EFFECT OF PILOCARPINE ON MINOR SALIVARY GLANDS OF RABBITS: A HISTOLOGICAL STUDY

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بسم الله الرحمن الرحيم

إن في السماوات والأرض لآيات للمؤمنين (3) وفي خلقكم وما يبث من دابة

آيات لقوم يوقنون (4) واختلاف الليل والنهار وما أنزل الله من

السماء من رزق فأحيا به الأرض بعد موتها وتصريف الرياح آيات

لقوم يعقلون (5) .

صدق الله العظيم

(سورة الجاثية)

To

My Dearest Mum

and

my Father's soul

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CERTIFICATE

This is to certify that the work presented in this thesis represents original research carried out by ***HAMIDA MOHAMMED OMAR BUSHAALA*** submitted in partial fulfillment of requirements for the degree of Master of Science in Oral Biology according to the regulations of University of Benghazi.

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DECLARATION

This is to declare that I have not submitted the research work embodied in this thesis

“ EFFECT OF PILOCARPINE ON MINOR SALIVARY GLANDS OF RABBITS: A HISTOLOGICAL STUDY”

to any other university before.

Candidate:

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CONTENTS

CHAPTER 1 Pages

INTRODUCTION

CHAPTER 2 Pages

LITERATURE REVIEW

CHAPTER 3 Pages

AIM OF THE WORK

CHAPTER 4 Pages

MATERIALS AND METHODS

CHAPTER 5 Pages

RESULTS

CHAPTER 6 Pages

DISCUSSION

CHAPTER 7 Pages

SUMMARY AND CONCLUSION

CHAPTER 8 Pages

RECOMMENDATIONS

CHAPTER 9 Pages

REFERENCES

LIST OF TABLES

Tables **Pages**

LIST OF FIGURES

Figures **Pages**

Figures Pages

Graph 1 :

LIST OF ABBREVIATIONS

M.S.G.	Minor Salivary Gland
IgA	Immunoglobulin A
PAS	Periodic Acid Schiff's
CN	Cranial Nerve
M3 /M1	Muscarinic Receptors
AMP	Adenosine MonoPhosphate
VIP	Vasoactive Intestinal Peptide
AQP5	Aquaporins Channel 5
MAChRs	Muscarinic Acetylcholine Receptors
AV3V	Anteroventral Third Ventricle
BKC	Benzalkonium Chloride
LSG	Labial Salivary Gland
H&E Stain	Hematoxylin And Eosin Stain
SS	Sjögren's Syndrome
CNS	Central Nerves System

ABSTRACT

Back ground: Pilocarpine is a parasympathomimetic agent with mild β-adrenergic increase the rate of salivation. It is commonly used for treatment of patients with Sjögren's syndrome and post radiation salivary gland hypofunction. The intention of the present work was to describe the effects of Pilocarpine drug which is used as a drug with parasympathomimetic effect and the histological changes during treatment with it. **Methods:** Eighteen male rabbits were used in this experiment to show the effect of Pilocarpine . Different doses of drug 3mg/kg ,5mg/kg were given to treated groups A and B within the therapeutic limits. The experimental animals were injected intraperitoneally twice daily, for two and six weeks. Samples of the minor salivary glands were processed for light microscopy. Sections of the minor salivary glands were stained with H&E and PAS stains. 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 increased as the dose of the drug increased. **Conclusion**: Pilocarpine as sialogogues drug simulates the effect of parasympathomimetic drugs. The structural histological alterations noticed in this study substantiate the use of Pilocarpine as prophylactic and treated agents in patients suffering from xerostomia , patients receiving radiotherapy at head and neck malignancies, and some diseases like Sjögren's syndrome. **Key words:** Rabbits, minor salivary glands, histology, Pilocarpine, vacuolation.

