ASSOCIATION OF AMITRYPTILINE AND DIAZEPAM ON THE HISTOMORPHOMETRY OF RAT PAROTID GLANDS

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Summary

This study aimed to investigate the association of two psychotropic drugs, an antidepressant and an anxiolytic one, on morphometric dimensions and salivary flow rate of rat parotid glands. Thirty six male Wistar rats divided in 2 groups were daily treated during 30 days with saline solution (control group, CG, n=18) or the association of amitriptyline and diazepam (experimental group, EG, n=18).

Saliva samples were collected 30 hours after the end of the treatment. The specimens were submitted to routine hematoxylin and eosin histological processing and sections were analyzed with the software Image Pro Plus version 4.5. Statistical significant differences (p<0.05) were observed between CG and EG for the parameters "gland size", "salivary flow rate" and "volume of cells". No differences (p>0.05) were detected for "number of cells" and "gland weight". The association of amitriptyline and diazepam showed an anticholinergic effect with the decrease of the salivary secretion. Also, the hypertrophy of the serous cells of parotid glands followed by an increase in glands size was observed.

Key-words: salivary glands, hyposalivation, psychotropic drugs

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Introduction

Xerostomia is the subjective sensation of dry mouth and occurs when the salivary flow rate is less than the rate of fluid loss from the mouth by evaporation and by absorption of water through the oral mucosa (1). Studies have found the condition in 17 to 29 percent of sampled populations based on self-reports or measurements of salivary flow rates (2). In a cohort study, dry mouth has been reported to affect between 10 to 44 percent of older people (3).

The chronic use of medications is clearly associated with dry mouth in the elderly. Several classes of drugs are linked to this disorder, for instance antihypertensives, antidepressants, antipsychotics, anti-Parkinsonian, anxiolytics and psychotherapeutic agents (3).

It has been shown that both stimulated and unstimulated whole saliva flow rates decrease with the increasing number of medications (4). Because the majority of elderly patients take multiple psychotrophic drugs, studies analyzing the interaction between polypharmacy and dry mouth are of scientific interest.

Thereby, this study aimed to investigate the association of two psychotropic drugs, an anxiolytic and an antidepressant one, on morphometric dimensions and salivary flow rate of rat parotid glands.

Material and Methods

All of the experiments followed the guidelines for the Scientific Practice of Vivisection in animals, as well as the Ethical Principals for Animal Experimentation, in accordance with Statute 6.638, of May 8th, 1979. This study was approved by the Research Ethics Committee of Tuiuti University of Paraná, under the registration number CEP-UTP 55/2003.

The animal model enrolled in this investigation consisted of male rats (*Rattus norvegicus albinus,* Wistar strain) obtained from the Central Animal Facility of the Pontifical Catholic University of Paraná.

The animals were aged 120 to 150 days, weighed approximately 180g, and were maintained in cages with water and food *ad libidum,* on a light/dark cycle of 12 hours.

Experimental group delimitation

The animals were divided in 2 groups of 18 subjects each: control group (CG), received 0.1 mL of physiological saline solution by intraperitoneal route for 30 days; experimental group (EG) received 0.4 mg/Kg of amitriptiline (Tryptanol[®], Laboratórios Farmacêuticos Prodome, São Paulo, Brazil) in the morning and 0.2mg/Kg of diazepam (Diazepam[®], União Química Indústria Farmacêutica, São Paulo Brazil) in the afternoon, by intramuscular route for 30 days. The psychotropic drugs doses were based on the data of Allen et al. (5).

Salivary gravimetry

Saliva samples were collected 30 hours after the end of treatment (6). Two drops of 4% pilocarpine hydrochloride eye drops (Allergan pilocarpina[®] 4%, Allergan Produtos Farmacêuticos Ltda., Guarulhos, Brazil) were briefly instilled in the rats' mouths. After 2 minutes, the saliva samples were collected with the animals gently positioned in ventral decubitus on the operator hands. The whole saliva dropped from their mouths was collected in a pre-weighted sterile universal collection vial. Immediately, the flasks were transferred to BelMark[®] U210A precision scale (Bel Engeneering, Piracicaba, Brazil) and the saliva masses were determined. A specific gravity of 1.006 was assumed (7) and the results of salivary flow rates (SRF) were expressed as mL/min.

