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# INCREASE IN THE CELL VOLUME OF THE RAT SUBMANDIBULAR GLAND DURING POSTNATAL DEVELOPMENT

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## ABSTRACT

The increase in the volume of acinar and convoluted granular tubule cells of the rat submandibular gland during the first 96 days of postnatal development was studied by morphometry. Absolute glandular mass increased significantly by 2,364% during the period studied, whereas the relative glandular mass decreased 0.21-fold from day 2 to day 7, remained stable from day 7 to day 28, and showed a significant 0.31-fold decrease between 28 and 96 days of life. Acinar cell volume did not increase during the first two weeks of postnatal development, but showed a significant increase (168%) by the end of the study period. Convoluted granular tubule cell volume increased by 132% between 28 and 96 days of postnatal life. The cell volume growth rate (daily gain) was similar for both cell types (about 14.7 $\mu m^3$/day). These results show that there is a marked increase in the volume of acinar cells and of convoluted granular tubule cells during the postnatal development of the rat submandibular gland.

**Key words:** Acinus, convoluted granular tubule, submandibular gland development

## INTRODUCTION

The parenchyma of the rat submandibular glands consists of two structural secretory compartments: the acini and the convoluted granular tubules. The acini are the predominant structures and consist of seromucous cells, whereas the convoluted granular tubules, which are interposed between the intercalated and striated ducts, consist of serous cells [12,16,17,21,25,28].

At birth, the submandibular glands of the rat still lack these definitive secretory structures and are composed of transitory secretory units called terminal tubules which consist predominantly of type I and, to a lesser extent, of proacinar or type III cells, as well as an immature system of intercalated, striated and excretory ducts [1,3,4,8,13,15,16].

During the first month of postnatal life, the acinar cells differentiate from the proacinar or type III cells and show a marked growth, eventually replacing the terminal tubule or type I cells that gradually simplify their morphology and are incorporated into the intercalated ducts or die through apoptosis [1,4-6,8,14-16,30]. During the same period, the striated ducts grow substantially and become highly convoluted [16]. After day 21 of postnatal life, some of the striated duct cells start to exhibit small granules in the apical cytoplasm, an event that represents the beginning of cytodifferentiation of the convoluted granular tubule cells [7,11]. This transformation of striated duct cells into granular tubule cells is highly evident by the fourth week of development. The maturation and stabilization of this secretory cell population are complete after 12-14 weeks of development [23].

Kinetic studies of the growth of acinar and convoluted granular tubule cells using $^3$H-thymidine labeling have shown the important role of proliferative activity in the growth of these two glandular compartments [1,4,23]. In addition, in a previous study we observed a significant increase (181%) in the cytoplasmic volume of seromucous acinar cells of rat submandibular glands from day 15 to day 30 of postnatal life [26]. This increase resulted primarily from the accumulation of rough endoplasmic reticulum membranes and secretory material. This observation suggested that an increase in the individual cell volume of the various cell types could play an important role in the growth of the gland during postnatal development.

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In this study, we examined the contribution of increases in the volume of acinar and convoluted granular tubule cells to the growth of the rat submandibular gland during the first 96 days of postnatal life.

## MATERIAL AND METHODS

Forty-eight male Wistar rats (*Rattus norvegicus*) 2, 7, 14, 21, 28, 35, 70 and 96 days old (6 rats/group) were obtained from the colony maintained by the Central Animal House in Bauru School of Dentistry. The pups were reared from birth in litters of six and were weaned at 21 days of age. Pregnant rats, mothers with pups and weaned pups received pelleted Purina chow and water *ad libitum*. After anesthesia with ketamine hydrochloride (10 mg/100 g body weight) plus xylazine hydrochloride (1 mg/100 g body weight), each animal was weighed. The submandibular glands of each rat were then carefully dissected and rapidly removed and weighed. The glands were always collected between 10:00 and 12:00 a.m. to avoid circadian variations [2]. After weighing the glands were fixed in Bouin solution at room temperature for 4 h, rinsed overnight in 80% ethanol, dehydrated with ethanol, cleared with xylene and embedded in Histosec™ (paraffin + plastic resin) (Merck). Alternate 5-µm thick sections were cut at 50 µm intervals with a Leitz-Jung™ microtome and stained with hematoxylin-eosin.