Chapter 1

INTRODUCTION

INTRODUCTION

Saliva is produced in and secreted from salivary glands [1]. It is sterile when leaves the salivary glands but ceases to be so as soon as it mixes with the crevicular fluid, remains of food, microorganisms and their products, desquamated oral epithelial cells, etc [2] . Saliva is rich in mechanisms and functions that are important, not only for oral health, but also for general health and well-being [3]. Saliva represents an increasingly useful auxiliary means of diagnosis of many systemic diseases. Sialometry and sialochemistry are used to diagnose systemic illnesses, monitoring general health, and as an indicator of risk for diseases creating a close relation between oral and systemic health [4]. Whole mouth saliva also contains small amounts of other fluids and products of the mucosal surface, this complex fluid that has digestive, lubricating, and protective functions [5].

In humans, there are three pairs of large salivary glands: parotid, submandibular, and sublingual glands. In addition, 600-1,000 minor salivary glands line the oral cavity and oropharynx, contributing a small portion of total salivary production , these glands secrete 10% of the total volume of saliva, but they account for approximately 70% of the mucus secreted [6,7]. Mammalian salivary glands mainly consist of two type of epithelial cell, the acinar cells that responsible for secreting the salivary fluid as well as most of the salivary proteins, and the ductal cells that secrete some proteins and modify the ionic composition of the saliva as they convey it to the mouth [8].

While in minor salivary glands lack a branching network of draining ducts. Instead, each salivary unit has its own simple duct, where they exist as small discrete aggregates of secretory tissue in submucosa throughout most of oral cavity and concentrated in the buccal, labial, palatal, and lingual regions. In addition, they may be found at the superior pole of the tonsils (Weber's glands), the tonsillar pillars, and the base of tongue (von Ebner's glands) [9].The minor salivary glands are mucous or mixed with serous demilunes but predominantly mucous[10]. Which they are innervated by postganglionic nerve fibers of the sympathetic and parasympathetic divisions of the autonomic nervous system, and there are two main types of cells in the secretory units: serous cells, mucous cells, that are associated with myoepithelial cells [11]. These are contractile cells associated with the secretory end pieces and intercalated ducts of the salivary glands [12]. That are star-shaped cells lying between the basal lamina and the acinar and ductal cells, these cells structurally resemble epithelial cells and smooth muscles [13] . In the minor salivary glands myoepithelial cells put in their processes around acini and continuing onto intercalated ducts [14].

Microanatomy of the salivary glands:

The secretory unit (salivary unit) consists of the acinus, myoepithelial cells, intercalated duct, striated duct, and excretory duct. All salivary acinar cells contain secretory granules; in serous glands, these granules contain amylase, and in mucous glands, these granules contain mucin. Acini, responsible for producing the primary secretion, are divided into 3 types:

1) Serous (protein-secreting)=spherical cells rich in zymogen granules
2) Mucous (mucin-secreting)=more tubular shaped cells; mucinogen granules are washed out on H&E preparations giving an empty cell appearance
3) Mixed=serous demilunes, or predominantly mucous acinar cells capped by a few serous acinar cells [15] .

Myoepithelial cells are contractile cells send numerous processes around the acini and proximal ductal system (intercalated duct), moving secretions toward the excretory duct [16].

The lumen of the acinus is continuous with the ductal system, made up of the intercalated duct, the striated duct, and the excretory duct, the intercalated duct is lined by low cuboidal epithelial cells rich in carbonic anhydrase, these cells secrete bicarbonate into the ductal lumen and absorb chloride from the lumen[15]. The striated duct is lined by simple cuboidal epithelial cells proximally with infoldings of the basal and basolateral plasma membrane and associated mitochondria, these cells absorb Na from the lumen, secrete K into the lumen, and produce an increasingly hypotonic fluid. Excretory ducts are lined by simple cuboidal epithelium proximally and stratified cuboidal or pseudostratified columnar epithelium distally, these cells do not perform any modification of the saliva [15].

