Histol Histopathol (1997) 12: 729-734

Histology and Histopathology

Expression of argyrophilic nucleolar organizer regions (AgNORs) in the Harderian gland of male and female hamsters during postnatal development

A. Fernández-Suárez[1], S. Carbajo[2], J.M. López[1] and E. Carbajo-Pérez[1]

[1]Department of Morphology and Cell Biology, Faculty of Medicine, University of Oviedo, Oviedo and

[2]Department of Human Anatomy and Histology, Faculty of Medicine, Universidad de Salamanca, Salamanca, Spain

Summary. The expression of argyrophilic nucleolar organizer regions (AgNORs) was studied in the different secretory cell types of the Harderian gland of male and female Syrian hamsters during postnatal development. Mean AgNOR area was calculated for each cell type in paraffin sections from 7-, 14-, 21-, 28-, 45- and 90-day-old animals. AgNOR content was similar in male type I-cells and in female cells, decreasing in both cell types from the 7th to the 14th day, increasing afterwards at the 21st day, and remaining at relatively stable levels from that point to the end of the study. AgNOR content of male type II-cells was greater than in any other cell type studied, and was greater in 45- and 90-day-old animals than in 28-day-olds. Changes of AgNOR content in type I-cells of male and female hamsters during the first two weeks seem to be related to changes in proliferative activity while metabolic activity might be responsible for changes taking place later on. Our results also support that male type I- and type II-cells have a different biological behaviour and that type II-cells are far from being degenerating cells.

Key words: AgNOR, Harderian gland, Postnatal development, Hamster

Introduction

Nucleolar organizer regions (NORs) can be identified in the nuclei of interphase cells by a silver impregnation that selectively stains some acidic proteins (NORAPs) associated to ribosomal genes (Ploton et al., 1986). Variations in the expression of argyrophilic nucleolar organizer regions (AgNORs) have been related to a number of important biological events; they have been found to parallel proliferative activity (Derenzini et al., 1990; Carbajo et al., 1993a; Carbajo-Pérez et al., 1993), differentiation or maturation in different cell types (Smetana and Likovsky, 1984; Carbajo et al., 1993b) as well as metabolic activity (Jotsa et al., 1993).

The Harderian gland is an orbital gland which is particularly well developed in rodents. Marked sex differences regarding both structure and function are found in the Harderian gland of adult Syrian hamsters, while glands of both males and females closely resemble each other during the first three weeks of postnatal life (review by Payne, 1994). A number of morphological studies have been made to characterize the postnatal development of this gland (López et al., 1992a,b, 1996). Despite the effort made by different researchers, some important questions concerning the development of the Harderian gland remain to be clarified. From a morphological point of view, only one type of glandular cell (type I-like cells) is present in the gland of mature female hamsters, while two types of secretory cells (type I- and type II-cells) are present in the adult male gland. Type II-cells, cells only present in male glands, appear during the fourth week of postnatal life.

It is well documented that the population of type II-cells is an androgen-dependent population (Payne et al., 1977; Sun and Nadakavukaren, 1980) but the origin and fate of these cells is controversial. Early theories considered type II-cells as degenerating cells that could be secreted *in toto* to the lumina (Hoffman, 1971). More recent studies show that type II-cells constitute a well differentiated and self maintained cell population (Fernández-Suárez et al., 1996). At present only, hypothesis exist on the functional role of type II-cells.

Similarly, differences in the metabolic activity of the male and female gland have been demonstrated by some reports. Differences in protein content (Hoh et al., 1984), lipidic synthesis (Lin and Nadakavukaren, 1982) and in the activity of porphirin (Thompson et al., 1984) and melatonin synthesizing enzymes (Menéndez-Peláez et al., 1987) have been reported between male and female glands, but no morphological correlates have been proposed.

