figure 3 – A – Immunohistochemistry of capybara testis (*H. hydrochaeris*) for estrogen receptor antibody β. Square: Leydig cell (Nucleus ++), triangle: Sertoli cell (Cytoplasm ++), arrow: Myoid cell (No marking), circle: Spermatogonia (No marking), star: Spermatocyte (Cytoplasm +), arrow empty: Spermatid (Cytoplasm +). Hematoxylin. Increase 400x. B – Immunohistochemistry of capybara testis (*H. hydrochaeris*) for the P450 aromatase enzyme antibody. Square: Leydig Cells (Membrane +++, Cytoplasm +++), Triangle: Sertoli Cell (Nucleus +), Arrow: Myoid Cell (Cytoplasm +), Circle: Spermatogonia (Membrane +++, Cytoplasm +++), star: Spermatocyte (Membrane ++), empty arrow: Spermatid (Cytoplasm +). Hematoxylin. 400x magnification.

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4. Discussion

This is the first study describing the expression of androgen and estrogen receptors and aromatase in *H. hydrochaeris* testis, which provides knowledge about the cells responsive to these critical factors related to spermatogenesis regulation. This work characterized Sertoli cells, Leydig cells, and seminiferous tubule cells in capybara testis for the presence and intensity of staining for estrogen receptors α and β, cytochrome P450 aromatase enzyme, and androgens. For the first time, the immunostaining of α and β estrogen receptors and androgens in capybaras were noted, although with different intensities, while there are already reports of the enzyme aromatase cytochrome P450 in capybara tests, but without the description of its immunostaining.

In capybaras, marking for α estrogen receptors was observed in all cells of the seminiferous epithelium except for myoid cells. As in other animals, this location for estrogen receptors α may suggest that in capybaras, it is also involved in the complete maturation of spermatozoa and formation of the acrosomal (Carreau et al., 2011; Gould et al., 2007; Delves, 2004; Berensztein, 2006). Moderate immunostaining for α estrogen receptors in the cytoplasm and cell membrane of capybara Sertoli cells is similar to that observed in mice (Lin et al., 2014). In post-pubertal horses, however, marking occurred only in the nucleus (Peal et al., 2011), and in pigs, it was not observed (Rago et al., 2004). In Leydig cells from capybaras, weak staining was noted both in the cell membrane and the cytoplasm for the estrogen receptor α, while in mice, this staining was strong in the nucleus, suggesting a different role in these cells (Zhou et al., 2002). In pigs, these cells show moderate immunoreactivity for estrogen receptor α (Rago et al., 2004), and in horses, estrogen receptor α appears in all Leydig cells (Pearl et al., 2011).

Regarding germ cells, capybaras are also similar to horses that show active ERα receptors in all germ cells (Peal et al., 2011). Pigs also have α estrogen receptor staining in spermatogonia and intense staining in spermatocytes (Rago et al., 2004). While mice were positive for this receptor only in Leydig and myoepithelial cells. (Sugita, 2021)

The expression of β estrogen receptors was noted with moderate intensity in the cytoplasm of capybara Sertoli cells, unlike what was found in mice (Levallet et ala, 1998) and post-pubertal horses (Pearl et al., 2011) in which the estrogen receptor β appears well marked in the nucleus of these cells. In pigs, β estrogen markings are not observed in Sertoli cells (Rago et al., 2004). Estrogen receptors (Erα and Erβ) are linked to the control of proliferation and apoptosis of Sertoli cells, so the investigation of their immunolocalization is the first step toward a better understanding of the reproductive physiology of capybaras (Lucas et al., 2008) (Lucas et al., 2011). In capybara, Leydig cells, moderate marking of β estrogen receptors was noted in the nuclear region. However, the same has been described in mice (Zhou et al., 2002) (Pelletier et al., 2000) and swine (Rago et al., 2004). These receptors were not observed in mouse Leydig cells. In horses, the estrogen receptor β is concentrated in the cytoplasm (Pearl et al., 2011). In rabbits, however, marking was observed only in round spermatids for estrogen receptors α and β (Dewaele et al., 2022).

The staining of β estrogen receptors in adult capybaras was weak in the cytoplasm of spermatocytes and spermatids and negative in spermatogonia and myoid cells. In pigs, there is intense staining in spermatogonia and spermatocytes, while spermatids are negative (Rago et al., 2004). In dogs, cats (Nie et al., 2002), and horses (Pearl et al., 2011), markings for α and β estrogen receptors are not observed in the seminiferous epithelium, contrary to capybaras. In dogs, positive but weak signals were noticed in germinative, Leydig, and Sertoli cells of healthy animals (Galuszka et al., 2021). Concerning these two receptors, capybaras are more similar to wild boars that present markings in all their cells except elongated spermatids (Mumtembei et al., 1995).