Parotidean gland exsiccation and size measurement

All the steps bellow followed the protocols proposed by Onofre et al. (1997) (6), with modifications. Glands were obtained from each group right after the saliva collection. Rats were weighted and anaesthetised by intraperitoneal administration of 100 mg/kg sodium thiopental

(Thionembutal®, Abbott Laboratories) and killed. The right and left parotid glands were dissected and adipose tissue and adjacent lymph nodes were carefully removed.

Fresh gland masses were determined with a BelMark® U210A precision scale (Bel Engeneering, Piracicaba, Brazil). For the size measurements, the glands were laid out onto a flat glass surface and let for five minutes in order to accommodate the organs. After this, the millimetric longitudinal dimensions were achieved using a high-precision digital calliper Mitutoyo 500 Mical® (Mitutoyo Co., Tokyo, Japan).

Histomorphometry of parotid glands

The material was fixed in Helly's fluid for 3 hours and rinsed overnight with running water. On the subsequent day, the glands were submitted to dehydration in alcohol of increasing concentration (80, 95, and 100%), clearing in xylene, and embedding in paraffin. Semiserial 5 μm sections were cut and stained with haematoxylin and eosin.

Processed gland volume (vp) was calculated for each animal using the following equation $Vp=m/ d \times rf$, where m is fresh mass, d is density and rf is the shrinkage caused by histological processing. For these calculations, we used $d=1.089$ g/cm^3 and $rf=0.7$ by the method of Onofre et al. (1997).

For the stereological evaluation of acinar volume density (V_{vi}) and total volume (V_{ti}) it was used a magnification of 1000× in oil-immersion microscopy (Olympus BX50®, Olympus Co., Tokyo, Japan) with an objective PLAN 10×/0.25 (Olympus Co., Tokyo, Japan). All the images were taken by a CCD-IRIS® camera (Sony Co., New Jersey, USA) that were transferred to a interface of Image Pro Plus® 4.5 for Windows 98 (Media Cybernetics Inc., Georgia, USA) software.

We randomly captured 40 histological fields per gland and counted the points coinciding with the images of acini (P_i)

and the total number of points (P_t) on the gland. Volume density (V_{vi}) was calculated by the equation $V_{vi} = P_i/P_t$.

Having obtained the V_{vi} and processed gland volume (V_p) values, we calculated the total acinar volume (V_{ti}) by the formula $V_{ti} = V_{vi} \times V_p$.

Nuclear volume was determined from the measurement of the orthogonal diameters of 100 nuclei per gland using a microscopy technique as stated before. We calculated the mean radius of the geometric mean diameter by $r^2 = d_1 \times d_2$ and the nuclear volume by the formula for the volume of a sphere: $V = 4/3 \times \pi \times r^3$.

To calculate cytoplasmic volume densities of the nucleus and of the cytoplasm of acinar cells by point volumetry were determined and corrected the error due to the Holmes effect. In this respect, it was counted the points over nuclei (P_n) and over the cytoplasm (P_{cyti}) in 40 histological fields of the cells under study. The corrected nuclear volume density (p_{ncorr}) was calculated by the equation $p_{ncorr} = (P_n/P_n+P_{cyti})/K_o$, where K_o is the correction factor for the overestimation due to the Holmes effect. K_o is calculated by the formula $K_o = 1+3t/2d$, where d is the mean nuclear diameter and t is section thickness.

The corrected cytoplasm volume density is $p_{cyticorr} = 1 - p_{ncorr}$. By dividing $p_{cyticorr}$ by p_{ncorr} it was obtained the cytoplasm/nucleus ratio ($R_{C/N}$) of the acinar cells. On the basis of nuclear volume (V_{ni}) and the C/N ratio, it was calculated the cytoplasmic volume (V_{cyti}) by the equation $V_{cyti} = V_{ni} \times R_{C/N}$. This then permitted to calculate the cell volume by $V_c = V_{ni} + V_{cyti}$.

