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Interstitial Cells in the Pineal Gland of Pregnant and Nonpregnant Viscachas *(Lagostomus maximus maximus)*: A Morphometric and Biochemical Study

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Lagostomus · Pregnancy · Pineal gland · Interstitial cells · Immunohistochemistry

Abstract
The pineal gland of mammals undergoes morphological and biochemical changes throughout the gestation period. In viscachas, a seasonal breeding rodent, pregnancy lasts approximately 154 days and 3 stages can be defined, i.e., early, mid, and late pregnancy. The purpose of this study is to analyze morphometric variations in the expression of S-100 protein, glial fibrillary acidic protein (GFAP), and vimentin in the interstitial cells (IC) in pregnant and nonpregnant viscachas by immunohistochemistry (IHC). We also aim to evaluate a probable relation between glandular activity and pregnancy. The immunopositive percentage area (%IA) for the studied proteins and the number of immunoreactive cells against the S-100 protein with a visible nucleus (nº IC-S-100) were analyzed. Estradiol and progesterone serum levels were also determined by RIA. Variations in the expression of the S-100 protein and GFAP, as well as changes in the nº IC-S-100 related to serum hormone levels, were found between pregnant and nonpregnant viscachas. Viscachas in mid pregnancy exhibited the highest values of %IA for the analyzed proteins, followed by females in late and early pregnancy, while the nonpregnant ones showed the lowest values for all of the groups studied. Likewise, the nº IC-S-100 also varied following the same pattern. Thus, these variations seem to indicate a direct relationship between glandular activity and gonadal hormone levels. On these grounds, we may conclude that IC undergo changes in relation to ovarian hormone levels and participate in the regulation of glandular activity during pregnancy. However, further research is necessary to elucidate this relationship.

Introduction

In the pineal gland of mammals, 3 main cell types have been described, i.e., pinealocytes or principal cells, supportive or interstitial cells (IC), and pigmented cells [Vollrath, 1981; Calvo et al., 1992]. To date, IC have re-

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ceived different names, such as type II pinealocytes, supportive cells, astrocytes, and glial cells. They have also been studied in different species, like the rat [Møller et al., 1978; Schachner et al., 1984; López-Muñoz et al., 1992; Borregón et al., 1993; Suzuki and Kachi, 1995], the mouse [Schachner et al., 1984], the golden hamster [Huang et al., 1984], the dog [Boya and Calvo, 1993], the cat [Boya and Calvo, 1993; Boya et al., 1995], the sheep [Franco et al., 1997; Redondo et al., 2001, 2003; Regodón et al., 2001], the monkey [Girod and Durand, 1985], and the donkey [Safwat, 2012]. These cells provide support and nutrition, phagocytosis, secretion of several cellular mediators, and formation of an intercellular communication network. However, analyses of the role of IC in different physiological processes still needs further development.

IC have been studied using different glial markers. The S-100 protein is the most commonly used marker protein for the identification of glial cells in the central nervous system, especially in astrocytes, neural progenitor cells, maturing oligodendrocytes, and some types of neurons. In other cell types, such as pituitary folliculostellate cells, Langerhans cells, melanocytes, and chondrocytes, expression of the S-100 protein has also been reported [Haimoto et al., 1987; Böni et al., 1997; Rambotti et al., 1999; Acosta et al., 2010; Acosta and Mohamed, 2013]. S-100 protein is exclusively expressed in vertebrates and performs several intracellular functions, as well as some extracellular ones. Within cells, it regulates the activity of different enzymes, the phosphorylation of specific proteins, calcium homeostasis, and the assembly-disassembly of several cytoskeleton proteins and multiple transcription factors, thus participating in proliferation, differentiation, and migration processes, energy metabolism, and apoptosis. Considering the extracellular level, the S-100 protein has an autocrine and/or paracrine function in cellular proliferation, differentiation, migration, inflammation, and tissue repair, and it also acts as a toxic and trophic agent [Santamaria-Kisiel et al., 2006; Donato et al., 2009, 2013].

Other cellular markers used for IC study are glial fibrillary acidic protein (GFAP) and vimentin. Both proteins belong to the intermediate filament protein family class III [Oshima, 2007]. GFAP is the main intermediate filament protein in mature astrocytes in the central nervous system [Eng, 1985; Eng et al., 2000], while vimentin is principally found in immature glial cells [Dahl et al., 1981] and mesenchymal cells [Lehtoen et al., 1985]. GFAP is involved in modulation of astrocyte motility and shape, providing structural stability to different astrocytic processes. Some reports indicate that GFAP participates in the modulation of some neuronal functions [McCall et al., 1996; Shibuki et al., 1996] and performs a role in maintenance of a normal central nervous system white matter architecture and blood-brain barrier integrity [Liedtke et al., 1996]. On the other hand, vimentin participates in a number of critical functions which involve attachment [Nieminen et al., 2006], migration [Gonzales et al., 2001; Nieminen et al., 2006], cell signaling [Lopez-Egido et al., 2002; Runembert et al., 2002; Ivaska et al., 2005], apoptosis and immune defense [Yang et al., 2005; Schietke et al., 2006], and regulation of genomic DNA [Tolstonog et al., 2000, 2001].