The presence of cytochrome P450 aromatase enzyme in Sertoli cells has been noted in mice (Van Pelt et al., 1999). Its presence is also noted in capybaras but only in the nucleus of these cells. In rats, it is already known that the Sertoli cell is a source of estrogen during the fetal phase (Carreau et al., 2011), and the presence of this enzyme in the Sertoli cells of adult capybaras may suggest a continuous production of this hormone. Intense immunostaining for cytochrome P450 aromatase was observed in the cytoplasm and membrane of capybara Leydig cells and horses (Sipahutar et al., 2003). Humans and Anubis baboons (Papio annubis) add italics also had markings throughout the Leydig cell for this enzyme (Berensztein et al., 2006) (Bonagura et al., 2011). In addition, in rabbits, inside seminiferous tubules, in germ cells with large nuclei and spermatocytes in pachytene, animals that knockout for the gene responsible for its production were noted, showing lower sperm motility in addition to malformation in these cells, evidencing the importance of aromatase in reproduction maintenance. (Dewaele et al. 2022).

In capybara, the enzyme aromatase P450 was observed in solid intensity in the cytoplasm and membrane of spermatogonia, while in spermatocytes and spermatids, there was moderate intensity only in cytoplasm and membranes, respectively. In myoid cells, weak staining was noted in the cytoplasm. In other animals, this enzyme has proven indispensable for spermatogenesis. In rats, for example, there is a decrease in spermatogenesis when the cytochrome P450 enzyme is inhibited, which can be observed in the decrease in testicular diameters, primary spermatocytes, and spermatids (Soto, 2012). In capybaras, the presence of the cytochrome P450 enzyme in testicular cells has already been evidenced (Miranda, 2003) without distinguishing the cellular location or intensity of immunostaining.

In adult mice, the cytochrome P450 aromatase enzyme can be observed in testicular germ cells, from spermatocytes in the pachytene phase to elongated spermatids. The cells of the spermatogenic lineage are also responsible for converting testosterone into estrogen (Nittat et al., 1993). In D. ecaudata, the expression of aromatase P450 was noted only in Sertoli cells (Silva et al., 2019).

In rats, androgens facilitate endocytosis, the recycling of proteins from the cell membrane surface to the cytosol, as well as their recycling from the cytosol back to the cell surface evolution of spermatogenesis (Yan et al., 2008) (França et al., 2016). It is also known that androgens influence the sustaining capacity of these cells through androgen receptors. In addition, in animals lacking

androgen receptors in Sertoli cells, a blockade of spermatogenesis is observed, compromising the immune privilege of the adluminal compartment due to problems in the permeability of the blood testicular barrier (Smith et al., 2015).

In Leydig cells from capybaras, intense staining for androgen receptors was observed in the cytoplasm and nucleus. In rats and horses, the staining was only nuclear (Pelletier et al., 2000) (Meseeha et al., 2016) (Van Pelt et al., 1999), while in mice, immunostaining occurs moderately in the cytoplasm and firmly in the nucleus (Zhou et al., 2002) (Sugita et al., 2021). Moreover, it was noted in D. ecaudata that the expression of androgen receptors is more often in Sertoli cells than in Leydig cells (Silva et al., 2019).

In capybaras, immunostaining for androgen receptors was observed in spermatogonia, spermatocytes, spermatids, and myoid cells, unlike horses (Pearl et al., 2011) (Bilinska et al., 2003), rats (Bremmer et al., 1994) (Madhabananda et al., 1990) and humans (Madhabananda et al., 1990), where these receptors are not found. In mice, immunostaining is found only in spermatogonia (Zhou et al., 1996). in rats with the blockage of the cascade of androgen signaling, the diameter of tubules was reduced by 10% despite all the cells being present (Kamińska et al., 2020). This absence of androgen hormone marking in the seminiferous tubules of so many animals confirms the hypothesis that androgens act indirectly on the cells of the germinal epithelium, but in the capybara, accentuated marking was observed in these cells (Walker, 2011). As in pigs, marking for these receptors can also be observed at a reduced frequency and intensity compared to capybaras (Srisuwatanasagul et al., 2018). The hormonal control of spermatogenesis varies between species (Schalatta and Ehmcke, 2014). However, studies have shown that there is no functionality for androgen receptors in cells of the spermatogenic lineage (Smith and Walker, 2015); this work showed very marked markings in these cells. Thus, further studies are needed to evaluate the function of this species.

In capybaras, it is known that Leydig cells, the leading producers of androgens, occupy more than 30% of the testicular parenchyma, leading to a very high production of these hormones, which seems to be related to the need to ensure the hierarchical position within the herd (PAULA et al., 2006). Knowing that, in mice, even without marking in germ cells, testosterone deprivation causes damage to spermatogenesis, such as loss of spermatocytes and incomplete meiosis (O'Shaughnessy, 2014), it is assumed that capybaras require direct action of androgen hormones in the germinal tubule for the completion of spermatogenesis, unlike other animals.

5. Conclusion

Capybaras have shown α and β estrogen receptors, cytochrome P450 aromatase, and androgens in Sertoli cells. Leydig cells and seminiferous tubule cells differed in the intensity of this marking and location in the mentioned cells. It is believed that there may be some differences related to the physiological mechanisms of control of spermatogenesis and that these should be studied in greater detail.

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