Total acinar-cell number was determined by the protocols proposed by Onofre et al. (1997) (6). For determination of the total number of cell nuclei it was used an integrated virtual grid generated by the Image Pro Plus® 4.5 for Windows 98 (Media Cybernetics Inc., Georgia, USA) software. In 40 microscopic fields selected at random for each gland, it was counted the number of nuclear images (n) and the

number crossing (c) between the margins of the profiles of the nuclear images and the parallel lines of the test system. Knowing the total area examined in mm^2 (A), the distance between the lines of the test system (d), the thickness of the secretion (t), and gland processed volume (V_p), we obtained the total number of acinar-cell nuclei using the equation $N = 2n \times V_p/A(c/n \times d+2t)$.

The number of nuclei per unit gland volume (N_{vi}) is calculated by the formula $N_{vi} = n' \times t/(t+2r-2K)$, where N_{vi} is the number of nuclei/mm^3, n' is the number of nuclear slices and fragments/mm^3, t is the section thickness, r is the mean radius, and K is the correction factor for nuclear spherical segments that escape counting and correspond to the vertical length of the smallest fragment observed. Here K is calculated by $r^2 = (r-k)^2+rf^2$ is the radius of the smallest nuclear spherical fragment observed. By multiplying N_{vi} by V_p the total number (N) of acinar cells in the gland was obtained.

Statistical analysis

Data normality for each group was tested by Kolmogorov-Smirnov test and Levene homogeneity of variances. Student test was used to analyze the relationship between macroscopic and microscopic aspects, and groups. The Mann-Whitney test was used to test differences between groups without data normality. The level of significance was set at 5% for all tests.

Results

Sialometric parameters

For the variables salivary mass and salivary flow rate (Table 1), the control group (CG) showed higher average values in relation to group treated with diazepam plus amitriptiline (EG) ($p < 0.0001$ and $p < 0.0001$, respectively).

Gland Dimensions

Table 1 shows the mean values of the investigated parameters. Gland size was statistically significant larger for EG than the CG (p = 0.0008).

Table 1 – Mean and standard deviation values of the investigated aspects.

Parameters	CG	EG	% change	p
Gland weight (mg)	0.176±0.028	0.174±0.038	1%	0.8827 [ns]
Gland size (mm)	12.139±1.277	13.881±1.558	14%	0.0008*
Salivary flow rate (mL/min.)	0.075±0.022	0.020±0.012	-73%	< 0.0001*
Number of cells (x10^6)	51.053±10.041	50.337±7.073	1%	0.8063 [ns]
Volume of Cells (mm^3)	1288.808±46.805	1725.093±76.105	34%	< 0.0001*

* Statistical significant difference between groups (p<0.05).
[ns] No-significant correlation
Source: PUCPR/CURITIBA, 2003

Volume of cells was statistically significant higher for the group EG when compared to group C (p < 0.0001). The CG group exhibited a statistical significant higher salivary flow rate than the experimental group (p < 0.0001). Gland weight and number of cells showed no statistical significant differences between groups (p > 0.05).

Morphological results
Acini morphology of CG

In this group, glandular parenchyma was arranged in a typical lobular structure containing acini and ducts (Figure 1).
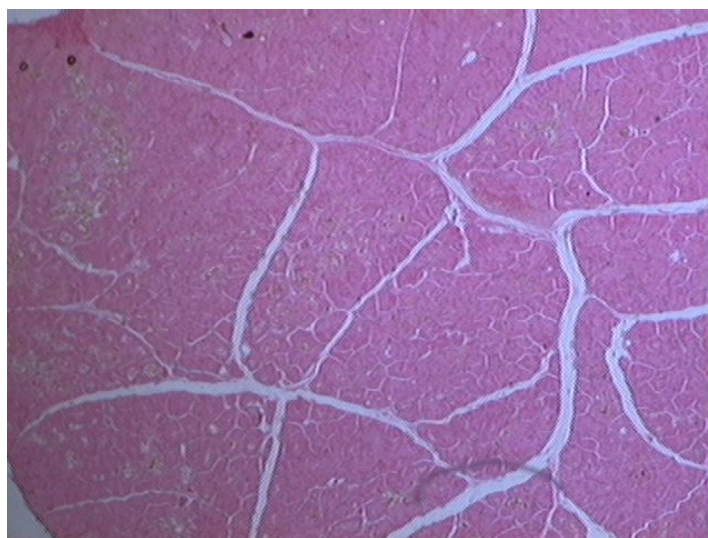
Fig. 1. Well-structured glandular parenchyma divided into lobes by the connective tissue septa (H.E.; original magnification 100X).

