# Histological Changes in the Parotid Salivary Gland of Rabbit Treated with Neostigmine

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## Abstract

**Back ground: Neostigmine** is parasympathomimetic drug. It is commonly used for treatment of myasthenia gravis, glaucoma, urinary retention, xerostomia, and post-operative ileus. Parasympathomimetic drugs increase the rate of salivation.

**Methods:** Twelve female rabbits were used in this experiment to show the effect of neostigmine. Therapeutic, double therapeutic and triple therapeutic doses of the drug were administered intramuscularly for two weeks. Samples of parotid salivary glands were processed for light microscopy. Sections of parotid were stained with H&E, PAS, and alcian blue. Statistical analysis was followed to measure the diameter of the secretory acini.

**Results**: The most noticeable changes were significant increase in the diameter of the secretory acini, and vacuolation with foamy appearance of the cells of the acini in treated groups. The PAS positive reaction in the acini was decreased as the dose of the drug increased. It had completely vanished in triple therapeutic dose of the drug. Negative reaction was obtained after alcian blue staining.

**Conclusion**: Neostigmine as sialogogues drug simulates the effect of parasympathomimetic drugs. The structural histological alterations noticed in this study substantiate the use of this drug in cases of xerostomia.

**Key words:** Rabbits, parotid gland, histology, neostigmine, vacuolation

## الخلاصة

**الخلفية** : يعتبر دواء النيوستجمين دواء ذو تأثير مماثل لتأثير الجهاز العصبى جار الودى من حيث زيادته لافراز اللعاب . لهذا الدواء استخدامات عديدة فى علاج الوهن العضلى والماء الازرق فى العين واحتباس البول وجفاف الفم والمغص المعوى المصاحب للعمليات الجراحية.

**طرق البحث** : استخدم فى هذه الدراسة اثناعشر ارنبة وشملت الدراسة الحقن العضلى لمدة اسبوعين لهذه الارانب بثلاث جرعات مختلفة من دواء النيوستجمين وتشمل هولاء الجرعات الجرعة العلاجية وضعف الجرعة العلاجية وثلاث امثال الجرعية العلاجية.تم اخذعينات من الغدد اللعبية النكفية لاجل الحصول على مقاطع نسجية ليتم دراستها بالمجهر الضوئى. صبغت المقاطع بالصبغات الاتية: صبغة الهيماتوكسلين والايوسين وصبغة الباز وكذلك صبغة الالسيان الزرقاء وشملت هذه التجربة ايضا دراسة احصائية لغرض قياس قطر الوحدات الافرازية.

**ألنتائج:** تبين من خلال الدراسة زيادة ملحوظة مضطردة فى قطر الوحدات الافرازية مع ظهور فجوات فى داخل خلايا الوحدات الافرازية للغدة اللعابية النكفية .كما لوحظ ايضا تثبيطا لحدة التفاعل مع صبغة الباز فى الوحدات الافرازية مع الزيادة المضطردة لجرعة الدواء حيث يختفى التفاعل كليا عند وصول الجرعة المعطاة الى ثلاث اضعاف الجرعة العلاجية . لم تضهر النتائج اى نوع من انواع التفاعل النسيجى مع صبغة الالسيان الزرقاء.

**ألاستنتاج** : ان دواءالنيوستجمين دواء مسيل للعاب يجانس فى تأثيره تأثير الجهاز العصبى الجار ودى وتؤكد النتائج المتحصلة من هذا البحث فعالية استخدام هذا الدواء فى علاج جفاف الفم .

## Introduction

Saliva is essential for alimentation, remineralisation of teeth, and the protection and lubrication of oral mucosal tissues, and diminished output can have deleterious effects on oral and systemic health [1 - 4] .Many drugs of medical applications either directly or indirectly cause joint size increasing of exocrine gland secretions. Among these drugs is the neostigmine, which is parasym-pathomimetic drug, It is commonly used as anticholinestrase of carbamate ester type for treatment of myasthenia gravis, glaucoma, urinary retention, xerostomia, and post operative ileus [5 -8 ].Neostigmine reversibly inhibit the acetylcholinestrase enzyme by acting as a substrate, so inhibiting the breakdown of acetylcholine in enhancement of acetylcholine activity at parasympathetic postganglionic synapses leads to increased secretion of salivary, lacrimal, bronchial, and gastrointestinal glands, increased peristaltic activity, papillary constriction, bronchoconstricion, bradycardia and hypotension, fixation of accommodation for near vision and fall in intraocular pressure [8 -12].As the mechanisms underlying the histological structural changes induced in rabbit parotid glands neostigmine have been overlooked, the present study was undertaken to investigate the participation of neostigmine in these alterations.