*Determination of the nuclear and cytoplasmic volume of acinar and convoluted granular tubule cells*

The orthogonal diameters of 50 nuclei of each cell type per rat were measured in 5 µm thick sections using a 10X Olympus Ramsden type eyepiece and a 100X immersion objective. Only intact nuclei or transections with the equator of the nucleus within the section were selected by changing the focus. The orthogonal measurements $D_1$ and $D_2$ of each nucleus converted into µm were used to calculate the geometric diameter (D) of the nucleus: $D = \sqrt{D_1.D_2}$ . The mean radius (r) for each rat was then calculated based on the arithmetic mean of the geometric diameters, which in turn was used to calculate the nuclear volume (Vn) using the formula for the volume of a sphere: $V_n = 4/3\pi \, . \, r^3$ [25].

The nuclear volume density ($\rho_n$), i.e., the cell volume fraction occupied by the nucleus, was determined by point-counting volumetry [29]. The number of points over the nucleus ($P_n$) and the cytoplasm ($P_{cyt}$) of each cell type was scored in 50 random histological fields and the nuclear volume density ($\rho_n$) was calculated using the relationship: $\rho_n = P_n/(P_n+P_{cyt})$. Since most nuclear images in histological sections represent transections covered or superimposed by cytoplasm, and since nuclei are more densely stained than cytoplasm, the nuclear volume density ($\rho_n$) is overestimated in point-counting because of the Holmes effect. This overestimation was corrected by applying the correction factor Ko calculated using the formula Ko = (1+3t)/2D [29], where t is the section thickness and D is the mean diameter of the nucleus. Thus, the corrected nuclear volume density ($\rho_{n\,corr}$) was $\rho_{n\,corr} = \rho_n/Ko$ and the corrected cytoplasmic volume density ($\rho_{cyt}$), $\rho_{cyt} = 1 - \rho_{n\,corr}$. Based on the nuclear volume ($V_n$), nuclear volume density ($\rho_{n\,corr}$) and cytoplasmic volume density ($\rho_{cyt}$), the cytoplasmic volume of the cells ($V_{cyt}$) was calculated using the equation $V_{cyt} = (V_n.\rho_{cyt})/\rho_{n\,corr}$.

*Statistical analysis*

All data were compared between age groups by analysis of variance (ANOVA) and a pairwise multiple comparison test (Student-Newman-Keuls method) using the Sigma Stat software (Jandel Scientific) for Windows, with the level of significance set at 5%. The data were also analyzed by a two-variable linear regression ($y = a_0 + a_1x$ type) using the ARCUS software. The goodness of fit was assessed using the coefficient of determination ($r^2$) calculated for the corresponding equations.

## RESULTS

The changes in body mass, absolute glandular mass, relative glandular mass and in nuclear and cell volume of the acinar and convoluted granular tubule cells during postnatal development are shown in Figures 1-3. Figure 4 shows the appearance of the acinar cells in 2-, 14-, 28- and 96-day-old rats and of the convoluted granular tubules cells in 28- and 96-day-old rats.

The body mass of the rats increased linearly by 4,430% from 6.6 g on day 2 to 299 g on day 96 (Fig. 1). During the same period, glandular mass also increased linearly by 2,364% from 18.4 mg to 453.3 mg (Fig. 2A). The relative glandular mass [(glandular mass x 100)/body mass] decreased 0.21-fold from day 2 to day 7, remained stable from day 7 to day 28, and showed a significant 0.31-fold decrease between 28 and 96 days of life (Fig. 2B).

