Effects of Benzodiazepine and Pilocarpine on Rat Parotid Glands: Histomorphometric And Sialometric Study

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Abstract: Benzodiazepines are among the most frequently prescribed drugs and are often related with dry mouth. Pilocarpine is a cholinergic agonist that increases salivary flow rate and has been used to treat xerostomia. This study aimed to measure salivary flow rate of rats under chronic treatment with benzodiazepine (Diazepam®), to analyze by histomorphometry the effects of the drug in the parotids glands and to verify the effect of the pilocarpine in glandular parenchyma and in the salivary flow rate. Seventy-two male Wistar rats were allocated to four groups. Control groups received saline during 60 days (C60) and pilocarpine (Pilo) during 60 days. Experimental groups were dealt with Diazepam® associated with saline (DS), and Diazepam® associated with pilocarpine (DP) during 60 days. The stimulated salivary flow rate was obtained by using the gravimetric method. After the animals were killed, parotid glands were removed and mass and size were determined. The specimens were processed and stereological analysis revealed cell volume. Mean values of size and salivary flow rate varied from 9.007 mm and 0.015 mg/min in DS to 7.854 mm and 0.029 mg/min in DP, respectively. ANOVA showed statistically significant differences between groups for size (p=0.0028) and salivary flow rate (p=0.0003). Psychotropic drugs caused hyposalivation in rats and acinar hypertrophy in their parotid glands. Pilocarpine, a cholinergic agonist with topical appliance, showed significant secretagogue action in the treatment of hyposalivation induced by Diazepam® chronic use.

Key Words: Benzodiazepine, diazepam, saliva, hyposalivation.

INTRODUCTION

The benzodiazepines were first introduced in 1960. After their introduction into the market, they quickly substituted the barbiturates, becoming the most used medicine with sedative properties. Several clinical studies showed the effectiveness of the benzodiazepines short-term treatments for acute anxiety and insomnia, and, long-term treatments, for controlling well defined anxiety disorders, such as panic disorder or agoraphobia [1]. In dentistry, these kinds of drugs are indicated to decrease the anxiety of noncooperative patients in ambulatorial treatment.

Drugs are the most common cause of reduced salivation. The drugs often related with the cause of dry mouth are the tricyclic antidepressants, antipsychotics, beta blockers and antihistamines. Therefore, the complaint of dry mouth is particularly common in patients treated for hypertensive, psychiatric or urinary problems [2].

Pilocarpine is a cholinergic agonist that increases salivary flow and has been used to treat xerostomia. Oral intake is the most frequent route of administration. Adverse effects are dose-dependent and include sudoresis, facial blushing and increased urinary frequency [3].

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The association of two psychotropic drugs, an antidepressant (amitryptiline) and an anxiolytic one (Diazepam), on morphometric dimensions and salivary flow rate of rat parotid glands, showed an anticholinergic effect with the salivary secretion decrease. It was also observed the hypertrophy of the parotid glands serous cells followed by an increase in glands size [4].

Thus, this study aimed to investigate the effect of a psychotropic drug, an anxiolytic (Diazepam), and the association between Diazepam and pilocarpine on the salivary flow rate and on morphometric dimensions of rat parotid glands.

MATERIAL AND METHODS

All 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, Brazil. This study was approved by the Research Ethics Committee of Tuiuti University of Paraná (# 55/2003). The animal model used 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 four groups of eighteen animals each: group C60 received 0.1 mL of physiological

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saline solution by i.p. route during 60 days; group P received 0.05 mL of Pilocarpine by topic route during 60 days; group DS received 0.2 mg/Kg of Diazepam (Diazepam, União Química Indústria Farmacêutica, São Paulo Brazil) by i.p. route during 30 days and for 30 more days received 0.1 mL of physiological saline solution by i.p. route; group DP received 0.2mg/Kg of Diazepam by i.p. route during 60 days and in the last 30 days, it received simultaneously 0.05 mL of Pilocarpine by topic route. The psychotropic drugs and the saline solution were administered every day at 9:00 a.m. and the topic pilocarpine was administered every day at 10:00 a.m. The psychotropic drugs doses were based on literature [5].

Salivary Gravimetry

Saliva samples were collected 30 hours after the end of the 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-weighed sterile universal collection vial. The flasks were immediately 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 (SFR) were expressed as mL/min.