The parotid gland is a purely serous, while the submandibular gland is mixed, with predominantly serous, and the sublingual gland is mixed, with predominantly mucous. The sublingual gland utilizes a simple system of transport, whereas the parotid and submandibular glands involve elaborate networks that consist of all components of the duct system [17]. The minor salivary glands distributed widely in the oral cavity and oropharynx, in submucosa and between muscle fibers, and their secretions directly wash the tissues. Most of minor salivary glands are mucous and some of them are mixed with serous or seromucous demilune. The lumen of the acinus is continuous with the ductal system which is consist of intercalated ducts, intralobular ducts usually lacking basal striations, and excretory ducts opening directly through the mucosa [18].

Connective tissue:

Surrounding each major salivary gland is a connective tissue capsule, that demarcates the gland from adjacent structures, and from it septa extend inward from the capsule divide the gland into lobes and lobules and carry the blood vessels and nerves that supply the parenchymal components and the excretory ducts that convey saliva to the oral cavity[11].The minor salivary glands don't have connective tissue capsules. instead of that there are stroma which are rich in lymphocytes and plasma cells, and responsible for the production of IgA [19].

Saliva :

Saliva is a viscous, transparent liquid that is secreted by cells of the salivary glands with average of one liter of saliva is produced within the body each day, where factors like sight, smell, thought, taste, or actual presence of food in the mouth causes an autonomic stimulation of the salivary glands that increases production of saliva and stimulates its release into the oral cavity[20,21]. Although the major component is water, saliva also contains ions, mucus, enzymes, and antibodies[22]. Beside salivary secretions, saliva contains other components like gingival crevicular fluid, oral mucosa transudate, in addition to mucous of the nasal cavity and pharynx, non-adherent oral bacterial, desquamated epithelial and blood cells, as well as traces of medications or chemical products, viruses, micro-organisms, and food debris [23].

Saliva is a complex secretion. 93% by volume is secreted by the major salivary glands and the remaining 7% by the minor glands [22],with volume: 600-1000 ml/day, resting flow rate: 0.2-0.4 ml/min, stimulated flow rate: 2.0-5.0 ml/min, and saliva Ph: (6.7-7.4) [11]. Approximately about 99% of salivary fluid is water, remaining part presented by a variety of electrolytes(sodium, potassium, calcium, chloride, magnesium, bicarbonate, phosphate) and proteins, represented by enzymes, immunoglobulins and other antimicrobial factors mucosal glycoproteins, traces of albumin and some polypeptides and oligopeptides of importance to oral health. there are also glucose and nitrogenous products, such as urea and ammonia[4].

Functions of saliva:

The most important function is the protection of oral cavity in many ways, which Individuals with a deficiency of salivary secretion experience difficulty eating, speaking, and swallowing and become prone to mucosal infections and rampant caries so saliva is an important dental caries inhibitor, which protect against hard-tissue loss and affects both sides of the control of demineralization and the promotion of remineralization[(24)]. Other functions of saliva enhance taste, begin the digestive process and it has a buffering action[(11)]. It also plays a major role in lubricating oral tissues by mucoglycoprotein substant, this lubrication reduces trauma to soft tissues during mastication, swallooowing and speaking, also it has antibacterial and antifungal effects so it contains lysozyme, lactoferrin, peroxidase, thiocynate, and others [(23)].

There are important roles played by different salivary components in protecting the soft and hard tissues of the mouth, so saliva like other mucosal fluids contain protected components, such as mucin 5B and mucin 7 and secretory IgA (sIgA) as well as components which appear to be less widely detected on other surfaces or may be peculiar to saliva (e.g. histatins, agglutinin) [(5)]. Specific property of saliva, that contains a range of components that interact with and protect teeth (e.g. p roline rich proteins, statherins). Saliva is essential portion in maintaining the integrity of the oral mucosal surface and in preserving an ecological balance [(5)].

Saliva has a complex composition and versatile physical properties, that make it fit to facilitate the taste and detection of foods nutritious to the body, also protect the mucosa from infection by the ever-present micro biota present in the mouth. The viscoelasticity of salivary fluid competent it of many roles, such as acting as a lubricant and an antimicrobial, preventing the dissolution of teeth, aiding digestion, and facilitating taste [25].