## Materials and Methods

**I-Experimental animals:** The present experiment was conduct on twelve apparently healthy female rabbits, 6 months old, of local mixed breed, weighing between 800-1000 gm. The experimental animals were kept under controlled laboratory conditions for one week for acclimatization of animals to the laboratory environment. The animals were allowed unrestricted access to food and water. According to the types of the drugs used, and the duration of administration of the drugs, the rabbits were divided into four groups, viz: G1; G2;G3 and G4 ,consisting of three rabbits each. The first three groups were treated groups, while the G4 has served as a control group.

**II- Drugs used:-**The drug used in the present work was neostigmine methyl sulfate (Neostigmine, ROTEXMEDICA/ Germany); it is a synthetic quaternary ammonium parasympathomimetic agent. This drug was available as 0.5 mg vial or ampoules served for an injection. Neostigmine was stored at room temperature and protected from light.

**III-Calculation of the drug dose**:-

**a)**

**eostigmine**: The drug dose used in this experiment was calculated according to Pagat and Barnus formula [13]**.** Human dose of neostigmine is o.o5 mg/kg body weight.For human with 70 kg body weight, the required dose is 0.05× 70 =3.5 mg once a day. According to Paget and Barnus formula, the dose for rabbit weighing 1.5 kg = Human dose (70 kg) × 0.07= 0.245 mg; so the therapeutic dose used in this study was 0.147mg of neostigmine once a day.

**IV-Administration of the drug and duration of treatment:-** The three experimental groups were given an intramuscular injection of neostigmine for **two weeks** as follows:

1- **G 1 group** (3 animals) has received an injection of therapeutic dose of neostigmine (0.147 mg) once a day.
2- **G 2 group** (3 animals) has received an injection of double therapeutic dose of neostigmine (0.294 mg) once a day.
3- **G 3 group** (3 animals) has received an injection of triple therapeutic dose of neostigmine (0.441 mg) once a day.
4- **G 4 group** (3 animals) served as a control, they have received equivalent injections of isotonic saline for the same period of time.

**V- Collection and processing of samples:-** Under general anesthesia (chloroform inhalation anesthesia), the

rabbits were sacrificed. Samples of parotid salivary glands obtained from the experimental animals were fixed in neutral buffered formalin, embedded in paraffin and stained with hematoxylin and eosin (H&E); periodic acid Schiff (PAS) stain [14] with and without pretreatment with diastase in order to differentiate between glycogenic and nonglycogenic mucuosubstances [14] alcian blue (AB) pH 1 and 2.5 [14] for acidic mucuosubstances.

**VI- Statistical analysis:** Histometric evaluations of diameter of secretory end pieces were conducted on ten slides stained by Haematoxylin and Eosin stain. For the measurement, an eyepiece equipped with graticule having standard scale was used. The readings obtained were multiplied by a factor to get the result in micrometer. Data, expressed as mean ± SEM, were analyzed by ANOVA and multiple comparison tests. Probability less than 0.05 is considered significant.

## Results

*** Clinical observation:**

**G 1 group**: Injected with therapeutic dose of neostigmine: 10 minutes after injection the following observation were noticed: Excitation of the animals followed by relaxation, weakness and excessive salivation.

**G 2group**: Injected with double therapeutic dose of neostigmine:

10 minutes after injection there were excitation followed by relaxation, weakness, excessive salivation, diarrhea and excessive urination.

**G 3 group:** Injected with triple therapeutic dose of neostigmine:

10 minutes after injection there were excitation followed by relaxation, weakness, excessive salivation, diarrhea, excessive urination, and difficulty in breathing (wet breathing sound).

The reaction of the neostigmine treated rabbits was subsided and relieved after two hours.