So far, only morphological criteria can be used to

Offprint requests to: Dr. Eduardo Carbajo Pérez, Departamento de Morfología y Biología Celular, Facultad de Medicina, C/ Julián Clavería s/n, Oviedo E-33006, Spain

distinguish the different cell types of the hamster Harderian gland. No immunocytochemical markers or markers of any other kind have been described for these cells. This indeed does not pose a serious problem to the cytologist when describing the gland of mature animals, but it is a serious drawback when trying to trace morpho-functional changes of this gland either during development or as a consequence of variations in the androgenic environment.

The present work was carried out in the hope that AgNOR quantification in the different cell types of the hamster Harderian gland might cast some light into some obscure areas of the postnatal development of this gland, such as the differentiation of type II-cells in the male gland, and in the hope that AgNOR quantification might serve as a marker of the putative differential metabolic activities of type I- and type II-cells.

Materials and methods

Animals

Pregnant Syrian hamsters (*Mesocricetus auratus*) were housed individually in plastic cages under conditions of controlled temperature (24±1 ºC) and maintained on a 14 hr light: 10 hr dark regimen (lights on at 06.00). Animals were provided with water and food *ad libitum*. For breeding purposes, pregnant females were checked daily after the 14th day *postcoitum*. The day of birth of the pups was designated as day 1 of postnatal live. After weaning, animals were caged according to sex (four males and four females each group) and sacrificed by decapitation under ether anesthesia on either the 7th, 14th, 21st, 28th, 45th or 90th postnatal day. Handling of animals an procedures used in this study were in accordance with those established in Spain by the «Real Decreto» 223/1988 of 14th March and the «Orden Ministerial» of 13th October 1989.

Tissue preparation and AgNOR staining

The right Harderian glands were removed, fixed in Carnoy's solution for 30 minutes, dehydrated and embedded in Paraplast. Five μm sections were obtained at 200 μm intervals. Sections were stained with silver as described by Ploton et al. (1986) with minor modifications. After deparaffinization, two glass slides per animal were thoroughly washed in distilled water and incubated for 15 min at 40 ºC in the dark with the silver staining solution. The staining solution was prepared by dissolving gelatin at 20 g/l aqueous formic acid and mixing with twice the volume of a 50% aqueous solution of $AgNO_3$. Samples were washed, lightly counterstained with methyl green, and mounted with DPX.

Data processing

For each cell type the AgNOR content was quantified in twenty five cells from adjacent microscope fields per slide in two slides per animal (50 cells from each cell type and animal were recorded). Measurements were done by using a MIP image analyzer. A x100 aberration-free lens was used. The image projected onto the CCD used for image capture was mapped into a 512x512x24 bit memory. The content of the frame memory is equivalent to 3235 μm^2 of the tissue preparation. Mean AgNOR area per cell for each cell type and time point were calculated in each animal.

Statistics

Comparison of AgNOR-derive parameters from female glands and type I-cells in males at different stages of development was performed by a two way analysis of variance (two way ANOVA, cell type and age). A two way ANOVA (cell type and age) for paired data was performed for data obtained from type I- and type II-cells in males older than 29 days. When significant difference was found according to age, a one way ANOVA followed by a Student-Newman-Keuls test was performed for two group comparisons. When significant interaction between the two variables tested by the two way ANOVA was found, comparisons between cell types at different ages were performed using a Student test.

Results

The AgNOR reaction resulted in clearly identifiable intranuclear black dots (Fig. 1). Regardless of sex and age, AgNORs appeared as small dots scattered throughout the nucleus. Direct observation at the light microscopy of samples from any of the experimental groups of our study showed that the number of AgNOR dots per nucleus was greatly variable (average three to five). AgNOR particles seemed to increased in size with age.

Numerical results of the morphometrical analysis of AgNOR content are shown in Table 1 and Fig. 2. Relevant data were as follows: 1) AgNOR content of male type I-cells and that of female cells was similar at

Table 1. Mean AgNOR area per nucleus (μm^2) in the hamster Harderian gland.