Therapeutic drugs consider the main factors responsible for a decreased flow rate, for example: (anticholinergics, hypnotics, antidepressants), particularly when multiple drugs are used . There are other factors like Sjögren syndrome; and radiation treatment for head and neck cancer [23].

Xerostomia:

Xerostomia is the subjective sensation and complaint of dry mouth, when the amount of saliva production is reduced, that are more prevalent in women [26]. while hyposalivation is the objective finding of a reduced salivary flow rate. It may occur with the use of medications, as a complication of connective tissue and autoimmune diseases, after radiation therapy to the head and neck [26]. Patients with low salivary flow may face a lot of problems like: xerostomia, an increase in caries, moreover reduced clearance of bacteria and food that leading to mucosal soreness, gingivitis, cheilitis, fissuring of the tongue and increase frequency of calculus deposition in the salivary ducts. In addition, recurrent yeast infections, difficulty in chewing, speaking and swallowing, burning mouth, and difficulty in retention of dentures [1].

Dry mouth can seriously impair oral health and diminish the quality of life [27]. Symptoms of a lack of saliva or oral dryness may be precipitated by dehydration of the oral mucosa, which occurs when output by the major and/or minor salivary glands decrease and the layer of saliva that covers the oral mucosa is reduced, making patients with xerostomia and hyposalivation generally deal with palliative treatment for the relief of symptoms and prevention of oral complications [28]. Oral Pilocarpine is the primary treatment to reduce xerostomia, which has some efficacy in the treatment of xerostomia after radiation therapy, Sjögren's syndrome and graft-versus-host disease [29].

Drugs affecting salivation :

Drug-induced sialorrhea:

At clinically significant degree, the hypersalivation can be caused by only a few drugs, one of major medication groups are antipsychotics; particularly clozapine, and direct and indirect cholinergic agonists that are used to treat dementia of the Alzheimer type and myasthenia gravis. Hypersalivation is also induced by certain heavy metal toxins such as (mercury and thallium), from exposure to irreversible acetylcholinesterase inhibitors (insecticides agents), and by a handful of other drugs (for example: yohimbine, mucosa-irritating antibiotics). The hypersalivation can be treated by medications that decrease saliva, (for example: atropine-related oral anticholinergics , sublingual ipratropium spray, clonidine patch). Recently, botulinum injections into the parotid gland have been used successfully to treat refractory cases [30].

Pilocarpine:

Pilocarpine is a parasympathomimetic agent with mild β-adrenergic stimulating properties. It has been proposed as a treatment for dry mouth for over 100 years. In previous clinical trials that examined the effects of Pilocarpine on dry mouth and salivary function in patients with Sjögren's syndrome and post radiation salivary gland hypofunction, doses of 5–10 mg 3 or 4 times daily, Pilocarpine can significantly improve symptoms of dry mouth and increase salivary output .The side effects of Pilocarpine are rare, such as sweating, flushing and urinary frequency, they are typically mild or moderate intensity and relatively short duration[31].

Chapter 2

LITERATURE REVIEW

LITERATURE REVIEW

Saliva is a complex fluid secreted from minor and major salivary glands, that are lining the oral cavity. The glands and their secretions are critical in maintaining both oral and systemic health[32]. Where the major salivary glands include three parts of glands, which communicate with the mouth: parotid, submandibular and sublingual gland. In the parotid gland, the spherical secretory end pieces are serous in adult, with pyramidally shaped acinar cells, which have a spherical, basally situated nucleus and surround a small central lumen, and mixed in infant and old age, intercalated and striated ducts are numerous[33]. The submandibular gland contains serous acini and mucous acini that are capped with serous demilunes; thus it is a mixed gland but the serous unit is predominate, the intercalated and striated ducts are less numerous than those in parotid gland[33]. The sublingual gland also is a mixed gland, but mucous secretory cells predominate. The mucous tubules and serous demilunes resemble those of the submandibular gland. The intercalated ducts are short and difficult to recognize, intralobular ducts are fewer in number than in the parotid or submandibular glands [33].