Parotid Gland Exsiccation and Size Measurement

Glands were obtained from each group right after the saliva collection. Rats were weighted and anesthetized by i.p. administration of 100 mg/Kg sodium thiopental (Thionembutal®, Abbott Laboratories) and sacrificed. The right and left parotid glands were dissected by carefully removing the adipose tissue and adjacent lymph nodes. Fresh gland masses (gland mass - M) were determined with a BelMark® U210A precision scale (Bel Engeneering, Piracicaba, Brazil). For the size measurements (gland size - S), the glands were laid out onto a flat glass surface and left there for five minutes in order to accommodate the organs. After that, the millimetric longitudinal dimensions were achieved using a high-precision digital calliper Mitutoyo 500 Mical® (Mitutoyo Co., Tokyo, Japan) [6].

Histomorphometry of Parotid Glands

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

Processed gland volume (V_p) was calculated for each animal using the following equation $V_p = \frac{m}{d \times rf}$, where m was fresh mass, d was density and rf was the shrinkage caused by histological processing. For these calculations, it was used d=1.089 g/cm^3 and rf=0.7 [6].

For the stereological evaluation of acinar volume density (V_{vi}) and total volume (V_{ti}) it was used a magnification of 1000X in oil-immersion microscopy (Olympus BX50®, Olympus Co., Tokyo, Japan) with an objective PLAN 10X/0.25 (Olympus Co., Tokyo, Japan). All the images were taken by a CCD-IRIS® camera (Sony Co., New Jersey, USA) and then analyzed using the 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 using the equation $V_{vi} = \frac{P_i}{P_t}$.

Based on the obtained V_{vi} and processed gland volume (V_p) values, the total acinar volume (V_{ti}) was calculated using the formula $V_{ti} = V_{vi} \times V_p$. The nuclear volume was determined from the measurement of the orthogonal diameters of 100 nuclei per gland using a microscopic technique, as stated before. The mean radius of the geometric mean diameter was calculated by $r = \sqrt{d_1 \times d_2}$ and the nuclear volume by the formula for the volume of a sphere: $V = \frac{4}{3} \times \pi \times r^3$.

In order to calculate the cytoplasmic volume densities of the nucleus and cytoplasm of acinar cells by point volumetry, we determined and corrected the error due to the Holmes effect. Hence, 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 following the equation $P_{ncorr} = \frac{P_n}{\frac{P_n + P_{city}}{K_o}}$, where K_o was the correction factor for the overestimation due to the Holmes effect. K_o was calculated by the formula $K_o = \frac{1+3t}{2d}$, where d was the mean nuclear diameter and t was section thickness. The corrected cytoplasm volume density was $P_{citycorr} = 1 - P_{ncorr}$. By dividing $P_{cyticorr}$ by P_{ncorr}, the cytoplasm/nucleus ratio ($R_{C/N}$) of the acinar cells was obtained. The cytoplasmic volume (V_{cyti}) was calculated following the equation $V_{city} = V_{ni} \times R_{C/N}$. Thereby, it was possible to calculate the cell volume (CV) by $V_c = V_{ni} + V_{cyti}$.

Statistical Analysis

Data normality for each group was subjected to Kolmogorov-Smirnov test and Levene homogeneity of variances. The analysis of variance (Anova) showed significant differences between the mean values obtained for the groups. Tukey's test identified the difference between treatments. The level of significance was set at 5% for all tests.

RESULTS

All the groups presented data distribution normality for the variables S, M, SFR and CV for the evaluated glands ($p>0.05$), which showed homogeneity of variance ($p>0.05$).

Table 1. Mean and Standard Deviation Values for the Investigated Aspects

Groups	C60 n = 18	P n = 18	DS N = 18	DP n = 18	p
S (mm)	8.242 ± 1.201	8.857 ± 1.154	9.007 ± 0.917	7.854 ± 0.631	0.003 *
W (mg)	0.094 ± 0.013	0.095 ± 0.015	0.098 ± 0.015	0.091 ± 0.014	0.495[ns]
SFR (mL/min)	0.020 ± 0.009	0.028 ± 0.009	0.015 ± 0.007	0.029 ± 0.014	0.000 *
CV(mm^3)	1347.72 ± 854.99	1463.99 ± 617.32	1762.06 ± 781.01	1880.16 ± 547.23	0.095[ns]

* Statistically significant difference.

[ns] No statistically significant difference.

Table **1** shows the mean values of the investigated parameters.

Gland size (S) presented a statistically significant difference between groups P and DP (p=0.019); DS and DP (p=0.005).

For the SFR (Table **1**), there was a statistically significant difference between groups P and DS (p=0.004), DS and DP (p=0.0007).

Gland mass (M) and CV showed no statistically significant differences between groups (p > 0.05).

