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ALLOMETRIC ANALYSIS OF THE POSTNATAL GROWTH OF RAT SUBMANDIBULAR GLAND

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ABSTRACT

The rat submandibular gland grows significantly during the first 10 weeks of postnatal life. During this growth, there is differentiation and maturation of the definitive glandular structures, (acini, intercalated ducts, convoluted granular tubules, striated ducts and excretory ducts) within a highly vascularized stroma. In this study, the absolute volume of each glandular component during postnatal development was determined morphometrically. The increases in gland mass and component volumes were analyzed allometrically relative to the growth of body mass, using Wald's non-parametric method. The allometric growth of gland mass was monophasic and negative ($k<1$), with $k = 0.86$. The absolute volumes of the acini plus terminal tubules, intercalated ducts, striated ducts and excretory ducts all showed a biphasic pattern, with the first phase occuring from day 2 to day 28 and the second phase from day 28 to day 96. In the first phase, all of the structures showed positive allometric growth ($k>1$), with k values from 1.09, 1.15, 1.49 and 1.17, for the acini plus terminal tubules, intercalated ducts, striated ducts and excretory ducts, respectively, while in the second phase, all showed negative allometrtic growth ($k<1$), with k values of 0.72, 0.33, 0.77 and 0.82, respectively. The convoluted granular tubules showed a single phase of positive allometric growth ($k>1$) between 28 and 96 days of age, with $k=1.28$, whereas the stromal volume showed negative allometric growth ($k<1$) from day 14 to day 96, with $k=0.77$.

Key words: Allometry, development, morphometry, rat, submandibular gland

INTRODUCTION

At birth, the submandibular gland of the rat is in a relatively rudimentary state. The morphological components that can be identified in disto-proximal sequence include well-defined terminal tubules, intercalated ducts in formation, immature striated ducts and excretory ducts [4,12,15].

The greatest change that occurs in the adenomer of the submandibular gland during the first 4 to 6 weeks of postnatal development is the substitution of the terminal tubules, which are transitory structures not found in the adult gland, by definitive acini [1,3,12]. This process is initiated in the first five days after birth, when sero-mucous acinar cells arise from proacinar cells involving the terminal tubule cells and afterwards they arrange themselves into typical acini [1,3,16].

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During the first month of life, the number of terminal tubule cells declines significantly, while the population of acinar cells increases at a constant rate, mainly through the mitotic activity of pre-existing acinar cells [1,3,17]. At the end of this period, the terminal tubule cells totally disappear through apoptosis and by a gradual transformation and incorporation into the intercalated ducts [1,3,6,9,10,12,16].

This stage of acinar development is followed by a phase of differentiation and development of the convoluted granular tubules [12]. The convoluted granular tubules are ductal segments made up of serous secretory cells and located between the intercalated and striated ducts. The convoluted granular tubule cells begin to differentiate from pre-existing striated duct cells on the 15th postnatal day and become very evident by the 3rd to 4th week of development [14]. The final maturation and numeric stabilization of the population of convoluted granular tubule cells occurs during the 4th to 14th week [14].

Although there have been numerous reports on the postnatal development of the submandibular gland of

rats and mice, there has been no allometric analysis of this development. In this study, we morphometrically assessed the absolute volume of each morphological component of the rat submandibular gland during postnatal development. These data, and those of gland mass, were examined by bivariate allometric analysis using Wald's non-parametric method modified by Bartlett [2] as a function of the growth of body mass. Using this approach, the growth parameters (k coefficient) between the various parts (gland mass, acinar volume, volume of the striated ducts, and others) and the total body mass, as well as the allometric growth pattern, were determined.

