A Seasonal and Age-Related Study of Interstitial Cells in the Pineal Gland of Male Viscacha *(Lagostomus maximus maximus)*

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ABSTRACT

The pineal gland of viscacha exhibits histophysiological variations throughout the year, with periods of maximal activity in winter and minimal activity in summer. The aim of this work is to analyze the interstitial cells (IC) in the pineal gland of male viscachas in relation to season and age. The S-100 protein, glio-fibrillary acidic protein (GFAP), and vimentin were detected in adult and immature animals by immunohistochemistry (IHC). Double-IHC was also performed. The S-100 protein was localized within both, IC nucleus and cytoplasm. GFAP was present only in the cytoplasm. Vimentin was expressed in some IC, besides endothelial cells, and perivascular spaces. In the adult males, the morphometric parameters analyzed for the S-100 protein and GFAP exhibited seasonal variations with higher values of immunopositive area percentage in winter and lower values in summer, whereas the immature ones showed the lowest values for all the adult animals studied. Colocalization of S-100 protein and GFAP was observed. The IC exhibited differential expression for the proteins studied, supporting the hypothesis of the neuroectodermal origin. The IC generate an intraglandular communication network, suggesting its participation in the glandular activity regulation processes. The results of double-IHC might indicate the presence of IC in different functional stages, probably related to the needs of the cellular microenvironment. The morphometric variations in the proteins analyzed between immature and adult viscachas probed to be more salient in the latter, suggesting a direct relationship between the expression of the S-100 protein and GFAP, and animal age. Anat Rec, 300:1847–1857, 2017.

Key words: lagostomus; pineal gland; interstitial cells; seasonal activity; immunohistochemistry

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In seasonal breeders, the pineal gland acts as a neuroendocrine interface, transforming ambient light changes into hormonal variations: the synthesis and release of nocturnal melatonin (Hernández Díaz et al., 2001). Three main cell types have been described: pinealocytes or principal cells, supportive or interstitial cells (IC), and pigmented cells (Vollrath, 1981; Calvo et al., 1992). In lower proportion, there are other cell types, such as macrophages, fibroblasts, and neurons. The presence of pineal calcifications (cerebral grit, *corpora arenácea*) has also been reported (Beltrán, 2001).

The IC have been studied using immunohistochemistry (IHC) in several species such as rat (Møller et al., 1978; Schachner et al., 1984; López-Muñoz et al., 1992; Borregón et al., 1993; Suzuki and Kachi, 1995), mouse (Schachner et al., 1984), golden hamster (Huang et al., 1984), monkey (Girod and Durand, 1985), dog (Calvo and Boya, 1993), cat (Calvo and Boya, 1993; Boya et al., 1995), sheep (Franco et al., 1997; Redondo et al., 2001; Regodón et al., 2001), and donkey (Safwat, 2012). The S-100 protein, glio-fibrillary acidic protein (GFAP), and vimentin were used as markers. The S-100 protein was present in glial cells of the central nervous system (CNS), especially in astrocytes, maturing oligodendrocytes, neural progenitor cells, and some types of neurons. S-100 expression has been documented in other cell types such as kidney epithelial cells, pituitary folliculostellate cells, chondrocytes, melanocytes, Langerhans cells, and myoblasts (Haimoto et al., 1987; Böni et al., 1997; Rambotti et al., 1999; Acosta et al., 2010; Acosta and Mohamed, 2013). Intracellularly, the S-100 protein has been involved in the regulation of proliferation, differentiation, inflammation and migration, Ca^{2+} homeostasis, energy metabolism, and apoptosis. These functions have been described through interactions with a variety of target proteins including enzymes, receptors, cytoskeletal subunits, transcription factors and nucleic acids. Extracellularly, the S-100 protein has an autocrine and/or paracrine function on cell proliferation, differentiation, migration, inflammation and tissue repair, and/or antimicrobial activity (Donato et al., 2013). GFAP and vimentin were found to be members of the intermediate filament protein family, specifically of the intermediate filament protein family Class III (Oshima, 2007). GFAP is the main protein constituent of cytoskeleton intermediate filaments of mature astrocytes in CNS (Eng, 1985; Eng et al., 2000). GFAP is considered important in modulating astrocyte motility and shape by providing structural stability to astrocytic processes. Studies by Liedtke et al. (1996) have suggested a role for GFAP in normal CNS white matter architecture and blood-brain barrier integrity. Some reports indicate that GFAP also participates in the modulation of some neuronal functions (McCall et al., 1996; Shibuki et al., 1996). Vimentin is the main constituent of cytoskeleton intermediate filaments of immature glial cells (Dahl et al., 1981) and mesenchymal cells (Lehtonen et al., 1985). Vimentin emerges as an organizer of a number of critical proteins involved in attachment, migration and cell signaling. Besides maintaining the structural integrity of cells and tissues (Nieminen et al., 2006) and participating in adhesion and migration processes (Gonzales et al., 2001; Nieminen et al., 2006), vimentin is involved in signal transduction (Lopez-Egido et al., 2002; Runembert et al., 2002; Ivaska et al., 2005; Yang 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).

Our experimental model is the viscacha (*Lagostomus maximus maximus*), the largest member of the *Chinchillidae* family. This rodent inhabits the southern hemisphere from Paraguay through central Argentina (Jackson et al., 1966; 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, melatonin (Dominguez et al., 1987; Fuentes et al., 2003; Aguilera-Merlo et al., 2005; Filippa et al., 2005). The cellular activity of pinealocytes and the serum values of melatonin have been reported to be highest during winter (short photoperiod) and lowest in summer (long photoperiod) (Piezzi et al., 1984; Dominguez et al., 1987; Fuentes et al., 1991, 2003; Cernuda-Cernuda et al., 2003). However, the seasonal- and age-related study of the S-100 protein, GFAP, and vimentin has not been performed yet. The aim of this work is to analyze the IC in the pineal gland of male viscachas in relation to season and age.

TABLE 1. Seasonal environmental conditions

Conditions	Summer	Autumn	Winter	Spring
Heliophany (h)	9.38	7.09	6.82	9.09
Precipitation (mm)	90	27	11	58.5
Temperature (°c)	22	13	12	19.66

MATERIALS AND METHODS

In this work, a seasonal and age-related study was conducted.