*** Histological findings:**

**Control group (G 4)**

The parotid gland in rabbits of control group (G4) was composed of purely serous acini. The acinar cells contained apical acidophilic part with acidophilic granules. Their nuclei tend to be displaced toward the basophilic distal third of the cells.The intralobular duct system comprised of fairly long intercalated ducts, which in turn empty into the striated ducts. The striated ducts were lined by a single layer of columnar epithelium. Their cells have eosinophilic cytoplasm, and large nucleus located in the central basal part. These ducts were surrounded by connective tissues (figure 1 A) in which blood vessels were observed. The striated ducts end in the interlobular ducts. The latter ducts mainly located in the extralobular connective tissue. They were lined by pseudostratified columnar epithelium. For the PAS stained sections, with and without pretreatment with diastase, the results were the same. Positive reaction was identified in the acinar cells (figure 2A and figure 3A). No reaction with alcian blue pH 1.0 and 2.5 were observed.

**Neostigmine treated groups**

In sections stained with the H&E stain, of the neostigmine treated group (G1 group) the most noticeable changes were the vacuolation and foamy appearance of the cells of their secretory acini (figure 1 B). The transformation was partial in the G1 group. With the increasing of the dose of the drug, as in groups G2 and G3, the transformation was more obvious. It was represented by increase in rate of vacuolation. In addition to that, the striking changes in the intralobular duct system were represented by filling of their lumen with mucous like material (figure 1 B, C and D). The diameter of the acini dramatically increased in the treated groups (see table 1; figs.4 and 5).The reaction for PAS stain (with and without pretreatment with diastase) was showing the same results. The reaction was moderate in the secretory acini and intralobular duct system of G1 group. In

G2 group the reaction has become mild. In G 3 group there was a dramatic loss of the reaction (figure 2 and 3).The statistical analysis revealed a change in the diameter of the acini of the parotid gland (see tables 1, 2, 3 and figs. 4 and 5). There was a difference between the three treated subgroups and the control with P value ≤ 0.05.

## Discussion

This study has demonstrated that there was pronounced increase in the diameter of the serous acini with striking vacuolation in the cytoplasm of neostigmine treated groups Neostigmine as a parasympathomimetic drug, has been used to overcome the problem of compliance of hyposalivation associated with certain diseased conditions [15], and medication [7] .The evaluation of neostigmine as sialogogues drug simulate the effect of parasympathetic nervous system [7] is very important.In factneostigmine and other parasym-pathomimetic drugs have been used in many pharmacological approaches as prophylactic agents in patients receiving radiotherapy at head and neck malignancies, who were suffering from diminished salivary gland output [15, 16]. The clinical approaches of the latter authors were in support to the histological results of this work. Hakim et al., [15] have noticed an improvement in compliance of the patients receiving radiotherapy after the use of the drug. In fact neostigmine like other parasympathomimetic drugs was important to induce compound exocytosis to the secretory granules [1 7], a process that was considered radioprotective in previous study [18 -21].

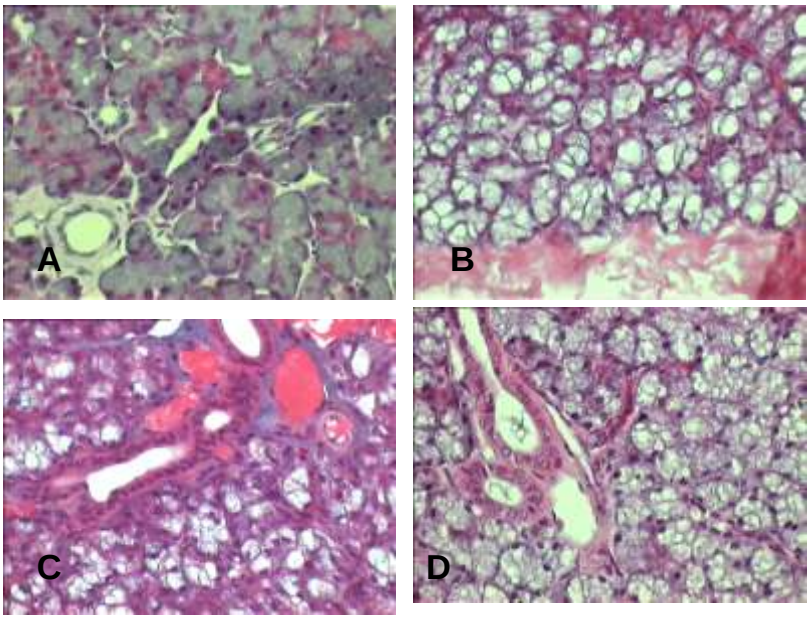


Figure 1. The micrograph of H & E stained sections of rabbit parotid gland:
**A**- The photomicrograph shows normal structure and appearance of secretory acini and duct system in the control subgroup. **G 4 group**. Original magnification × 200.
**B**- The photomicrograph shows acini of parotid gland in therapeutic dose treated rabbits. Note the presence of vacuolation in the cytoplasm.
**G 1 group .**original magnification × 200.
**C**- The photomicrograph shows clear vacuolation of cytoplasm and congestion of blood vessels in double therapeutic dose of neostigmine injected rabbits **G2 group**. Original magnification × 200.
**D**-. The vaccuolation the cytoplasm is much more pronounced in this subgroup of rabbits injected with triple therapeutic dose of neostigmine. **G 3 group**. Original magnification × 200.