Studies in the pineal gland during pregnancy have been carried out in different species. Ultrastructural changes have been described in the pineal gland of rats [Satodate et al., 1980; Karasek et al., 1982], moles [Pevet and Smith, 1975], guinea pigs [Vollrath and Schmidt, 1969], and sows [Wyrzykowski et al., 1987]. Likewise, pineal activity has been related to pregnancy by biochemical studies performed in rats [Tigchelaar and Nalbandov, 1975] and humans [Pang et al., 1987]. It has been suggested that the pineal gland in rats may be involved in the synthesis and release of gonadotropins. Furthermore, this gland might also modify gonadal hormone levels during the last stage of gestation [Nir et al., 1979; Nir and Hirsschmann, 1980]. Most studies indicate an enhanced activity of the gland during the gestational period [Lew, 1987; Bishnupuri and Haldar, 2000a, b]. However, the relationship between the gland and pregnancy remains to be clarified.

Our experimental model, the viscacha (*Lagostomus maximus maximus*), is a hystricomorph rodent with seasonal reproductive patterns. This rodent lives in extensive burrows and exhibits a nocturnal behavior, emerging at dawn and dusk to feed [Redford and Eisenberg, 1992]. In its natural habitat, the adult male exhibits an annual reproductive cycle synchronized by the environmental photoperiod and modulated by the pineal gland and its main hormone, i.e., melatonin [Dominguez et al., 1987; Fuentes et al., 2003; Aguilera-Merlo et al., 2005; Filippa et al., 2005]. Due to restricted light exposition, the viscacha constitutes an interesting model for studying the pineal gland and the processes in which it is involved. In female viscachas, pregnancy lasts approximately 154 days [Weir, 1971] and 3 stages can be defined: early, mid, and late pregnancy. They usually have an estrous period in early autumn and a large number of pregnant animals can be found in winter, although pregnant females have also been found in others seasons [Jackson, 1989]. The gestation period ends in spring, after which 2 well-devel-

oped offspring are usually born [Weir, 1971]. In viscachas, variations in pineal melatonin levels and in their enzyme activity have been reported during pregnancy [Gil et al., 2005]. Moreover, these researchers also found changes in progesterone and androstenedione levels in the ovary and serum [Gil et al., 2007]. Regarding IC, in our previous work we carried out a seasonal and age-related study in male viscachas [Busolini et al., 2017], while in this investigation the IC of females is researched during pregnancy.

Considering the relevance of pineal gland function in the reproductive process of *Lagostomus*, the purpose of this study is to analyze morphometric variations in the expression of S-100 protein, GFAP, and vimentin in the IC of pregnant and nonpregnant viscachas by IHC. We also aim to evaluate a probable relation between glandular activity and pregnancy.

Materials and Methods

Experimental Design

Adult female viscachas, weighing 2–4 kg, were captured in their habitat near San Luis, Argentina (33°20' south latitude, 769 m altitude). In this research, 16 female viscachas (12 pregnant and 4 nonpregnant) were used. The age and reproductive condition of viscachas were carefully assessed on the bases of: (1) body weight [Llanos and Crespo, 1954; Branch et al., 1993] and (2) sexual maturity estimated by ovary histological analysis. Additionally, the uterine horns were examined to evaluate the presence of embryos and fetuses. The viscachas captured in summer (February) were nonpregnant. All females captured from early autumn to spring (April to September) were pregnant. According to the number and size of embryos or fetuses, pregnancy stages were classified as follows: early pregnancy (April), 2 or more 1- to 3-cm embryos; mid pregnancy (July), two 9- to 11-cm fetuses; or late pregnancy (September), 2 fetuses >19 cm. This classification was established according to previous reports in our laboratory [Gil et al., 2007; Filippa et al., 2010a, b].

After capture, the animals were immediately taken to the laboratory, anesthetized with a combination of ketamine (Ketamina 50; Holliday-Scott®, Buenos Aires, Argentina) and xylazine (PharmaVet® S.A., Santa Fe, Argentina) at a dose of 12 and 0.4 mg/kg, respectively. The blood was collected by cardiac puncture for evaluation of the serum hormone concentration. The viscachas were quickly sacrificed by intracardiac injection of euthanyle (0.25 mL/kg of body weight, sodium pentobarbital, and sodium diphenylhydantoin; Brouwer S.A., Buenos Aires, Argentina). The brain was rapidly exposed and the pineal gland was excised, fixed in Bouin's fluid, processed for light microscopy, embedded in paraffin, and then sectioned (3 μm). The hematoxylin-eosin stain was used. The sections were examined using an Olympus BX-40 light microscope.

The experimental design was approved by the local ethics committee and it was in agreement with the guidelines of the National Institutes of Health (NIH, USA) for the use of experimental animals. Moreover, the Biodiversity Control Area of the Environmental Ministry of San Luis (Argentina) approved a study protocol to carry out scientific research in the province (resolution No. 47-PBD-2015).