AGE (days)	FEMALE	MALE	
		Type I-cells	Type II-cells
7	2.52±0.06	2.94±0.14	
14	2.14±0.11	2.38±0.07[1]	
21	3.33±0.01[1]	3.35±0.25[1]	
28	3.41±0.04	3.63±0.08	4.14±0.03[2]
45	3.67±0.18	3.57±0.09	4.94±0.35[1,2]
90	3.72±0.28	3.68±0.13	5.13±0.25[2]

Each point represents the mean value obtained from 4 animals±SEM. [1]: $p<0.05$ when compared to the value of preceding age (one way ANOVA, Student-Newman-Keuls test). [2]: $p<0.05$ when compared to the other cell types in animals of the same age (Students test).

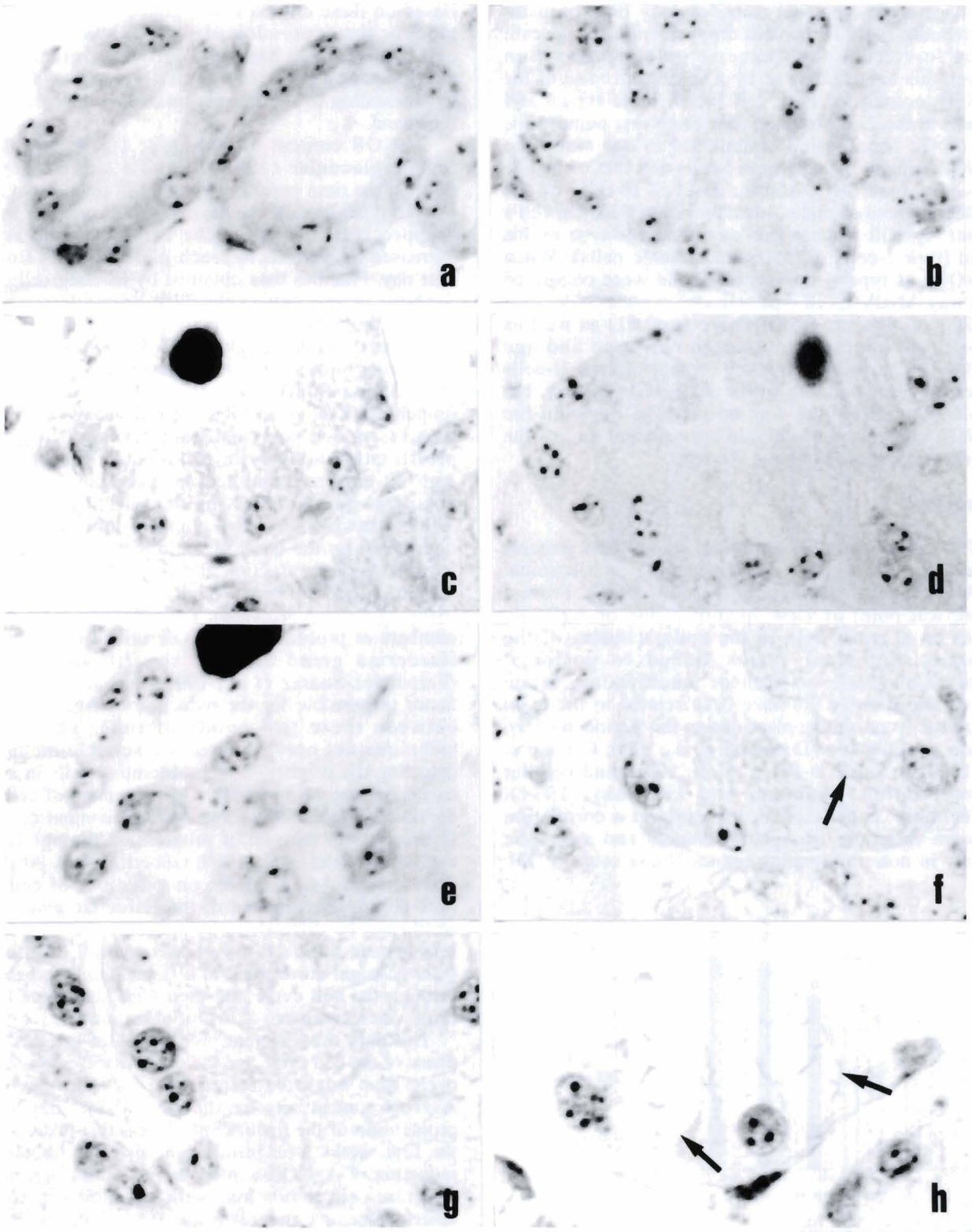

Fig. 1. AgNOR impregnation in paraffin sections of the Harderian gland from 14- (**a**, female; **b**, male), 21- (**c**, female; **d**, male), 28- (**e**, female; **f**, male) and 45-day-old hamsters (**g**, female; **h**, male). Note that cells showing big lipidic droplets (type II-cells) are present in section from 28- and 45-day-old males (f, h; arrows). Varying numbers of AgNOR dots per nucleus (3 to 5) are seen in some of these sections. Note that the silver-stained dots tend to be greater in the older animals. x 900

any stage of development considered. 2) Both in males and females AgNOR content dropped from the seventh to the fourteenth day (decrease only significant in males), this being followed by a significant raise of the AgNOR content of both cell types (male type I and female) at the 21st postnatal day. From that point to the end of the time period considered in our study the AgNOR content of male type I-cells and that of female cells remained fairly stable. 3) Type II-cells of the Harderian gland of males, once they appeared, showed a greater AgNOR content than any other cell type in this gland (type I-cells of males and female cells). When AgNORs of type I- and type II-cells were compared (two way ANOVA, cell type and age) in males