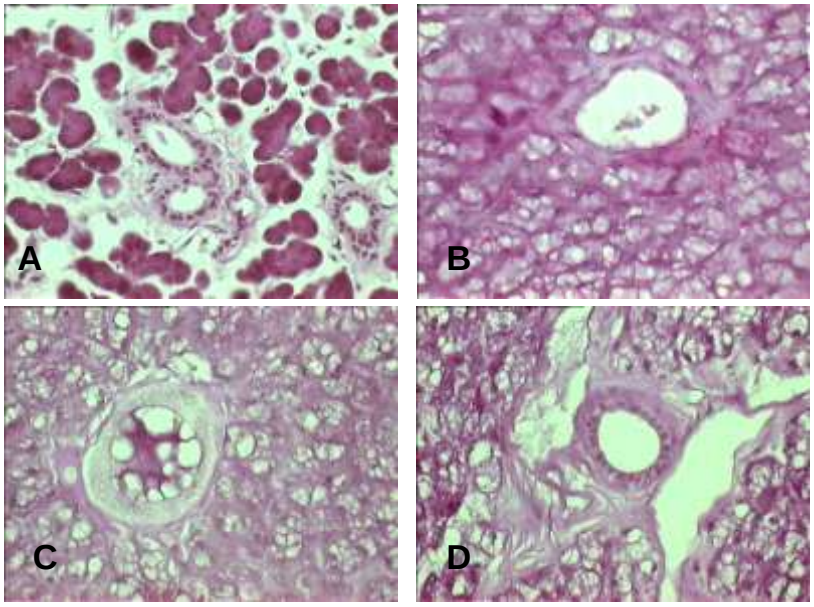


***Figure 2: PAS stained sections without diastase***
***( Neostigmine treated group)***

The photomicrograph shows normal **positive** PAS reaction in the control subgroup. **G 4 group**. Original magnification × 200.
B- The photomicrograph shows **moderate** reactivity of mucosubstances with PAS. **G 1group**. Original magnification × 200.
C- The photomicrograph shows **mild** reactivity of mucosubstances with PAS.**G 2 group**. Original magnification × 200.
D- The photomicrograph shows **negative** reaction of mucosubstances with PAS. **G 3 group**. Original magnification × 200.

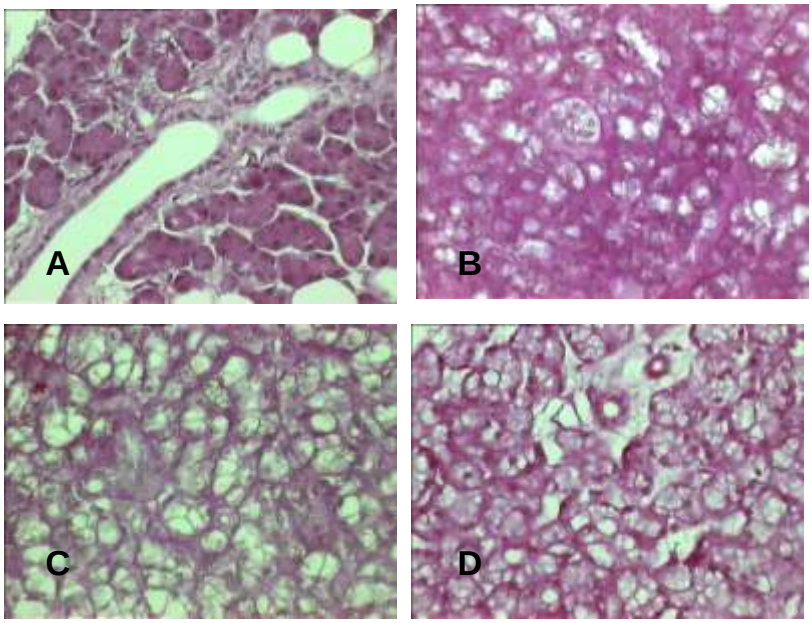


***Figure 3: PAS stained sections with diastase (Neostigmine treated group)***

A-The photomicrograph shows **positive** PAS reaction in the acini of control subgroup. **G 4 group**. Original magnification X 200.
B- The photomicrograph shows **moderate** reactivity of mucosubstances in the acini with PAS. **G 1 group**. Original magnification X 200
C- The photomicrograph of acini and duct system showing **mild** reactivity of mucosubstances with PAS. **G 2** group. Original magnification X 200

