Braz. J. morphol. Sci. (2000) ***17,*** *93 - 100*

A MORPHOMETRIC STUDY OF SEASONAL INFLUENCES ON THE DAILY CYCLE OF RAT SUBMANDIBULAR GLANDS

Gerson Francisco de Assis, Tania Mary Cestari, Antonio Carlos Marconi Stipp, Renato Massaharu Hassunuma and Rumio Taga

Laboratory of Histology, Department of Biological Sciences, Faculty of Dentistry of Bauru, University of São Paulo, Bauru, SP, Brazil.

ABSTRACT

The submandibular glands of rats raised at room temperature show daily and seasonal variations. To quantify these variations, the submandibular glands of male Wistar rats were collected at 4 h intervals over a 24h period in the summer and winter and processed for histological analysis. Gland weight decreased 37% ($P<0.01$) in the winter and 13% in the summer. The volume of the acinar compartment decreased 73% ($P<0.01$) between midnight and 4 a.m. in the winter and 38% ($P<0.01$) between 8 p.m. and midnight in the summer. In contrast, the volume of the stroma increased about 164% ($P<0.01$) between midnight and 4 a.m. in the winter and by 10% ($P<0.01$) between 8 p.m. and midnight in the summer. The volume of the duct system generally remained constant, although in the winter the convoluted granular tubules diminished 44% ($P<0.01$) between midnight and 4 a.m. Although the volume of the intercalated ducts remained constant over 24 h in both seasons, there was a 49% decrease in winter compared to summer ($P<0.01$). These results indicate that the large changes in gland weight were caused mainly by variation in the volume of the acini, convoluted granular tubules and stroma. The morphological variations were much more pronounced in winter than in summer and this affected the daily saliva production.

Key words: Circadian, morphology, rat, seasonal, stereology, submandibular gland

INTRODUCTION

Animals and isolated cells and tissues show regular oscillations or biological rhythms in their behaviour and physiology over time. Biological rhythms are classified according to their duration as being ultradian (<24 h), circadian (~24 h), infradian (>24 h) and seasonal or circannual (~1 year) [34].

Many circadian rhythms act in synchrony with the external day/night cycle through the cyclical release of melatonin, a hormone produced by the pineal gland. The blood melatonin level is controlled by the amount of exposure to light which, in higher animals, acts indirectly through a nervous circuit that involves retinal connections to the suprachiasmatic nucleus in the hypothalamus. The latter is in turn connected to the pineal through the sympathetic system [21,23,24]. Blood melatonin levels are high during the night and low during the day [10,11,23], but can be influenced by variations in the photoperiod during the summer and winter.

The circadian rhythm in humans may be affected by the photoperiod [8,12,17,33] and the responses to variations in the photoperiod are similar to those in other mammals [16]. Individuals who experience changes in their "biological rhythm" as a result of night jobs or trips depend on melatonin to adjust to the altered cycle. Melatonin is usually measured in blood samples but may also be quantified in saliva, although the levels are 30% lower than in blood [31].

The morphophysiological variations in the large salivary glands of rats accompany the daily melatonin cycle which peaks at night and reaches a minimum during the day. These variations are also related to the ingestion of solid food and to chewing activity [14,15]. Over a 24-h period, the content of secretory material increases and decreases, in parallel with the mass of the glands [15,27]. Many studies [1-5,13-15,27,28,30] have shown circadian variations in the biochemistry, physiology and ultrastructural morphology of salivary glands, particularly the parotid gland. Field *et al*. [9] have shown that such variations also occur in minor salivary glands.

In this paper, we examined the 24-h variations in submandibular gland mass and morphology in adult male Wistar rats during the winter and summer.

MATERIAL AND METHODS

One hundred 80-day-old male Wistar rats *(Rattus norvegicus)* from the Central Animal House of the Faculty of Dentistry, Bauru were used in this study. The rats were divided into four groups (two groups with 30 rats each and two with 20 rats each).

From the time of birth, the rats were kept in groups of 6 per litter

Correspondence to: Dr. Rumio Taga or Dr. Gerson Francisco de Assis - Laboratório de Histologia, Departamento de Ciências Biológicas, Faculdade de Odontologia de Bauru. Al. Octávio Pinheiro Brisolla, 9-75, CEP 17012-901, Bauru, SP, Brasil.
Tel: (55) (14) 235-8259, E-mail: gfassis@fob.usp.br

and weaned at 21 days old. Water and food (Purina® rat and mouse chow) were furnished *ad libitum*. The rats were housed in a well-ventilated room, in which temperature and humidity were not controlled and there was no artificial lighting. During the study, the highest temperature recorded was 32ºC in the summer and 26ºC in the winter, and the lowest temperatures were 23ºC and 17ºC, respectively. The relative humidity was approximately 73%rF in the summer and 61%rF in the winter. The photoperiod in winter was 11 h, and 13 h in the summer.

The two largest groups of rats were subdivided into 6 groups of 5 animals each. The glands were collected at 4 h intervals (noon, 4 p.m., 8 p.m., midnight, 4 a.m. and 8 a.m.) in the winter and summer. Each sample collection lasted 30 min.