Seasonal Study

Sixteen adult male viscachas (body weight above 5 kg) were captured in their natural habitat near San Luis, Argentina (33° 20' south latitude, 760 m altitude), using traps placed in their burrows, during the following periods: February to March (summer; N = 4), April to May (autumn; N = 4), July to August (winter; N = 4), and November to December (spring; N = 4).

Age-Related Study

Four immature male viscachas (body weight 2–3 kg) were captured in autumn and carefully classified as sexually immature according to body weight and light microscopy observations of testes (Llanos and Crespo, 1952; Muñoz et al., 1998).

Values of solar irradiation expressed as heliophany and seasonal mean values of precipitation and temperature were provided by the Servicio Meteorológico Nacional San Luis (www.smn.gov.ar). The lowest values of heliophany, precipitation, and temperature were observed in winter (Table 1).

After captured, 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.

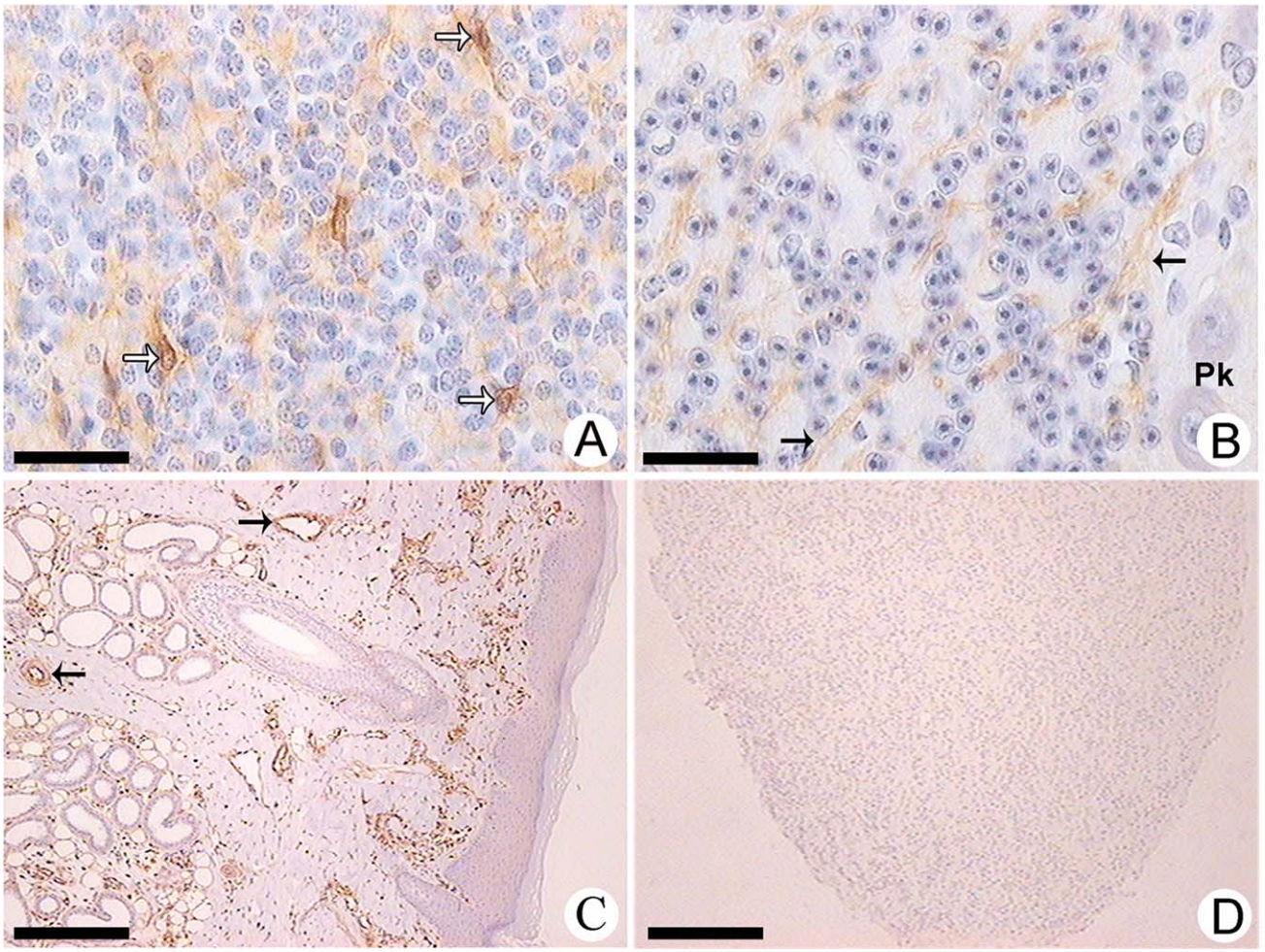

Fig. 1. IHC positive controls for S-100 **(A)**, GFAP **(B)** and vimentin **(C)**, negative control **(D)**. (A) The immunostaining for S-100 (white arrows) is expressed in the cytoplasmic processes and nuclei of glial cells in rat cerebellum. (B) The immunostaining for GFAP (black arrows) is shown in cytoplasmic processes near Purkinje cells and cells of glomerular layer in rat cerebellum. (C) Rat skin section immunostained with anti-vimentin. Immunolabeling (black arrows) is observed in dermis, where the cells show strong positivity. (D) Viscacha pineal gland section used as negative control for immunohistochemical reaction. Scale bars: (A,B) 25 μm; (C,D) 250 μm.

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 performed. The sections were examined using an Olympus BX-40 light microscope.