older than 28 days, a significant difference ($p<0.01$) as well as significant interaction between cell type and age ($p<0.05$) were found. AgNOR content of type II-cells increased from the 28th to the 90th postnatal day, but significant difference was only found between the AgNOR content of 28-day-old animals and that of the other two age groups (45 and 90 days).

Discussion

The AgNOR proteins are acidic, non-histone proteins associated with loops of DNA encoding for ribosomal RNA (Fakan and Hernández-Verdún, 1986). From a theoretical point of view, quantification of AgNORs might be of great help in the understanding of the complex biological events linked to postnatal maturation. This is so because modifications in the expression of AgNORs have been related to the main biological events taking place during this period, namely, cellular proliferation (Derenzini et al., 1990; Carbajo et al., 1993a,b; Carbajo-Pérez et al., 1993) and cellular differentiation (Smetana and Likovsky, 1984). Furthermore, experimental data support a correlation between variations in AgNOR content and metabolic activity in non proliferating tissues (Jozsa et al., 1993).

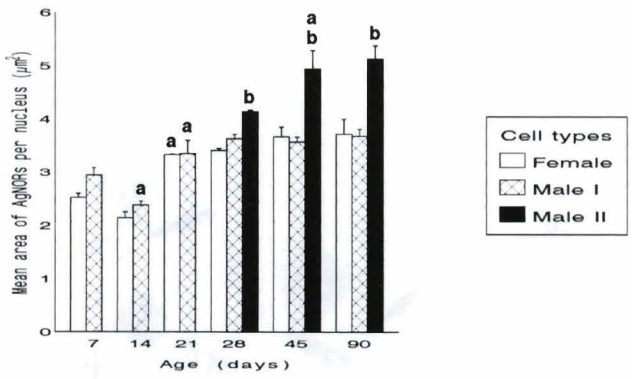

Fig. 2. Expression of AgNORs in the different cell types of the hamster Harderian gland during postnatal development. Values are means± SEM. [a]: statistically different from the value of the same column in the preceding age group; [b]: significantly different from the values of the other columns of the same age group. ($p<0.05$).

Based on these criteria it was considered worthwhile to monitor the expression of AgNORs in the different cell types of the hamster Harderian gland during development, thus trying to give a step forward in the understanding of the morphofunctional development of this gland.

