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Light and Ultrastructural Immunocytochemical Study of Somatotropic Cells (GH Cells) in Ovine Adenohypophysis: Lactation and Weaning Influences

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With 4 figures and 1 table

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Summary

The influence of lactation period and weaning on the distribution, number, and structure of somatotropic cells (GH cells) in ewes was studied using immunocytochemical procedures for light and electron microscopy, as well as morphometric and stereological techniques. The adenohypophyseal gland of 12 ewes of the Segureña breed in different stages of milk production and weaning was studied, while three ewes in anoestrus served as controls. The size of secretory granules was heterogeneous in all stages, suggesting that this characteristic is not related to functional activity. During lactation and weaning the size of GH cells decreased, while the number of 'synthesis cells' increased. The rough endoplasmic reticulum and Golgi complex appeared more developed and small secretory granules showed lower gold particle labelling. These data suggest that GH cells are more active during lactation, particularly during late lactation.

Introduction

Growth and milk production, two of the most important activities of sheep farming, depend, respectively, on the secretion of two hormones, somatotropin (GH) and prolactin (PRL). These hormones are synthesized by two different cell types, somatotropic cells (GH cells), which secrete GH and mammotropic cells (PRL cells), which secrete PRL (Kurosumi, 1991).

Galactopoietic activity has been attributed to GH cells [after the administration of growth hormone-releasing factor (GRF) or exogenous GH] in cattle, sheep (Hart et al., 1985; Enright et al., 1986, 1988, 1989; Peel and Bauman, 1987) and goats (Mepham et al., 1984). These reports corroborate previous suggestions of Cowie (1971) and Hart and Flux (1973) concerning the influence of GH on milk production in goats. Other authors (Vasilatos and Wangsness, 1981; Gluckman et al., 1987) have suggested that the secretion of GH during peak milk production has a functional meaning. On the other hand, the decreased plasma levels of GH during lactation in rats (Saunders et al., 1976) are due, according to Porter et al. (1990) and Scheitauer et al. (1990), to a decrease in GH cell number and activity. These findings suggest that GH does not influence milk production in rats (Saunders et al., 1976; Porter et al., 1990) and humans (Scheitauer et al., 1990).

In this study we tested the hypothesis of a direct influence of GH in milk production in sheep, by assessing the expected increase in the number and activity of GH cells during lactation, and their gradual decrease during weaning, using immunocytochemical techniques for light and electron microscopy.

Materials and Methods

The adenohypophysis of 15 ewes of the Segureña breed was studied. The individuals were distributed into three groups. The first consisted of three 1 year old ewes in anoestrus (control). The second group was composed of six ewes in lactation; three of them 7 days into lactation (early lactation) and the other three in the third month (late lactation). These last animals were sacrificed immediately after suckling. The third group comprised six ewes after weaning, three animals in the third month of lactation and 1 day after weaning and three animals 7 days after weaning.

The animals were anaesthetized with Penthotal and the head was perfused through both carotids under direct systolic pressure for 15–20 min. Then a rinsing solution was introduced for 1 min (0.8% sodium chloride + 0.4% dextrose + 0.8% sucrose + 1% sodium nitrite in phosphate-buffered saline (PBS) 0.12 M, pH 7.4) followed by a fixing solution of 4% paraformaldehyde and 0.5% glutaraldehyde in PBS 0.12 M, pH 7.4. The adenohypophysis was sectioned into two halves along the sagittal plane, with one being used for light microscopic study and the other for an ultrastructural study. All samples were obtained between April and July.