Histological procedure

The rats were sacrificed by ether inhalation in a closed glass chamber and immediately weighed. The submandibular glands were then carefully removed and weighed before fixation in Bouin solution (75 ml of saturated picric acid, 25 ml of formaldehyde and 5 ml of glacial acetic acid) for 4 h at room temperature. The glands were subsequently dehydrated in ethanol, diaphanized in xylol and embedded in Paraplast (paraffin + resin). Semi-serial sections (5 µm) were obtained with a Leitz-Jung microtome at 50 µm intervals. The sections were stained using Masson's trichromic method.

Volume of the processed gland

The volume of the processed gland (Vpg), i.e., the gland volume after all histological procedures, was calculated using the relationship Vpg = (m/gd)x Sf, where m is the mass of the fresh gland, gd is the gland density and sf is a factor to correct for shrinkage caused by histological processing.

The density of the submandibular gland (gd) was determined in the third group of rats using Scherle´s method [26], based on the recommendations of Pardini and Taga [22]. The shrinkage factor (Sf) was determined in the fourth group of rats, as described by Taga and Sesso [29]. The intervals used were midnight and 4 a.m. in the winter and 8 p.m. and midnight in the summer.

Stereological evaluation of the volume density and total volume of the gland compartments

The initial data for these parameters were obtained using a 100 x immersion objective, a Kpl 8 x ocular and a Zeiss® II integration grid composed of 10 parallel lines and 100 points per quadrangular area. The grid image was successively superimposed on 50 histological fields selected by systematic sampling [32], and the number of points (Pi) that fell over each submandibular gland structure (i) and over the entire gland (Pt) was recorded. The reliability of systematic sampling was confirmed using the chi-square test included in the SigmaStat software package (V. 1.0, Jandel Corporation). The volume density (Vvi) of each glandular compartment was calculated as Vvi=Pi/Pt [32].

The volume density (Vvi), and the volume of the processed gland (Vpg) were used to calculate the total volume (TVi) of each compartment as TVi=Vvi x Vpg.

Statistical analysis

Where appropriate, the results were expressed as the mean ± S.E.M. of the number of rats indicated. The data were compared by analysis of variance (ANOVA) using SigmaStat software (V 1.0, Jandel Corporation). Volume densities were compared after arcsin transformation of the original data. A value of $P < 0.05$ was considered to indicate significance.

RESULTS

Morphological results

Winter period: Figure 1A-C shows histological sections of adult rat submandibular glands sacrificed at midnight, 4 a.m. and 8 a.m., respectively, in the winter.

At midnight (Figure 1A), the epithelial structures were more developed than at other times. The acini were generally formed by cells with an ample cytoplasm and a large quantity of secretory material in the mid and apical regions. The small round nucleus was located in the basal portion of the cell. The convoluted granular tubules were very coiled and had a small lumen. The cells of these tubules were upright and had a round nucleus in the basal portion, as well as a large amount of secretory material in the mid and apical regions. The striated ducts were formed by upright cubic cells with spherical nuclei in the central portion and characteristic striations in the basal plasmalemma. The intercalated duct consisted of a simple squamous epithelium. The excretory ducts had an ample lumen, surrounded by low cubic cells, with the nucleus occupying around 2/3 of the cell. The capsular and septal stroma were still well-organized.

At 4 a.m. (Figure 1B), the gland showed the greatest morphological modifications: the lobules were shrunken because of a reduction in the parenchyma, and there was an increase in the stroma. All the parenchymal structures, including the acini, were small and had a reduced lumen surrounded by a cluster of nuclei. The cytoplasm was almost unobservable and this made it difficult to identify intracellular structures by light microscopy. The excretory ducts also showed very little cytoplasm and the nuclei were very close to one another. This arrangement gave the appearance of a stratified epithelium.

At 8 a.m. (Figure 1C), there was still a large amount of stroma, but it was now possible to differentiate the acini more easily since there was an increase in the volume of cytoplasm. A similar response was seen in some convoluted granular tubules. The striated and intercalated ducts were more difficult to identify. The excretory ducts had cells with more cytoplasm and a larger lumen than at 4 a.m.

The parenchymal structures increased in size during the rest of the day, whereas the stroma decreased until the maximum gland size was reached at midnight.

Summer period: In the summer, the submandibular glands showed no drastic alterations in their morphology. Figure 1D and 1E shows histological sections of adult rat submandibular glands sacrificed at 8 p.m. and midnight, respectively, in the period of greatest variation.

At 8 p.m. (Figure 1D), the characteristics of the glandular structures were similar to those observed at midnight in the winter, except for the acinar cells which had ample cytoplasm and a small, round, densely colored nucleus

which occupied the basal portion of the cell.

At midnight (Figure 1E), decreased the cytoplasm and the acini shrank, with the result that the round nucleus now occupied a more central region in the cell. The remaining parenchymal structures showed practically no morphological alterations. The stroma was more noticeable than at 8 p.m.

Quantitative results: The gland weight, density volume and absolute volume of each glandular compartment are shown in Figures 2 - 4.

In the winter, gland weights at 4 a.m. and 8 a.m. were significantly lower than at midnight ($P<0.01$), with the greatest decrease occurring at 4 a.m. (Fig. 2). In the summer, gland weight was greatest at 8 p.m. and lowest at midnight. There were no significant variations in body weight or in the gland weight/body weight ratios in the winter or summer.