The nuclear volume of the acinar cells (type I cells + seromucous cells) remained stable during the first 21 days of life but increased by 55% after 96 days. In contrast, the cell volume of the acinar cells remained stable from day 2 (Fig. 4a) to day 14 (Fig. 4b), and showed a significant increase of 168% between 14 and

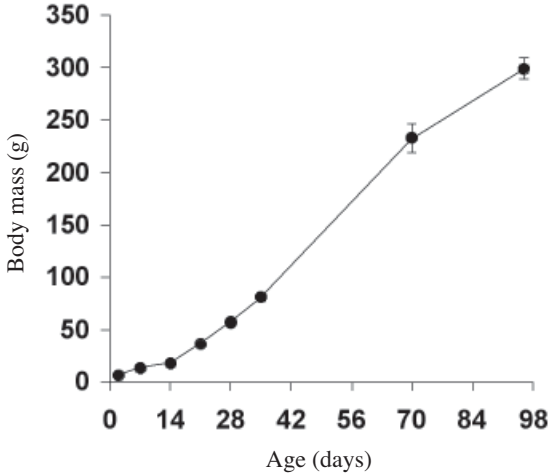


**Figure 1.** Increase in the body mass of rats during postnatal development. The points are the mean ± SEM of 6 rats.

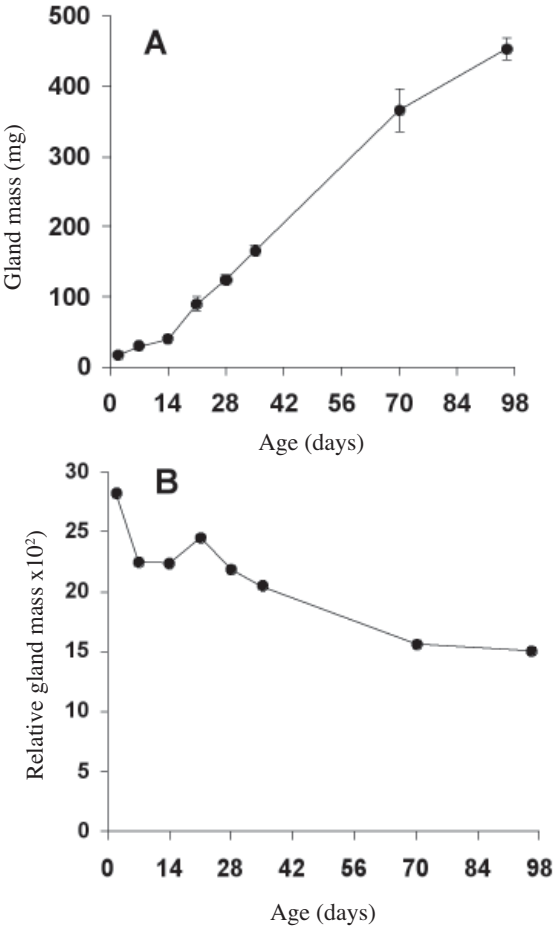


**Figure 2.** Absolute (**A**) and relative (**B**) submandibular gland mass during postnatal development. The points are the mean ± SEM of 6 rats.

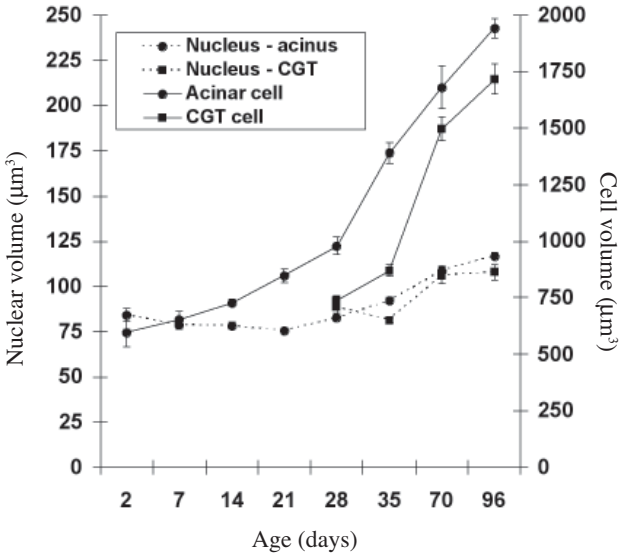


**Figure 3.** Changes in the nuclear and cell volume of acinar and convoluted granular tubule (CGT) cells during postnatal development. The points are the mean ± SEM of 6 rats.

