Changes in the cell population and in the absolute volume of the stroma of rat submandibular glands during postnatal development

Modificações na população de células e no volume absoluto do estroma de glândulas submandibulares de rato durante o desenvolvimento pós-natal

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The changes that occur in the parenchyma of an epithelial organ during its ontogenesis are controlled by the interaction with its stroma of mesenchymal origin. The objective of this work was to evaluate a possible relation between the evolution of the absolute volume and the number of cells in the stroma and in the parenchyma of rat submandibular glands during postnatal development. From days 2 to 70 the glandular mass increased 1822%, due to great increases 3593% and 1211%, respectively, in the absolute volume of the parenchyma and the stroma. On the other hand, the number of cells for the same compartments increased 1033% and 1203%, respectively. These results indicate that the increase in the individual cell volume of the epithelial cells also plays an important role in the growth of the parenchymal volume. In the stroma, the data indicated that a proportional growth occurs in the number of cells and of the extracellular matrix. The stromal volume to parenchymal volume ratio decreases from 2 to 28, and 35 to 70 days respectively, stabilizing during the period of 28 to 35 days of age. The ratio between the number of cells in the stroma and in the parenchyma increased substantially in the period of 28 to 35 days due exclusively to the increase in stroma cell number, showing stability in the other periods. It should be pointed out that during the period of 28 to 35 days of age, a great process of transformation of the striated duct cells into serous secretory cells of the convoluted granular tubules occurs, which characterizes the beginning of the ductal phase in the rat submandibular gland post-natal development. The changes detected in the stroma during the same period could be related to this fact.

Uniterms: Submandibular gland, development; Morphometry; Stroma; Rat.

Introduction

Rat submandibular glands consist of sero-mucous acini, intercalated ducts, convoluted granular tubules, striated ducts and excretory ducts immersed in a highly vascularized connective tissue [11, 18].

During the first days after birth, these glands are morphologically immature, made up of transitory epithelial structures designated of terminal tubules, and of immature ducts, immersed in an abundant stroma of loose connective tissue [1, 5]. During the first two months of post-natal life, these transitory structures are replaced by permanent secretory structures, the acini and convoluted granular tubules [1, 3, 5, 17]. Concomitant with the enormous growth of the glandular parenchyma, the interparenchymatous spaces of connective tissue undergo a reduction.

It has long been accepted that, during the development of an organ, a crucial interaction occurs between the epithelial tissue responsible for the formation of the parenchymatous structures and the adjacent mesenchyma. This interaction is important for the occurrence of morphogenesis and cytodifferentiation [6, 14].

In fully developed skin and oral mucosa, this interaction between the epithelium and the underlying connective tissue seems to be essential for the epithelium to develop in an orderly fashion and to express its own phenotypes (ref. in Ten-Cate, 1998).

The objective of the present study was to evaluate the changes that occur in the volume density, absolute volume and total number of cells in the stroma of rat's submandibular glands in relation to the parenchyma during the post-natal development.

Material and Methods

In the present study, 42 male Wistar rats (*Rattus novergicus)* were used, furnished by the Central Bioterium of the School of Dentistry, University of São Paulo, Bauru, Brazil. The animals were divided by age into groups of 6 animals: 2, 7, 14, 21, 28, 35 and 70 postnatal days.

Histological procedure

The animals were anesthetized with ethyl ether and their body masses were measured on a Mettler® Toledo PG5002-S scale, and afterwards, the submandibular glands of each animal were dissected and removed and their fresh masses were rapidly determined on a Mettler Toledo® AT261 Delta Rang analytical scale. All the glands were collected between 11:00 and 12:00 a.m.

The glands were fixed in Bouin liquid for 4 hours at room temperature and kept in 80% ethanol overnight. On the subsequent day, the glands were submitted to standardized dehydration procedures in ethylic alcohol, clarification in xylol and embedded in Histosec-Merck(paraffin + plastic resin).

Semi-serial cuts of 5μm thick sections were obtained with a Leitz-Jung® 2045 Multicut microtome, with a 50μm interval between sections. The sections were stained by the Masson-trichrome method, modified by Goldner [12].

Evaluation of processed submandibular gland volume

In morphometry, in order for relative numerical data to be transformed into absolute data, for example volume density of parenchyma into the absolute volume of parenchyma within the organ, it is necessary to determine the gland's processed volume (Vp), that is, its volume after all the histological procedures, as described above.