The volume density of each glandular compartment over time in winter and summer is shown in Figure 3. The greatest changes occurred in the acinar and stromal compartments between midnight and 4 a.m. in the winter, and between 4 p.m. and midnight in the summer. At midnight in the

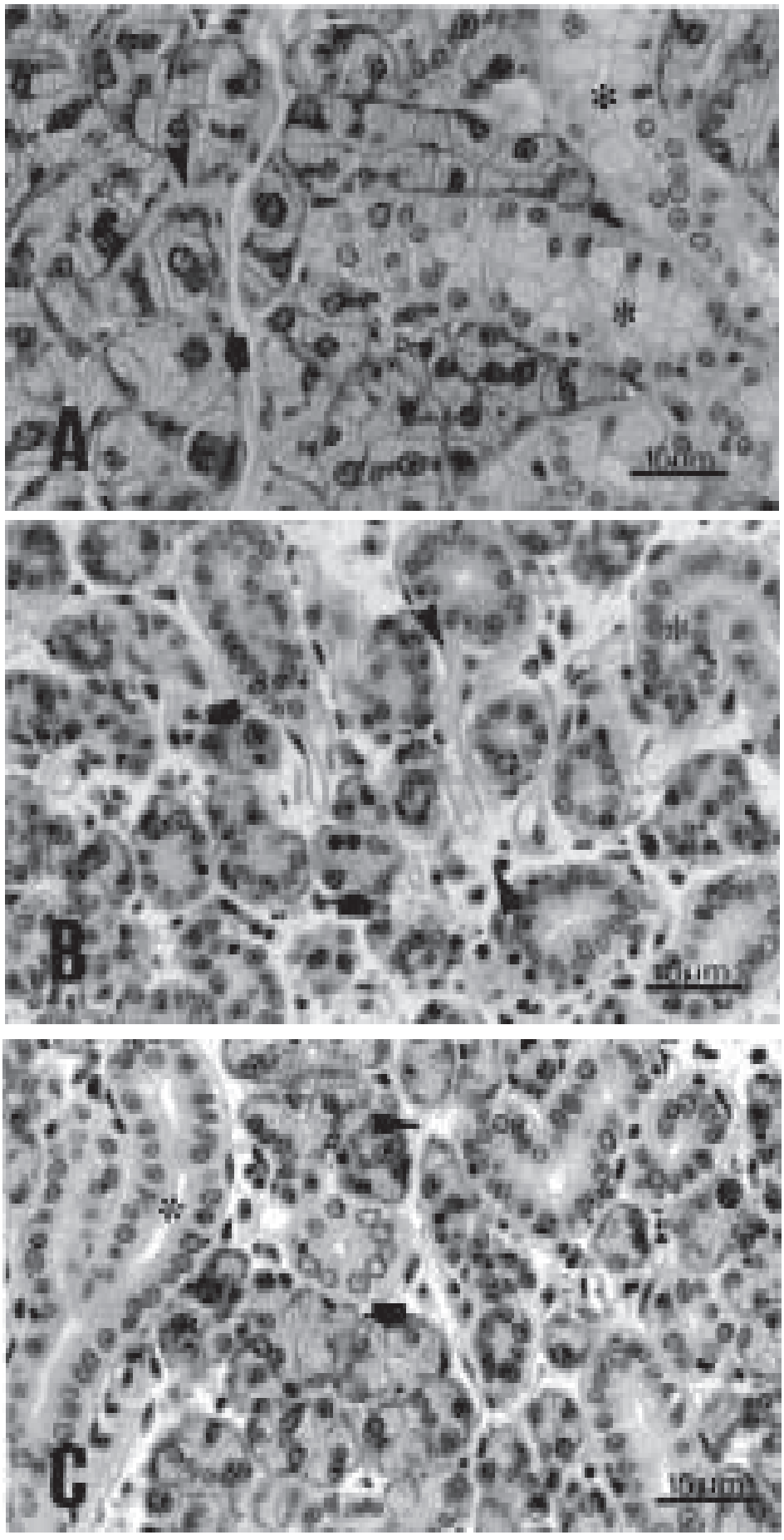
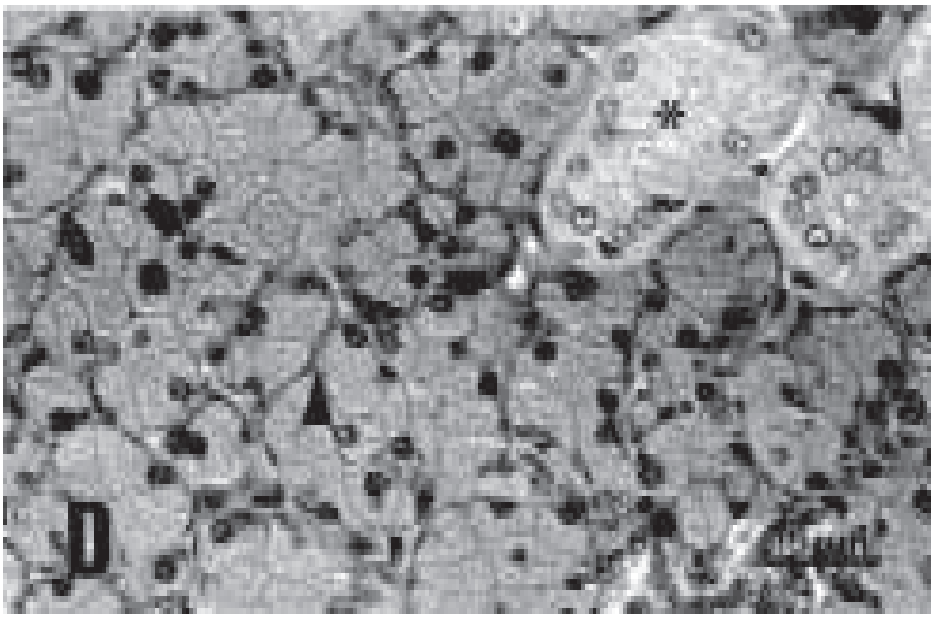