The experimental design was approved by the local Ethics Committee and was in agreement whit the guidelines of the National Institute 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: Polyclonal anti-S-100 protein (AR058–5R; BioGenex, San Ramon, CA), polyclonal anti-GFAP (AR020–5R; BioGenex, San Ramon, CA), and monoclonal anti-vimentin (Clone V9 AM074–5M; BioGenex, San Ramon, CA). These antisera have been previously used with successful results in viscacha (Rodriguez et al., 2007; Acosta and Mohamed, 2013). 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 at full power 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, rinsed with distilled water, and phosphate-buffered saline (PBS, 0.01 M, pH 7.4). Non-specific binding sites for immunoglobulins were blocked by 15 min incubation with 0.25% casein in PBS, washed in PBS, and incubated with the primary antibody (12 hr in a humidified chamber at 4°C). The slides were subsequently washed three times for 10 min each in PBS. Immunohistochemical visualization was carried out using the *Super*

Sensitive Ready-to-Use Immunostaining Kit (QD000–5LE; BioGenex, San Ramon, CA) at 20°C. Sections were incubated for 30 min with biotinylated anti-IgG, and after being washed three 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, San Ramon, CA), resulting in a brown precipitate. Sections were counterstained with Harris' hematoxylin for 10 sec, washed 2 min in running water, dehydrated in increasing concentration 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 controls procedures included replacing primary antibody with normal goat serum and omitting the primary antibody. No positive structures or cells were found in these sections (Fig. 1).

Double-IHC for S-100 and GFAP

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 DAB chromogen solution in PBS and 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 by New Fuchsin Chromogen Kit (Catalog no. HK 183–5K; BioGenex, San Ramon, CA), 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). The sections were counterstained with Harris' hematoxylin for 10 sec, washed for 2 min in running water, and mounted with a permanent aqueous mounting medium (SuperMount, BioGenex San Ramon, CA). Labeling was assessed using an Olympus BX-40 light microscope.

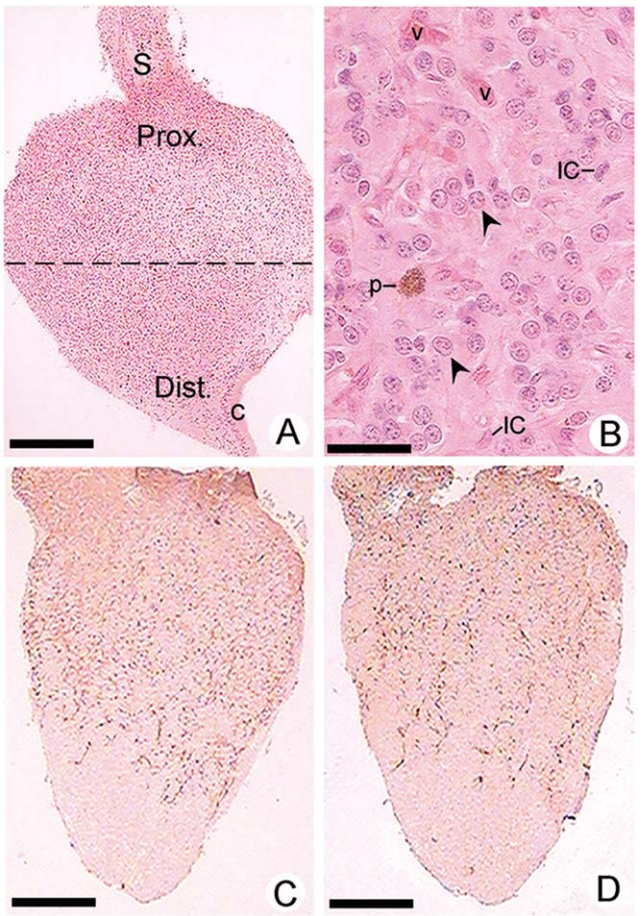

Fig. 2. Pineal gland of adult male viscacha. **(A)** The gland presents a typical pine-cone shape covered by a connective tissue capsule (c), a stalk (S), and two regions: the proximal (Prox.) region (close to the stalk) and the distal (Dist.) region (away from the stalk). **(B)** The image shows a homogeneous distribution of cells in the parenchyma and numerous blood vessels (v). The pinealocytes (arrowheads) exhibit big and round nuclei, open chromatin, and evident nucleolus. Elongated nuclei with dense chromatin are displayed in IC. A pigmented cell shows brown pigment granules (p). Stains: (A,B) Hematoxylin-Eosin; (C,D) Immunostaining for the S-100 protein and GFAP, respectively, in a winter section. Abundant immunolabeling is observed in the proximal region for both proteins, which decreases towards the distal region. Scale bars: (A,C, and D) 500 μm; (B) 25 μm.

Morphometric Analysis

Morphometric parameters were measured using a computer-assisted image analysis system consisting of an Olympus BX-40 binocular microscope interfaced with a computer. The images were captured by a Sony SSC-DC5OA camera and processed with the software Image Pro Plus 5.0 under control of a Pentium IV computer. The software allowed the following processes: acquisition, automatic analogous adjustment, distance calibration, background subtraction, thresholding, area measurement, and data logging. Briefly, the image was displayed on a color monitor; a standard area of 18,141.82 μm^2 (reference area) was defined, and the distance calibration was performed using a slide with a micrometric scale for microscopy (Reichert, Vienna, Austria). The morphometric study was carried out as follows: for each group studied, four pineal glands were analyzed. Three regularly spaced serial tissue sections (50 μm each) from a pineal 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 (10 from the proximal region and 10 from the distal region, Fig. 2A). Finally, 120 microscopic field measurements per region were made in each group. The formula used to determine the percentage of immunopositive area (%IA) was $\%IA = (\sum IA/\sum RA) \times 100$.

IA, immunopositive area in each microscopic field (image); RA, reference area; %IA, immunopositive area percentage).