The processed volume of the submandibular gland of each animal was calculated based on the fresh mass of the organ (m), the density of the organ (d) and the retraction caused by the histological processing (Rf). The processed volume was calculated using the formula: Vp=m/dxRf.

The density (d) of the gland was evaluated in groups of 6 animals by age: 14, 21, 35 and 70 postnatal days, on a Mettler Toledo® AT261 Delta Rang precision scale, containing the accessories to determine the density of solids. Analysis of variance ANOVA) obtained by SigmaStat – Jadel scientific software for windows 1.0 (Jadel Corporation) showed that between 14 and 21 days and between 35 and 70 days not occurred statistical difference (P>0,05). The average density obtained for the groups at 14 and 21 days was 1.056mg/mm^3, and for the groups at 35 and 70 days, 1.07mg/mm^3.

The shrinkage caused by the histological procedures, was evaluated in 12 animals, subdivided into 2 groups at 21 and 70 days old, respectively, using the method described by Taga and Sesso [16]. Analysis of variance (ANOVA) obtained by SigmaStat – Jadel scientific software showed that there was no statistical difference between days 21 and 70. The average retraction obtained was 53.75%, and the retraction factor was 0.4625.

Morphometric evaluation of volume density, total volume and absolute cell number of parenchyma and stroma

These morphometric parameters were evaluated with

a 100X imersion objective and an 8X Zeiss® Kpl eyepiece containing a Zeiss® II integration grid with 10 parallel lines and 100 points in a quadrangular area. Fifty histological fields per animal were selected by systematic-stratified sampling [20], and the following parameters were counted: a) number points (Pi) above the images of parenchyma and stroma and above the entire gland (P); b) number of nuclear images (n) of parenchyma and stroma; and c) number of crossing (Li) of the nuclei of the parenchyma and the stroma with the grid lines.

Knowing the processed glandular volume (Vp), the distance between the grid lines(d), the total area examined(A) and the tickness of the histological section(t), the following morphometric parameters were calculated for the parenchyma and the stroma [2, 20]: a) volume density (Vvi), Vvi=Pi/P; b) total volume (Vti), Vti=Vvi x Vp; and c) absolute cell number (Ni),

Ni= 

Statistical analysis

All the quantitative data obtained for each group were compared pairwise by the analysis of variance (ANOVA) using SigmaStat – Jadel scientific software for windows version 1.0 (Jadel Corporation) . For volume density, the tests were applied after arcsin transformation of the original data. The data concerning glandular mass, total volume and absolute cell number were submitted to curve fitting by linear and exponential regression: $y=a_o+a_1x$ and $y=a.e^{kx}$, respectively, using ARCUS professional statistical analysis software version 2.0 XTc. (Eain E Buchan, 1992) The quality of curve fittings were verified examining the values of determination coefficient (r^2) of the equations.

Results

The evolution of the post-natal body mass, glandular mass and volume density, absolute volume and the total number of cells in the stroma and parenchyma of developing rat submandibular glands are represented in Table 1 and in Figures 1 - 6.

The analysis of the data presented from days 2 to 70, showed that:

a) the body mass grew 3446% (P<0.01) (Table 1 and Figure 1) and the gland mass 1882% (P<0.01) (Table 1 and Figure 1);

b) a growth of volume density of 1.16 times (P<0.01) occurred in the parenchyma (Table 1 and Figure 2). The stroma decrease of 0.66 times (P<0.01) in two phases, one between 2 and 28 days and the other between 35 and 70 days; between 28 and 35 days there was stabilization (P>0.05);

c) the absolute volume of the parenchyma grew 3592% (P<0.01), whereas that of the stroma increased 1203%(P<0.01) (Table 1 and Figure 3). The ratio between stroma and the parenchyma (Figure 4) calculated for this parameter decreased substantially throughout the period analyzed, varying from 0.465 at 2 days to 0.264 at 70 days, that is, a reduction of 43.2% (P<0.01) indicating that the growth in stromal volume was proportionally smaller than in relation to the parenchymal volume;

d) the total number of cells in the gland grew 1066% (P<0.01) (Table 1 and Figure 5), where the number of cells of the parenchyma (Table 1 and Figure 5) increased 1030% (P<0.01) in two phases: the first from 2 to 28

