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Effects of Pilocarpine Hydrochloride on the Hyposalivation Induced by Psychotropic Drugs

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Abstract: The aim was to study the secretagogue action of pilocarpine on the murine parotid glands submitted to chronic treatment with psychotropic drugs by salivary flow rate determinations and histological alterations. Fifty four male Wistar rats were equally divided in three groups: C group (control) received saline solution for 30 days; AD group (n = 18) received Amitriptyline® and Diazepam® for 30 days, and ADP group (n = 18) received Amitriptyline® and Diazepam® for 30 days and Amitriptyline®, Diazepam® and pilocarpine for further 30 days, resulting in 60 days of treatment. Saliva samples were collected 30 h after the end of treatment. Parotids were removed and processed for hematoxylin-eosin histological analysis. Dedicated software for image processing allowed the determination of cell number and volume. Significant differences between paired-groups C-AD ($P < 0.01$) and AD-ADP ($P < 0.01$) were observed for glands size and weight. The volume of serous cells was greater in AD, suggesting a hypertrophy of the salivary glands. For salivary flow rate, C group showed the highest average. The number of serous cells was similar between groups ADP and C, with the lowest average being found in AD group ($P < 0.05$).

Key words: Salivary glands, pilocarpine, psychotropic drugs, hyposalivation.

1. Introduction

Pilocarpine is an alkaloid present in the leaves and calluses of Brazilian Rutacea *Pilocarpus jaborandi* Holmes that has been used by different South-American native tribes [1] and by cabocle population. Etymologically, the word jaborandi derives from the tupy-guarany term *yaborã-di* and means “saliva inducing plant”.

The therapeutic properties of this alkaloid are due to its direct and unselective action on cholinergic muscarinic receptors [2], where pilocarpine acts qualitatively as similar acetylcholine in the parasympathetic pathway.

Modern city living people have experienced a stressing way-of-life. In some instances, it may lead to depressive pictures that require treatment with antidepressant drugs. Among the undesirable effects, the antidepressant molecules have affinity for the adrenergic receptors present in the salivary glands leading them to produce a more viscous secretion with low flow rates. A reduction in the salivary flow can lead to many complications, including dry and burning mouth, as well as difficulty in speaking, swallowing, eating and sleeping [3, 4]. In addition, it has been associated with an increase in the prevalence and severity of periodontal disease and an increase in the susceptibility to root caries, erosion and dental hyperaesthesia, chronic mucositis, trauma of the soft tissues and oral candidosis [5, 6].

Despite the well-known pharmacological effects of pilocarpine on salivary glands, almost nothing concerning to the histological aspects was yet published. Also, it was never proposed a protocol to the restitution of salivary flow rates in patients

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undergoing depression and receiving antidepressant medication. Therefore, the present study aimed to evaluate histologically the secretagogue action of a galenic formulation of pilocarpine hydrochloride on the parotid glands of rats treated chronically with two psychotropic drugs.

2. Materials and Methods

2.1 Animals

All of the experiments followed the guidelines for the Scientific Practice of Vivisection in animals, as well as the Ethical Principal for Animal Experimentation, in accordance with Statute 6638, 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 used here was the male rat (*Rattus norvegicus albinus*, Wistar strain) with 120 to 150 days and approximately 180 g. Water and food were offered *ad libidum*, on a light/dark cycle of 12 h.

2.2 Pilocarpine Hydrochloride Gel Handling

An ora-base gel was prepared with 1% pilocarpine hydrochloride (Gerbras Chemical and Pharmaceutical, São Paulo, Brazil). According to the manufacturer, the alkaloid was entirely extracted from *Pilocarpus jaborandi* Holmes leaves. Throughout the experiments, the gel was keep at room temperature.

2.3 Experimental Groups' Delimitation

The animals were divided to three groups with eighteen subjects each: control group (C), received 0.1 mL of saline solution by intraperitoneal route for 30 days; AD group received 0.4 mg/kg of amitriptyline (Prodome Pharmaceuticals, São Paulo, Brazil) in the morning and 0.2 mg/kg of diazepam (União Química Pharmaceuticals, São Paulo, Brazil) in the afternoon, by intramuscular route for 30 days [7]; ADP group received the psychotropic drugs treatment for the first 30 days and, after this period, the animals still received the medication plus the topical oral application of 1% pilocarpine gel.