The minor salivary glands (M.S.Gs.):

The development of salivary glands begins during the sixth to eighth embryonic week when oral ectodermal outpouchings extend into the adjacent mesoderm and serve as the site of origin for major salivary gland growth. Upper respiratory ectoderm gives rise to simple tubuloacinar units, they develop into the minor salivary glands during the 12th intrauterine week [15].

The minor salivary glands arise from oral ectoderm and nasopharyngeal endoderm. They develop after the major salivary glands [9]. They are classified by their anatomical location as buccal, labial, palatal, palatoglossal glands which are located in the region of the pharyngeal isthmus, the palatal glands lie in both the soft and hard palate, while the anterior lingual glands are embedded within muscle near the ventral surface of the tongue, and have short ducts opening near the lingual frenulum, the posterior glands are located in the root of the tongue, both groups are mucous, while the von Ebner glands are serous, empty into the trench of the circumvallate papillae [33]. These none capsulated glands are distributed throughout the oral mucosa and submucosa, with secretory end pieces are mostly mucous or have a small serous component (demilunes) , the cuboidal to columnar mucous cells have flattened basal nuclei, lymphocyte aggregates are commonly observed within minor salivary glands [6]. Secretory end pieces that are composed of serous cells are typically spherical and consist of 8 to12 cells surrounding a central lumen, the cells are pyramidal in shape with basally spherical nuclei. Secretory end pieces that are composed of mucous cells typically have a tubular configuration, and surrounding a central large lumen [11].

Minor gland saliva varies between different oral sites so buccal saliva flow is higher than labial saliva flow, which in turn is usually higher than the palatal gland secretion rate. M.S.Gs. exhibit a continuous slow secretory activity, and thus have an important role in protecting and moistening the oral mucosa, especially at night when the major salivary gland are mostly inactive. The secretion from these glands seems also important for subjective feelings of dry mouth and general well-being. It is generally agreed that minor gland saliva is important for the whole saliva composition, and especially for the secretory immunoglobulin A and mucins [7,11].

The minor salivary glands secrete highly glycosylated mucins, containing blood group determinants. The mucus constituent of saliva facilitates the lubrication of food particles during the act of chewing, and probably active in tissue lubrication and bacterial aggregation, the lingual serous (van Ebner's) glands secrete digestive enzymes and proteins with possible taste perception functions [18]. They also secrete several antimicrobial proteins and immunoglobulins, where secretory immunoglobulin A is the predominant immunoglobulin in secretions of labial minor salivary gland, and its mean concentration is four times higher in these secretions than in parotid gland secretion [34]. The minor salivary glands can produce 30 to 35 percent of the immunoglobulin A that enters the oral cavity, this, together with the potential accessibility of these glands to antigenic stimulation, suggests that they may be an important source of the immune factors that are involved in the regulation of the microorganisms in the oral environment [34].

Histochemistry of M.S.G.:

The Mucous cells of the mixed glands of the lips, buccal mucosa, and floor of the mouth synthesize neutral, carboxylated and sulphated glycans which are present in varying amounts. Where mucigen granules are poorly stain in H&E, so special histochemical stains like a periodic acid Schiff's (PAS) and alcian blue are used to reveal the carbohydrate component of mucous secretory product with positive reaction [35].

The mucins present in mixed seromucous/mucous and pure mucous minor salivary glands are comparable to those encountered in major sublingual and submandibular glands. Seromucous demilunes are encountered more often in association with neutral and carboxy-mucin laden acini whereas highly sulphated acinar clusters are often devoid of terminal demilunes. Variability in mucin heterogeneity was encountered from gland to gland and from different individual cases of the same type of glands. Pure mucous glands of the palate and retromolar regions elaborate greater proportions of sulphated glycans than mucous cells present within mixed gland acini [(35)].