**Table 1.** Linear equations for the increase in gland mass and cell volume (in µm³) in the acini + terminal tubules and convoluted granular tubules of rats from day 2 to day 96 of postnatal life.

| Parameter | Period (days) | Equation | $r^2$ |
|---|---|---|---|
| Gland mass<br>Cell volume | 2 to 96 | y = -7.36 + 4.95x | 0.99 |
| Acini + terminal tubules | 14 to 96 | y = 617.1 + 14.62x | 0.92 |
| Convoluted granular tubules | 28 to 96 | y = 359.0 + 14.80x | 0.97 |

$r^2$: Coeficient of determination

96 days of life (Fig. 3; see also Fig. 4b-d). The nuclear volume of the convoluted granular tubule cells increased by 20% from day 28 to day 70, while the cell volume increased significantly by 132% between 28 and 96 days of postnatal life (Fig. 3; see also Fig. 4e-f).

The linear regression equations obtained for the gain in glandular mass from day 2 to day 96, for the increase in acinar cell volume from day 14 to day 96, and for the increase in convoluted granular tubule cell volume from day 28 to day 96 are shown in Table 1.

## DISCUSSION

The postnatal development of the rat submandibular gland has been extensively studied morphologically and in terms of its growth kinetics, as reviewed elsewhere [8,11,15,22]. At birth, the gland is small and completely immature, but marked growth is observed during the initial months of postnatal life when the gland gradually acquires the morphology of the mature organ [1,4-8,15,16,23].

As shown here, a significant increase (2,364%) in glandular mass was observed between 2 and 96 days of postnatal life. Since analysis of the graph indicated a linear growth pattern, the data were fitted by linear regression analysis and the equation y = -7.36 + 4.95x ($r^2$ = 0.99) was obtained. Based on this equation, the growth rate or daily accumulation of mass in the rat submandibular gland from 2 to 96 days of development was 4.95 g/day. This marked growth resulted from an increase in the number and/or size of all structures comprising the glandular parenchyma, particularly the acini, during the first month of postnatal development [1,4,16], and by the acini and convoluted granular tubules during the second and third month [16,23].

The growth of an organ during postnatal life is the result of two basic mechanisms, namely, an in-