Light microscopy

The sagittal half of each gland was washed in cacodylate buffer (0.2 M, pH 7.2 with 1% sucrose) for 1 h, refixed in sublimate formalin (Gerad's liquid), and embedded in poliwax (Difco, MVS, Buckingham, UK). Sixty sagittal serial sections (4–5 μm thick) were mounted on slides. Double immunolabelling was carried out by combining the indirect immunoalkaline phosphatase technique (AP) with the ABC immunostaining technique (AP-ABC). In the first immunoreaction, the samples were incubated with rabbit anti-ovine GH (1:1000, UCB, Brussels, Belgium) for 60 min at 32°C, after treatment with normal goat serum (Sigma, St Louis, USA) at 1:30 dilution. Biotinylated swine anti-rabbit IgG (DAKO, Santa Barbara, USA) was used as the second antibody at 1:250 dilution in Tris-buffered saline (TBS; 0.05 M pH 7.6) for 20 min. The slides were then rinsed and incubated for 30 min with the anti-biotin AP complex (Sigma) diluted 1:100 in the same buffer for 30 min. After rinsing with TBS, AP was developed with naphthol-AS-MX-phosphate

(0.1 M Tris buffer pH 8.2 containing 0.24 mg levamisol (Sigma) and 1 mg fast blue salt (Sigma)). After the first immunoreaction the sections were then treated for 1 h at 60°C with paraformaldehyde vapour. Samples from the second immunoreaction were incubated with rabbit anti-ovine PRL (1:1000, UCB) for 60 min at 32°C. Swine anti-rabbit IgG (Sigma) was used as the second antibody at 1:250 dilution. The reaction was then continued with PAP soluble complex (Sigma) at a dilution of 1:100. The reaction was visualized with 3-3′ diaminobenzidine tetrahydrochloride (DAB, Sigma). The slides were not counterstained, and were mounted with glycer gel (Dako).

Electron microscopy

The remaining half of each gland was divided into three portions: anterior, middle and posterior. Samples were taken from the central and peripheral regions of each portion, and then fixed by immersion in 3% glutaraldehyde in PBS pH 7.4, and post-fixed in 1% osmic tetroxide for 2 h at 0°C. The tissue was then dehydrated in graded alcohol and propylene oxide, and embedded in Epon.

The immunocolloidal gold complex method of Roth (1983) was followed. Ultra-thin sections were etched for 30 min with sodium metaperiodate (Bendayan and Zollinger, 1983). After washing with TBS, the sections were successively incubated at 32°C in the following solutions diluted in TBS: normal goat serum, 1:30 for 20 min (Sigma); rabbit anti-ovine GH, 1:1000 for 60 min; goat anti-rabbit immunoglobulin coupled to Au 10 nm (1:40, Serva, Germany) for 30 min. Ultra-thin sections were treated for 1 h at 60°C with paraformaldehyde vapour after the first immunogold stain according to a slight modification of the method by Wang and Larsson (1985). The second immunogold reaction was completed using the alternative primary antibody (anti-ovine PRL, 1:5000) and a different size of colloidal gold particle (20 nm). The grids were routinely contrasted with uranyl acetate and lead citrate.

For both light and electron immunocytochemistry the specificity of the staining was checked as follows: (1) incubation of normal rabbit serum instead of specific anti-serum; (2) adsorption of the specific anti-serum with its corresponding homologous hormone. No immunostaining was recognized in these controls.

Morphometry

The numerical densities of GH cells were calculated in five fields of 10 000 μm^2 per section chosen randomly from 10 sections of the *pars distalis*, separated from each other by 50 μm. A total of 50 fields per animal was evaluated.

The other parameters (cell area and diameter for light microscopy, and numerical density of the secretory granules and granular diameter for electron microscopy) were calculated with an Image Analyser Computer (IMCO 10 Kontron Bildanalyse, Eching, Germany), using the Microm Image processing software (Microm, Madrid, Spain). Twenty immunoreactive cells of each animal were chosen randomly from the different sections separated from each other by 50 μm, in order to determine the cell area and diameter. Twenty micrographs per group of whole GH cells were analysed at a final magnification of $\times$14 800, in order to determine the size of a minimum of 20 secretory granules per hormone and cell, and the numerical density (Nv) of the secretory granules in μm^3. Nv was estimated according to the point-counting method (Weibel and Gomez, 1962), in which $Nv = K/\beta \times Na^{3/2}/Vv^{1/2}$, Na being the number of secretory granules per unit of cytoplasmic area, Vv the density volume of the secretory granules, and K and β are constants calculated according to Weibel and Gomez (1962) ($K = 1.05$ and $\beta = 1.38$ for this study). The density of granule volume in μm^3 (Vv) was determined from photographs magnified $\times$11 500, following Weibel's (1979) stereological method of area analysis, in which $Vv = Ao/At = Vo/Vt$; Ao corresponds to the total area of the profiles due to a particular secretory granule, whereas At is the total area of the section, Vo is the volume occupied in the section, and Vt is the total volume of the section.