Immunohistochemistry

Tissue sections were stained using the streptavidin-biotin-peroxidase complex method at 20 °C. For the IC study, the following primary antisera were used: rabbit polyclonal anti-S-100 protein (AR058-5R; BioGenex, San Ramon, CA, USA), rabbit polyclonal anti-GFAP (AR020-5R; BioGenex), and mouse monoclonal anti-vimentin (Clone V9 AM074-5M; BioGenex). These antisera have previously been used with successful results in viscachas [Rodríguez et al., 2007; Acosta and Mohamed, 2013; Busolini et al., 2017]. The tissue sections were first deparaffinized with xylene and hydrated through decreasing concentrations of ethanol. Microwave pretreatment (antigen retrieval) was performed by microwaving the sections twice for 3 min each time at full power (800 W) in a sodium citrate buffer (0.01 M, pH 6.0). To inhibit endogenous peroxidase activity, sections were incubated for 20 min in a solution of 3% H_2O_2 in water, and rinsed with distilled water and phosphate-buffered saline (PBS; 0.01 M, pH 7.4). Nonspecific binding sites for immunoglobulins were blocked by 15 min of incubation with 0.25% casein in PBS, washed in PBS, and incubated with the primary antibody (12 h in a humidified chamber at 4 °C). The slides were subsequently washed 3 times for 10 min each in PBS. Immunohistochemical visualization was carried out using a Super Sensitive Ready-to-Use Immunostaining Kit (QD000-5LE; BioGenex) at 20 °C. Sections were incubated for 30 min with biotinylated anti-IgG, and after being washed 3 times for 5 min each in PBS, they were incubated for 30 min with horseradish peroxidase-conjugated streptavidin and finally washed in PBS. The reaction sites were revealed by 100 μL 3, 3'-diaminobenzidine tetrahydrochloride (DAB) chromogen solution in 2.5 mL PBS and 50 μL H_2O_2 substrate solution (catalog No. QD000-5LE; BioGenex), resulting in a brown precipitate. Sections were counterstained with Harris' hematoxylin for 10 s, washed for 2 min under running water, dehydrated in increasing concentrations of ethanol, cleared in xylene, and mounted with Entellan® (Merck, Darmstadt, Germany). Labeling was assessed using a light microscope (BX-40; Olympus Optical, Tokyo, Japan).

Positive controls for S-100 and GFAP immunohistochemical staining were carried out on rat cerebellum sections, and vimentin-positive controls were assayed on rat skin sections, as recommended by the supplier. To confirm the specificity of the immunoreactions, negative control procedures included replacing the primary antibody with rabbit or mouse negative control sera (both from BioGenex) and omitting the primary antibody. No positive structures or cells were found in these sections (Fig. 1).

Double IHC for S-100 and GFAP

The double IHC was performed with the objective of examining colocalization in the expression of S-100 and GFAP in the IC. The reaction sites of the first primary antibody (GFAP) were revealed by the DAB chromogen solution in PBS and the H_2O_2 substrate solution, resulting in a brown precipitate. For the second labeling, the slides were incubated with the second primary antibody (against S-100 protein). The reaction sites were revealed using a New Fuchsin Chromogen Kit (catalog No. HK 183-5K; Bio-

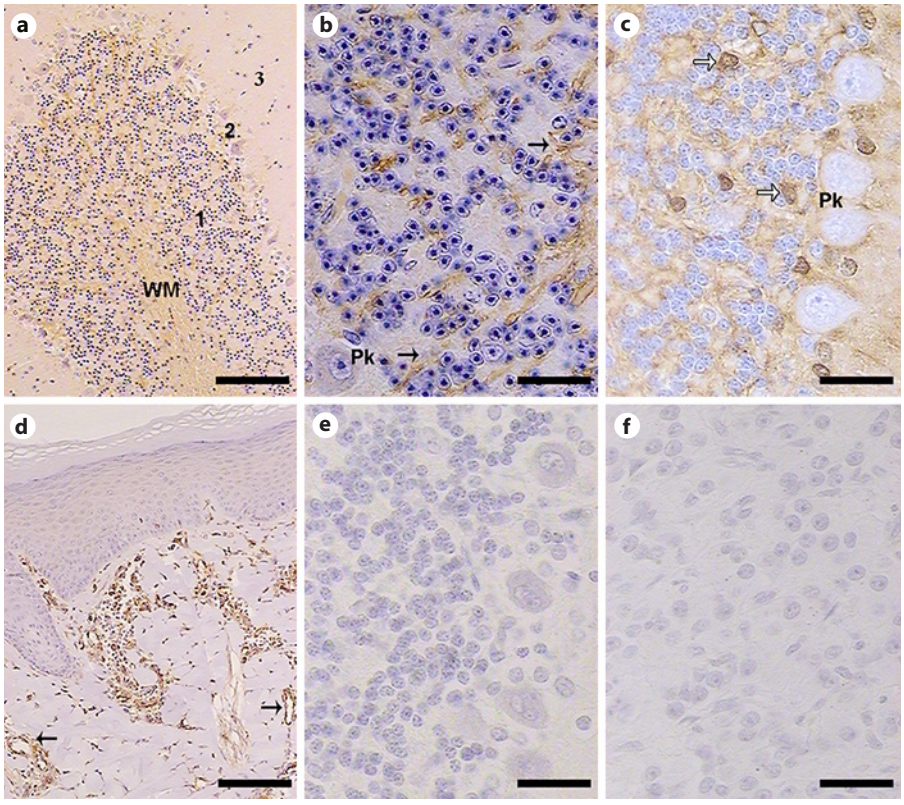

Fig. 1. IHC positive controls for GFAP (**a**, **b**), S-100 protein (**c**), vimentin (**d**) and negative controls (**e** and **f**). **a** The photomicrograph shows a rat cerebellum section immunostained with GFAP. White matter (WM) and layers of gray matter: glomerular cell layer (1), Purkinje cell monolayer (2), and molecular layer (3). **b** GFAP is observed in cytoplasmic processes (arrows) near Purkinje cells (Pk) and cells of the glomerular layer. **c** S-100 protein is expressed in the nuclei and cytoplasmic processes of glial cells (arrows). **d** A strong positivity for vimentin is displayed in the rat skin dermis (arrows). **e**, **f** Rat cerebellum and viscacha pineal gland sections used as a negative control for the immunohistochemical reaction. No positive structures or cells were found. Scale bars, 100 (**a**, **d**) and 25 μm (**b**, **c**, **e**, **f**).