D- The photomicrograph of acini shows **negative** reaction of mucosubstances with PAS. **G 3 group**. Original magnification × 200

Table 1. Describe the means and standard deviations for Group 1 treated with different doses of neostigmine and their control group.

| Group | Number of readings | Mean±Standard Deviation(SD) | Minimum | Maximum |
|---|---|---|---|---|
| G1 | 150 | 47.975±7.143 | 30.00 | 63.75 |
| G2 | 150 | 48.282±9.222 | 33.75 | 71.25 |
| G3 | 150 | 51.672±9.954 | 33.75 | 75.00 |
| G4 | 150 | 39.751±6.797 | 26.25 | 56.25 |

Table 2: ANOVA test applied on the diameters of the acini between the subgroups of group 1 that treated with neostigmine.

| | ***F value*** | ***P value*** |
|---|---|---|
| *Between groups* | *54.687* | *0.000* |

***P value ≤0.001 means significance***

***Table 3:*** *describe the degree of significance between the diameters of G1d subgroup (control) and G1a, G1b, and G1c subgroups (neostigmine treated) using* ***multiple comparison test****.(ANOVA)*

| Control(I) | Treated(J) | Mean Difference (I-J) | Std.Error | P value |
|---|---|---|---|---|
| G4 | G1 | -8.2233* | 0.9648 | ≤0.0001 |
| | G2 | -8.5300* | 0.9648 | ≤0.0001 |
| | G3 | -11.9200* | 0.9648 | ≤0.0001 |

**The mean difference is significant if P value ≤ 0.05.*

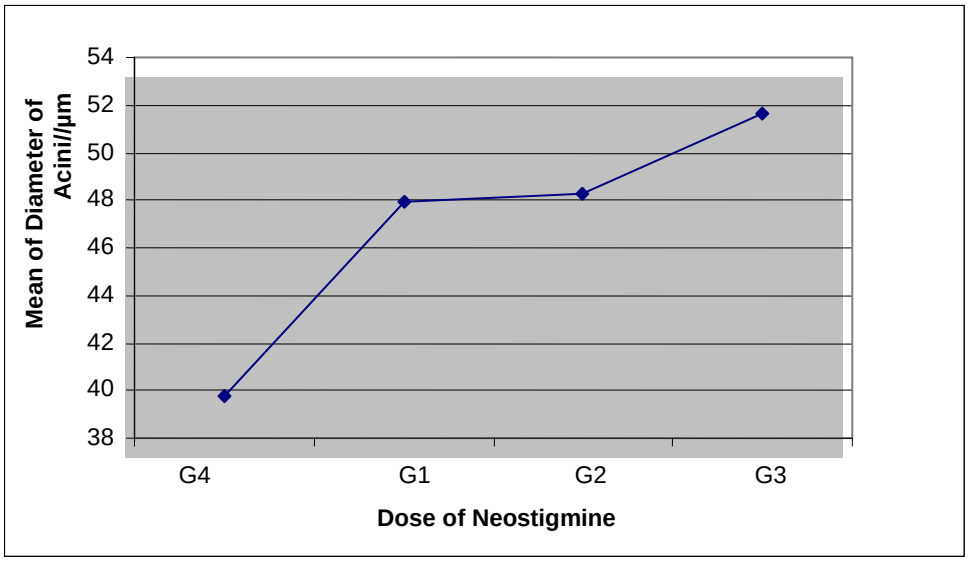


Fig. 4: showing the relation between the diameter of the acini and the dose of the neostigmine.