The secretory granules were classified, according to their diameter, into three classes: small (<300 nm), medium (small and large granules) and large (>400 nm). GH cells were considered to be of the 'synthetic' type when the rough endoplasmic reticulum (RER) and Golgi complex (GC) were moderately or well developed and the granule content was medium or low. The 'storage' type corresponds to cells with the RER and GC poorly or moderately developed but with a high granule content. The 'immature or inactive' type corresponds to a poorly developed RER (and GC), and a low granule content. The same criteria were previously used by Racadot (1963) and Sánchez et al. (1992, 1993).

For the quantification of gold particle labelling density (number of gold particles per unit of sectional area of the granules; Bendayan, 1984), the number of gold particles was calculated automatically with the Image Analyser computer in at least 30 randomly selected granules from 16 GH cells of each group using micrographs at a final magnification of $\times$113 800.

The statistical analyses were carried out with the STATISTIX 3.5 program (Analytical Software, Florida). The differences between means were determined by applying the Scheffe and Tukey test and significance is reported at the level of $P < 0.01$.

Results

Light microscopic

GH cells were distributed homogeneously throughout the *pars distalis* of the adenohypophysis in all of the animals. They were found near large blood vessels except in the rostral and medial portions of the gland.

GH cells generally formed groups or 'palisades' in the dorsocaudal portion of the gland, but appeared isolated or clustered into small groups in the ventrocaudal portion. Cell area and diameter decreased from anoestrus to the following stages (Table 1), suggesting an increasing activity of this cell type from anoestrus onwards. There were no significant differences in the cellular density between lactating and post-weaning groups. The GH cells varied in shape but were mostly spherical or oval.

Ultrastructural study

The RER and GC typical of 'synthetic cells' were seen in 10% of the cells during anoestrus and early lactation, in 35% of the cells during late lactation (Figs 1, 2), and in 40% of cells after weaning (Fig. 3). The other cells corresponded to the 'storage' type, as the typical RER and GC of 'immature or inactive' cells were not seen. RER was made up of narrow cisternae (Fig. 1) sometimes slightly dilated (Fig. 2), or concentric cisternae distributed throughout the cytoplasm with scarce secretory granules after weaning (Fig. 3). The volume density of the secretory granules decreased in early lactation and weaning although not significantly, while the numerical density only increased in early lactation (Table 1).

Table 1. Mean ± standard error of different somatotropic cell (GH cell) parameters of adult ewes in anoestrus, lactation (early (EL) and late (LL)) and weaning (1 day (AW1) and 7 days (AW7) after weaning)

	Anoestrus	EL	LL	AW1	AW7
CD	13.52 ± 2.44**	11.71 ± 2.24	11.37 ± 2.36	11.08 ± 1.92	11.71 ± 3.23
CA	90.10 ± 23.91**	61.43 ± 17.02	60.40 ± 14.16	62.52 ± 13.57	56.43 ± 20.07*
NvC	20.74 ± 9.96	18.84 ± 5.86	21.46 ± 7.75	20.22 ± 7.76	22.00 ± 6.92
GD	421 ± 84.50**	380 ± 85.30	378 ± 78.98	365 ± 91.03	363 ± 82.42
VvG	15.29 ± 5.57	12.38 ± 6.22	16 ± 7.89	12.08 ± 12.20	9.86 ± 5.41
NvG	1.70 ± 0.58	2.27 ± 0.88*.**	1.73 ± 0.45	1.61 ± 0.81*	1.89 ± 0.48
LD	395 ± 78.24**	325.9 ± 32.99	318.5 ± 12.65	346.4 ± 34.50*	333.3 ± 43.41