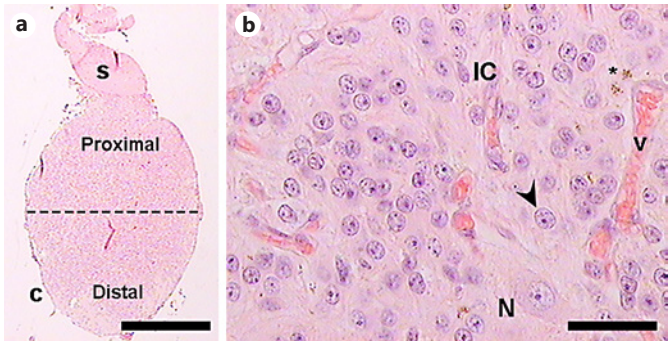

Fig. 2. Image of a viscacha pineal gland in mid pregnancy. **a** A well-developed parenchyma ensheathed by a connective tissue capsule (c) is observed in the gland. A stalk (s), a typical pine cone shape, and 2 regions can be seen (proximal and distal regions). **b** The image shows numerous blood vessels (v) and a homogeneous cell distribution. A big and round nucleus, open chromatin, and an evident nucleolus are observed in the pinealocyte (arrowhead). An elongated nucleus with dense chromatin is exhibited in an IC. Some brown pigment granules (*) can be found near a blood vessel. N, Neuron. Hematoxylin-eosin. Scale bars, 500 (**a**) and 25 μm (**b**).

Genex), resulting in a fuchsia precipitate. DAB and new fuchsin were selected as chromogens to visualize the antigens, since this combination is known to provide good contrast [Acosta et al., 2010; Busolini et al., 2017]. The sections were counterstained with Harris' hematoxylin for 10 s, washed for 2 min under running water, and mounted with a permanent aqueous mounting medium (SuperMount; BioGenex). Labeling was assessed using an Olympus BX-40 light microscope.

Morphometric Analysis

The morphometric study was carried out in pregnant and nonpregnant viscachas. For this purpose, 4 pineal glands per group were analyzed. Three regularly spaced serial tissue sections (50 μm each) from each gland were used and the microscopic fields were examined under a 40× objective. In each section, 20 microscopic fields were randomly selected throughout the pineal gland (10 microscopic fields from the proximal region and 10 from the distal region; Fig. 2a). Finally, 120 microscopic field measurements per region were taken in each group. The formula used to determine the percentage of immunopositive area (%IA) is: $\%IA = (\Sigma IA/\Sigma RA) \times 100$, where IA is the immunopositive area in each microscopic field (image) and RA is the reference area.

Two morphometric parameters were measured, i.e., %IA-S-100 (percentage of immunopositive area for S-100 protein) and %IA-GFAP (percentage of immunopositive area for GFAP). Vimentin was expressed in IC and other structures such as blood vessels; thus, the morphometric analysis was not performed for this protein.