AgNOR content of male type I-cells and that of female glandular cells followed a similar trend all through the time period considered in the present study. In both types of cells the mean AgNOR area per nucleus dropped from the 7th to the 14th postnatal day and increased afterwards to reach plateau levels from the 21st day. Previous data obtained by labeling cells at the S-phase of the cell cycle with bromodeoxyuridine showed that proliferative activity of glandular cells of the hamster Harderian gland follows a steep downward slope from birth to the 14th postnatal day both in males (Fernández-Suárez et al., 1995) and females (unpublished data), and that from that day on there is a progressive and non significant decline of the overall proliferative activity in this gland. The decline in AgNOR content found here between the 7th and 14th postnatal day matches the decline of proliferative activity between these two points of time, and could be interpreted on the basis of the direct correlation found between S-phase fraction and AgNOR content both in normal (Carbajo-Pérez et al., 1993) and neoplastic tissues (Croker et al., 1988). The reduction in the relative numbers of proliferating cells described in the hamster Harderian gland between the 7th and 14th day (Fernández-Suárez et al., 1995) may not be the only factor responsible for the reduction of AgNOR content between these two points of time. The AgNOR technique not only represents a suitable method for detecting the proportion of proliferating cells in a given tissue, but can also provide a measurement of cell cycle duration (Trérè, 1993) since AgNOR content correlates inversely with the rate of progression through the cell cycle (Derenzini et al., 1989; Gilberti, 1995). At present there are no data available on variations of cell cycle time during development of the Harderian gland, but it would not be difficult to hypothesize that in younger, less mature animals, the greater overall proliferative activity might correspond to a faster rate of progression through the cell cycle and then with shorter cell cycle times when compared to that of older animals.

Not only the different proportions of cells at the S-phase of the cell cycle and the putative differences in cell cycle time might be responsible for the reduction of AgNOR content between the 7th and 14th day, but the progression of the maturation process that takes place in the first weeks after birth might also contribute to the reduction of AgNOR content as previously described in different cell populations with advancing maturation stages (Smetana and Likovski, 1984; Yan and Stanley, 1988; Zatsepina et al., 1988). In good agreement with this, studies of the expression of AgNORs in the rat anterior pituitary during development (Carbajo et al., 1993b) have shown that a good correlation between

AgNOR expression and proliferative activity exists, but it was stressed in that report that, taking into account the absolute numbers of proliferating cells in a given tissue sample, it would be difficult to assume that proliferative activity could be the only factor responsible for the expression of AgNORs in developing organs.

In any case, the reasoning followed above cannot be applied to the results obtained in animals older than 14 days since the parallelism between AgNOR expression and proliferative activity is broken down by an increase in AgNOR content between the 14th and 21st postnatal day. Consequently, neither proliferative activity nor cell differentiation can explain by themselves the changes of AgNOR content that take place during the third week of postnatal life. It would be then reasonable to assume that another variable such as metabolic activity might be the phenomenon underlying these modifications.

Recently, Josza et al. (1993) have reported sound experimental evidence supporting that the quantity of AgNORs is a marker of ribosomal protein synthesis activity in non-proliferating cells. Based on this, the rise in AgNOR content of type I-cells between the 14th and 21st postnatal day might reflect changes in metabolic activity which, after rising during the third week of postnatal life, would remain at relatively stable levels from that point of development up to adulthood. Both changes in AgNOR content and the likely changes in metabolic activity would have ultrastructural correlates. Between the 7th and 21st postnatal day type I-cells of males and females resemble each other when studied either under the light or electron microscope, but ultrastructural features of these cells diverge in both sexes from the 21st day. From that point, tubular bodies strongly develop in type I-cells of males while lamellar bodies regress and disappear. On the contrary, strong development of lamellar bodies and disappearance of tubular bodies takes place in the glandular cells of females (López et al., 1992a). Although no morpho-functional correlates have been so far made it is tempting to suggest that when ultrastructural features of type I-cells of males and females begin to settle in their definitive form around the 21st day after birth, the functional potential of these cells is accomplished, and that this corresponds to a stabilization of AgNOR expression.