*Significant differences between lactation or weaning groups; ** significant differences between anoestrus and lactating or weaning groups (* $P < 0.01$). CD, cellular diameter in μm; CA, cellular area in μm^2; NvC, numerical density of GH cells in 10 000 μm^2; GD, granular diameter in nm; VvG, volume density of secretory granules in μm^3; NvG, numerical density of secretory granules in μm^3; LD, labelling density or number of gold particles per unit of sectional area of the secretory granule.

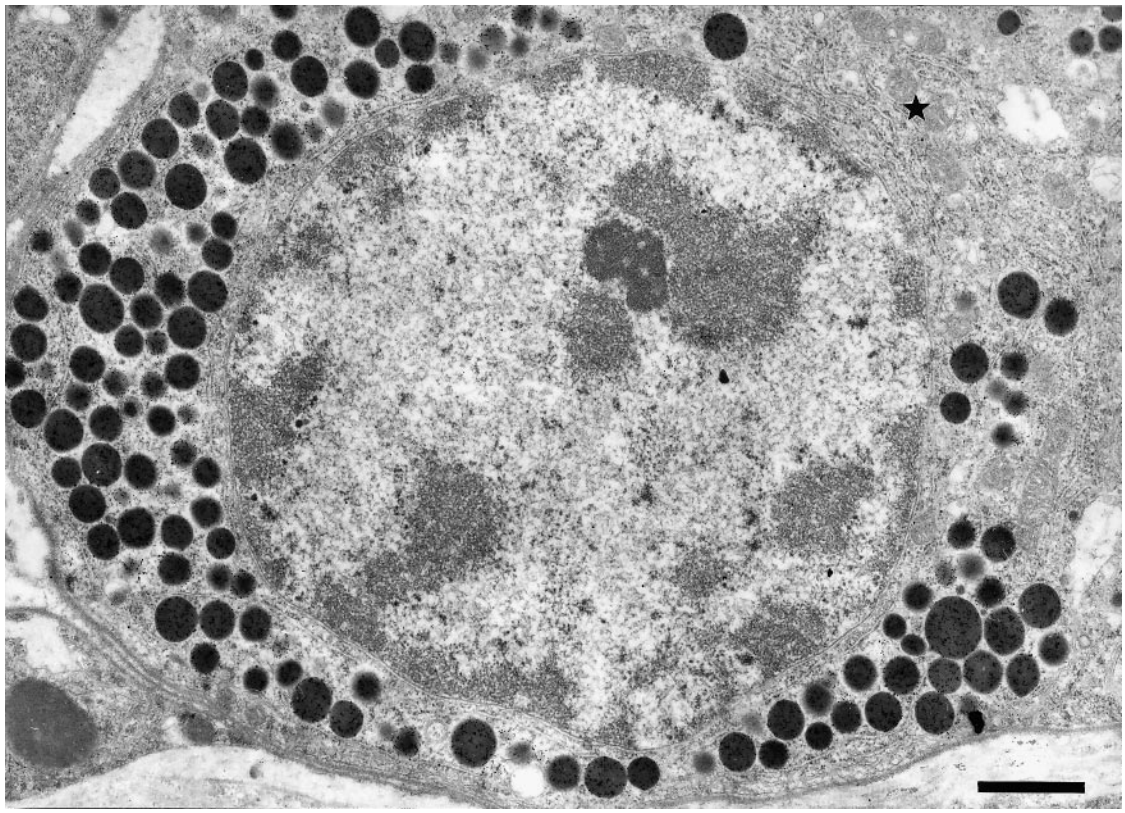

Fig. 1. Somatotropic cell (GH cell) with moderate 'synthetic' activity and moderately developed RER, parallel cisternae (star) and a moderate density of secretory granules. Ewe, 3 months in lactation. Bar = 1 μm.