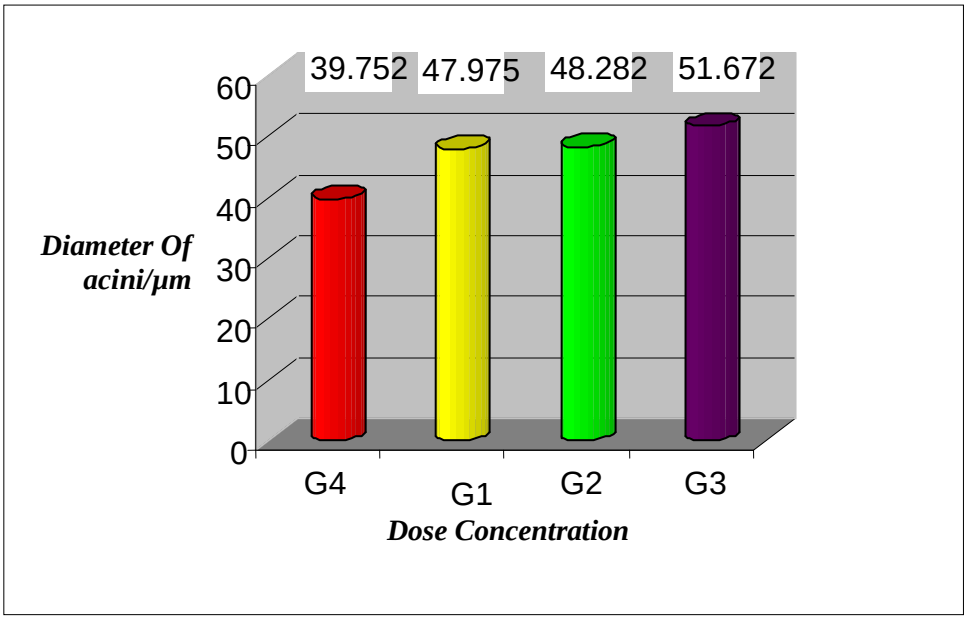


Fig.5: Showing the relation between the diameter of the acini and the dose of the neostigmine

Compound exocytosis is essential process in production of excessive amount of saliva by the parotid salivary gland [22]. Similar results were obtained for amifostine in experimental studies on rat and rabbit models [23] and clinical trials which provide encouraging results.

In neostigmine treated rabbits, the most striking features were the increase in size of the secretory acini and the vacuolation of their acinar cells. In parasympathetic denervated gland in rabbit there was an extensive cellular change in the glands [24]. The acini were reduced in size and the degree of vacuolation has decreased. On contrary, our study has demonstrated an increase in incidence of the degree of vacuolation in acinar cells of rabbit parotid gland. Infact a quick glance to the sections of the present work and the pictorial evidences presented by Cope and Williams [25] clearly demonstrate the structural changes seen in the acinar cells induced by supersensitiztion by neostigmine. There has been awareness that vacuolation, after the use of parasympathomimetic drugs, can occur at time in exocrine serous cells of the trachea [26]**;** pancreas [27,28]. Early in vitro studies on rat parotid gland [29] showed that stimulation with muscarinic and adrenergic agonists caused movement of water and vacuole formation. It has been sometimes considered that vacuole formation is an essential part of water secretion [30]. In rat parotid [31] , prolonged strong parasympathetic stimulation has increased the tendency for acinar vacuolation that was quite evident in triple dose treated rabbits of the present work. Thesupersensetivity at the acinar cells of rabbits of the present study was manifested by increase rate of salivation. That was in agreement with the findings of Ikeno et al., [32] who proposed that sensitization of the serous acini by parasympathetic stimulation was a necessary condition for secretion occurring. The presence of same degree of reaction after PAS (with and without pretreatment with diastase) suggested the presence of nonglycogenic mucuosubstances. On the other hand, the absence of the reaction in the serous acini after PAS staining, especially in triple dose treated rabbits, was in contrast to the striking features in the serous acinar cells in parasympathetic denervation in the gland of rabbit. In the latter cells there has been an accumulation of glycogen **[24]** Thus, our results inclined to agree with the latter authors who pointed out that only the normally functioning serous cells expressed no reserve of glycogen

granules in their cytoplasm. On the other hand the absence of reaction after AB staining suggested the absence of acidic mucuosubstances, which are demonstrated in the mucous cells of the salivary glands [33] .The production of endogenous saliva is of greatest benefit to patient both for its convenience and the importance of natural saliva to oral function. The artificial saliva does not replace the many macromolecules critical to protective and other functions of saliva. Stimulation of gland function also may help prevent ascending infection of salivary glands and retard the formation of mucous plug [34] .The structural histological alterations noticed in this study substantiate the use of this drug in cases of xerostomia.

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