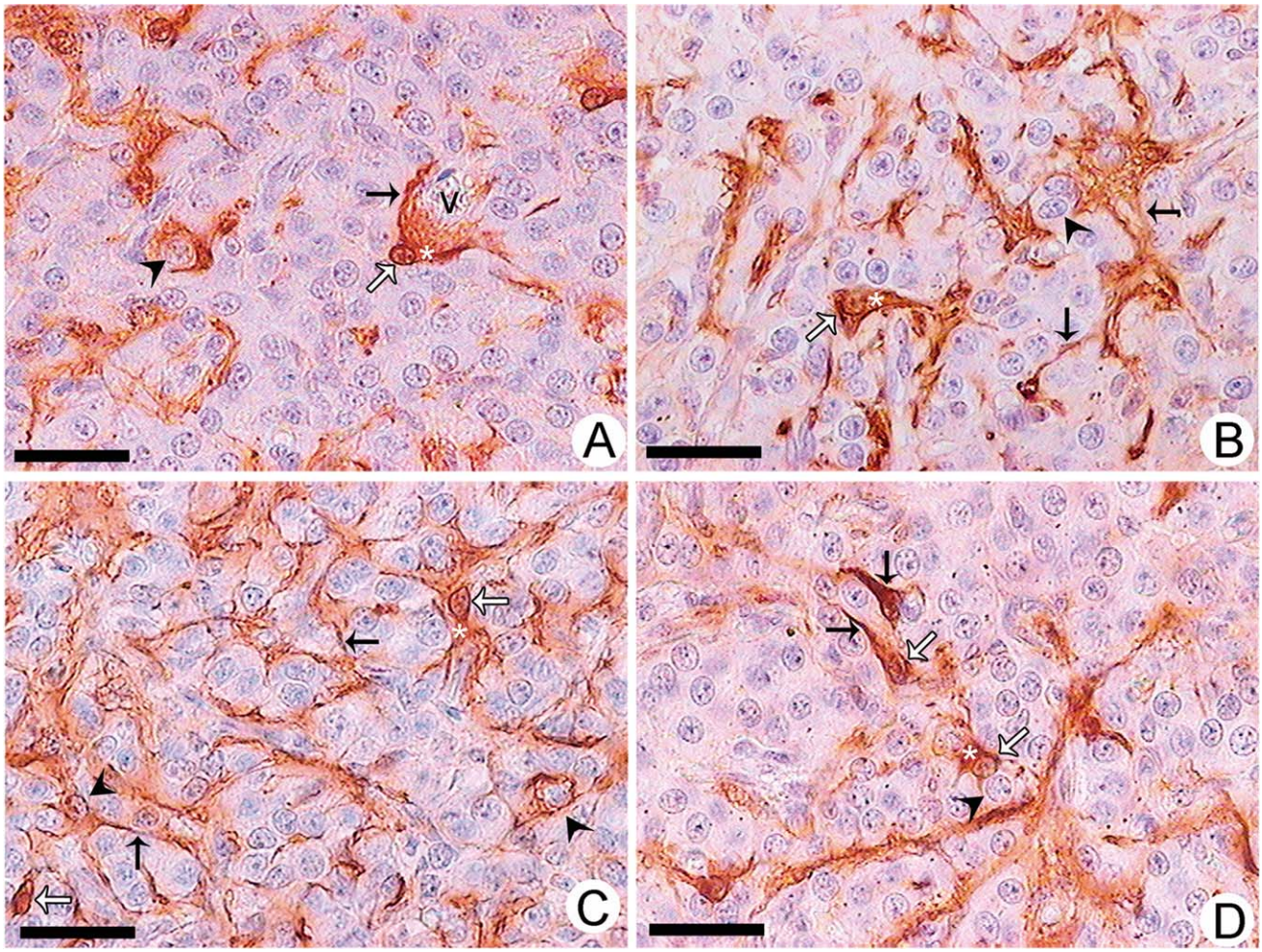

Fig. 3. Seasonal immunolabeling for S-100 protein in viscacha pineal gland. **(A)** summer, **(B)** autumn, **(C)** winter, and **(D)** spring. The S-100 protein is expressed in IC nuclei (white arrows), perinuclear cytoplasm (*), and cytoplasmic processes (black arrows). A close interaction between cytoplasmic process, pinealocytes (arrowheads), and blood vessels (v) is present. A significant increase of the immunostaining for this protein is observed in winter. Scale bars: (A–D) 25 μm.

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

The number of immunoreactive cells against the S-100 protein with a visible nucleus (no. 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 formula $A/(A+B) \times 100$. Each field contained between 90 and 110 cells. The number of S-100 immunoreactive cells (A) and the number of nuclei in immunonegative cells (B) were counted.

Statistical Analysis

Results were expressed as mean ± standard error of the mean (SEM) for all data sets. The data were analyzed using the Kruskal-Wallis test. Differences between two glandular regions (proximal region vs. distal region) were evaluated by means of the Mann–Whitney test. A value of $P < 0.05$ was considered statistically significant. InfoStat software (Di Rienzo et al., 2011) was used for statistical analysis of morphometric measurements.

RESULTS

Seasonal Study

The pineal gland of adult viscacha presented an oval-like morphology, elongated, with a stalk connected to the brain. Two different regions in the parenchyma were observed. The proximal region (close to the stalk) was wider and rounded, while the distal region (away from the stalk) was thinner and edged resulting in a characteristic pine-cone shape. The gland was covered by a connective tissue capsule (Fig. 2A). The vascularization was abundant and largely distributed within the gland. The pinealocyte was the main cell type, with large nucleus, spherical in shape, exhibiting a visible nucleolus and open chromatin. The IC was the least frequent cell type, with elongated nucleus, and dense chromatin. Few pigmented cells were observed in close association