Basically, the parotidean acini were composed by serous pyramidal-type cells. These cells were observed on the periphery forming a central lumen, with nuclei localised in the basal portion and cytoplasm rich in serous granules (Figure 2).

Inside the lobules, intercalary ducts were covered by cuboidal cells while striated ducts were found covered by columnar cells. Surrounding the gland, a fine capsule of connective tissue was observed, which was continuous with the interior of the gland and generating the interlobular connective septa.
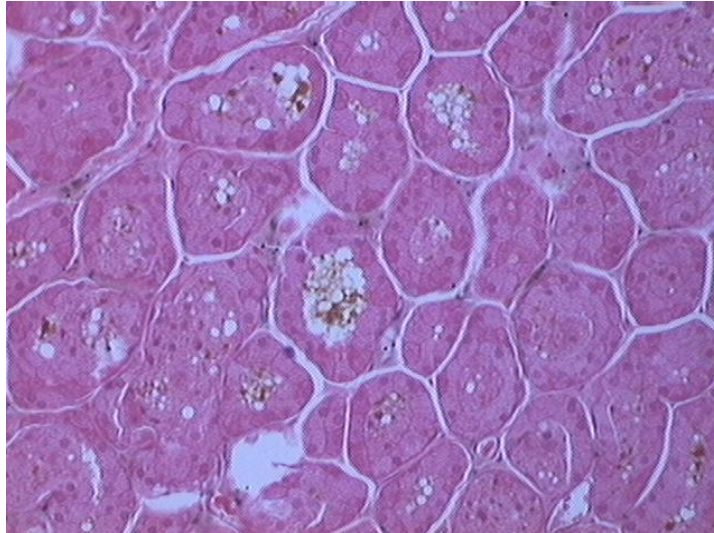
Fig. 2. Glandular lobules with serous acini and central lumen surrounded by serous cells. (H.E.; original magnification 200X).

Acini morphology EG group

The parotid glands of rats treated with amitriptiline plus diazepam exhibited a parenchymal disorganisation (Figure 3).
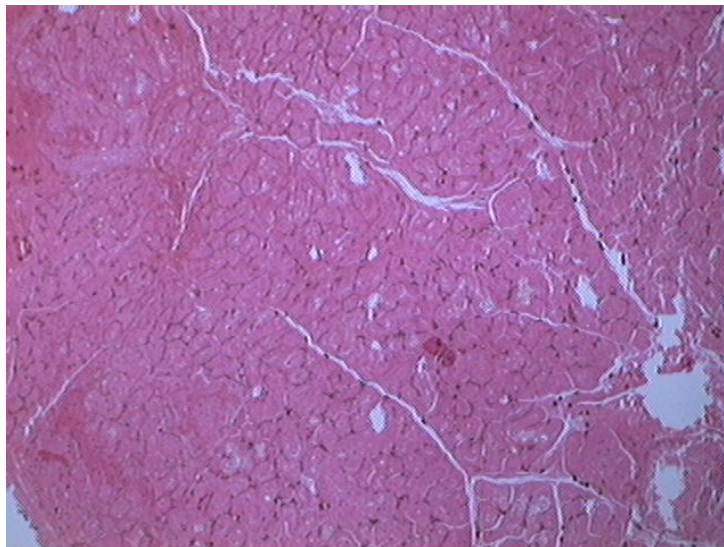

Fig. 3. Disorganized glandular parenchyma with lost of acini limits. (H.E.; original magnification: 100X)