In study aim to investigate histochemical structures of three major salivary glands in the adult local rabbits, that are parotid, submandibular and sublingual glands. Histochemical staining procedures were indicated significant reactions of the glandular acini of these glands toward PAS stain, where parotid acini were reacted positively with PAS stain, Similarly, submandibular showed PAS positive reaction by the appearance of the magenta color, It indicated the presence of acidic and neutral mucins. Sublingual glands showed positive reaction with the PAS stain characterized by magenta color formation of its mucinous contents [(36)].

The minor salivary glands (M.S.Gs.) in rabbit :

Species variation:

There are number of differences existed between various mammalian species, such as variations in the proportion of serous to mucous cells or the extent of innervations .Other variations are in the structure and biochemistry of the parenchymal cells [37].

In rabbit, the weber's glands show as small tubular aggregations of mucous cells, mucous cells capping with serous demilunes and few number of serous acini .They are mixed glands, but predominantly mucous. The mucous cells displayed acidic mucouosubstances, while the serous cells contained neutral mucouosubstances [38].

There are a little variations between rabbits vonEbner's glands and many other mammals, where they appear as mixed glands rather than serous in nature as in human [39].

In the rabbits palatine salivary glands there are no or small differences between them and other mammals, these differences are due to anatomical variations, under electron microscopic palatine glands appeared as small lobuli separated by interlobular spaces containing numerous bundles of collagen fibers [40].

Control of salivary secretion:

The production of saliva is an active process occurring in 2 phases:

- 1) Primary secretion – occurs in the acinar cells. This results in a product similar in composition and osmolality to plasma.
- 2) Ductal secretion – results in a hypotonic salivary fluid. It also results in decreased sodium and increased potassium in the end product.

The degree of modification of saliva in the ducts turns heavily on salivary flow rate. Fast rates result in a salivary product more like the primary secretion (but it should be noted that saliva is always hypotonic to plasma). Slow rates result in an increasingly hypotonic and potassium rich saliva [(9)].

The unstimulated flow rate averages 0.3 to 0.4 milliliter per minute. Unstimulated flow rates of less than 0.1 ml/minute are considered evidence of hypo-salivation. Many factors influence The salivary flow rate, including the degree of hydration, body position, exposure to light, previous stimulation, circadian and circannual rhythms, gland size and drug use [(23)].

Also taste plays a prominent role, among physiologic stimuli to regulate the salivary secretion. Different taste component stimulate parotid secretion in a specific way: fructose increases amylase content in parotid saliva (beta-adrenergic pathway), citric acid increases salivary flow rate (cholinergic pathway). Beside contributing roll of gut and neuropeptides in the regulation of human salivary secretion [(41)].

Autonomic innervation

Autonomic nerves control the secretion of salivary fluid and proteins. where all salivary glands are supplied by cholinergic parasympathetic nerves which release acetylcholine that binds to M3 and (to a lesser extent) M1 muscarinic receptors, then saliva secretion evoked by acinar cells in the endpieces of the salivary gland ductal tree. Also a variable innervations of sympathetic nerves supply most salivary glands, which released noradrenaline from which tends to evoke greater release of stored proteins, mostly from acinar cells but also ductal cells [5].

The parasympathetic nervous system is the primary instigator of salivary secretion, and its stimulation results in an abundant, watery saliva. And cholinergic nerves regulate primarily the salivary flow rate and Na secretion. While stimulation by the sympathetic nervous system results in a scant, viscous saliva rich in organic and inorganic solutes. And salivary protein secretion (i.e. parotid amylase) is mediated by adrenergic mechanisms [9, 41].

Salivary flow increases tenfold in respond to taste or chewing stimuli, while at the rest, reflex salivary flow occurs at a low rate and for short periods of the day [5].

Three type of efferent nerves, secretory, motoric and vasomotoric fibers that are involved in the regulation of salivary secretion, afferent sensoric nerves are a further component of the salivary gland innervations [41].

the acinar and granular tubule cells showed extensive degranulation, after parasympathetic nerve stimulation, at frequencies varying from 1 to 10 Hz. This effect of stimulation by parasympathetic nerve caused a profuse flow of saliva. After stimulation, there was a marked loss of acidic mucosubstances from the acinar cells and an almost complete loss of neutral mucosubstances from the granular tubule cells [(42)].