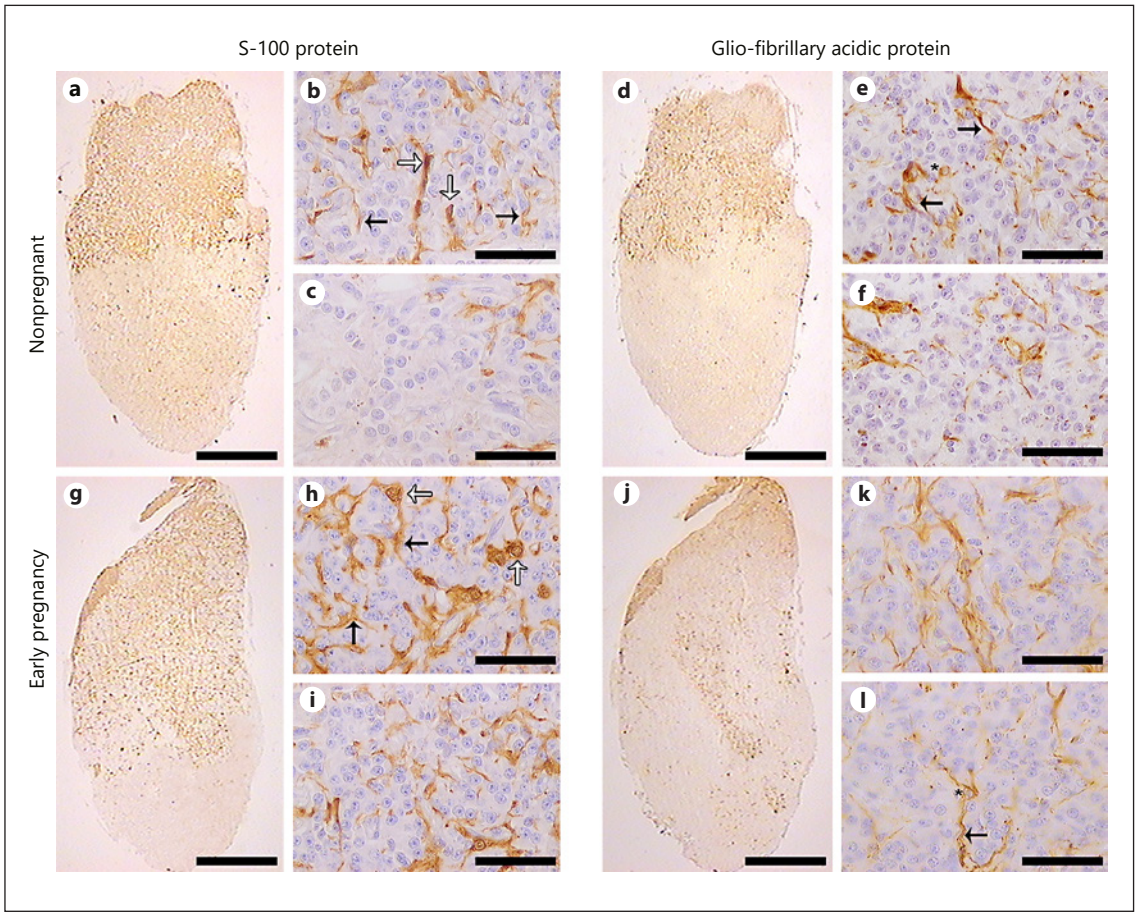

Fig. 3. Sections immunostained with anti-S-100 protein and anti-GFAP in nonpregnant (**a–f**) and early pregnancy (**g–l**) viscachas. Immunostaining for the S-100 protein (**a–c**, **g–i**) is exhibited in the IC nuclei (white arrows) and cytoplasmic process (black arrows), whereas GFAP (**d–f**, **j–l**) is displayed only in the cytoplasmic process and perinuclear spaces (*). At each pregnancy stage, different immunolabeled areas are observed in relation to the analyzed protein and to each region, respectively. In nonpregnant viscachas the proteins under analysis are found in the proximal region, while in the distal region they are scarce or absent. In early pregnancy, the proteins are also exhibited in the proximal region, but the distal region showed an increase in immunostaining. Nonpregnant (**a**, **d**). Early pregnancy (**g**, **j**). Proximal region (**b**, **e**, **h**, **k**). Distal region (**c**, **f**, **i**, **l**). Scale bars, 500 (**a**, **d**, **g, j**) and 25 μm (**b**, **c**, **e**, **f**, **h**, **i**, **k**, **l**).

The number of immunoreactive cells against the S-100 protein with a visible nucleus (IC-S-100) was counted in 120 microscopic fields per region in each group. Per field, the percentage of immunoreactive cells was obtained according to the following formula: A/(A + B) × 100. Each field contained between 90 and 110 cells. The numbers of S-100 immunoreactive cells (i.e., A) and the number of nuclei in immunonegative cells (i.e., B) were counted.

Serum Levels of Estradiol and Progesterone

Blood samples were incubated at 37 °C for 30 min in a water bath and centrifuged at 5,000 *g* for 10 min and the serum was removed. Serum estradiol and progesterone levels were quantified using RIA Estradiol (A21854; Immunotech s.r.o., Prague, Czech Republic) and RIA Progesterone (IM1188; Immunotech s.r.o.) kits from Beckman Coulter®, respectively. These tests were competitive radioimmunoassays for in vitro use.

Statistical Analysis

The results were expressed as means ± SEM. The groups were analyzed using the Kruskal-Wallis test. Differences between 2 glandular regions (proximal vs. distal regions) were evaluated by means of the Mann-Whitney test. $p < 0.05$ was considered statistically significant. InfoStat software version 2011 [Di Rienzo et al., 2011] was used for statistical analysis of morphometric measurements.

Results

The pineal gland of female viscachas exhibited a typical pine cone shape. Two distinctive regions or zones were defined in relation to the proximity to the stalk: the