MATERIALS AND METHODS

General histological procedures

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 Central Animal House of the Bauru School of Dentistry. Pregnant dams, mothers and pups weaned at 21 days of age received water and pelleted Purina food *ad libitum*. All of the glands were collected between 10:00 and 12:00 a.m. to avoid the influence of circadian variations. The study was approved by the institutional Committee for Ethics in Animal Experimentation and was done within the institutional guidelines for the use of laboratory animals. The body mass of each rat was determined after anesthesia with ketamine hydrochloride plus (intramuscular injection of 100 mg/kg of corporal mass) and xylazine hydrochloride (intramuscular injection of 3 mg/Kg of corporal mass) and, afterwards, the submandibular glands were carefully dissected and removed, and the fresh gland mass was determined on an analytical balance. The glands were fixed in Bouin solution for 4 h at room temperature and then stored overnight in 80% ethanol. The next day, the glands were dehydrated in ethanol, cleared in xylol and embedded in Histosec-Merck (paraffin plus plastic resin). Alternate 5 μm thick sections obtained with a Leitz-Jung microtome were stained using the Masson trichromic method.

Determination of processed submandibular gland volume

The processed volume (Vp) of the submandibular gland of each rat was calculated based on the fresh gland mass (m), the gland density (δ) and the correction factor (RF) for the shrinkage caused by the histological processing, using the formula $Vp = (m/\delta) \times RF$. The density (δ) of the gland was evaluated in 24 rats at 14, 21, 35 and 70 days of age (6 rats/group) using a Mettler Toledo AT261 Delta Rang precision balance fitted with the appropriate accessories to allow the density determinations. "F" statistic analysis showed that there was no difference between the 14- and 21-day-old groups and between the 35- and 70-day-old groups. The average density obtained for the groups at 14 and 21 days and at 35 and 70 days was 1.06 mg/mm^3 and 1.07 mg/mm^3, respectively. The retraction caused by the histological procedure was evaluated in 21- and 70-day-old rats (6 rats/group) using the method described by Taga and Sesso [18]. In this method, linear measurements are obtained from the square trimmed fresh organ (Sf) and after histological processing (Sp). The averages of the Sf and Sp linear values are raised to the third power. The retraction factor (Rf) was estimated using the relationship: $Rf = Sp^3/Sf^3$. "F" statistic analysis showed that there was no difference ($P>0.05$) between the 21- and 70-day-old rats. The average retraction was 53.7%, and the correction factor was 0.4625.

Morphometric determination of the volume density (Vvi) and total volume (Vti) of each gland component

The volume density (Vvi) of each submandibular gland component was determined using a digital image analysis system that consisted of a Zeiss Axioskop microscope with a 100X immersion objective, a CCD-IRIS RGB – Sony camera and Kontron KS300 software (Kontron Electronic GMBM) installed on an IBM computer. A total of 50 histological fields per rat were taken and selected by systematic sampling [19]. For each field, the area occupied by each structure (Ai) (acini plus terminal tubules, intercalated ducts, convoluted granular tubules, striated ducts, excretory ducts and stroma) was determined and the total gland area (A) was calculated. The area density of each structure was calculated using the relationship: $Avi = Ai / A = Vvi$. The volume of each gland component (Vti) was calculated using the formula: $Vti = Vvi \cdot Vp$, where Vvi is the volume density and Vp is the processed gland volume.

Since precise identification of the terminal tubules was difficult in histological sections of glands embedded in paraffin plus plastic resin, these structures were included in the morphological compartment of the acini.

Allometric analysis

The gland mass and volume of each gland component were studied by allometric analysis as a function of the increase in body mass. The allometric relationship between two ponderal or volumetric parameters in a system subject to modifications is represented by the coefficient "k" of an equation of the type $y = bx^k$ [11]. For this type of analysis, three growth situations may occur: a) when $k=1$, the growth between the two variables is isometric, i.e., the unit growth of y is equal to that of x, b) when $k>1$, the allometric growth is positive, i.e., the growth of y is greater than that of x, and c) when $k<1$, the allometric growth is negative, i.e., the unit growth of y is lesser than that of x.

In this study, the coefficient of allometry k was calculated with Wald's non-parametric method, modified by Barlett [2], programmed on an IBM microcomputer. This method allowed calculation of the coefficient of allometry, k, the confidence intervals for k, and the t value for the linearity.

Statistical analysis

All of quantitative data obtained for each group were compared by analysis of variance (ANOVA) followed by a paired, multiple comparisons test (Student-Newman-Keuls) using the Sigma Stat-Jandel™ Scientific Software for Windows, with the level of significance set at 5%. The volume density data were arc-sin transformed before statistical analysis. The data for gland mass and total volume of each gland component were analyzed by linear regression using the equation $y = a_0 + a_1x$ and Sigma-Stat software.