Morphological Results

In group C60, glandular parenchyma was arranged in a typical lobular structure containing acini and ducts. Basically, the parotidean acini were composed by serous pyramidal-type cells. These cells were observed on the periphery, forming a central lumen with nuclei localized in the basal portion and cytoplasm rich in serous granules (Fig. **1**). Interlobular connective septa were observed.

The salivary glands of the group P showed similar characteristics to the samples of the group C60.

The parotid glands of rats treated with Diazepam plus physiological saline solution exhibited a parenchymal disorganization, with the loss of acini limits. The disappearance of the serous cells limits plus the reduction or the disappearance of the central lumen was also noticed (Fig. **2**).

Parotid glands of group DP showed severe disorganization of the parenchyma and total disappearance of the central lumen (Fig. **3**).

DISCUSSION

Benzodiazepines, such as Diazepam, Clonazepam, and Nitrazepam, have hypnotic, anxiolitic, and anticonvulsant actions mediated *via* the central-type benzodiazepine receptors [8]. On the other hand, these drugs have been known to induce xerostomia as a serious oral side effect [9].

The side effects of the benzodiazepines can occur due to their high liposolubility, since these drugs have an inherent capability to cross over the blood-brain barrier, and are hardly excreted by the kidneys. Besides that, the high serum half-life of Diazepam makes them detectable in saliva for long periods of time [10], probably enhancing their anticho-

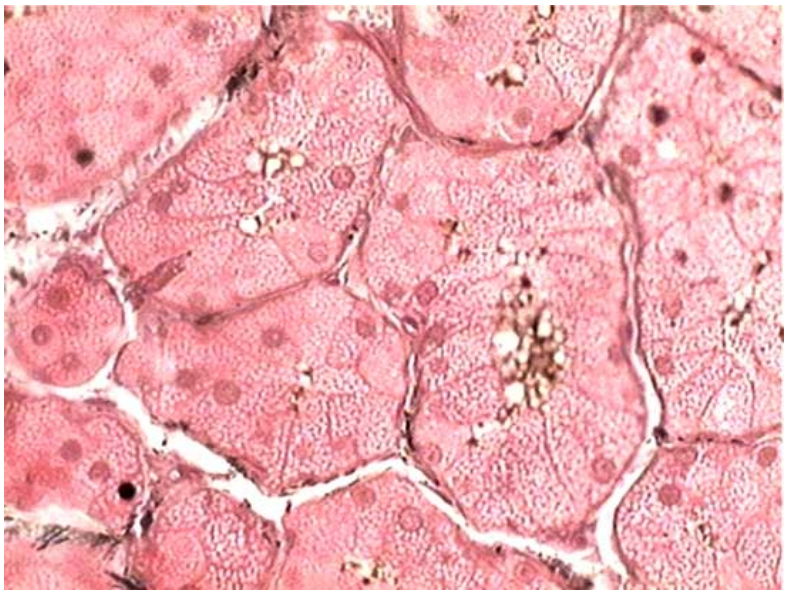

Fig. (1). Photomicrograph of rat parotid gland of the group C60, pointing out well delimited acinar cells, circumscribing the acinar lumen. (H.E.; original magnification 400 X). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this paper)

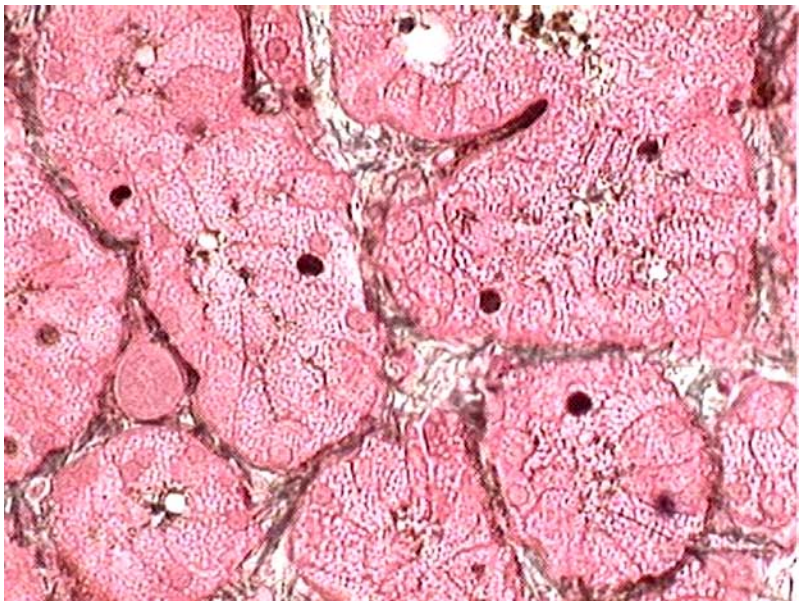

Fig. (2). Disorganized glandular parenchyma with loss of acini limits in rat of group DS. (H.E.; original magnification 400 X). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this paper)

linergic effect on the salivary glands. This effect has been also shown in our study, once the SFR decreased in the groups that received Diazepam plus saline (Table **1**).