Our study shows that the mean AgNOR area per nucleus is greater in male type II- than in male type I-cells and than in glandular cells of females. The higher AgNOR content of type II-cells can be interpreted in different ways which, in any case, are far from the theories sustaining that type II-cells are dying or degenerating cells (Hoffman, 1971). The greater AgNOR content of type II-cells would closely correlate with recently reported data pointing to type II-cells as a self-maintained cell population with a higher renewal rate than any other glandular cell of the hamster Harderian gland (Fernández-Suárez et al., 1996). A strong secretory activity involving synthesis and release of great amounts of lipids might also underlie the great expression of AgNORs in type II-cells. In this sense, the age-related increase of AgNOR content found in type II-cells could parallel secretory activity, which presumably increases from the 28th-day, when type II-cells have just appeared, to the 45th and 90th days when these cells are a well established cell population. In any case, the biological meaning of type II-cells is still an obscure question.

Our attempt to trace changes in the expression of AgNORs through postnatal development in the cell populations of the hamster Harderian gland, where important maturative changes and significant modifications in the proliferative rate take place, confirms that interpretation of variations in the expression of AgNORs in normal developing tissues is rather difficult. Our expectations on the application of this technique have not been fully satisfied, mostly because based on functional data a different AgNOR expression in type I-cells of adult males and females might have been expected. However, it seems that the nucleolar activity of type I-cells of either sex have a similar level although this may be involved in different physiological processes. This activity level seems to be achieved at the end of the third week of postnatal life coinciding with the settling of the ultrastructural features proper to male and female type I-cells. It is also clear from the present study that type I- and type II-cells have a different biological behaviour and that type II-cells are by no means degenerating cells. Given that clear differences in AgNOR content are described in type I- and type II-cells, early changes in AgNOR content might serve to monitor early changes in the population of type I- and type II-cells, or even the transition between these two types of cells without using time-consuming electron microscopy techniques in situations such as changes of androgen environment, where important phenotypic changes take place in this gland.

Acknowledgements. This work was supported by the grant (DF94/218-3) from the University of Oviedo. A. Fernández-Suárez was supported by a predoctoral grant (1995) from the University of Oviedo.

References

Carbajo S., Orfao A., Vicente-Billardón J.M. and Carbajo-Pérez E. (1993a). Expression of silver-stained nucleolar organizer regions is coupled to cell cycle in rat thymic cells. Cytometry 14, 46-52.

Carbajo S., Carbajal J.C., Rodríguez J. and Carbajo-Pérez E. (1993b). Cellular proliferation and/or differentiation can condition modifications in the expression of AgNORs in rat pituitary cells during growth. Histol. Histopathol. 8, 317-321.

Carbajo-Pérez E., Alberca V., Vicente-Villardón V., FLores T. and Carbajo S. (1993). Expression of argyrophilic nucleolar organizer regions (AgNORs) can be used to assess cellular proliferation as shown in rat thymic sections. Histochem. J. 25, 548-553.

Croker J., Macartney J.C. and Smith P.J. (1988). Correlation between DNA flow cytometric and nucleolar organizer regions data in non-Hodkin's lymphomas. J. Pathol. 154, 151-156.

Derenzini M., Pession A., Farabegoli F., Trerè D., Badiali M. and Dehan P. (1989). Relationship between interphasic nucleolar organizer regions and growth rate in two neuroblastoma cell lines. Am. J. Pathol. 134, 925-932.

Derenzini M., Pession A. and Trerè D. (1990). Quantity of nucleolar silver-stained proteins is related to proliferative activity in cancer cells. Lab. Invest. 63, 131-137.

Fakan S. and Hernández-Verdún D. (1986). The nucleolus and the nucleolar organizer regions. Biol. Cell 56, 189-206.

Fernández-Suárez A., López J.M., Carbajo S. and Carbajo-Pérez E. (1995). Proliferación celular de la glándula de Harder durante el desarrollo postnatal en el hamster macho. In: IV Congreso de la Sociedad Ibérica de Citometría. Abstracts book. Oviedo. p 228.

Fernández-Suárez A., López J.M., Carbajo S., Alvarez-Uría M. and Carbajo-Pérez E. (1996). New insights into the cytodynamics of the hamster Harderian gland as provided by the bromodeoxyuridine-labelling method. Histol. Histopathol. 11, 351-355.