2.4 Salivary Gravimetry

Saliva samples were collected 30 h after the end of treatment, according to the protocols of Ref. [8]. Briefly, two drops of 4% pilocarpine hydrochloride eye drops (Allergan Pharmaceuticals, Guarulhos, Brazil) were instilled in the rats' mouths. After 2 min, the saliva was collected with the animals positioned in ventral decubitus. The whole saliva was collected in pre-weighted sterile universal collection vials that were transferred to a precision scale (Bel Engeneering, Piracicaba, Brazil). A specific gravity of 1.006 was assumed [9] and the results of SRF (salivary flow rates) were expressed as mL/min.

2.5 Exsiccation and Size Measurement of Parotidean Glands

Rats were weighted and anaesthetised by intraperitoneal administration of 100 mg/kg sodium thiopental (Abbott Laboratories, São Paulo, Brazil) 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 precision scale (Bel Engeneering, Piracicaba, Brazil). The glands were laid out onto a flat glass surface and let for 5 min in order to accommodate the organs. The longitudinal dimensions were achieved using a digital calliper (Mitutoyo Co., Tokyo, Japan).

2.6 Histomorphometry of Parotid Glands

After fixation in Helly's fluid for 3 h and rinsing overnight with running water the glands were submitted to dehydration in alcohol of increasing concentration from 80% to 100%, cleared in xylene, and embed in paraffin. Semiserial 5 μm sections were cut and stained with haematoxylin and eosin.

Processed gland volume (V_p) was calculated for each animal using the following Eq. (1):

$$V_p = m/d \times rf \tag{1}$$

where, m is fresh mass; d is density; and rf is the shrinkage caused by histological processing. For these calculations, it was used $d = 1.089$ g/cm^3 and $rf = 0.7$ [7].

For the stereological evaluation of acinar volume density (V_{vi}) and total volume (V_{ti}), it was used a magnification of 1,000× 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.

The study was 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 Eq. (2):

$$V_{vi} = P_i/P_t \qquad (2)$$

Having obtained the V_{vi} and processed gland volume (V_p) values, we calculated the total acinar volume (V_{ti}) by the Eq. (3):

$$V_{ti} = V_{vi} \times V_p \qquad (3)$$

Nuclear volume was determined from the measurement of the orthogonal diameters of 100 nuclei per gland using a microscopy technique as stated before. Then it was calculated the mean radius of the geometric mean diameter by the Eq. (4):

$$r = \sqrt{d_1} \times d_2 \qquad (4)$$

The nuclear volume was calculated by the Eq. (5) for the volume of a sphere:

$$V = 4/3 \times \pi \times r^3 \qquad (5)$$

To calculate cytoplasmic volume, we initially determined the 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 Eq. (6):

$$p_{ncorr} = (P_n/P_n + P_{cyti})/K_o \qquad (6)$$

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 Eq. (7):

$$V_{cyti} = V_{ni} \times R_{C/N} \qquad (7)$$

This then permitted to calculate the cell volume by the Eq. (8):

$$V_c = V_{ni} + V_{cyti} \qquad (8)$$

Total acinar-cell number was determined by the protocols proposed by Onofre et al. [8]. 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), it was obtained the total number of acinar-cell nuclei using the Eq. (9):

$$N = 2n \times V_p/A(c/n \times d + 2t) \qquad (9)$$

The number of nuclei per unit gland volume (N_{vi}) is calculated by the Eq. (10):

$$N_{vi} = n' \times t/(t + 2r - 2K) \qquad (10)$$

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 the Eq. (11):

$$r^2 = (r - k)^2 + rf^2 \quad (11)$$

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.

2.7 Statistical Analysis

The normality of the data for each group was tested using Lilliefors' test and Bartlett's test of homogeneity of variances. ANOVA was applied to detect the differences between the means of the groups, at a probability level of 5%.

3. Results

3.1 Sialometric Parameters

For the variables salivary mass and salivary flow rate (Table 1), the control group (C) showed higher average values in relation to group treated with amitriptyline plus diazepam (AD) ($P < 0.0001$ and $P < 0.0001$, respectively). Regarding to the comparison of the same variables for groups control (C) and with amitriptyline plus diazepam plus pilocarpine (ADP), the average values did not show great differences ($P = 0.9231$).

3.2 Gland Dimensions

Table 2 shows that the parotidean glands' masses of animals from AD group were superior to those found in animals from groups C ($P < 0.0001$) and ADP ($P < 0.0001$). Also, none statistical difference was observed between the last two groups ($P = 0.8057$).