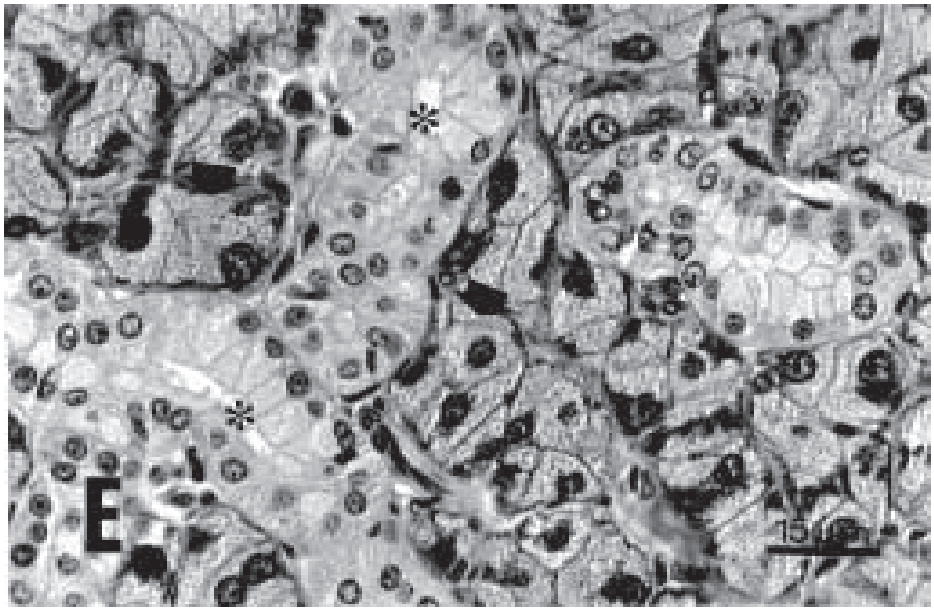
Figure 1. Circadian alterations in the histology of rat submandibular glands in the winter and summer:

A - Midnight, winter: note the acinar cells with their cytoplasm full of secretory material. The convoluted granular tubule cells are high and there is little stroma.

B - 4 a.m., winter: the acini and convoluted granular tubules occur as small cells with the nuclei very close to one another; the stroma occupies a large part of the gland.

C - 8 a.m., winter: the acini and convoluted granular tubules are still small but more easily recognized and the stroma is less marked than at 4 a.m.





D - 8 p.m., summer: the acinar cells have ample cytoplasm with a small nucleus in the basal region of the cell. The cells of the granular convoluted tubules are similar to those seen at midnight in the winter (reduced stroma).

E - 4 a.m., summer: the acini are smaller than at 8 p.m. (summer) and their nuclei are located more centrally.
In all cases, note the acini (arrows), convoluted granular tubules (asterisks) and stroma (arrowheads). Masson's trichrome.

winter, the acini represented 67.7 ± 3.75% of the glandular volume, and the stroma only 11.8% ± 3.01%. At 4 a.m., the acini decreased to 29.5 ± 2.5% of the volume, whereas the stroma occupied 49.2 ± 3.18% of the gland. During the summer, the acini and the stroma accounted for 68.0 ± 0.7% and 10.1 ± 0.9% of the gland, respectively, at 4 p.m. and 58.0 ± 1.6% and 18.0 ± 0.6% at midnight. The duct system showed no significant variations.

Figure 4 shows the absolute volume of each glandular compartment at different times of the day in winter and summer. For all of the components there were significant differences at some time point in the winter and summer. The greatest acinar size occurred at midnight in the winter and at 8 p.m. in the summer. The changes in the stroma were generally the opposite of these in acini.

The absolute volume of the duct compartment differed significantly from that of the convoluted granular tubules at various intervals (midnight - 4 a.m., 4 a.m. - 4 p.m. and 4 p.m. - 8 p.m) in the winter. The absolute volume of the intercalated ducts in the summer was always significantly greater than in the winter. The striated and excretory ducts showed no significant daily differences.

DISCUSSION

Most mammals show seasonal variations in many aspects of their physiology and behaviour. The causes of these oscillations include changes in temperature, humidity and water availability, but alterations in the photoperiod are the most important [10]. Seasonal variations also interfere with the circadian rhythm and act in conjunction with the normal day-night cycle.