RESULTS

The changes in body mass, gland mass, volume density and absolute volume of each morphological component of the submandibular gland are shown in Figures 1-3.

The body mass increased by 4,551%, from 6.6 g on day 2 to 299 g on day 96. In the same period, the gland mass grew 2,353%, from 18.5 mg to 453.3 mg (Fig. 1). Graphical analysis indicated that the gland mass grew linearly.

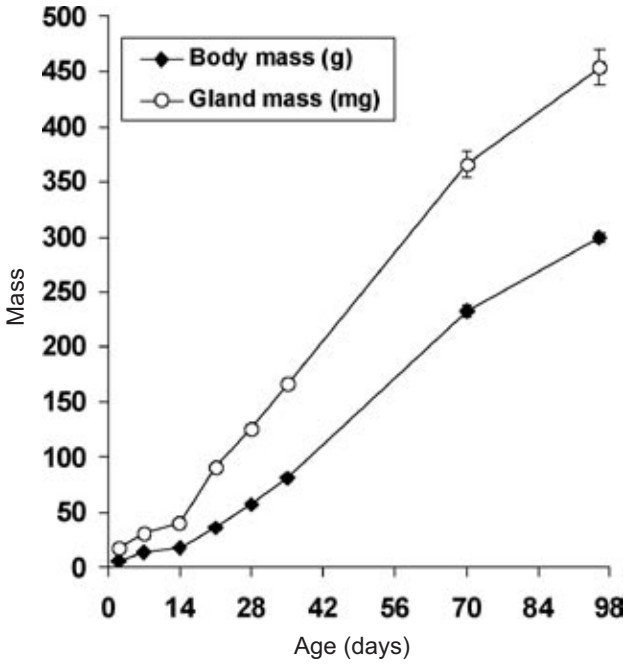

Figure 1. Body mass and submandibular gland mass of rats from day 2 to 96 of postnatal life. Bars = standard error of mean.

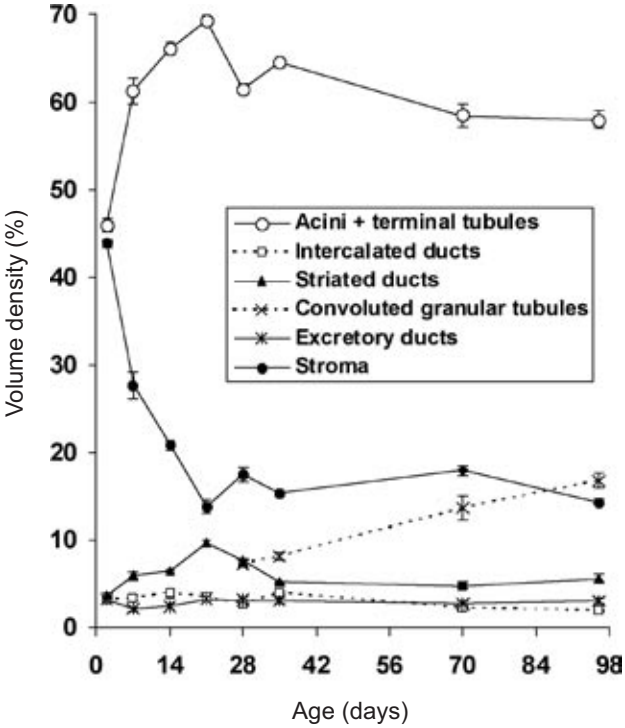

Figure 2. Volume density of each morphological component of the submandibular gland from day 2 to 96 of postnatal life. Bars = standard error of mean.