TABLE 1- Evolution of body mass(in g), gland mass(in mg) and volume density(in %), total volume(in mm^3) and total cell number(x10^6) of the parenchyma and stroma of rat submandibular gland, during the first 70 days of postnatal life

Period	2	7	14	21	28	35	70
Body mass	6,57±0,128*	13,55±0,333	18,03±0,868	36,03±1,546	57,55±1,567	81,30±2,178	233,00±5,639
Gland mass	18,46±0,430	30,78±0,625	40,27±2,323	91,49±3,589	125,49±2,607	166,58±2,970	366,29±12,169
Volume density							
Parenchyma	68,25±0,402	71,80±0,241	73,70±0,216	75,80±0,140	76,77±0,254	76,99±0,835	79,16±1,193
Stroma	31,75±0,402	28,20±0,241	26,30±0,216	24,20±0,140	23,23±0,254	23,01±0,835	20,84±1,193
Compartimental total volume							
Parenchyma	5,30±0,110	9,18±0,201	11,62±0,529	29,14±1,260	40,50±0,858	53,95±1,328	121,83±3,594
Stroma	2,47±0,788	3,60±0,693	4,15±0,230	9,30±0,393	12,26±0,284	16,09±0,573	32,18±1,669
Total cell number							
Gland	6,57±0,227	15,03±0,239	18,74±0,794	36,13±1,266	43,09±1,157	48,64±1,070	99,98±2,658
Parenchyma	6,93±0,213	12,18±0,192	16,36±0,638	29,22±0,975	34,77±0,997	37,75±0,994	78,34±2,422
Stroma	1,65±0,058	2,85±0,0540	3,39±0,175	6,91±0,300	8,32±0,191	10,89±0,387	21,63±0,599

* mean ± standard error of mean

TABLE 2- Linear and exponential regression equations of the growth of the rat submandibular glands during the first 70 days of postnatal life

Parameter	Regression equation	Determination coefficient (r^2)	Daily accumulation (Da) Duplication time (Dt)
Gland mass	y = -13.758 + 5.284x	0.987	Da = 5.28 mg
	$y = 25.687e^{(0.043341x)}$	0.886	Dt = 16.0 dias
Compartimental total volume			
Parenchyma	y = -6.013 + 1.772x	0.985	Da = 1.77 mm^3
	$y = 7.552e^{(0.0454727x)}$	0.880	Dt = 15.2 dias
Stroma	Y = - 0.04425 + 0.454x	0.989	Da = 0.45 mm^3
	$y = 3.0733e^{(0.038062x)}$	0.891	Dt = 18.2 dias
Total cell number			
Gland	y = 4.406 + 1.351x	0.992	Da = 1.35 x 10^6
	$y = 12.413e^{(0.033894x)}$	0.872	Dt = 20.4dias
Parenchyma	y = 4.0821 + 1.051x	0.990	Da = 1.05 x 10^6
	$y = 10.115e^{(0.038244x)}$	0.867	Dt = 18.1 dias
Stroma	y = 0.363 +0.300x	0.992	Da = 0.30 x 10^6
	$y = 2.308e^{(0.036337x)}$	0.885	Dt = 19.1 dias

days and the second from 35 to 70 days; in the period from 28 to 35 days, there was a stabilization (P>0.05). The number of cells of the stroma increased 1211% (P<0.01) in only one phase(Table 1 and Figure 5). The ratio between the stromal cell number and parenchymal cell number (Figure 6) exhibited stabilization (P>0.05) between 2 and 28 days, that is, the increase in the number of cells in the parenchyma and the stroma in this period occurred in proportionality; in the period of 28 to 35 days, an increase of 20.5% (P<0.01) occurred in this ratio, indicating a greater growth in the stromal cell number in relation to the parenchymal cell number. During the period of 35 to 70 days, stabilization (P>0.05) also occurred in this regard.

The linear equations type $y = a_o + a_1x$., and exponential equations type $y = a.e^{-Kx}$ corresponding to the evolution of glandular mass, absolute volume and number of stromal and parenchymal cells, obtained by curve fitting on microcomputer are presented in Table 2 with the corresponding coefficients of determination (r^2). Both of the equations, linear and exponential, yielded a good adjustment for all the parameters analyzed. This table also presents the average daily accumulation (Ad) and the duplication time (Td) for each parameters analyzed. The average daily accumulation was obtained directly from the term a_1 of the linear equation, and the duplication time (Td) was calculated from the relation Td = ln2/k, where ln2 is the natural logarithm of 2 and k is the exponent from the exponential equation.