Some studies [15,27] have reported that the weight of the large salivary glands varies according to the time of the day. As shown here, rat submandibular glands underwent daily and seasonal changes in size. These alterations were more accentuated in the winter, probably because of an increase in the consumption of solid food to maintain temperature and metabolism. This increase in food consumption would lead to a greater release of secretory products by the glands. In the winter, the submandibular glands showed a 38% decrease in weight from midnight to 4 a.m., while in the summer, the decrease was 15% from 8 p.m. to midnight and coincided with the period of glandular secretion when the rats were feeding. The subsequent synthesis and accumulation of secretory material eventually restored gland weight. Sreebny and Johnson [27], Sreebny *et al.* [28] and Vermouth *et al.* [30] observed that these

daily cycles were related to the regulatory mechanisms that exist between the synthesis, storage and release of secretions. Similar responses were observed in von Ebner's salivary gland [9]. These mechanisms depend on mastication and the quantity of solid food ingested.

At midnight in winter, the acinar compartment represented 67.7% of the gland volume (absolute volume of 119.3 mm^3), while the stromal compartment occupied 11.8% of the gland (absolute volume of 20.5 mm^3). Morphologically, all of the parenchymal structures were well-defined, and the acinar and convoluted granular tubule cells were full of secretion. The interlobular stroma was almost invisible because of the increase in the size of the lobules.

At 4 a.m. in the winter, the acinar compartment occupied only 29.5% of the glands, and the stroma 49.2% (absolute volumes of 32.4 mm^3 and 54.0 mm^3, respectively). By this time, the acini had already released their secretions and had decreased drastically in size, which made it difficult to identify them. A similar response was seen in the convoluted granular tubules, which decreased in volume by 44% from 21.6 mm^3 to 12.1 mm^3. The absolute volumes of the other interlobular ducts showed no significant variations. As the lobules decreased, the stroma became more evident because of an increase in absolute volume caused by hydration from plasma transudation. The increase in stroma volume did not involve cells, fibers or other tissue elements.

Beginning at 8 a.m. in the winter, the acini and duct system were more defined. The acini recuperated their volume from 4 a.m. to midnight. Recuperation in the convoluted granular tubules was similar but slower. Although the gland at midnight was well-developed in winter, it still had not reached 70% of the volume observed at 8 p.m. in the summer. In the rat parotid, α-amylase only begins to appear in glandular tissue at midnight [30], its highest levels occurring at 10 p.m. Thus, in this gland, the secretory material still increases in this period (10 p.m.), and consequently raises the secretory cell volume and gland weight.

The gland weight was higher in the summer, probably because of the lower consumption of solid food in this period. At 8 p.m., the acinar compartment occupied 67.2% (128.2 mm^3) of the gland and the stroma 11.2% (20.5 mm^3). Morphologically the acini were larger and their cells were full of secretory products. The nuclei, which were small and heterochromatic, occupied the basal region of the cell, and the stroma was smaller. At midnight, after rat had eaten, the gland weight reached its minimum (386.3 mg), and the acinar cells had less cytoplasm, because of the release of some of their granules. These cells occupied only 58% of gland volume (absolute volume of 94.3 mm^3). Albergger *et al.* [2,3] reported that the secretory granules in rat parotid glands showed changes in their number, shape and size in the daily cycle. With the release of some of their granules, the acinar cell nuclei became euchromatic and spherical

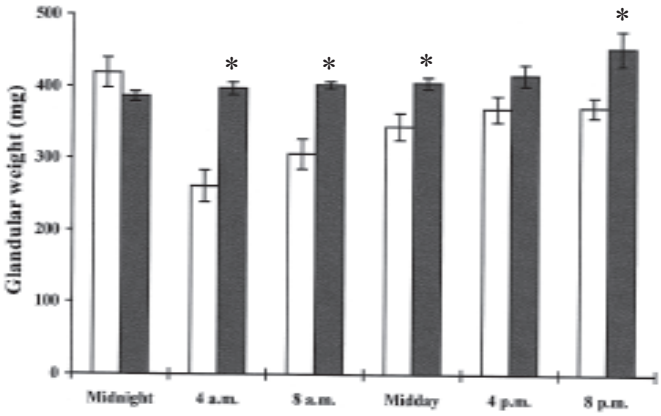


Figure 2. Rat submandibular gland weight in the winter (empty columns) and summer (filled columns). The results are the mean ± S.E.M. of 6 rats per group. $P<0.05$.

but still occupied the basal region. However, by 8 p.m. the acini had recovered their characteristic form following a period of synthesis and accumulation of secretory material. None of the structures in the duct system showed significant morphological or volumetric alterations.

Comparison of the behaviour of rat submandibular glands in the summer and winter revealed significant alterations in the acini and convoluted granular tubules in the winter, indicating that these structures are involved in releasing secretion in this period. In the summer, only the acinar component was affected. The cyclical behavior of the intercalated duct system was similar in the summer and winter. However, the absolute volume was about 40% less in the winter.

At 4 a.m. in the winter, mitosis was observed in the acinar cells. The replacement of these cells probably occurred during this period of the day. Since acinar cells contained little secretory material at 4 a.m., they were smaller and had fewer cytoplasmic structures, which facilitated mitotic division. By 8 a.m., the cells had recuperated their organelles and began their synthesis of secretory material.