In a similar manner, the glandular sizes of animals belonging to AD group were higher than both C ($P < 0.0001$) and ADP ($P < 0.0001$) groups. Differences between groups C and ADP were not stated ($P = 0.1627$).

3.3 Acinar Cells Morphometry

The average numbers of serous cells were considered similar between groups ADP and C ($P = 0.8126$), by the ANOVA. However, the same variable for AD group was considered lower when compared to groups C ($P = 0.0251$) and ADP ($P = 0.0435$) (Table 2). The cell volume data were heterogeneous among the three ($P < 0.0001$) groups with the AD group expressing the higher values.

3.3.1 Acini Morphology of Control Group

In this group, glandular parenchyma was arranged in a typical lobular structure containing acini and ducts (Fig. 1A). 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 (Fig. 1 detail a). Inside the lobules, intercalary ducts were covered by cuboidal cells while striated ducts were found covered

Table 1 Mean values for the sialometric variables.

Groups	Mass of saliva	SFR (mg/min)
C	0.1529 ± 0.0960^a	0.076^a
AD	0.0358 ± 0.0112^b	0.018^b
ADP	0.0830 ± 0.0072^a	0.041^a

Groups followed by the same letter did not differ from one another ($P > 0.01$). C = control group; AD = amitriptyline-diazepam group; ADP = amitriptyline-diazepam-pilocarpine group; SFR = salivary flow rate.

Table 2 Mean values for the glandular parotidean variables.

Groups	Mass (mg)	Size* (mm)	CV (μm^3)	N ($\times 10^6$)
C	0.1594 ± 0.0126^a	11.52 ± 0.49^a	1288.80 ± 46.80^a	51.05 ± 10.04^a
AD	0.1806 ± 0.0147^b	13.99 ± 0.89^b	2462.50 ± 43.19^b	44.12 ± 9.61^b
ADP	0.1567 ± 0.0124^a	11.98 ± 0.79^a	1725.09 ± 76.10^c	50.33 ± 7.07^a

* Size expressed in millimetres of the longitudinal axis. Groups followed by the same letter did not differ from one another ($p > 0.01$). C = control group; AD = amitriptyline-diazepam group; ADP = amitriptyline-diazepam-pilocarpine group; SFR = salivary flow rate; CV = cell volume; N = cell number.

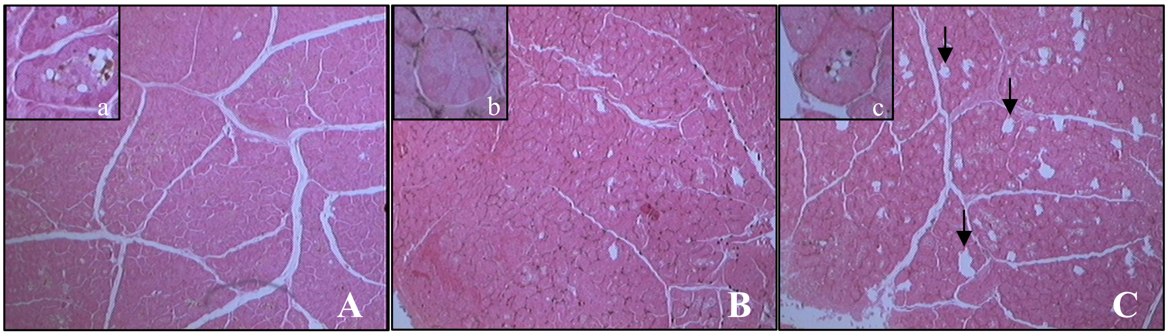

Fig. 1 Histological aspects of parotidean glands. (A) Control: Well-organized parenchyma dived into lobules by the connective tissue septa. (B) Psychotropic treatment: Disorganized parenchyma with loss of acini limits. In the detail (b), notorious retention of saliva within the serous cells. (C) Psychotropic plus pilocarpine treatment: Partial restoration of glandular parenchyma by the alkaloid. The arrows indicate probable loss of acini due to osmotic shrinkage induced by the pilocarpine. Pictures A, B and C (HE-40×); Upper details a, b, and c (HE-200×).

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.

3.3.2 Acini Morphology AD Group

The parotid glands of rats treated with amitriptyline plus diazepam exhibited a parenchymal disorganisation (Fig. 1B). 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 (Fig. 1 detail b).