Gilberti M.F.P., Metze K. and Lorand-Metze I. (1995). Changes of nucleolar organizer regions in granulopoietic precursors during the course of chronic myeloid leukemia. Ann. Hematol. 71, 275-279.

Hoffman R.A. (1971). Influence of some endocrine glands, hormones and blinding of the histology of the Harderian glands of golden hamsters. Am. J. Anat. 132, 463-478.

Hoh J.H., Lin W. and Nadakavukaren M.J. (1984). Sexual dimorphism in the Harderian gland proteins of the golden hamster. Comp. Biochem. Physiol. 77, 729-731.

Josza L., Kannus P., Jarvinen M., Isola J., Kvist M. and Lehto M. (1993). Atrophy and regeneration of rat calf muscle cause reversible changes in the number of the nucleolar organizer regions. Evidence that also in non-proliferating cells the number of NORs is a marker of protein synthesis activity. Lab. Invest. 69, 231-237.

Lin W.L. and Nadakavukaren M.J. (1982). Delta-aminolevulinate synthase activity in the Harderian glands of male and female golden hamsters. Comp. Biochem. Physiol. 71, 539-540.

López J.M., Tolivia J. and Alvarez-Uría M. (1992a). Postnatal development of the Harderian gland in the Syrian golden hamster (*Mesocricetus auratus*): a light and electron microscopy study. Anat. Rec. 233, 597-616.

López J.M., Tolivia J. and Alvarez-Uría M. (1992b). An ultrastructural study of myoepithelium maturation during postnatal development of the hamster Harderian gland. Anat. Embryol. 186, 573-582.

López J.M., Carbajo-Pérez E., Fernández-Suárez A. and Alvarez-Uría M. (1996). Postnatal development of cell types in the hamster Harderian gland. Microsc. Res. Tech. 34, 48-54.

Menéndez-Peláez A., Howes K.A., González-Brito A. and Reiter R.J. (1987). N-acetyltransferase activity, hydroxyindole-O-methyl-transferase activity and melatonin levels in the Harderian gland of female Syrian hamster: Changes during the light-dark cycle and the effect of 6-parachlorophenylalanine administration. Biochem. Biophys. Res. Commun. 145, 1231-1238.

Payne A.P. (1994). The harderian gland: a tercentennial review. J. Anat. 185, 1-49.

Payne A.P., McGadey J., Moore M.R. and Thompson G.G. (1977). Androgenic control of the Harderian gland in the male golden hamsters. J. Endocrinol. 75, 73-82.

Ploton D., Menager M., Jeaesson P., Himber G., Pigeon F. and Adnett J.J. (1986). Improvement in the staining and in the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level. Histochem. J. 18, 5-14.

Smetana K. and Likovsky Z. (1984). Nucleolar silver-stained granules in maturing erythroid and granulocytic cells. Cell Tissue Res. 237, 367-370.

Sun C.Y. and Nadakavukaren M.J. (1980). Effect of testosterone on the female hamster Harderian gland pigmentation and ultrastructure. Cell Tissue Res. 207, 511-517.

Thompson G.G., Hordovatzi X., Moore M.R., McGadey J. and Payne A.P. (1984). Sex differences in haem biosynthesis and porphyrins content in the Harderian gland of the golden hamster. Int. J. Biochem. 16, 849-852.

Trérè D. (1993). Critical analysis of the methods commonly employed in the assessment of cell proliferation: advantages of the NOR silver-stainign technique in routine cyto-histopathology. Anal. Cell Pathol. 5, 191-201.

Yan Y.S. and Stanley W.S. (1988). Effect of differentiating agents on nucleolar organizer region activity in human melanoma cells. Cancer Genet. Cytogenet. 321, 253-262.

Zatsepina O.V., Chelidze P.V. and Chentsov Y.S. (1988). Changes in the number and volume of fibrillar centres with the inactivation of nucleoli at erythropoiesis. J. Cell Sci. 91, 439-448.

Accepted January 28, 1997