Several studies have shown the synchronisation and regulation of cell proliferation by circadian rhythms in mammalian tissues. Such rhythms have important implications for the circadian control of the cell cycle in the treatment of cancer and other pathologies [6,7,25]. The circadian rhythm can directly influence the proliferation of the bone marrow and gut cells, and this is related to a circadian expression of cell cycle proteins in oral mucosa [6].

Litynska [18-20] reported seasonal effects on the daily acid phosphatase and β-acetyl-glucosaminidase activities in adult mouse submandibular glands at ambient temperature, humidity and photoperiod, and suggested that this phenomenon represented a genetic memory controlling hormonal background in these animals.

In conclusion, the results of this study show that the circadian rhythm in the rat submandibular gland is sensitive to seasonal changes that can affect the daily saliva

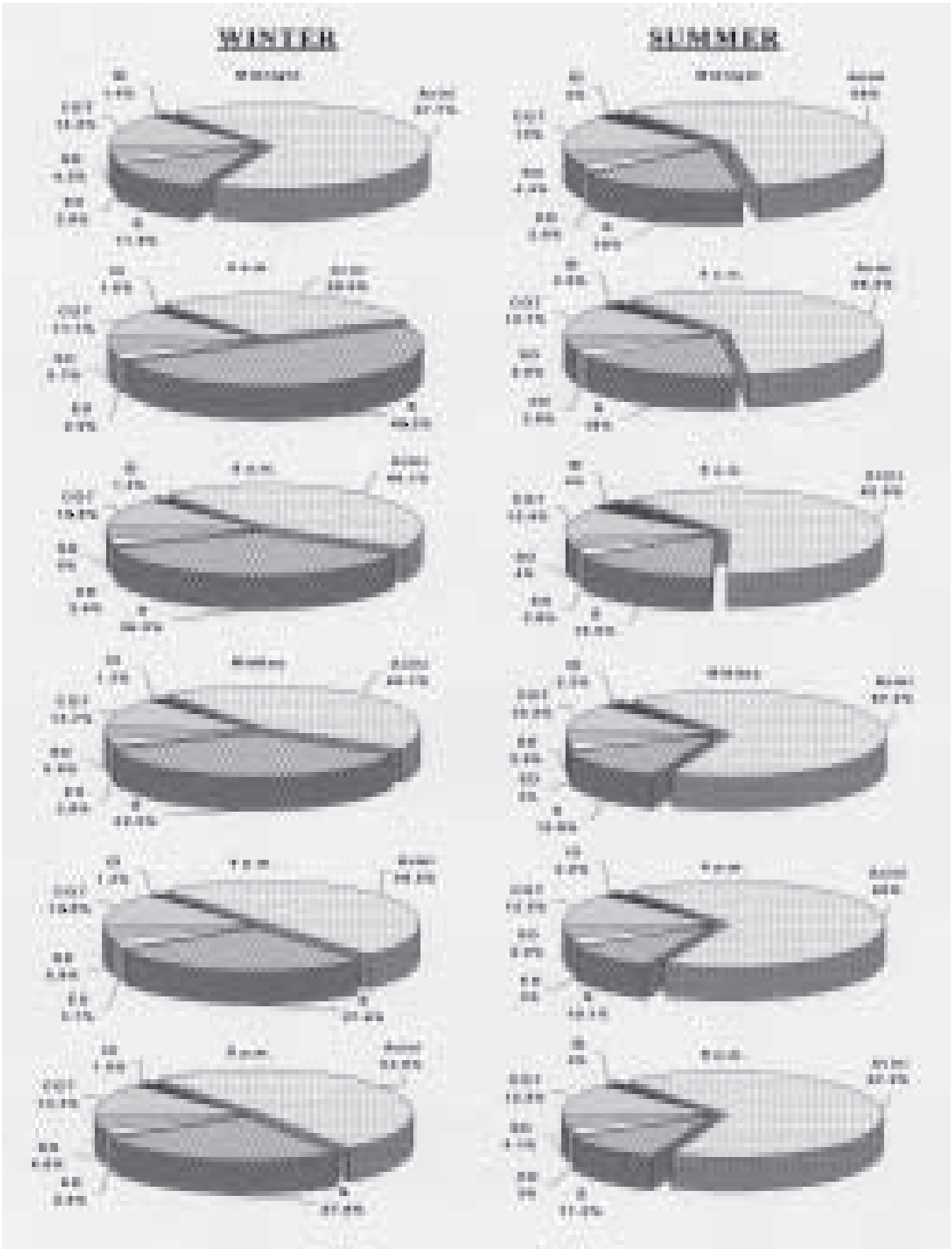


Figure 3. The volume density of rat submandibular gland compartments in winter and summer. ID - intercalated duct, SD - striated duct, CGT - convoluted granular tubule, ED - excretory duct, S - stroma.