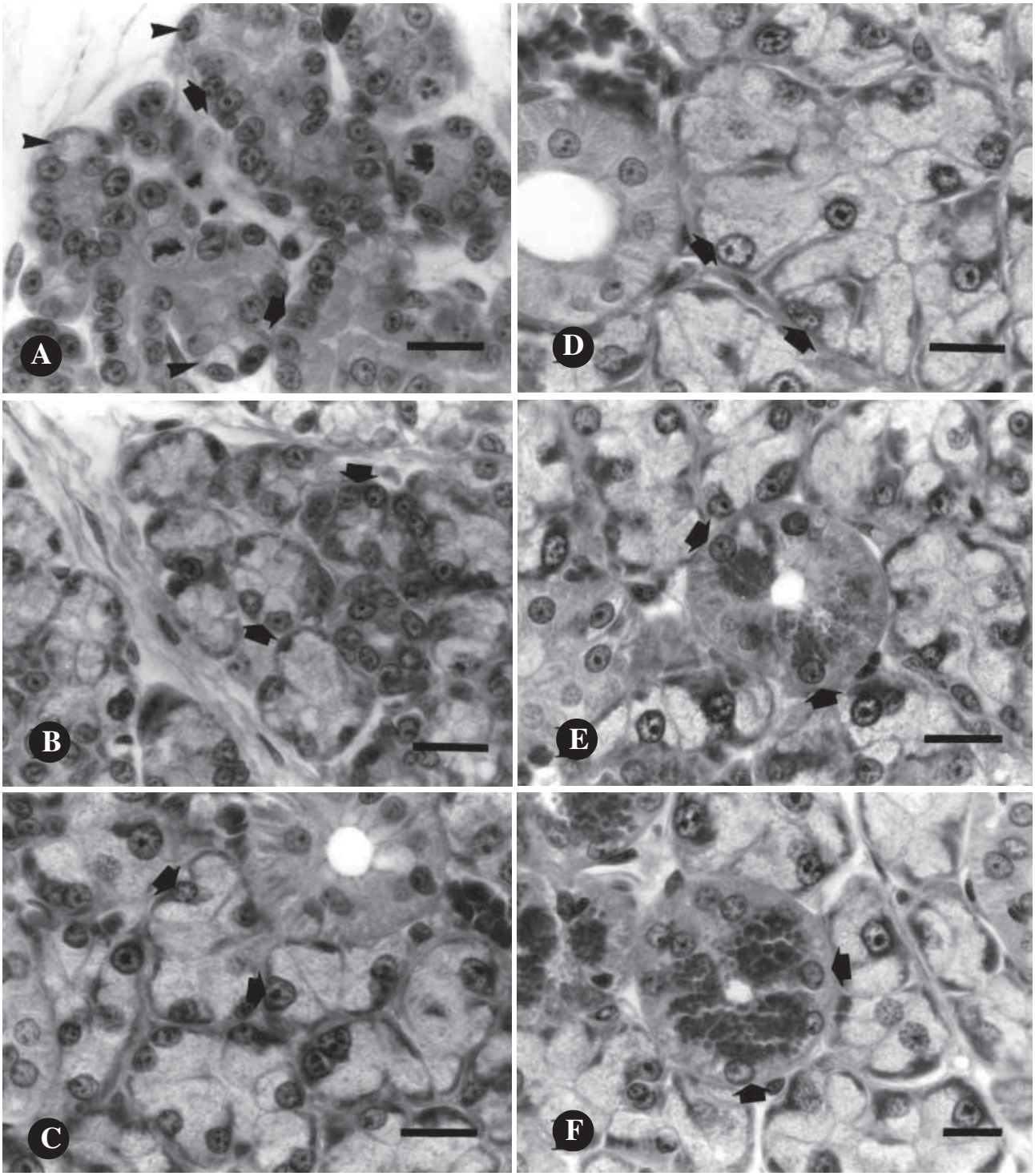


**Figure 4.** Acinar cells at 2 (**A**), 14 (**B**), 21 (**C**) and 96 (**D**) days, and convoluted granular tubule cells at 28 (**E**) and 96 (**F**) days of postnatal development. Note that between 2 and 14 days the acinar (arrows) and terminal tubule or type I (arrowheads) cells were small in size (panels **A** and **B**). The terminal tubule or type I cells were not seen at 21 days, and the volume of the acinar cells increased substantially up to day 96 (panels **C** and **D**). Between 28 and 96 days, the convoluted granular tubule cells (arrows) increased significantly in volume because of a large accumulation of secretory granules (compare panels **E** and **F**). Bar = 20 μm.

crease in the absolute number of cells through mitotic activity and an increase in individual cell volume [10,19]. Radiographic studies using $^3$H-thymidine have shown the importance of the proliferative activity of each cell population (terminal tubule cells, acinar cells, cells of the intercalated, striated and excretory ducts, convoluted granular tubule cells, and stromal cells) in the growth of the rat submandibular gland during postnatal development [1,4,23,27].

Although in a previous study we demonstrated a significant increase in acinar cell volume during postnatal life, the importance of this mechanism in final gland growth was not analyzed. In this study, we assessed the role of an increase in the volume of acinar and convoluted granular tubule cells (the most frequent cells in the adult rat submandibular gland) in the glandular growth during the first 96 days of postnatal development.