3.3.3 Acini Morphology of ADP Group

In the parotid glands of rats treated with amitriptyline plus diazepam plus pilocarpine, it was observed a partial re-organisation of the glandular parenchyma with some normal aspects (Fig. 1C). The acini returned to exhibit a central lumen and the edges of the serous cells could be seen again (Fig. 1 detail c).

4. Discussion

Hyposalivation is the most common complaint among users of psychotropic drugs and brings with it numerous other problems. The saliva has several important functions in the mouth, including protection of the oral mucosa, chemical buffering, digestion, taste, antimicrobial action and maintaining the integrity of the teeth. Due to its glycoprotein contents saliva has a viscous aspect that protects the oral mucosa by the formation of a barrier against noxious stimuli, microbial toxins and minor trauma. Its fluid nature facilitates the removal of cell debris and non-adherent bacteria [10].

Despite the well-state knowledge that psychotropic drugs induce mild-to-severe reduction in salivary secretion rates, until the present date, no studies prospected the histological alterations in salivary glands. To our knowledge, this is the first report that evaluated the architectural alterations provoked by antidepressants and anxiolytics in such glands.

In this study, the histomorphometric results revealed that, after the long-term treatment with amitriptyline plus diazepam the parotid glands exhibited a disorganised parenchyma with loss of inter-lobular limits and a decrease and/or the disappearance of the central lumen. This anomalous architecture plus the results of acinar cell counts that did not vary suggest an increase in the size of the serous cells (Table 1). These findings led to the idea of hypertrophy and refute the supposition of hyperplasia. It can be postulated that such hypertrophy derives from the anticholinergic effect of the psychotropic drugs administered, via interaction with M3 muscarinic receptors, which reduces the release of saliva and leads to its retention within the gland. Such inference is reinforced by the similar cell counts ($P >$ 0.05) observed in the group that received psychotropic medication plus pilocarpine and in the control group.

Also, the antidepressant drugs possess affinity for adrenergic receptors. A study [8] involving the use of isoproterenol, a β2-sympathomimetic agent that acts in a similar manner to antidepressant drugs in salivary glands, showed that the interaction of this drug with glandular β2-receptors let the acinar cells to a state of hypertrophy, once the cell number did not vary and the parotid gland increased in volume.

The systemic drug therapy of hyposalivation with muscarinic cholinergic agonists is limited due to the secretagogue action of these molecules on other organs causing several side-effects [11]. A prescription of a gel basis for pilocarpine could facilitate its topical application, enabling it to be easily spread over the oral tissues, including the parotid glands. Furthermore, the topical use may enhance the local drug absorption and does not let to undesirable side-effects, as diarrhoea, excessive sweating, dyspepsia, asthenia, increase in urinary frequency and hypotension [12].

The animals that received only the psychotropic drugs exhibited reduced salivary rates and the concomitant administration of pilocarpine to the other group put under psychotropic regimen (group ADP) restored the salivary flow rates (Table 1). It may be explained by the inductive interaction of this alkaloid with M3 muscarinic receptors and subsequent secretagogue effect. Noteworthy, it was observed a release of the stored saliva greater than expected; since the group that received pilocarpine gel exhibited glands whose sizes and masses were lower when compared to the other groups (Table 1).

Beside the syalometric parameters here evaluated, it is important to point out that in the ADP group the structural architecture of lobular parenchyma did not diverge significantly in relation to the normal (non-treated) pattern. However, as it may be seen in Fig. 1 (arrows), some serous acini seemed to be elapsed. Such effect may be a result of an osmotic shrinkage subsequent to the intracellular free Ca^{2+} concentration ($[Ca^{2+}]$) that rises simultaneously at both the basolateral and luminal parts of the acinar cell after a luxuriant muscarinic cholinergic stimulation [13], here, induced by pilocarpine. It is reasonable to suppose that this shrinkage occurred in function of an excessive exposition to the parasympathetomimetic agent and this may be a serious inconvenient side-effect.

At now, our group is centering efforts to identify the beneficial effects of pilocarpine and to verify if it shows side effect. In this sence, we have been evaluated the histological and sialometric effects of the concomitant administration of psychotropics and muscarinic agonist drugs and verify a benefic results without side effect.

5. Conclusion

To conclude, 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, besides that the pilocarpine can modify this scenery.

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