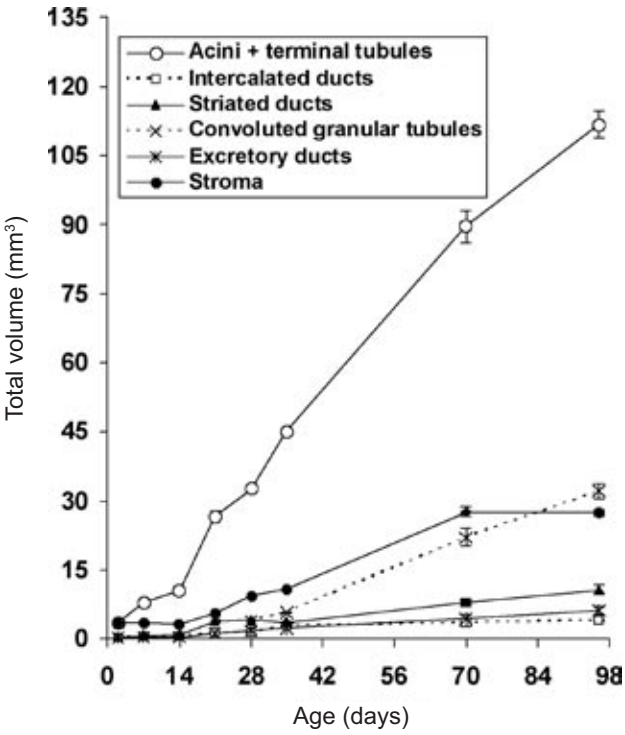

Figure 3. Absolute volume of each morphological component of the submandibular gland from day 2 to 96 of postnatal life. Bars = standard error of mean.

The acinus plus terminal tubule volume density increased by 1.50 fold, from 46.5% on day 2 to 69.8% on day 21, while in the same period, the stromal volume density decreased by 0.32 fold, from 44.3% to 14.0%; both parameters remained stable in the subsequent periods. Graphical analysis showed an inverse relationship between the volume density of the acini plus terminal tubules and of the stroma during the first three weeks of postnatal life. The volume density of the convoluted granular tubules increased by 2.31 fold from 7.3% on day 28 to 16.8% on day 96. The volume densities of the other duct types showed no significant variations from day 2 to day 96 of postnatal life (Fig. 2).

The absolute volumes of the acini plus terminal tubules, intercalated ducts and excretory ducts increased linearly from day 2 to day 96 by 3,029% (from 3.6 mm^3 to 111.7 mm^3), 1,492% (from 0.2 mm^3 to 4.0 mm^3) and 3,311% (from 0.2 mm^3 to 6.1 mm^3), respectively. The absolute volume of the striated ducts increased by 4,140% between 2 and 96 days, in two phases, one from day 2 to day 28 with an increase of 1,532% (from 0.2 mm^3 to 4.1 mm^3) and the other from day 35 to day 96 with an increase of 188% (from 3.7 mm^3 to 10.6 mm^3); there was no statistically signicant difference from day 28 to day 35. During the first two weeks of postnatal life, the absolute volume of the stroma remained stable, with a value of approximately 3.4 mm^3 and increased by 836% between days 14 and 70 (from 3.3 mm^3 to 27.4 mm^3) (Fig. 3).

The linear equations obtained through regression analysis for the gland mass and the absolute volumes of the various glandular components are shown in Table 1.

Allometric analysis

The results of the allometric analysis of the growth of gland mass and of the absolute volumes of the acini plus terminal tubules, intercalated ducts, striated ducts, convoluted granular tubules, excretory ducts and stroma relative to the growth of body mass from day 2 to day 96 of postnatal life are shown in Table 2 and in Figures 4 and 5 A-F.

Table 2 shows the values for the coefficient of allometriy (k), the *t* values and the confidence limits for k. The *t* value is important for testing the hypothesis that the two variables are associated with a straight line. If the *t* value is less than the critical *t* values, then the null hypothesis cannot be rejected. In Figures 4 and 5, the points represent the original