Discussion

In the present research, the glandular mass increased 1882% in the interval from 2 to 70 days, with average daily accumulation of 5.28mg/day obtained by the linear equation, and average duplication time of 16.0 days obtained by the exponential equation. The analysis of

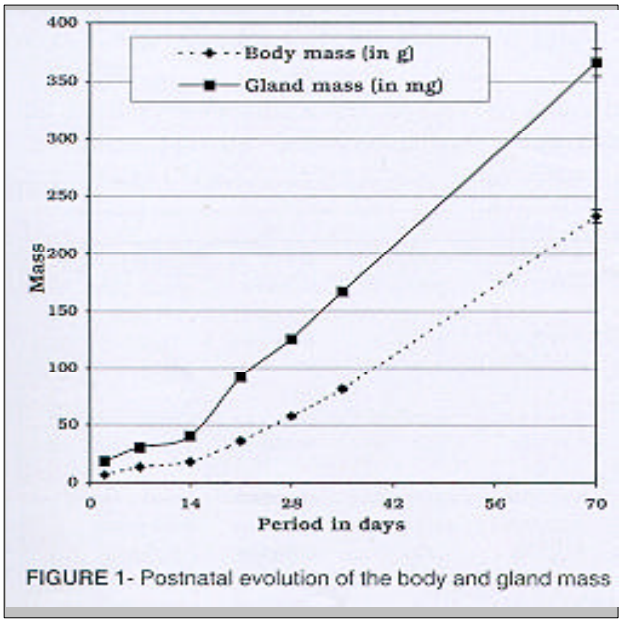

FIGURE 1- Postnatal evolution of the body and gland mass

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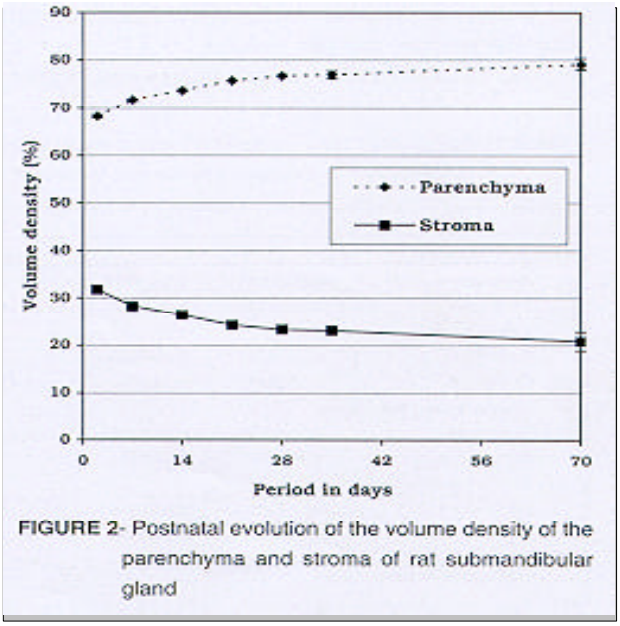

FIGURE 2- Postnatal evolution of the volume density of the parenchyma and stroma of rat submandibular gland

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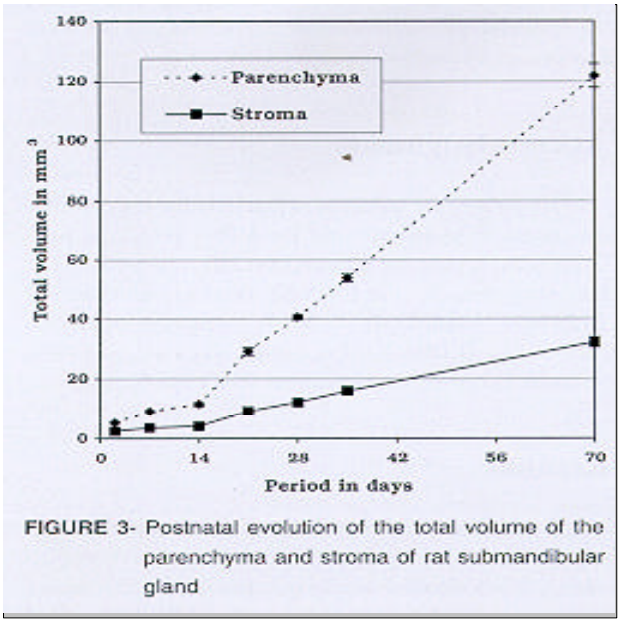