Half the GH cells were well granulated in anoestrus, 30% moderately granulated and 20% poorly granulated (Fig. 4). The number of well-granulated cells increased (70%) in early lactation and decreased in late lactation (35%), while the number of moderately granulated cells increased (65%) in the latter period (Fig. 4). One day after weaning these average numbers were similar to those observed in anoestrus, while 7 days after weaning the number of moderately granulated cells increased to 50% (Fig. 4).

The diameter of the secretory granules decreased in the lactating and weaning groups, in which they showed significant differences from ewes in anoestrus (Table 1), with a prevalence towards medium- and large-sized granules (> 300 nm).

The average labelling density decreased during lactation and increased slightly 1 day after weaning, before decreasing again 7 days after weaning. There were significant differences between the anoestrus group and both the lactation and weaning groups (Table 1). Significant differences also occurred between both subgroups of weaning animals (Table 1).

Lysosome-like structures with a moderately electron-dense content were abundant in lactation, and their number and size increased 7 days after weaning. Crinophagy figures and lipid drops were also frequent 7 days after weaning.

Discussion

The distribution of GH cells observed in lactating and weaning ewes was similar to that observed in anoestrus, with a predominance in the dorsocaudal regions of the hypophysis, as described by Tassell and Kennedy (1972), Ortman (1987) and

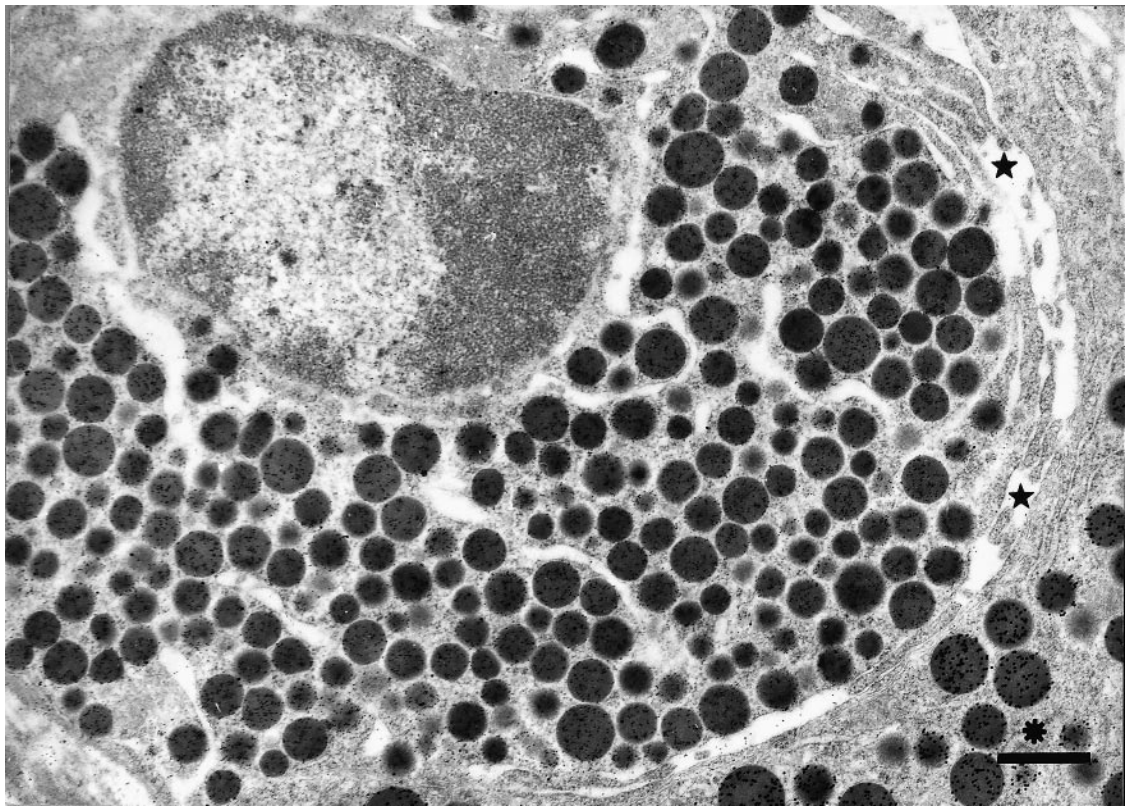

Fig. 2. 'Synthetic' somatotropic cell (GH cell) at the beginning of 'storage' with moderately developed RER (star) and abundant granules. Mammotropic cell (PRL cell) to the right (asterisk). Ewe, 3 months in lactation. Bar = 1 μm.