During the first days after birth, the immature acini consist of two distinct types of secretory cells: the predominant terminal tubule cells [16] or type I cells which have serous secretory characteristics and secrete a 89 kDa protein; and the proacinar [30] or type III cells, which are less common and are characterized by the expression of immunoreactive $B_1$ protein [13,18]. In the latter cells, the $B_1$ protein genes are repressed during the second week of postnatal life, and these cells differentiate into seromucous acinar (definitive) cells that express the secretory proteins of the adult animal [18].

The absolute number of terminal tubule or type I cells increases up to the end of the third week of development, and during the following two weeks, these cells are progressively exceeded in number by seromucous acinar cells [1,4]. Although the disappearance of some type I cells involves a simplification of cellular morphology, a reduction in size and subsequent incorporation into the initial portion of the intercalated ducts, most of these cells disappear by atrophying and dying through apoptosis between postnatal days 25 and 30 [14,15].

The mean volume of acinar cells (type I cells + seromucous cells) increased linearly by 168% between 14 and 96 days of life. Linear regression analysis of these date yielded the equation $y = 617.1 + 14.6x$ ($r^2 = 0.92$). The growth rate (daily gain) of acinar cell volume from day 14 to day 96 was 14.6 $\mu m^3$/day. No significant increase in cell volume was observed between 2 and 14 days of age. The proliferation rate of acinar cells (type I and definitive cells) is high during the first 15 to 20 days of postnatal life [1,4]. The stability of cell volume during the first two weeks of life observed here indicated that from birth to the beginning of a change in feeding habits, the gain in gland mass was predominantly through an increase in the number of cells, notably acinar cells. From the second week until the end of the study period, the increase in mean acinar cell volume (14.6 $\mu m^3$/day) played an important role in glandular growth. In addition, since the proliferative activity of this cell type decreases after weaning and remains at a markedly low level [23], we concluded that the post-weaning glandular growth associated with acinar cells was predominantly through an increase in cell volume.

The growth of the acinar cell population, termed the acinar phase of development by Jacob and Leeson [16], starts at birth and lasts up to approximately the 8$^{th}$ week, when the number of seromucous acinar cells stabilizes [23]. Although this period is quite long, the essential biological events leading to the formation of the acini, i.e., cytodifferentiation of the proacinar or type III cells into definitive acinar cells, occur within the first two weeks of postnatal life. After the depletion of type III cells, new acinar cells are formed exclusively by the proliferation of pre-existing differentiated cells until the end of the acinar phase.

The acinar phase of postnatal development of the rat submandibular gland is followed by the ductal phase [16], which is characterized by the appearance and maturation of convoluted granular tubules. The beginning of the morphological differentiation of the first secretory cells of these tubules from striated duct cells is observed by postnatal day 15, but becomes more evident only by week 4 [7,23]. The development and maturation of the convoluted granular tubule cells continue until postnatal week 12 to 14 [23].

Kinetic studies of the growth of submandibular gland cells in rats and mice using $^3$H-thymidine have shown a significant increase in the absolute number of convoluted granular tubule cells during the ductal phase. This increase was initially due to the differentiation of pre-existing striated duct cells, followed by mitotic activity of recently differentiated cells of the compartment itself, and the proliferation of intercalated duct cells that migrated and differentiated into secretory cells of the convoluted granular tubules [9,23,31].

Our results showed a significant increase in the volume of these secretory cells during the period from 28 to 96 days of life. In contrast, in mice, the volume of these cells is already stable by postnatal day 35 [20]. Since analysis of the data indicated a linear growth pattern, the data were fitted using linear regression and the equation $y = 359.0 + 14.8x$ ($r^2 = 0.97$)

was obtained. The daily volume gain calculated for the convoluted granular tubule cells was 14.8 $\mu m^3$/ day from day 28 to day 96 of development.

The results described here demonstrate that the increase in the volume of acinar (from day 14 onwards) and convoluted granular tubule cells (from day 28 onwards) contributes to the gain in mass of the rat submandibular gland during postnatal development, with a similar growth rate being observed for the two cell populations up to the end of the period studied.

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