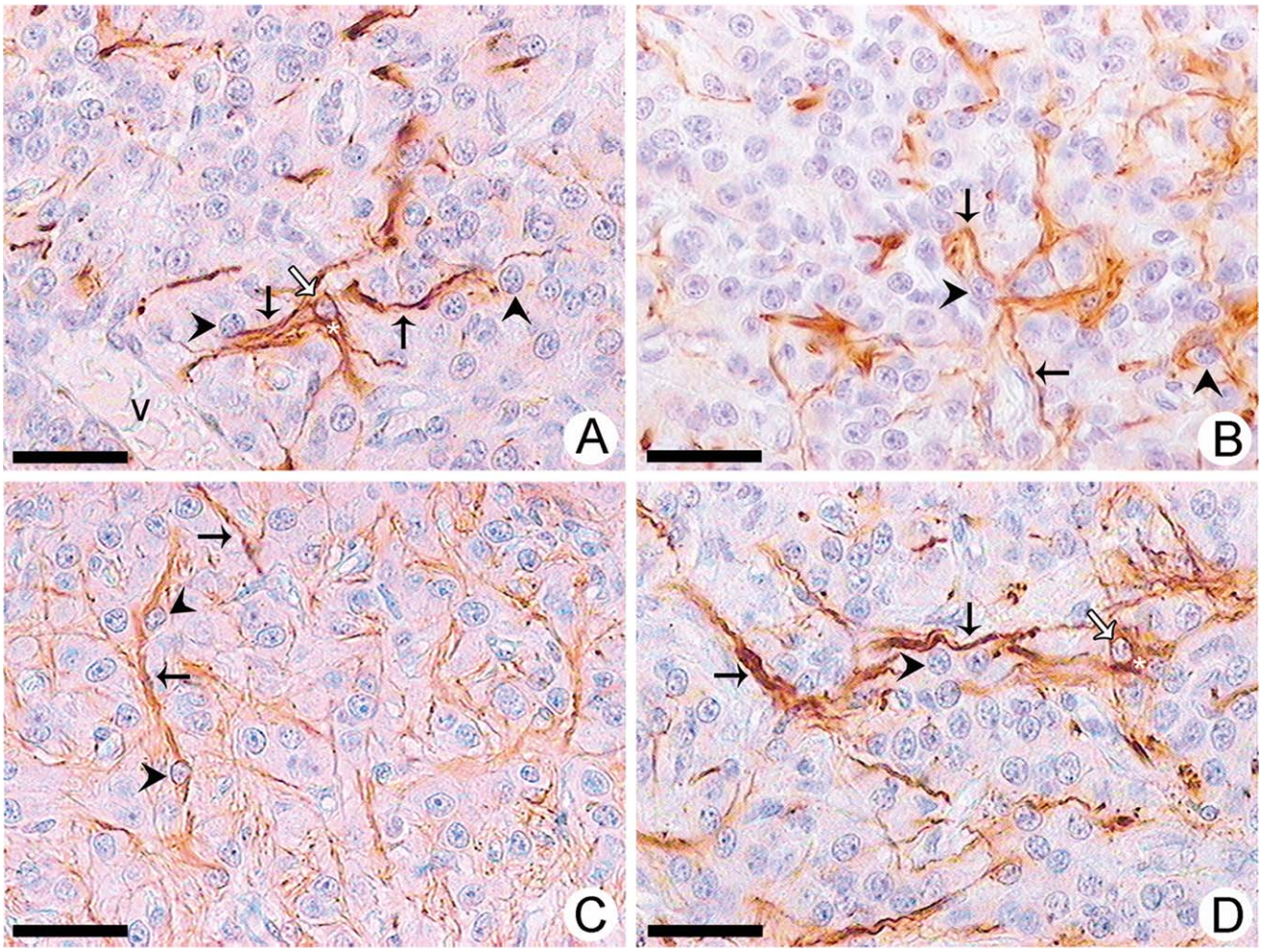

Fig. 4. Seasonal immunolabeling for GFAP in viscacha pineal gland. **(A)** summer, **(B)** autumn, **(C)** winter, and **(D)** spring. The GFAP is expressed in IC cytoplasmic processes (black arrows) and perinuclear cytoplasm (*). The nuclei (white arrows) remain immunonegative. The IC cytoplasmic processes are in contact with pinealocytes (arrowheads) and blood vessels (v). During winter, a significant increase of the immunostaining for this protein is observed. Scale bars: (A–D) 25 μm.

with blood vessels. They exhibited pigment granules, light to dark brown in color (Fig. 2B).

IHC and double-IHC. The immunolabeling for the S-100 protein and GFAP revealed that IC were localized mainly in the proximal region, and they were scarce or absent in the distal region (Fig. 2C,D). No expressions of these proteins were detected in pinealocytes or endothelial cells. The S-100 protein showed a pattern of nuclear and cytoplasmic immunolabeling. These cells exhibited a stellate-like shape, irregular cell body with abundant fine cytoplasmic processes, which surround the pinealocytes individually or in small groups, and some of them reach the blood vessels. The seasonal immunohistochemical study, showed higher expression for this protein during winter (Fig. 3). The immunolabeling for GFAP was expressed only in the IC cytoplasm, around the nucleus, and cytoplasmic processes. The seasonal expression for this protein was also higher in winter (Fig. 4). The double-IHC technique revealed that some IC expressed colocalization for both proteins (S-100 and GFAP), while others expressed exclusively only one of them (Fig. 5). Vimentin was expressed in the cytoplasmic processes of some IC, and this protein was also observed in endothelial cells and perivascular spaces (Fig. 6).

Morphometric analysis. The morphometric analysis revealed that the %IA-S-100 in the proximal region was higher in animals captured in winter compared with those captured in summer. The %IA-GFAP in the proximal region was higher in animals captured in winter and spring in relation to those captured in summer. In the distal region, the %IA-S-100 was lower in animals captured in summer and autumn in regard to those captured in winter. In contrast, the %IA-GFAP was lower in animals captured in spring compared with those captured in autumn and winter (Table 2).

Regarding the no. IC-S-100, no significant differences in the proximal region were observed in the adult animals studied (summer: 2.59 ± 0.22; autumn: 3.09 ± 0.30; winter 3.31 ± 0.20 and spring: 2.84 ± 0.24). In the distal region, a significant difference ($P < 0.01$) was found between summer and winter values (summer: 1.07 ± 0.13; autumn: 1.23 ± 0.25; winter 1.67 ± 0.06; and spring: 1.37 ± 0.23).