On the other hand continuous sympathetic stimulation at 8–10 Hz caused intense vasoconstriction in the gland, only a small increase in fluid secretion occurred; it became thick and tended to block the cannula. It was reported that, under natural reflex conditions, sympathetic impulses can increase the amount of acinar mucosubstance secreted [(43)].

In recent years it has become evident that elicitation of salivary secretion may not be only caused by cholinergic or adrenergic agonists but also by peptides, injected into the bloodstream [(44)].

The autonomic secretory function of the upper aerodigestive tract minor salivary glands is controlled by the following ganglia: the sphenopalatine (pterygopalatine) ganglia, innervate the paranasal sinuses, nasal cavity, portions of the palate, and the upper most pharynx, the otic ganglia, innervate the buccal mucosa, the submandibular ganglia, innervate the floor of the mouth and the anterior tongue, and finally, the pharyngeal plexus innervates the pharynx [(45)]. The oral cavity region itself determines the blood supply and venous and lymphatic drainage of the glands [(15)].

Parasympathetic impulses play important role to maintain normal parenchymal cells, where as parasympathetic denervation caused atrophy in both acinar and granular tubule cells, and a substantial reduction in gland wet weight. In addition to that unusual features appeared on the secretory cells , and striated duct cells tended to accumulate glycogen. While there is no obvious effect in the secretory cells, due to chronic sympathectomy, and there was no significant change in wet weight [46].

Hormonal control:

The salivary flow rate regulated by several hormones of endocrine systems, also they are responsible for protein concentration, and protein composition [47]. Both progesterone and contraceptive hormones affect on submandibular glands, which caused a decrease in the diameter and number of granular tubules [48].

Markedly decrease in stimulated submandibular salivary flow rate after Ovariectomy. A decrease in the parenchymal structures and acinar cells, an increase in the interstitial connective tissue and fatty degeneration of the gland were observed. The changes in gland morphology may be responsible for the decrease in salivary flow rate in young adult rats [49]. Following hypophsectomy practice on experimental rats, obvious histological alterations occurred in the rat submandibular glands, these alterations consists in part of a decrease in both the size of the serous tubules and their granular content. And there is decrease in proteolytic and amylolytic activity. A partial restoration in size and granular content of the serous tubules in young adult thyroidectomised rats treated with thyroxine, while testosterone plus thyroxine were fully effective in restoration of size and granular content after thyroidectomy [50].

Salivary gland function improves by hormone replacement therapy in peri- and postmenopausal women, this improvement happened in both the quantity and the quality [51].

Intravenous injection of vasoactive intestinal peptide (VIP) provoke a flow of saliva from both the parotid and the submaxillary gland in the rat, but Parotid saliva was very viscous and less than that secreted from the submaxillary gland [52].

Age changes :

Features such as acinar atrophy, fatty change, fibrous replacement, ductal dilatation, periacinar callus formation and inflammatory infiltration were noted in the aged glands. [53, 54]. The seromucous acinar cells are the most affected fraction in labial salivary glands by age-related changes, that showed marked decrease in the mean volume accompanied by a lesser decrease in the mean volume fraction of mucous acinar cells, that may explain age-related changes in labial salivary glands (LSG) secretion [55].

As age increased there was decrease in acinar volume proportion and an increase in ductal and connective tissue volume proportion is recorded. A foci of lymphorecticular cells and hyperplasia mostly in lobules affected by parenchymal atrophy, affecting females earlier than males. [56].