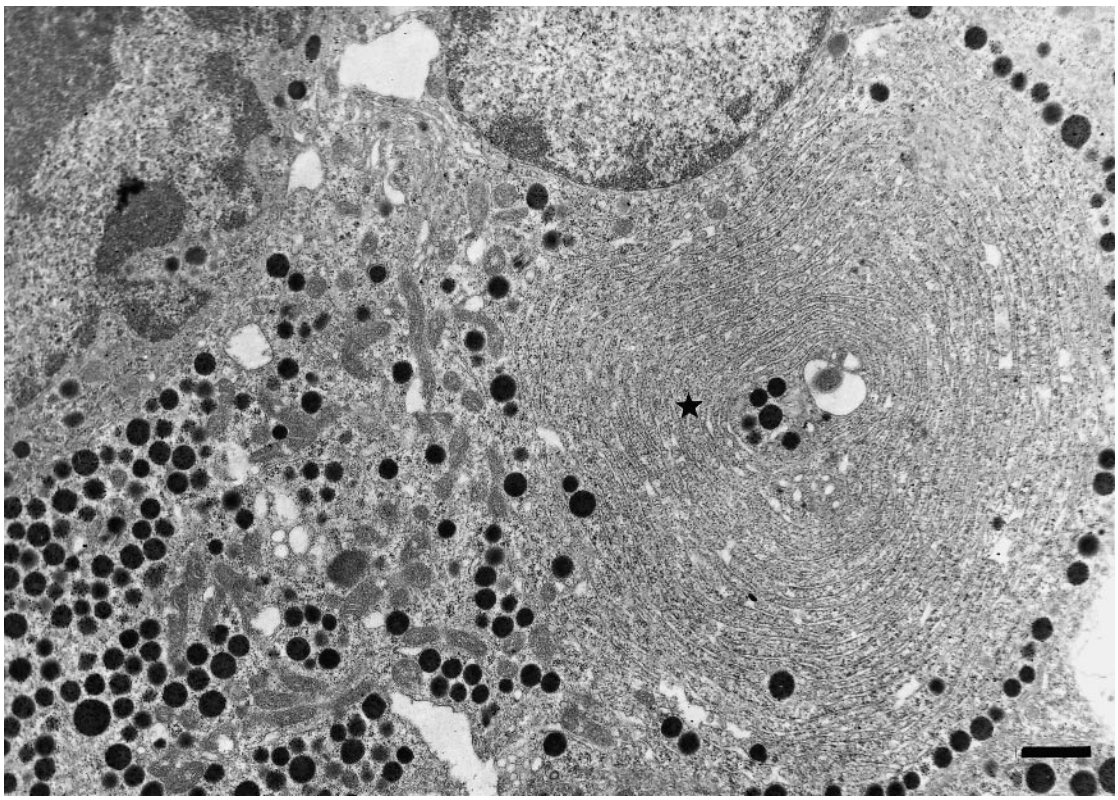

Fig. 3. Beginning of 'synthetic' activity of somatotropic cell (GH cell) with scarce granules and well-developed RER (star) close to a well-granulated GH cell (left). Ewe, 3 months in lactation and 1 day after weaning. Bar = 1 μm.