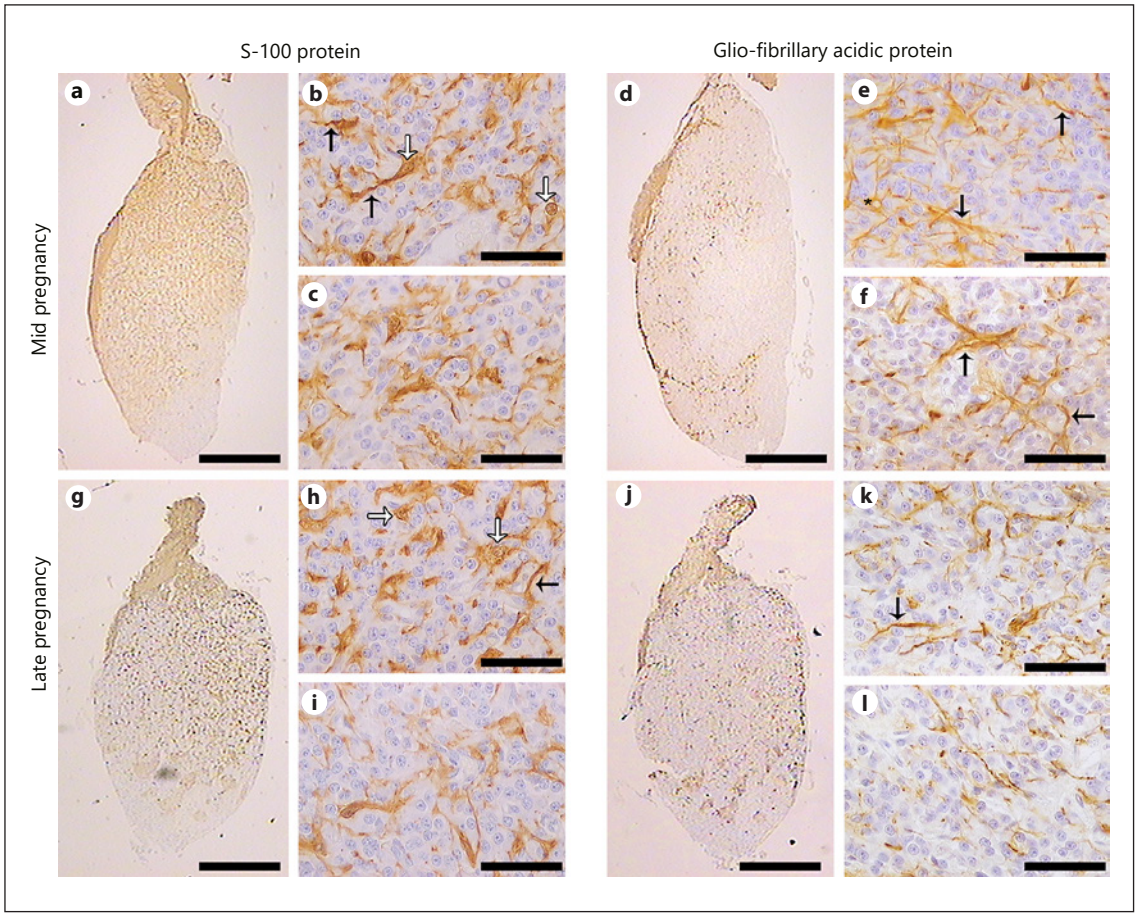

Fig. 4. Sections immunostained with anti-S-100 protein and anti-GFAP in mid (**a–f**) and late (**g–l**) pregnancy viscachas. S-100 protein is displayed widely in both regions of mid pregnancy females (**a–c**), while in late pregnancy the cytoplasmic process decreases into the distal region (**g–i**). GFAP also varies following the same pattern (mid pregnancy: **d–f**, late pregnancy: **j–l**), but the covered areas are smaller. Mid pregnancy (**a**, **d**). Late pregnancy (**g**, **j**). Proximal region (**b**, **e**, **h**, **k**). Distal region (**c**, **f**, **i**, **l**). Scale bars, 500 (**a**, **d**, **g**, **j**) and 25 μm (**b**, **c**, **e**, **f**, **h**, **i**, **k**, **l**).

proximal region was rounded and wider, while the distal region was thinner and edged. The gland was ensheathed by a connective tissue capsule and it presented abundant vascularization throughout the parenchyma. Pinealocytes were the main cell type; the nucleus was large and spherical, exhibiting a visible nucleolus and open chromatin. IC were the least frequent cell type, with an elongated nucleus and dense chromatin. Abundant pigmented cells displaying brown pigment granules were observed in close association with blood vessels (Fig. 2b).

IHC and Double IHC

Immunolabeling for the S-100 protein and GFAP revealed abundant expression in the IC of the proximal region, while in the distal region the expression of these proteins significantly varied in relation to the pregnancy stage. No expression of these proteins was detected in pinealocytes or endothelial cells.

S-100 protein was localized within the IC nucleus and cytoplasm. IC showed a stellate-like shape with an irregular cell body and abundant cytoplasmic processes. These processes were large, thick, and irregular. Several of them surrounded the pinealocytes either individually or in small groups, while others reached the blood vessels. On the other hand, immunolabeling for GFAP was expressed only in the cytoplasm, the perinuclear space, and cytoplasmic processes. These processes were smaller, thinner, and more delicate in relation to the S-100 protein cytoplasmic processes (Fig. 3, 4).

The double IHC technique revealed that some IC expressed colocalization for both proteins. S-100 protein was mainly observed in the nucleus, while GFAP was observed in the cytoplasmic processes. Other IC expressed exclusively only one of them (Fig. 5a, b). Vimentin was expressed in the proximal and distal regions of the viscacha pineal gland in each pregnancy stage. Immunolabeling was observed in the cytoplasmic processes of some IC, endothelial cells, and perivascular spaces. No differences were found between pregnant and nonpregnant viscachas (Fig. 5c, d).

Morphometric Study

In nonpregnant viscachas, the expression of S-100 protein and GFAP was abundant in the proximal region, while in the distal region they were scarce or absent. In early pregnancy, the main expression for these proteins was also found in the proximal region, but the distal region showed increased expression for the proteins in contrast to nonpregnant animals (Fig. 3). Viscachas in mid pregnancy exhibited an intense immunolabeling pattern for the analyzed proteins in both regions. The proximal region was heavily marked and almost covered by these proteins. Regarding the distal region, a significantly covered area was also found. In late pregnancy, immunostaining for the S-100 protein and GFAP was primarily found in the proximal region, while in the distal region the expression of these proteins was lower than in mid pregnancy but higher than in early pregnancy (Fig. 4). The morphometric parameters (%IA-S-100 and %IA-GFAP) varied according to the pregnancy stage (Table 1).

Regarding the n° IC-S-100, significant differences in the proximal region were observed between nonpregnant animals and those in early pregnancy compared to mid pregnancy females (nonpregnant, 3.10 ± 0.13; early pregnancy, 3.25 ± 0.15; mid pregnancy, 3.92 ± 0.09; and late pregnancy, 3.43 ± 0.14). In the distal region, a significant difference was found between nonpregnant and mid pregnancy viscachas (nonpregnant, 0.71 ± 0.16; early pregnancy, 1.70 ± 0.23; mid pregnancy, 2.67 ± 0.25; and late pregnancy, 1.84 ± 0.24).

In mid pregnancy, the %IA-S-100 and %IA-GFAP, along with the nº IC-S-100, were the highest in relation to the other pregnancy stages, while the nonpregnant viscachas presented the lowest values for all of the parameters analyzed.