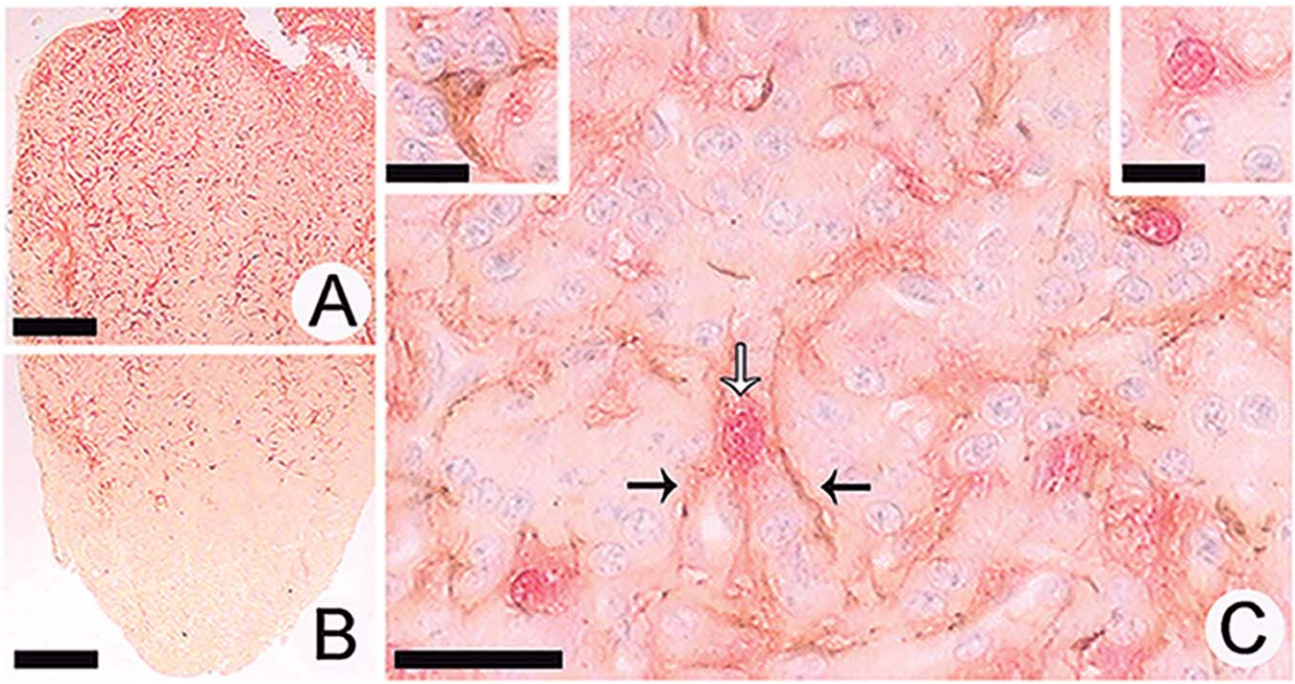

Fig. 5. Double IHC for S-100 protein and GFAP in winter. **(A,B)** Double-IHC for the S-100 protein and GFAP is shown in the proximal and distal regions of adult male viscachas, respectively. Abundant immunolabeling is observed in the proximal region, decreasing towards the distal region. **(C)** Some IC exhibit colocalization for both proteins, the S-100 protein is mainly observed in the nuclei (white arrow) while GFAP is exhibited in the cytoplasmic processes (black arrows). Others expressed only one of them. Left inset shows immunopositivity only for GFAP (brown), and right inset only for S-100 (fuchsia). Scale bars: (A,B) 125 μm; (C) 25 μm. Inset Scale Bars: 12.5 μm.

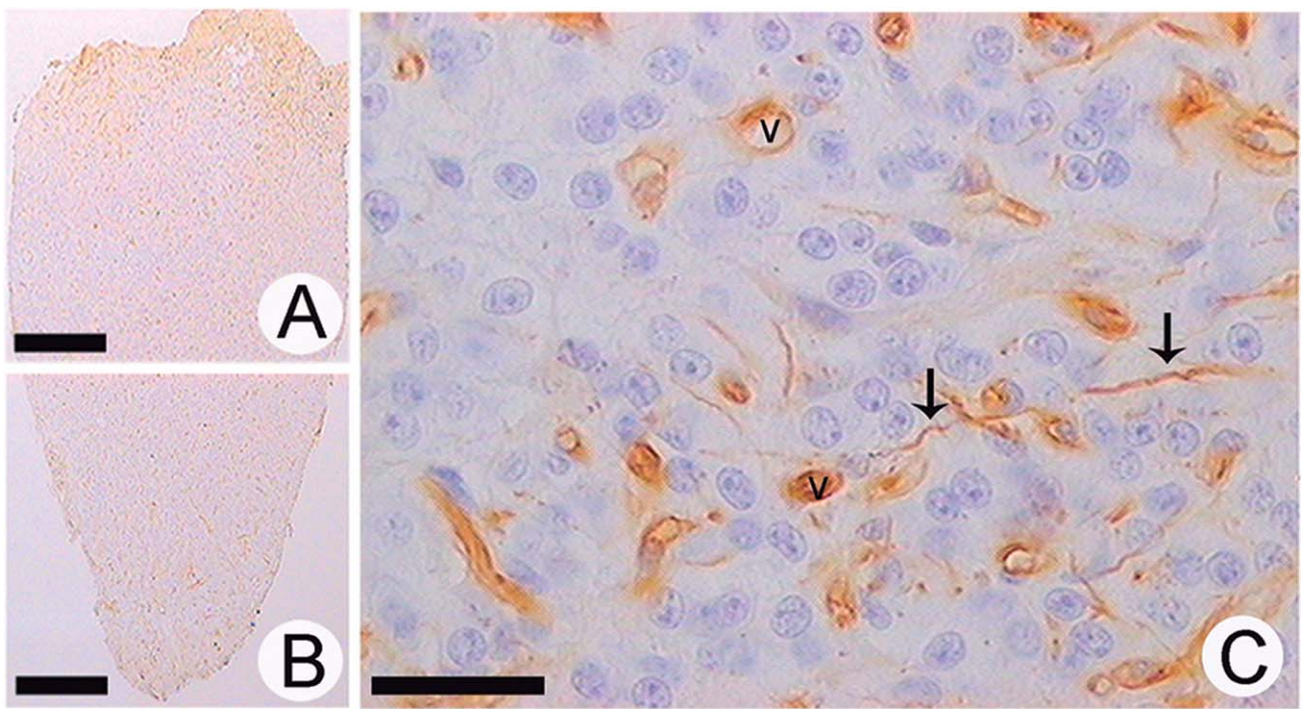

Fig. 6. IHC for vimentin in adult viscacha. **(A,B)** Vimentin is present in the proximal (A) and distal (B) regions of viscacha pineal gland. **(C)** Few IC show immunostaining for vimentin in their cytoplasmic processes (black arrows). Immunolabeling for this protein can be seen in numerous blood vessels (v). Scale bars: (A,B) 125 μm; (C) 25 μm.