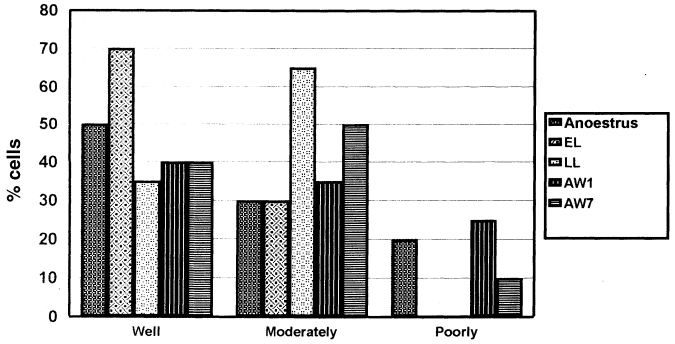

Fig. 4. Proportions of well-, moderately and poorly granulated somatotropic cells (GH cells) in ewes in anoestrus, early lactation (EL), late lactation (LL) and 1 (AW1) and 7 days after weaning (AW7).

Bernabé et al. (1995–1996). The number of GH cells remained constant throughout lactation, unlike those observed by Cinti et al. (1985) and Porter et al. (1990) in rats, and Navarro (1987) in goats, where the numbers decreased.

During lactation and weaning the size of the GH cells was smaller than during anoestrus, suggesting that the hormone is secreted during lactation. High levels of plasma GH during peak lactation is thought to reflect the effect of GH in stimulating galactopoiesis (Gluckman et al., 1987). Carretero et al. (1990) observed the opposite situation in ovariectomized rats, where a decrease in the plasma levels of GH was accompanied by an increase in the area and number of GH cells. The structural results reported here for GH cells agree with these conclusions regarding the galactopoietic effect of GH and its releasing factor in ruminants (Hart et al., 1985). In other species GH cells do not seem to influence lactation, such as rodents and humans, in which plasma levels of GH decrease during gestation and lactation. Additionally, the number of GH cells is lower which, in turn, secrete a smaller quantity of hormone (Porter et al., 1990; Scheitauer et al., 1990).

Morphological data suggest that in goats GH cells participate in early lactation. This is corroborated by the decrease in granule size and granule labelling density during early lactation, suggesting that the hormone is released to a greater extent during this stage. Data referring to the Vv and Nv of the secretory granules suggest that GH cells are much more heavily granulated during early lactation (70%), than during late lactation (35%) and weaning (40%), when the number of secretory granules decreases, and RER and GC increase in size. However, these changes do not seem to affect the biosynthetic activity during anoestrus and early lactation, as 10% of cells are 'synthetic' in both phases.

During late lactation and weaning the percentage of 'synthetic' cells increased, suggesting that synthetic activity is enhanced during late lactation. The fact that during this phase the hormone is stored in smaller granules suggests that the newly synthesized hormone is frequently released.

Labelling density was significantly lower during lactating and weaning than in anoestrus, suggesting that the levels of hormone stored during the former phases are lower, as observed by Gómez-Marín (1994) in pre-pubertal lambs.

These findings together with the decrease in cell area during lactation point to the participation of GH in lactation, particularly during late lactation. Sánchez et al. (1992) observed increased synthetic activity in lactating goats, which agrees with the increased plasma levels of hormone observed by Hart and Flux (1973) and Gluckman et al. (1987). In contrast to the releasing pattern found for PRL (Gómez-Marín, 1994), the suckling stimulus does not seem to influence the release of GH, as no degranulated cells were observed during lactation. This finding agrees with that put forward by Hart and Linzell (1977). This release of GH after weaning and the presence of degranulated cells remain unexplained. Future studies should test the influence of the high levels of PRL (Esquifino et al., 1989) at the beginning of weaning, or even the influence of sexual steroids (Gopinath and Kitts, 1984) which are associated with the beginning of oestrous, on the release of GH hormone. After weaning lysosomes and crinophagic figures, both of which contribute to regulating cell hormone levels, are frequently seen particularly after 7 days.

In conclusion, the morphological data observed in GH cells from ewes at the milk production stage indicate an increase in GH synthesis and release compared with the levels observed at anoestrus. The initial hypothesis is therefore partly corroborated, as no numerical increase in GH cell numbers was observed.

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