Biochemical Study

Estradiol and progesterone serum concentrations showed significant differences between pregnant and nonpregnant viscachas. In mid pregnancy, estradiol exhibited the highest serum concentrations and significant differences were found among nonpregnant viscachas and those in early and late pregnancy. Likewise, progesterone levels presented the highest serum concentrations in mid pregnancy and significant differences were also found among nonpregnant, early, and late pregnancy viscachas. Moreover, in early pregnancy, progesterone levels were significantly higher in relation to nonpregnant animals. In late pregnancy, significant differences were found in progesterone levels between nonpregnant viscachas and those in early pregnancy. All of the differences mentioned above were determined by the Kruskal-Wallis test (Table 1).

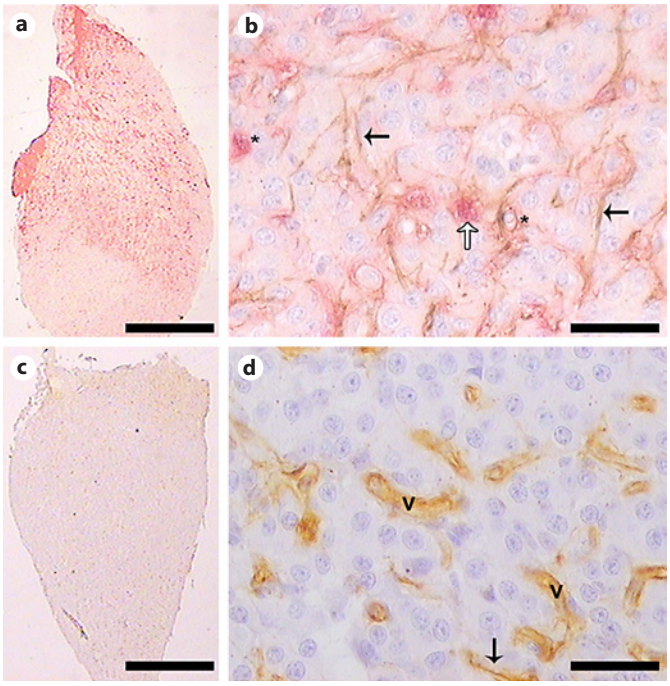

Fig. 5. Double IHC for the S-100 protein and GFAP in early pregnancy. Immunolabeling for vimentin in late pregnancy. **a** The photomicrograph shows the double IHC technique for the S-100 protein and GFAP in a pineal gland section during early pregnancy. **b** The image reveals some IC exhibiting colocalization for both proteins. S-100 protein is mainly observed in the nucleus (white arrow), while GFAP is observed in the cytoplasmic processes (black arrows). Other IC expressed exclusively only one of the proteins (*). **c** The photomicrograph shows immunolabeling for vimentin in both regions of the pineal gland during late pregnancy. **d** Immunostaining for this protein is observed in numerous blood vessels (v) and few IC present immunolabeling in their cytoplasmic processes (arrow). Scale bars, 500 (**a**, **c**) and 25 μm (**b**, **d**).

Table 1. Morphometric and biochemical study of the S-100 protein and GFAP in pregnant and nonpregnant viscachas

Stage	Morphometric study				Biochemical study	
	%IA S-100 protein		%IA GFAP		estradiol, pg/mL	progesterone, ng/mL
	proximal region	distal region	proximal region	distal region		
Nonpregnant	11.29±0.72	1.81±0.38	7.27±0.39	0.96±0.20	17.25±2.87	0.69±0.12
Early pregnancy	12.96±0.45	5.93±0.69	7.97±0.88	3.35±0.70	26.25±2.39	4.59±0.90[i]
Mid pregnancy	19.36±0.54[a]	10.69±1.34[c]	11.09±0.54[e]	6.49±0.71[f]	77.50±1.44[h]	55.05±1.81[j]
Late pregnancy	16.24±1.30[b]	8.19±1.47[d]	8.37±0.69	5±0.76[g]	23.75±1.75	19.03±1.94[k]

Values are expressed as means ± SEM ($n = 4$). Significant differences in the morphometric study were determined by the Kruskal-Wallis test. In addition, comparisons between regions (proximal vs. distal) for each pregnancy stage were done using the Mann-Whitney test. S-100 protein showed significant differences ($p < 0.01$ for each pregnancy stage). GFAP also showed significant differences ($p < 0.01$: mid pregnancy and nonpregnant; $p < 0.05$: early and late pregnancy). Significant differences in the biochemical study were determined by the Kruskal-Wallis test. [a] $p < 0.001$: mid pregnancy vs. early pregnancy and nonpregnant. [b] $p < 0.001$: late pregnancy vs. nonpregnant. [c] $p < 0.001$: mid pregnancy vs. early pregnancy and nonpregnant. [d] $p < 0.001$: late pregnancy vs. nonpregnant. [e] $p < 0.01$: mid pregnancy vs. late pregnancy, early pregnancy, and nonpregnant. [f] $p < 0.001$: mid pregnancy vs. nonpregnant. [g] $p < 0.001$: late pregnancy vs. nonpregnant. [h] $p < 0.05$: mid pregnancy vs. late pregnancy, early pregnancy, and nonpregnant. [i] $p < 0.05$: early pregnancy vs. nonpregnant. [j] $p < 0.001$: mid pregnancy vs. late pregnancy, early pregnancy, and nonpregnant. [k] $p < 0.001$: late pregnancy vs. early pregnancy and nonpregnant.