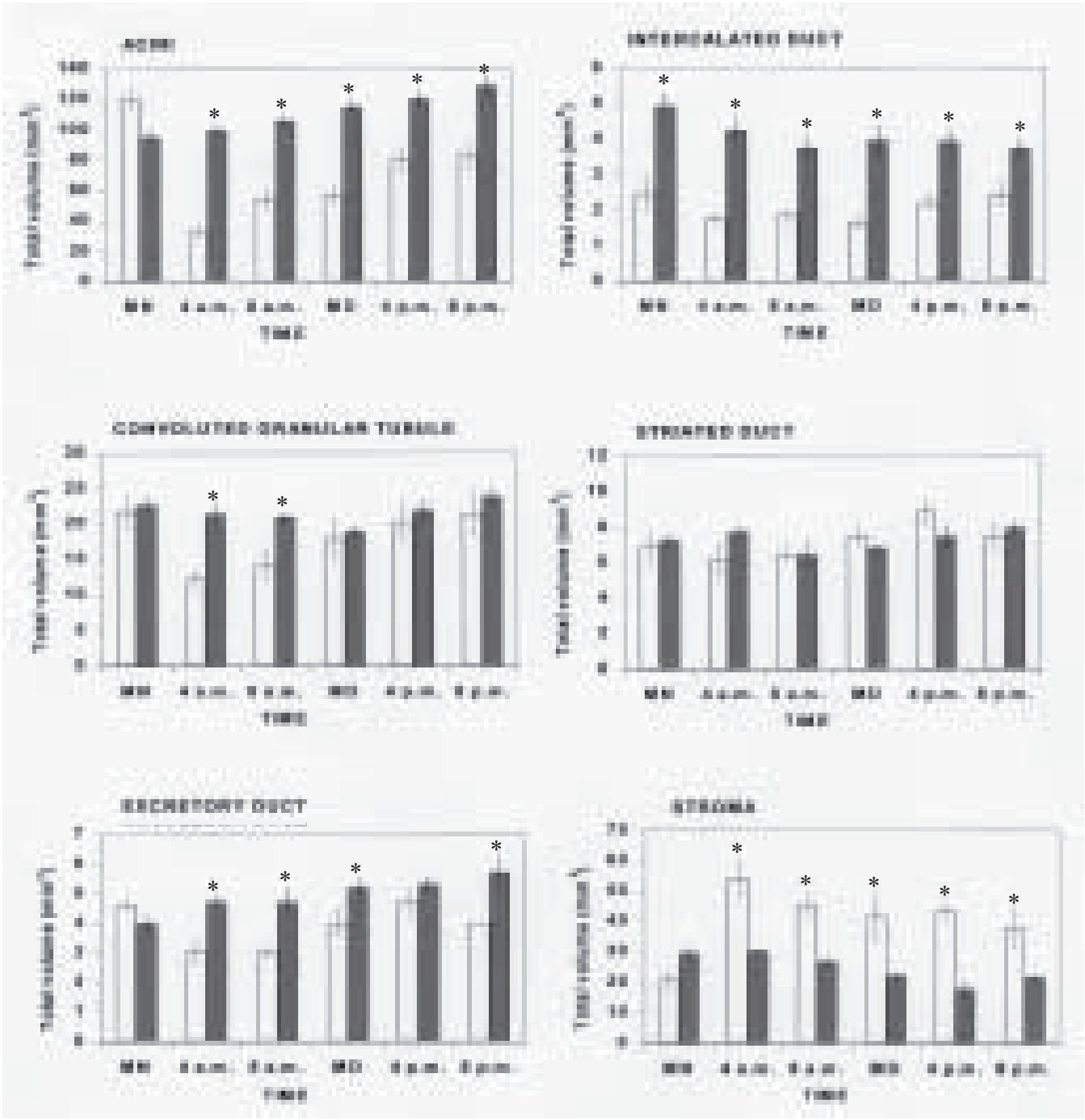


Figure 4. The total volume of rat submandibular gland compartments in winter (empty columns) and summer (filled columns). MN - midnight, MD - midday. The results are the mean ± S.E.M. of 6 rats per group. $P<0.05$.

production, with the morphological variations in the acini and convoluted granular tubules being much more pronounced in the winter.

REFERENCES

1. Albergger KW, Muller O, Albergger C (1975) Zur circadianstruktur der glandula parotis morphologische und morphometrische untersuchungen. *HNO* **23**, 233-236.
2. Albergger KW, Muller O, Albergger C (1977) Morphometric untersuchungen des physiologischen sekretions-zyklus' der glandula parotis der rate. *Mikroskopie* **33**, 142-147.
3. Barontini M, Romeo HE, Armando I, Cardinalli DP (1988) 24 hours change in catecholamine content of rat thyroid and submaxillary glands. *Neural Transm*. **71**, 189-194.
4. Bellavia SL, Gallara RV (1998) Modification of the beta- and alpha 2-adrenergic sensitivity of rat subman-