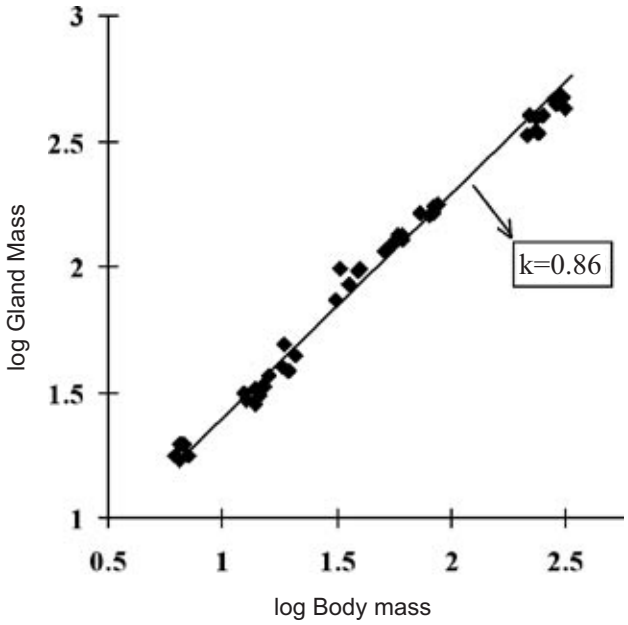

Figure 4. Allometric growth of the gland mass as a function of the body mass from day 2 to 96 of postnatal life.

Table 1. Linear equations for growth of the gland mass and total volume (mm^3) of acini plus terminal tubules, intercalated ducts, striated ducts, convoluted granular tubules, excretory ducts and stroma from day 2 to 96 of postnatal life.

Parameter	Period analized (days)	Linear regression equation	Coefficient of determination
Gland mass	2 to 96	y = -7.36 + 4.95x	0.99
Compartmental volume			
Acini plus terminal tubules	2 to 96	y = -0.41 + 1.21x	0.99
Intercalated ducts	2 to 96	y = 0.34 + 0.04x	0.90
Striated ducts	2 to 96	y = 0.28 + 0.10x	0.97
Convoluted granular tubules	28 to 96	y = -8.42 + 0.42x	0.99
Excretory ducts	2 to 96	y = -0.23 + 0.07x	0.99
Stroma	14 to 70	y = 3.43 + 0.44x	0.99

logarithms of the two variables, and the straight line of each figure was determined by using the respective equation.

The allometric growth of the gland mass in relation to the body mass was monophasic from day 2 to day 96, with k = 0.862 (Fig. 4). The absolute volumes of the acini plus terminal tubules, intercalated ducts, striated ducts and excretory ducts all showed a biphasic pattern of differential growth, with the first phase from day 2 to day 28, and the second from day 28 to day 96, the k coefficients were 1.086 and 0.717, 1.152 and 0.335, 1.491 and 0.774, and 1.172 and 0.818, respectively (Figs.5A-C,E). The convoluted granular tubule showed a positive allometric growth from day 28 to day 96, with k = 1.278 (Fig. 5D), whereas the stromal volume had a negative allometric growth from day 14 to day 96, with k = 0.767 (Fig. 5F).

DISCUSSION

In this study, the fresh gland mass increased more than 2,000% from day 2 to day 96 of postnatal development, with an accumulation rate of 4.95 mg/day. Allometric analysis of the growth of gland mass as a function of the increase in body mass showed a monophasic pattern of negative differential growth, with k = 0.86, i.e., the gland mass grew less than the body mass, and for each unit increase in body mass, the gland mass increased 0.86 fold. Hassunuma and Taga [8] also reported a monophasic pattern of differential growth, but of the isometric type (k = 1) for the growth of rat sublingual gland mass from day 2 to day 40 of postnatal life.The marked growth in submandibular gland mass resulted from an increase in the number and/or size of all of the glandular structures, particularly the acini in the first month [1,3,12], and of the acini and convoluted granular tubules in the 2nd and 3rd months of development [12,14].

As indicated earlier, the terminal tubules were included in the same morphological compartment of the acini in the quantifications reported here. Terminal tubules are transitory secretory units present at the distal extremity of the intralobular duct system of newborn rat submandibular glands. These units are formed of two secretory cell types, namely, the terminal tubule or Type I cells, which

Table 2. Allometric analysis of various parameters during the first 96 days of postnatal life.