TABLE 2. Seasonal morphometric study of S-100 protein and GFAP

Parameters		Summer	Autumn	Winter	Spring
%IA S-100	Proximal	3.44 ± 0.40	4.49 ± 0.38	5.49 ± 0.12[a]	4.41 ± 0.36
	Distal	1.45 ± 0.33	1.63 ± 0.32	2.75 ± 0.44[b]	2.01 ± 0.10
%IA GFAP	Proximal	2.80 ± 0.14	4.01 ± 0.40	4.94 ± 0.22[c]	4.19 ± 0.33[d]
	Distal	1.33 ± 0.14	1.39 ± 0.24	2.00 ± 0.54	0.75 ± 0.06[e]

The values are expressed as mean ± SEM (N = 4).
%IA S-100: [a]$P < 0.01$; winter vs. summer.
[b]$P < 0.01$; winter vs. summer and autumn.
%IA GFAP: [c]$P < 0.01$; winter vs. summer.
[d]$P < 0.01$; spring vs. summer.
[e]$P < 0.01$; spring vs. autumn and winter.
The significant differences were determined by Kruskal Wallis test.
For both proteins, per season, the comparison between regions (proximal vs. distal) shows significant differences ($P < 0.01$, Mann-Whitney test).

In each season, the %IA-S-100, the %IA-GFAP, and the no. IC-S-100 were significantly different ($P < 0.01$) between the two pineal regions (proximal vs. distal).

Age-Related Study

The organization and morphology of the pineal gland of immature male viscachas were similar to those of adult males. The IC exhibited immunostaining in all tested proteins. The immunolabeling for the S-100 protein and GFAP demonstrated that these cells presented the typical star-like shape, numerous shorter cytoplasmic processes, which surround the pinealocytes, and reach the blood vessels (Fig. 7A,B). Few IC exhibited colocalization for the S-100 protein and GFAP, while others presented only one of these proteins (Fig. 7C). Vimentin was observed in the cytoplasmic processes of some IC, endothelial cells, and perivascular spaces (Fig. 7D). In immature males, for both regions, the morphometric parameters (%IA-S-100 and %IA-GFAP) were lower than in adult viscachas. In the proximal region, the %IA-S-100 (2.96 ± 0.06) was significantly lower ($P < 0.01$) in relation to the adult values found in autumn, winter, and spring. Likewise, the %IA-GFAP in the proximal region (2.39 ± 0.14) was significantly lower ($P < 0.01$) compared with the adult values obtained in autumn, winter, and spring. In the distal region, the %IA-S-100 (0.92 ± 0.07) decreased significantly ($P < 0.01$) in regard to winter and spring values. The %IA-GFAP (0.59 ± 0.08) presented significant differences ($P < 0.01$) according to the adult values found in summer, autumn, and winter.

The no. IC-S-100 in the immature (1.50 ± 0.09) was significantly lower ($P < 0.01$) in the proximal region compared with all the adult animals. In contrast, in the distal region, the no. IC-S-100 (0.81± 0.04) was significantly lower ($P < 0.01$) with respect to the adult winter value.

The %IA-S-100, the %IA-GFAP, and the no. IC-S-100 in immature viscachas were significantly different ($P < 0.01$) between the proximal and distal regions.

DISCUSSION

In different species, the IC of the pineal gland showed immunostaining for S-100 protein, GFAP, and/or vimentin. Møller et al. (1978) demonstrated that the IC of rat pineal gland expressed S-100 and GFAP, and they were considered true macroglial cells. In gerbils, Welsh and Reiter (1978) confirmed a high activity of protein synthesis, according to the great development of the rough endoplasmic reticulum. Girod and Durand (1985) verified the expression of S-100 protein in IC of macaque pineal gland. Calvo et al. (1988b) reported astrocyte-like cells on the basis of the quantity of filaments in the pineal gland of dogs. A similar result was found in cats (Boya et al., 1995). In addition, Calvo and Boya (1984) and Calvo et al. (1988a) described the histological features of IC in rat pineal gland and showed the expression of the S-100 protein, GFAP, and vimentin. In sheep pineal gland, it was reported that GFAP and vimentin immunostaining occurred in cells with morphological characteristics of IC and these proteins are immunomarkers of IC in different stages of maturity (Franco et al., 1997; Redondo et al., 2001, 2003). An immunohistochemical study of IC during the postnatal development of rat pineal gland found a decrease in vimentin-positive cells, an increase of S-100 and GFAP positive cells with the age of the animals (Borregón et al., 1993). Moreover, these researchers described a regionalized distribution of those markers in the gland. In adult rats, Suzuki and Kachi (1995) demonstrated that S-100 positive cells were dispersed throughout the gland while the GFAP positive cells were detected only in the stalk and proximal region. In donkeys, Safwat (2012) established that both, S-100 and GFAP immunoreactive cells, showed a similar distribution and morphological characteristics in the pineal gland, but the immunostaining of S-100 cells was denser.

In viscacha, numerous studies have confirmed the relationship between photoperiod and melatonin on 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). The short photoperiod provokes biochemical and morphological changes in different components of the reproductive axis. An increase of pineal gland activity inhibited the gonadal and pituitary activities. During winter months, ultrastructural changes in pinealocytes and maximal serum levels of melatonin were reported (Dominguez et al., 1987; Dominguez, 1990; Cernuda-Cernuda et al., 2003). In this study, significant differences in the %IA of S-100 protein and GFAP may indicate that IC undergo seasonal variations in their biochemical properties, in relation to seasonal changes in the pinealocyte activity. It has been reported that S-100