Discussion

Numerous immunohistochemical studies have established the IC glial nature in different species. S-100 protein, GFAP, and/or vimentin have been used as markers. Several researchers have described relevant characteristics of IC, such as their histological and morphometric features [Møller et al., 1978; Calvo and Boya, 1984; Huang et al., 1984; Schachner et al., 1984; Girod and Durand, 1985; Calvo et al., 1988; Boya and Calvo, 1993; Franco et al., 1997; Safwat, 2012], the existence of coexpression of GFAP and vimentin [Girod and Durand, 1985; López-Muñoz et al., 1992], a regionalized distribution of S-100 protein and GFAP in the parenchyma [Susuki and Kachi, 1995; Safwat, 2012], a decrease in vimentin-positive cells, and an increase in S-100- and GFAP-positive cells in correlation with age [Borregón et al., 1993], and immunostaining of IC in different stages of maturity [Franco et al., 1997; Redondo et a., 2001, 2003]. All of these studies prove that S-100 protein, GFAP, and/or vimentin are reliable markers for IC analysis.

In viscachas, numerous investigations have confirmed the relationship between the environmental photoperiod and the pineal main hormone, i.e., melatonin, in relation to male reproductive function [Fuentes et al., 1991, 1993; Muñoz et al., 1997, 1999, 2001; Aguilera-Merlo et al., 2005, 2009; Chaves et al., 2011; Cruceño et al., 2013; Filippa et al., 2015; Rosales et al., 2016]. Biochemical and ultrastructural changes in pinealocytes and maximal serum levels of melatonin were previously reported in our laboratory [Domínguez et al., 1987; Domínguez, 1990; Cernuda-Cernuda et al., 2003]. In a previous work, we reported a seasonal and age-related study of IC in male viscachas. An increase in pineal gland activity during winter months was found and significant differences in the %IA of S-100 protein and GFAP were reported. There, we suggested that IC undergo seasonal variations in their biochemical properties, in agreement with the seasonal changes in pinealocyte activity. Thus, in winter, a higher expression of S-100 protein may reflect a greater activity of IC according to the pinealocyte activity, while the increased amount of GFAP was essential for maintenance of the cellular shape during this period of maximal glandular activity [Busolini et al., 2017].

In this work, the immunohistochemical pattern and morphometric variation of IC in the pineal gland of pregnant and nonpregnant viscachas also showed significant differences. Like in male viscachas, this study supports our previous hypothesis that IC have a neuroectodermal origin. Moreover, a few vimentin-positive cells were also found in female viscachas, suggesting the existence of a population of immature glial cells. Furthermore, double IHC results might indicate the existence of IC in different functional stages, probably related to cellular microenvironmental needs. Busolini et al. [2017] have reported in male viscachas that the IC cytoplasmic processes generate

an extensive communication network within the gland. In this study the network was also observed, but important differences were found between females and males. In nonpregnant viscachas, this network is localized mainly in the proximal region. During early pregnancy, it begins to extend into the distal region in relation to the increased level of hormones. In mid pregnancy, when estradiol and progesterone levels are higher, the network extends throughout the parenchyma, almost covering the distal region. In late pregnancy the network begins to decrease into the distal region as a response to lower hormonal levels. Apart from the changes observed in relation to the number, branching, and extension of IC cytoplasmic processes, the increase in the nº IC-S-100 during mid pregnancy may indicate an active proliferation of IC in relation to a higher glandular activity and gonadal hormonal levels. In contrast, the nº IC-S-100 decreased in agreement with the lower glandular activity and hormonal levels in nonpregnant viscachas. Further studies including PCNA expression by IHC and apoptosis techniques are needed to show the involvement of IC in the proliferation and apoptosis processes in the pineal gland during each pregnancy stage. These findings are important because they evidence that the pineal gland of the female viscacha is very sensitive to changes in ovarian hormone levels and responds to them consistently. On the other hand, the pineal gland of the male viscacha does not respond in the same way to androgen levels, but it is more sensitive to environmental seasonal changes. These findings were in agreement with the results reported by Bishnupuri and Haldar [2000a] in female Indian palm squirrels (*Funambulus pennanti*). These authors suggested a direct relationship between pineal gland activity and ovarian steroids during the gestation period. At the time of implantation, they found that plasma levels of estradiol and progesterone as well as pineal gland weight, protein content, and plasma melatonin levels were the lowest. After implantation, a gradual increase in plasma concentrations for both ovarian hormones was reported. In the same way, the pineal gland also showed a gradual increase in activity throughout the gestation period. Moreover, these authors also reported that, under constant light conditions, short and long photoperiods did not significantly affect the pineal gland activity during pregnancy [Bishnupuri and Haldar, 2000b].

In female viscachas, estradiol and progesterone levels are subject to variations during pregnancy. Our results are in agreement with the observations of Gil et al., [2007], who found a significant increase in both hormones in pregnant viscachas. However, their biochemical study did not take into consideration the different pregnancy stages. In our research, the analysis of the serum hormonal profile during the different stages of gestation showed fluctuations in estradiol and progesterone levels. These variations were in accordance with the changes observed in IC features for each stage of gestation. Therefore, it may be suggested that a high level of melatonin and ovarian steroids is required for maintenance of a normal physiology during the gestation period. Furthermore, the changes in the %IA of S-100 protein and GFAP, together with modifications in the nº IC-S-100, also indicate a direct relationship between glandular activity and gonadal hormone levels during pregnancy. Electron microscopy studies would be an important tool to verify at ultrastructural level the reported changes in the IC during the different pregnancy stages.

On these grounds, we could conclude that IC undergo changes in relation to ovarian hormone levels and participate in the regulation of glandular activity during pregnancy. However, further research is needed to elucidate this relationship.

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Disclosure Statement

We have no conflict of interest to disclose.

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