Parameter	Period (days)	K value	Confidence interval limits	t value
Gland mass x Body mass	2 to 96	0.862	S=0.881 I=0.844	1.640
Acinus plus terminal tubule volume x Body mass	2 to 28	1.086	S=1.096 I=1.076	0.299
	28 to 96	0.717	S=0.736 I=0.700	0.544
Intercalated duct volume x Body mass	2 to 28	1.152	S=1.277 I=0.944	1.289
	28 to 96	0.335	S=0.394 I=0.273	0.794
Striated duct volume x Body mass	2 to 28	1.491	S=1.524 I=1.459	0.447
	28 to 96	0.774	S=0.885 I= 0.668	1.456
Convoluted granular tubule volume x Body mass	28 to 96	1.278	S=1.334 I=1.221	1.628
Excretory duct volume x Body mass	2 to 28	1.172	S=1.265 I=1.082	1.418
	28 to 96	0.818	S=0.968 I=0.684	1.425
Stroma volume x Body mass	14 to 96	0.767	S=0.776 I=0.757	0.351

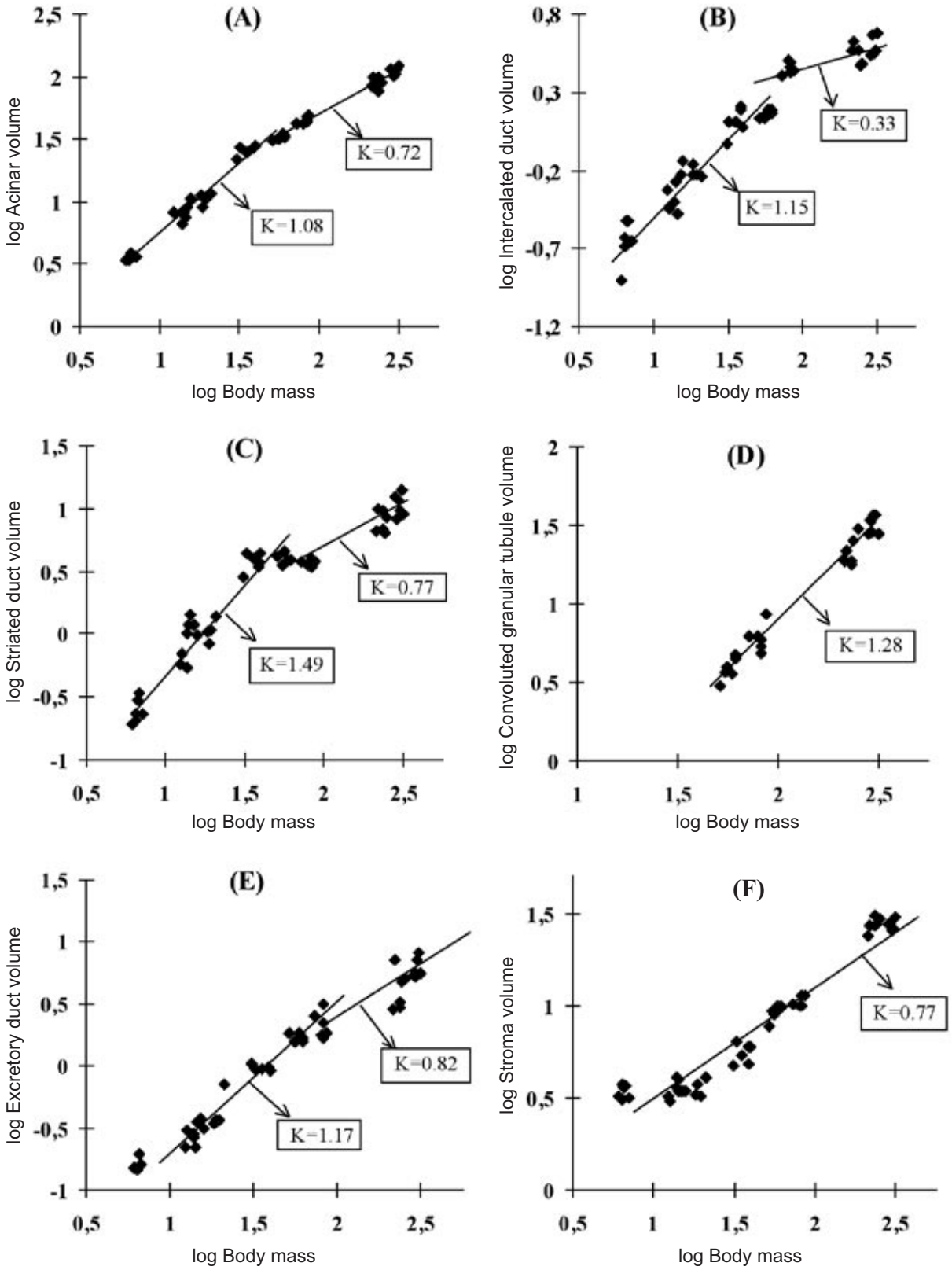

Figure 5. Allometric growth of the compartmental volume of the **(A)** acini, **(B)** intercalated ducts, **(C)** striated ducts, **(D)** convoluted granular tubules, **(E)** excretory ducts and **(F)** stroma as a function of the body mass from day 2 to 96 of postnatal life.

are the predominant type, and the proacinar or Type III cells, which are sparse. During the first month of postnatal development, the Type I cells increase in absolute number by mitotic activity and become the most abundant cell type by the end of the 3rd week after birth. Thereafter, the number of Type I cells decreases significantly and are progressively replaced by seromucous acinar cells derived from Type III cells, which form the definitive acini [1,3,7,12,13,16]. The loss of Type I cells occurs through apoptosis [9,10] and by transformation and incorporation into the intercalated duct compartment [1,3,6,13].

Analysis of the change in volume density of the different glandular components suggested that during this growth, the parenchymal structures, especially the acini, grew occupying gradually the connective tissue spaces. This was confirmed by the morphological analysis of histological sections, which showed a marked reduction in the interparenchymal stromal spaces during postnatal development.

All of the glandular structures showed a significant increase in absolute volume. Previous autoradiographic studies using ^{3}H-thymidine labeling showed that this growth occurred through the mitotic activity of each cell population in the developing submandibular gland, including the cell of the terminal tubules, during the first three weeks of postnatal life [1,3,14,17]. On the other hand, morphometric data of acinar and terminal tubule cells during the same period [16] suggest that the increase in individual cell volume may also have an important role in this compartmental growth.