There was a loss of borders to the serous cells, which were also increased in size, with a consequent reduction or disappearance of the central lumen (Figure 4).
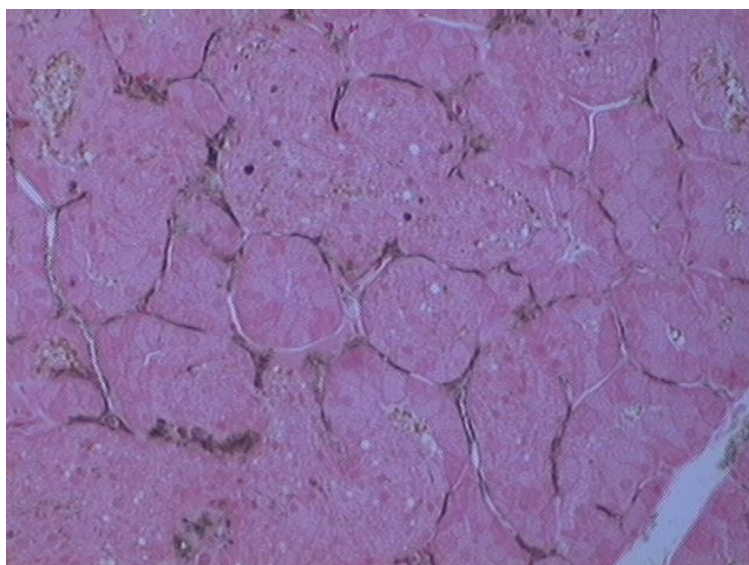

Fig. 4. Decrease/disappearance of the central lumen of serous cells. (H.E.; original magnification: 200X)

Discussion

Polypharmacy presents a formidable methodologic challenge for older people (3), with the majority of these patients taking more than one medication. In this study, the diazepam and amitriptyline drugs were used simultaneously in order to simulate polypharmacy in patients undergoing psychiatric treatment. Three out of the five parameters investigated in this study were affected by the associated administration of diazepan and amitriptyline: salivary flow rate, gland size and volume of cells.

Salivary secretion is complex and occurs subsequent to neurotransmitter stimuli. The principal control of secretion is derived from sympathetic and parasymphatetic innervation which regulates the secretory function on the acinar cell level and controls the reabsorption process in the striated ducts of

salivary glands (8; 9). Parasympathetic stimulation increases the volume of secreted saliva, whereas sympathetic stimulation mainly affects protein content and composition (8).

The salivary gland may serve as a model to determine the peripheral effects of different antidepressants on the monoaminergic and the cholinergic systems. Salivary gland function depends on the integrity of both parasympathetic and sympathetic innervation.

It has been shown that patients treated with amitriptyline exhibit a stimulated saliva secretion rate approximately 50% lower than that observed for the control group (7,10). In this study, the lower salivary flow rate (-73%) detected for the experimental group in comparison to the control group probably indicates a supplementary anticholinergic effect of diazepam associated to amitriptyline. This effect might be related to the known effect of the benzodiazepines enhancing the action of GABA (gamma aminobutyric acid), which works as an inhibitory neural mediator of the central nervous system reducing the influx of sympathetic and parasympathetic systems. Also, the high serum half-life of benzodiazepines makes them detectable in saliva after long periods of time (11), probably enhancing their anticholinergic effect over the salivary glands.

In this study, the gland size and volume of cells increased after treatment with diazepam and amitriptyline, while the number of acinar cells remained unchanged. On this basis, the increase in the gland size could only have been due to increased volume of cells, i.e. hypertrophy of cells already present in the gland by the retention of saliva inside the serous acini. This finding reinforces the anti-secretory action of the psychotropic drugs investigated, and was also observed by Onofre et al. (1997) (6) when studying the acinar growth of rat parotid gland induced by an anti-asthma drug (isoproterenol). The possible occurrence of cell hyperplasia leading to the increase of gland size is neglected because the number of serous cells counted for the group EG was even smaller than that counted for the control one.

Moreover, elderly patients exhibit age-associated changes in salivary gland structure with depletion of their secretory reserve (12). Adequate secretory function can still be maintained as long as no further stress, such as drugs causing hyposalivation, is placed on the secretory system (13). Thereby, the regular salivary gland hypofunction caused by psychotropic drugs could be more critical in elderly patients.

Conclusion

The association of amitriptyline and diazepam showed an anticholinergic effect with the decrease of the salivary secretion. Also, the hypertrophy of the serous cells of parotid glands followed by an increase in glands size was observed.

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