Basically, saliva consists of two components that are secreted by independent mechanisms. First, there is a fluid component that includes ions, produced mainly by parasympathetic stimulation, and, secondly, a protein component released mainly in response to sympathetic stimulation. Salivary gland secretion is mainly under autonomic nervous control, although various hormones may also modulate salivary composition. Secretion appears to be dependent on several modulatory influences which act *via* either a cyclic AMPc or a calcium dependent pathway [2].

The main control of secretion is derived from sympathetic and parasympathetic innervations, which regulates the secretory function on the acinar cell level and resorption process in the striated ducts of salivary glands [11].

Previous studies demonstrated that both central and peripheral-type benzodiazepine receptors exist in the rat salivary glands as well as in the brain [12,13].

It has been previously reported that benzodiazepines decrease salivary secretion, cause muscarinic receptor stimulation in rats and modify chloride transport and Ca^{2+} flux activity, which trigger the process of fluid secretion in rat parotid cells [14]. In our study, the histomorphometric results revealed that, after the long-term treatment with Diazepam, the

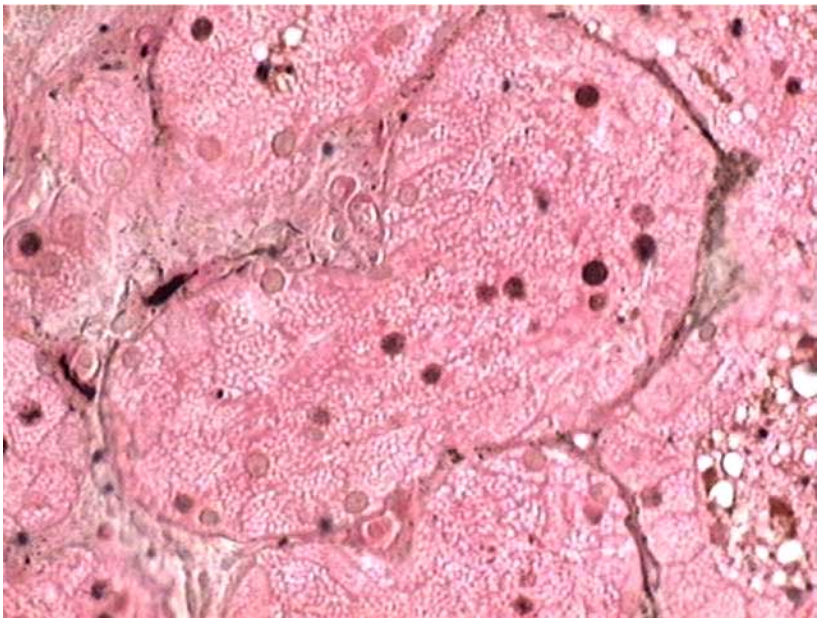

Fig. (3). Severe disorganization of rat glandular parenchyma of group DP (H.E.; original magnification 400 X). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this paper)

parotid glands exhibited a disorganized parenchyma with loss of inter-lobular limits and a decreasing and/or disappearance of the central lumen. This anomalous architecture associated with the high values of CV in groups DS and DP (Table **1**) suggests an increase on the size of the serous cells**.** This finding leads to the idea of hypertrophy, which is in accordance to the acinar hypertrophy observed in parotid glands of rats treated with Diazepam and Tryptanol [4]. 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.

Besides the volume increase of the acinar cells and the SFR decrease caused by Diazepam, it was also noticed a higher mass and a larger size of the parotid glands treated with such psychotropic, confirming thus, the saliva retention into the acinar cells (Table **1**).

Cholinergic agents stimulate acetylcholine receptors of the major salivary glands. The use of parasympathomimetic drugs, such as pilocarpine, can stimulate salivary gland secretion and has been shown to be effective for patients with Sjogren´s syndrome and for those who have had irradiation therapy or bone marrow transplantation [15]. These findings emphasize our results, since the group treated with Diazepam and pilocarpine showed an increase in the SFR values when compared to the groups treated only with Diazepam, suggesting that the pilocarpine can be used in the hyposalivation induced by benzodiazepines (Table **1**). Besides that, we noticed a decrease in the mass and size of the glands of rats treated with pilocarpine when compared to the rats treated only with Diazepam.