The growth rate of each gland component calculated using the respective adjusted linear equation, was 1.21 mm^3/day for acini plus terminal tubules, 0.44 mm^3/day for stroma, 0.42 mm^3/day for convoluted granular tubules, 0.10 mm^3/day for striated ducts, 0.07 mm^3/day for excretory ducts and 0.04 mm^3/day for intercalated ducts.

The increase in the volume of each type of structure was also submitted to bivariate allometric analysis as a function of the increase in body mass. The volumes of the acini plus terminal tubules, intercalated ducts, striated ducts and excretory ducts between 2 and 96 days of age showed a biphasic pattern of allometric growth, with the first phase occurring from day 2 to day 28 and the second phase occurring from day 28 to day 96. In the first phase, all of the structures showed positive allometry, i.e., all had greater unit growth than that of body mass. In the second phase, all showed negative allometry, i.e., the unit increase in volume of each structure was less than that of the body mass.

The convoluted granular tubules, the secretory cells of which trigger cytodifferentiation at 21 days of age [5], were initially quantified on day 28 when they were more evident. Between 28 and 96 days of age, the absolute volume of these tubules showed a monophasic pattern and positive allometric growth with k = 1.28, i.e., for each unit growth of body mass the absolute volume of the convoluted granular tubules grew 1.28 fold. In contrtast, the stromal volume showed a negative allometric growth from day 14 to day 96, i.e., a unit growth less than that of body mass.

In contrast to the submandibular gland described here, the mucous cells, demilune serous cells, ducts and stroma of rat sublingual gland all showed monophasic allometric growth in their absolute volumes, with k values of 1.11, 0.76, 0.86 and 1.00, respectively, between 2 and 40 days of postnatal development [8].

The growth parameters for the postnatal development of rat submandibular glands calculated here revealed two distinct phases in the allometric growth of the absolute volumes of the parenchymal structures, one between days 2 and 28 and the other between days 28 and 96, with a transition that coincided with the appearance of convoluted granular tubules cells. This change in differential growth between the two phases could be related to extrinsic factors such as hormones, a change in diet and weaning, which exert a greater influence at this time.

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