FIGURE 3- Postnatal evolution of the total volume of the parenchyma and stroma of rat submandibular gland

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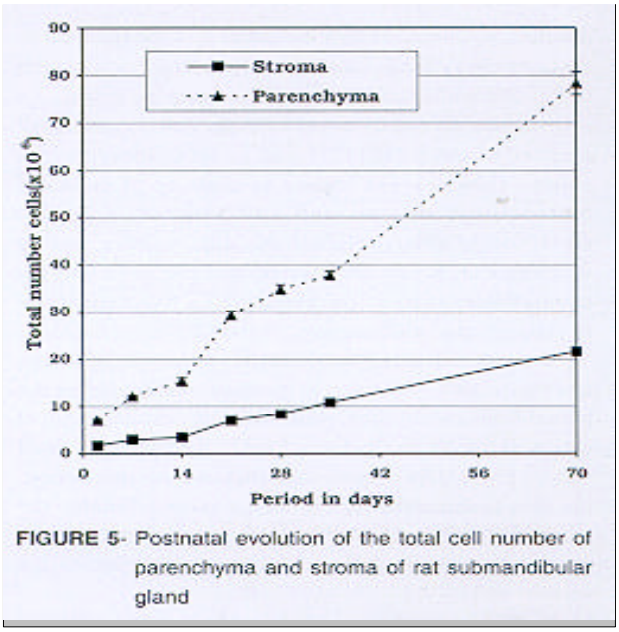

FIGURE 5- Postnatal evolution of the total cell number of parenchyma and stroma of rat submandibular gland

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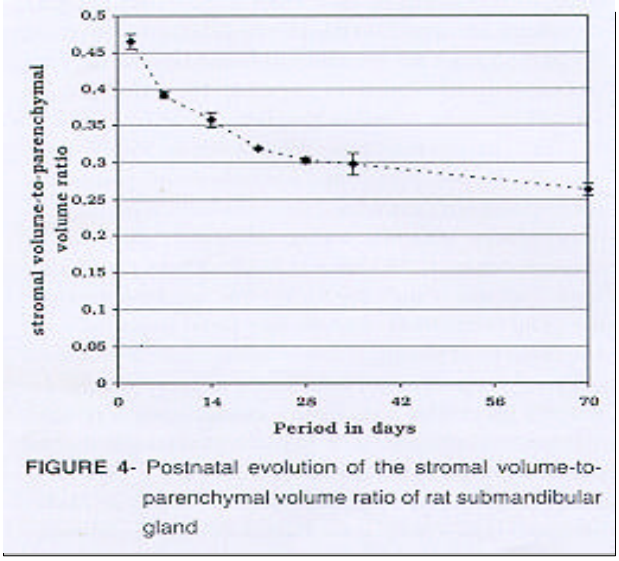

FIGURE 4- Postnatal evolution of the stromal volume-to-parenchymal volume ratio of rat submandibular gland

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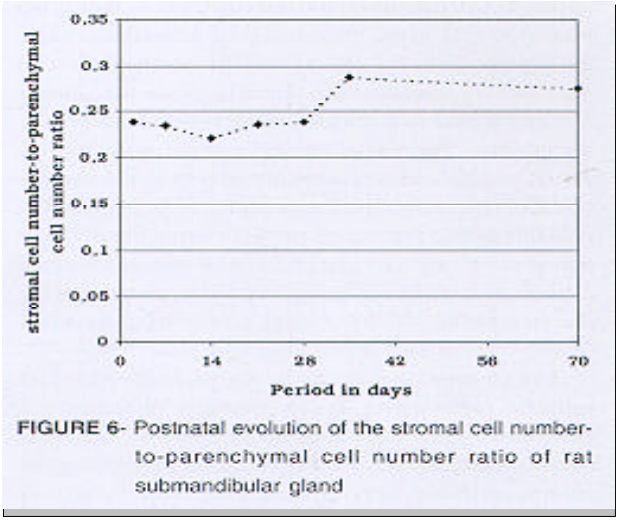

FIGURE 6- Postnatal evolution of the stromal cell number-to-parenchymal cell number ratio of rat submandibular gland

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the latest data showed that, duplication of the glandular mass occurred every 16 days. This marked growth in glandular mass resulted from the increase in the absolute volume of the parenchyma and the stroma.