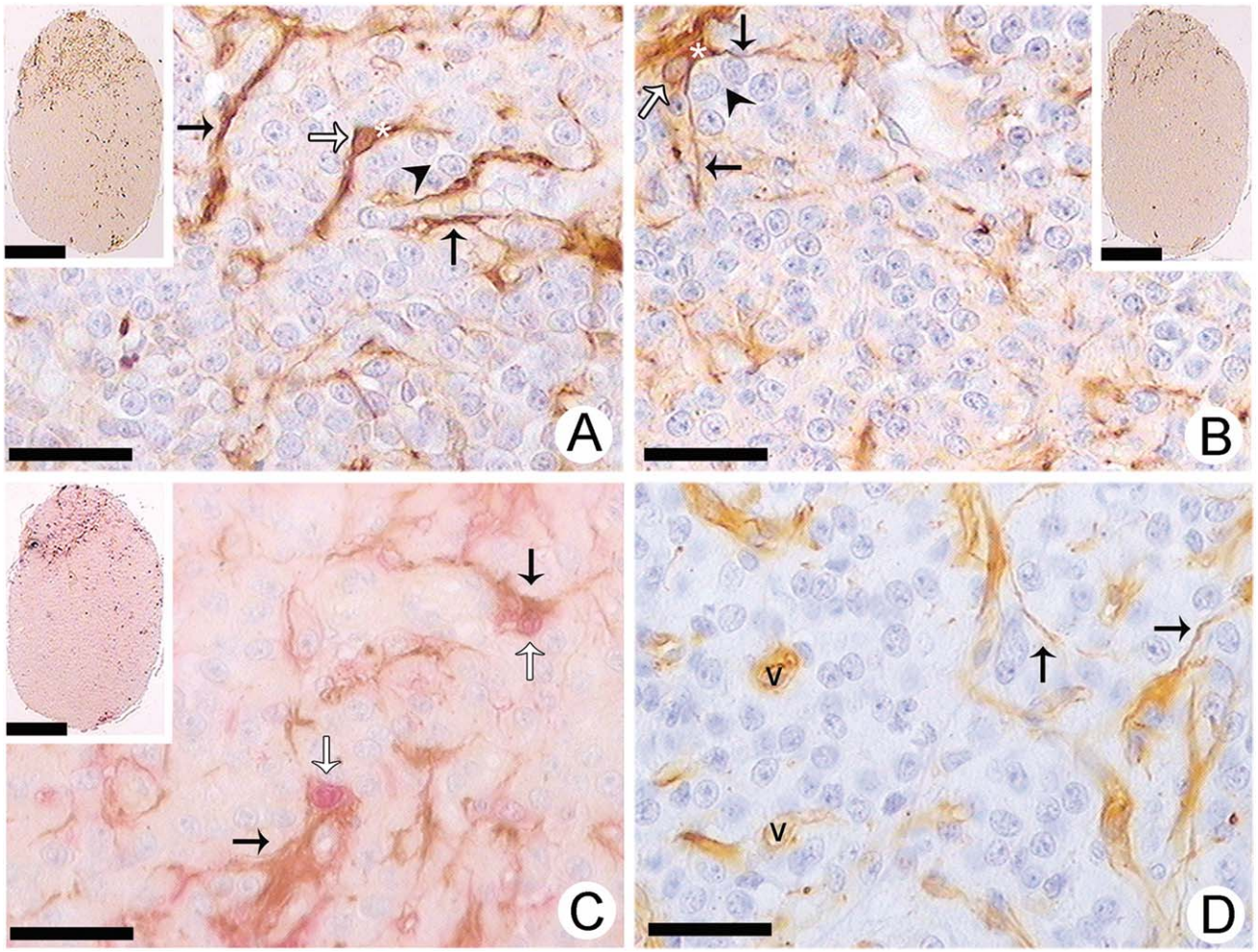

Fig. 7. Immunolabeling for S-100, GFAP, and Vimentin in immature viscacha pineal gland. **(A)** The S-100 protein is expressed in IC nuclei (white arrow), perinuclear cytoplasm (*), and cytoplasmic processes (black arrows). **(B)** GFAP is exhibited in IC cytoplasmic processes (black arrows) and perinuclear cytoplasm (*). The nuclei remain immunonegative (white arrow). For both A) and B), a close interaction between cytoplasmic processes and pinealocytes is shown (arrowhead) and a significant decrease of immunopositivity for both proteins is observed. **(C)** Colocalization for the S-100 protein (white arrow) and GFAP (black arrow) can be observed in immature IC, while others expressed only one of these proteins. **(D)** Some IC displayed in their cytoplasmic processes immunostaining for vimentin (black arrows). Many blood vessels (v) show immunolabeling for this protein. Scale bars: (A–D) 25 μm. Inset Scale Bars: 250 μm.

protein acts as a cytoskeleton dynamics regulator, intracellular signal transducer as well as paracrine regulator at extracellular level (Donato, 2003; Donato et al., 2009; Acosta et al., 2010). In viscachas, the higher expression during winter may reflect a major activity of IC according to pinealocyte activity. In addition, the increased amount of GFAP, a constituent of the intermediate filaments, was essential for the maintenance of cellular shape (Eng, 1985; Inagaki et al., 1994). Other authors have reported a regionalized immunoexpression of these proteins in the pineal parenchyma, suggesting a relationship with the IC maturity stages (López-Muñoz et al., 1992; Borregón et al., 1993; Suzuki and Kachi, 1995; Safwat, 2012).

Our results in viscacha pineal gland by IHC demonstrated that the IC were regionalized in the parenchyma, and the expression of S-100 protein and GFAP could indicate a neuroectodermal origin. Moreover, the presence of few vimentin-positive cells suggests the existence of a population of immature glial cells, which probably constitutes a reserve population. However, further studies are needed to confirm this relationship. On the other hand, the IC long and branched cytoplasmic processes may indicate the existence of an extensive communication network within the gland. Thus, it is likely that these cells participate in the glandular activity regulation processes. Besides seasonal changes in the number, branching and extension of the cytoplasmic processes, the increase in no. IC-S-100 during winter indicated an active proliferation of IC in relation to a higher glandular activity. In contrast, the no. IC-S-100 decreased in agreement with the lower glandular activity observed in summer. In immature males, the no. IC-S-100 was significantly lower compared with the adult values. This demonstrated a relation between cellular proliferation and glandular activity. Further studies including PCNA and apoptosis are needed to show the possible proliferative activity of the IC in immature and

adult pineal glands. The results of double-IHC might indicate the existence of IC in different functional stages, probably related to the needs of the cellular microenvironment. The morphometric variations in the proteins analyzed probed to be more salient in the adult males compared with the immature viscachas, indicating a direct correlation between the expression of the S-100 protein and GFAP, and animal age.

Although the IC, have been considered support and nutrition elements in different species, our results account for the participation of these cells in seasonal glandular activity. Therefore, the pineal gland of viscacha constitutes an interesting model for further studies of IC.

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