Patients using parasympathomimetic drugs, however, may experience a number of unpleasant side effects that may limit the efficacy of these medications [15]. A prescription of a gel basis for pilocarpine could facilitate its topical application, enabling it to be easily spread over the oral tissues. Drugs administered *via* the oral mucosal route are an alternative method of systemic administration for several classes of pharmaceutical agents, especially psychotropic drugs, vasodilators and antihypertensives. Furthermore, the topical use may enhance the local drug absorption also avoiding undesirable side-effects, such as diarrhea, excessive sweating, dyspepsia, asthenia, increase in urinary frequency, and hypotension [16].

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Hence, our study points out the acinar hypertrophy and hyposalivation caused by psychotropic drugs, as previously hypothesized [4,17]. Our results also suggest that the pilocarpine, a cholinergic agonist with topical appliance, has effectiveness against hyposalivation caused by benzodiazepines.

REFERENCES

[1] Kaplan, H.I.; Sadock, B.J.; Grebb, J.A. *Synopsis of Psychiatry,* Williams e Wilkins: Baltimore, **1994**.

[2] Scully, C. Drugs effects on salivary glands: dry mouth. *Oral Dis*., **2003**, *9*, 165-176.

[3] Bernardi, R.; Perin, C.; Becker, F.L.; Ramos, G.Z.; Gheno, G.Z.; Lopes, L.R.; Pires, M.; Barros, H.M. Effect of pilocarpine mouthwash on salivary flow. *Braz. J. Med. Biol. Res.,* **2002**, *35*, 105-110.

[4] Grégio, A.M.T.; Durscki, J.R.C.; Lima, A.A.S.; Machado, M.A.N.; Ignácio, S.A.; Azevedo, L.R. Association of amitryptiline and diazepam on the histomorphometry of rat parotid glands. *Pharmacologyonline*, **2006**, *2*, 96-108.

[5] Allen, D.G.; Pringle, J.K.; Smith, D.A. *Handbook of Veterinary Drugs***.** Lippincott Williams & Wilkins: Philadelphia, **2004**.

[6] Onofre, M.A.; de Souza L.B.; Campos, A.Jr.; Taga, R. Stereological study of acinar growth in the rat parotid gland induced by isoproterenol. *Arch. Oral Biol.,* **1997**, *42*, 333-338.

[7] Hunter, K.D.; Wilson, W.S. The effects of antidepressant drugs on salivary flow and content of sodium and potassium ions in human parotid saliva. *Arch. Oral Biol.,* **1995,** *40*, 983-989.

[8] Bormann, J. Electrophysiology of GABA A and GABA B receptor subtypes. *Trends Neurosci*., **1988**, *11*, 112-116.

[9] Sreebny, L.M.; Schwartz, S.S. A reference guide to drugs and dry mouth. *Gerodontology*, **1986**, *5*, 75-99.

[10] Concheiro, M., Villain, M., Bouchet, S., Ludes, B.; López-Rivadulla, M.; Kintz, P. Windows of detection of tetrazepam in urine, oral fluid, beard, and hair, with a special focus on drug-facilitated crimes. *Ther. Drug. Monit.,* **2005**, *27*, 565-570.

[11] Baum, B.J. Neurotransmitter control of secretion. *J. Dent. Res.,* **1987**, *66*, 628-32.

[12] Kawaguchi, M.; Yamagishi, H. Coupling of benzodiazepine and GABA(A) receptors in the salivary gland is a factor of drug-induced xerostomia. *Int. Acad. Biomed. Drug Res.,* **1996**, *11*, 291-296.

[13] Yamagishi, H.; Kawaguchi, M. Characterization of central- and peripheral-type benzodiazepine receptors in rat salivary glands. *Biochem. Pharmacol.,* **1998**, *55*, 209-214.

[14] Kawaguchi, M.; Ouchi, K.; Ohse, S. *In vitro* studies on receptive mechanisms for benzodiazepines in rat parotid gland. *Dent. Jpn.,* **1995**, *32*, 38-40.

[15] Guggenheimer, J.; Moore, P.A. Xerostomia: Etiology, recognition and treatment. *J. Am. Dent. Assoc.,* **2003**, *134*, 61-69.

[16] Gorsky, M.; Epstein, J.B.; Parry, J.; Epstein, M.S.; Le, N.D.; Silverman, S.Jr. The efficacy of pilocarpine and bethanechol upon saliva production in cancer patients with hyposalivation following radiation therapy. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.,* **2004**, *97*, 190-195.

[17] Martinez-Madrigal, F.; Micheau, C. Histology of the major salivary glands. *Am. J. Surg. Pathol.,* **1989,** *13*, 879-899.