Since the increase in the parenchyma was significantly greater than the stroma, in terms of volume density there was an inversely proportional relation in the evolution of the two compartments, that is, the parenchyma grew relatively occupying the connective tissue spaces, which can clearly be confirmed during the analysis of the histological sections.

In absolute terms, the parenchyma and the stroma grew 3592% and 1203%, respectively, with the average daily accumulation of volume being 1.77 and 0.45mm^3, and with average duplication time of 15.2 and 18.2 days, respectively. These numbers show that the large increase in glandular volume owed itself mainly to the growth of the parenchyma.

Regarding the number of cells, the number for the parenchyma grew 1033%, and for the stroma 1211%. In the case of the parenchyma, the fact that the percentual (1033%) is significantly less than the growth of its

absolute volume (3592%) indicated that the increase in the parenchymal cell volume has also a large participation in this compartmental growth. Regarding the stroma, an equivalence occurred between the growth percentual of number of cells (1211%) and in its compartmental volume (1203%). The stroma is made up of cells and extracellular matrix, and since the cells do not significantly change their individual volume during development, it can be inferred that the cells and the extracellular matrix of the gland connective tissue grew in proportion to each other.

The ratio calculated between the volume of the stroma and the volume of the parenchyma decreased during the period from 2 to 28 days, while the ratio between stromal cell number to parenchymal cells number remained stable. These data demonstrate that, during this period, the two compartments increased proportionally the number of cells, while the parenchymal cell presented a greater increase in cellular volume in relation to the stromal cells.

In the period of 28 to 35 days, the stromal volume–to-parenchymal volume ratio remained stable, indicating proportionality in the growth among themselves, while the number of stroma cell number–to- parenchymal cell number ratio increased substantially. Since the number of parenchymal cells did not increase during this period, the increase observed of the cell number ratio ion this period resulted entirely from the growth in the number of cells in the connective tissue. It should be pointed that is in this period that occurs the transformation of the in transporting cells of striated ducts in serous secretory cells of the convoluted granular tubules, characterizing the beginning of the ductal phase of post-natal development of the rat submandibular gland [5, 15].

The changes that occur in the parenchyma of an epithelial organ during its ontogenetic development are controlled by their interaction with connective tissue that constitutes its stroma. Various molecules present in the stroma, produced there or acquired, including growth factors absorbed in proteoglycans and the components of its extracellular matrix and of the basal membrane, affect these changes and the parenchymal organization, as well as, directing the formation and arrangement of collagen fibers [9, 10,13]. Some authors have demonstrated the importance of the type and organization of the collagen fibers in this epithelial-mesenchymal interaction [4, 7, 8].

The results of this work, therefore, showed that, the stroma of the rat submandibular gland has an inherent pattern of development in relation to the parenchyma during the post-natal life, with some changes that could be detected at certain periods, which may be related to the epithelial-connective tissue interaction that occurs in key phases of the development.

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Resumo

As mudanças que ocorrem no parênquima de um orgão epitelial durante o seu desenvolvimento ontogenético são controladas pela sua interação com seu estroma de origem mesenquimal. O objetivo do atual trabalho foi o de avaliar uma possível relação na evolução do volume absoluto e no número de células do estroma e do parênquima da glândula submandibular do rato durante o desenvolvimento pós-natal. No período de 2 a 70 dias de idade, a massa glandular aumentou 1822%, às custas de um marcante crescimento de 3593% e de 1211%, respectivamente, no volume absoluto do parênquima e do estroma. Por outro lado, o número de células nos mesmos compartimentos aumentou, respectivamente, 1033% e 1203%. Esses resultados indicaram que o aumento no volume celular individual das células epiteliais também tem papel importante no crescimento do volume do parênquima, e que no estroma, ocorreu proporcionalidade entre o crescimento do número de células e de matriz extracelular. A relação volume do estroma/volume do parênquima diminuiu nos períodos, respectivamente, de 2 a 28 e 35 a 70 dias, e manteve-se estável no período de 28 a 35 dias de idade, enquanto que, a relação número de células no estroma/número de células no parênquima, aumentou substancialmente no período de 28 a 35 dias devido exclusivamente ao aumento no número de células do estroma e exibiu estabilidade nos demais períodos. Convém salientar, que é nesse período de 28 a 35 dias, que ocorre o grande processo de transformação de células dos ductos estriados em células secretoras serosas dos ductos granulosos, o que caracteriza o início da fase ductal do desenvolvimento pós-natal da glândula submandibular do rato. As mudanças detectadas no estroma nesse período podem estar relacionados a este fato.