dibular glands by environmental stimuli and stress. *Arch. Oral Biol.* **43**, 933-939.
5. Bellavia SL, Sanz EG, Sereno R, Vermouth NT (1992) Alpha-amylase circadian rhythm of young rat parotid gland: an endogenous rhythm with maternal coordination. *Arch. Oral Biol.* **37**, 429-433.
6. Bjarnason GA, Jordan RC (2000) Circadian variation of cell proliferation and cell cycle protein expression in man: clinical implication. *Prog. Cell Cycle Res.* **4**, 193-206.
7. Bjarnason GA, Jordan RC, Sthoern RB (1999) Circadian variation in the expression of the cell-cycle proteins in human oral epithelium. *Am. J. Pathol.* **154**, 613-622.
8. Czeisler CA, Allan JS, Strogatz JM, Ronda JM, Sanchez R, Rios CD, Freitag WO, Richardson GS, Kronauer RE (1986) Bright light resets the human circadian pacemarker independent of the timing of the sleep-wake cycle. *Science* **233**, 667-671.
9. Field RB, Redman RS, Calloway AM, Goldberg WJ (1999) Effect of 24 hours light on circadian rhythms of secretory enzymes and morphology of rat von Ebner's glands. *Arch. Oral Biol.* **44**, 953-960.
10. Goldman BD (1983) The physiology of melatonin in mammalian**.** In: *Pineal Research Reviews.* Vol. 1. (RJ Reiter, ed). pp. 145-182. Alan R. Liss, Inc.: New York.
11. Goldman BD, Darrow JM (1983) The pineal gland and mammalian photoperiodism. *Neuroendrocrinology* **37**, 386-396.
12. Illnerová HM, Buresová J, Nedvidková J, Dvoraková M, Zvolsky P (1993) Maintenance of a circadian phase adjustment of the human melatonin rhythm following artificial long days. *Brain Res*. **626**, 322-326.
13. Ishikawa Y, Amano I, Chen C, Ishida H (1992) Diurnal variation of amylase secretion is coupled with alterations of beta-adrenoreceptores in the rat parotid gland. *Res. Exp. Med. (Berl)* **192**, 231-240.
14. Johnson DA, Sreebny LM (1971) Effect of food consistency and starvation on the diurnal cycle of the rat. *Arch. Oral Biol.* **16**, l77-185.
15. Johnson DA, Sreebny LM (1973) Effect of increased mastication on the secretory process of the rat parotid gland. *Arch. Oral Biol.* **18**, 1555-1557.
16. Laakso ML, Porkka-Heiskanen T, Alila A, Stenberg D, Johansson G (1994). Twenty-four-hour rhythms in relation to the natural photoperiod: a field study in humans. *J. Biol. Rhythms* **9**, 283-293.
17. Lewy AJ, Sack RL, Miller LS, Hoban TM (1987) Antidepressant and circadian phase-shifting effects of light. *Science* **235,** 352-354.
18. Litynska A (1984) Seasonal changes in the circadian activity rhythm of light-dark (LD) and completely dark (DD) regimen in the mouse submandibular gland in the presence of light-dark (LD) and completely dark (DD) regimen. *Physiol. Bohemoslov* **33**, 447-456.
19. Litynska A (1985) Influence of seasons on circadian rhythm of acid phosphatase and beta-acetylglucosaminidase activity in the mouse salivary gland and liver. *Acta. Physiol. Hung.* **65**, 389-397.
20. Litynska A (1987) Annual changes of beta-N-acetylglucosaminidase activity rhythm in mouse submandibular gland. *Acta. Physiol. Hung.* **69**, 211-216.
21. Menaker M, Moreira LF, Tosini, G (1997) Evolution of circadian organization in vertebrates. *Braz. J. Med. Biol. Res.* **30**, 305-313.
22. Pardini LC, Taga, R. (1986) Evaluation of the density of the mouse submandibular gland by the method of Scherle. *Rev. Fac. Odont. Ribeirão Preto, São Paulo* **23**, 206-207.
23. Reiter RJ (1993) The melatonin rhythm: both a clock and a calendar. *Experientia* **49**, 654-664.
24. Reuss S (1996) Components and connections of the circadian timing system in mammals. *Cell Tissue Res.* **285**, 353-378.
25. Smaaland R (1996) Circadian rhythm of cell division. *Prog. Cell Cycle Res.* **2**, 241-266.
26. Scherle WA (1970) Simple method for volumetry of organs in quantitative stereology. *Mikroskopie* **26**, 57-60.
27. Sreebny L M, Johnson DA (1969) Diurnal variation in secretory components of the rat parotid gland. *Arch. Oral Biol.* **14**, 397-405.
28. Sreebny LM, Johnson DA, Robinovirch MR (1971) Functional regulation of protein synthesis in the rat parotid gland. *J. Biol. Chem*. **246**, 3879-3884.
29. Taga R, Sesso A (1978) Avaliação do número de células de órgãos pela dosagem bioquímica de DNA em homogeneizados e por contagem direta através de métodos morfométricos. *Ciênc. Cult.* **30**, 1232-1236.
30. Vermouth N, Sanz EG, Bellavia SL (1985) Circadian rhythm of secretory and non-secretory proteins in parotid gland of rat. *Acta Odont. Latinoam.* **2**, 25-30.
31. Voutsios A, Kennaway DJ, Dawson D (1997) Salivary melatonin as a circadian phase marker: validation and comparison to plasma melatonin. *J. Biol. Rhythms* **12**, 457-466.
32. Weibel ER (1969) Stereological principles for morphometry in electron microscopic cytology. *Int. Rev. Cytol.* **26**, 235-302.
33. Wever RA, Polasek J, Wildgruber CM (1983) Bright light affects human circadian rhythms. *Pfügers Arch*. **396**, 85-87.
34. Wollnik F (1989) Physiology and regulation of biological rhythms in laboratory animals: an overview. *Lab. Anim.* **23**, 107-125.

Received: May 27, 1999
Accepted: March 06, 2001