Unitermos: Glândula submandibular, desenvolvimento; Morfometria; Estroma; Rato.

References

1- ALVARES, E.P.; SESSO, A . Cell proliferation, differentiation and transformation in the rat submandibulaar gland during early postnatal growth. A quantitative and morphological study. **Arch. Histol. Jap**.,v.38, p.177-208, 1975.

2- AHERNE, W.; DUNNILL, M.S. **Morphometry**. London, Edward Arnold,1982.

3- CHANG, W.W.L. Cell population changes during acinus formation in the postnatal rat submandibular gland. **Anat. Rec**.,v.178, p.187-202, 1974.

4- CUTLER, L.S. The role of extracellular matrix in the morphogenesis and differentiation of salivary glands. **Adv. dent. Res**.,v.4, p.27-33, 1990.

5- JACOBY, F.; LEESON, C.R. The post-natal development of the rat submaxillary gland. **J. Anat**., v.93, p.201-16, 1959.

6- GROBSTEIN, C. Analysis in vitro of the early organization of the rudiment of the mouse submandibular gland. **J. Morphol**.,v.93, p.19-43, 1953.

7- HARDMAN, P.; SPOONER, B.S. Alterations in biosynthetic accumulation of collagen types I and III during growth and morphogenesis of embryonic mouse salivary glands. **Int. J. Dev. Biol**., v.36(3), p.423-7, 1992.

8- HAYAKAWA, T.; KISHI, J.; NAKANISHI, Y. Salivary gland morphogenesis: possible involvement of collagenase. **Matrix suppl**., v.1, n.344-51, 1992.

9- HIEDA, Y.; NAKANISHI, Y. Epithelial morphogenesis in mouse embryonic submandibular gland: its relationships to the tissue organization of epithelium and mesenchyme . **Dev. Growth Differ**, v.39, n.1, p.1-8, 1997.

10- ITO, Y.; TAMURA, I. Age-related changes in collagen, laminin and tenascin in the infant rat submandibular gland. **J. Osaka Dent. Univ**., v.31, n.1/2, p.19-27, 1997.

11- LEESON, C.R. Structure of salivary glands. In: **HANDBOOK of Physiology**. Washington, American Physiology Society, 1967. v. 2, p. 463-95.

12- MARTOJA, R.; MARTOJA-PIERSON, M. Técnicas de histologia animal. Barcelona Toray-Masson, 1970.

13- NAKANISHI, Y.; ISHII, T. Epithelial shape change in mouse embryonic submandibular gland: modulation by extracelullar matrix components. **Bioessays**, v.11, n. 6, p.163-7, 1989.

14- RUTTER, W. J.; WESSELS, N.K.; GROBSTEIN, C. Control of specific synthesis in the developing pancreas. **J. nat. Cancer Inst**., v.13, p.51-65, 1964.

15- SRINIVASAN, R.; CHANG, W.W.L. The development of the granular convoluted duct in the rat submandibular gland. **Anat. Rec**., v.182, p.29-40, 1975.

16- TAGA, R.; SESSO, A. Avaliação do número de células de órgãos pela dosagem bioquímica de DNA em homogeineizados e por contagem direta através de métodos morfométricos. **Ciên. Cult**., v.30, p.1232-6, 1978.

17- TAGA, R.; ALVARES, E.P.; SESSO, A. Morphometric studies on terminal tubule and acinar cells in developing submandibular gland of the rat. **Arch. Histol. Cytol**.,v.56, n. 5, p.517-23, 1993.

18- TAGA, R.; ACHÔA, A.S.; PARDINI, L.C. Estudo morfométrico do sistema de ductos interlobulares de glândulas submandibulares de ratos machos e fêmeas. **Rev. FOB**, v.2, p.20-5, 1994.

19- TEN-CATE, A.R. Relações entre epitélio e mesênquima. In: **Histologia bucal.** Cap.5, pp.67-74, 2ª ed., Guanabara & Koogan SA: Rio de Janeiro, 1988.

20- WEIBEL, E.R. Stereological principles for morphometry in electron microscopic cytology. **Int. Rev. Cytol**., v.26, p.235-302, 1969.