Pilocarpine drug and its effect

Action and clinical pharmacology :

Pilocarpine HCl tablets are made from the naturally-occurring alkaloid Pilocarpine which is obtained from the leaflets of the South American shrub Pilocarpus jaborandi [(57)]. Pilocarpine HCl is a cholinomimetic (cholinergic parasympathomimetic) agent capable of exerting a broad spectrum of pharmacologic effects with predominant muscarinic action [(58)]. Oral intake is the most frequent route of administration, and due to properties of Pilocarpine as sialagogue drug has been used for the treatment of xerostomia, and promotes salivary flow, but has a number of unwanted side-effects due to its non-specific exocrine stimulation, such side-effects include sweating, wheezing, abdominal cramps, lacrimation, nausea, vomiting, diarrhoea, dizziness, headache palpitations, asthenia, chills, increased urinary frequency and rhinitis, these side-effects are generally mild and tolerable [(59)].

Among different stimulants of pharmacological secretagogues, such as the adrenergic agonists isoproterenol and adrenaline , peptidergic agonists like physalemin and substance P or other cholinergic agonists like carbachol, the Pilocarpine has been the favorite drug can be used, where the stimulation by pilocarpine results in an abundant and aqueous salivary secretion, in addition to that pilocarpine formulation was well-tolerated and resulted in significant improvements in symptoms of dry mouth and other xerostomic conditions[(60)].

Pilocarpine as a cholinergic parasympathomimetic agent is predominantly muscarinic M3 action that causes stimulation of residual-functioning exocrine glands [61]. It is available in an ophthalmic solution, gel and also as an oral tablet (Salagen) with dosages of 5 mg to 30 mg, divided into one to four oral daily doses, and taken time to reach peak concentrations following oral administration is approximately 1.25 hours. The duration of sialogogic effect is about two to three hours [61].

Indications and clinical use:

The effect of topical Pilocarpine solutions as mouthwashes on salivary flow is revealed in increasing salivary flow in healthy volunteers, with no adverse effects [58]. In treatment of the symptoms of xerostomia due to salivary gland hypofunction caused by radiotherapy for cancer of the head and neck, also in treatment of the symptoms of xerostomia and xerophthalmia (dry eyes) in patients with Sjögren's syndrome and graft-versus-host disease [29].

Contraindications:

Use of Pilocarpine is contra indicated in patients with uncontrolled asthma, narrow-angle glaucoma or acute iritis. Caution is advised with use in patients with cardiovascular disease [31]. The most common side effects are increased sweating and gastrointestinal intolerance. Hypotension, rhinitis, diarrhea and visual disturbances can also occur [61].

Effect of Pilocarpine:

The Pilocarpine mouthwash solutions revealed efficacy in increasing salivary flow in healthy volunteers. In study was performed to evaluate the effects of topical pilocarpine solutions as mouthwashes on salivary flow and their adverse effects on healthy subjects. Forty volunteers received 10 ml 0.5, 1 and 2% pilocarpine solutions or 0.9% saline in a randomized, double-blind, placebo-controlled manner. Salivation was measured before and 45, 60 and 75 min after mouth rinsing for 1 min with 10 ml of saline or pilocarpine solutions. Mouth rinsing with pilocarpine solutions at concentrations of 1 to 2% induced a significant objective and subjective dose-dependent increase in salivary flow [(58)].

Also simultaneously intake of Pilocarpine during radiotherapy treatment demonstrate encouraging results with regard to lowering salivary flow reduction and incidence of xerostomia, as well as of oral complications [(62)].

It probably revealed more effective in improving xerostomia[(63)]. There was a slightly higher increment in salivary secretion at the end of four weeks treatment with Pilocarpine, in compared to treatment performed with cevimeline [(64)]. The use of oral Pilocarpine in juvenile-onset, Sjögren's syndrome patients, seem to be safe and effective for treating xerostomia [(65)]. Beside it has some efficacy in the treatment of xerostomia after radiation therapy, and graft-versus-host disease [(29)].

In study performed To examine salivary function in patients with primary Sjögren's syndrome (SS) by assessing unstimulated and stimulated flows, where observed a significant increase in stimulated salivary flows using a solution of an ophthalmic 5% pilocarpine administered sublingually[66].

After radiotherapy treatment, clinical side effects and postradiation xerostomia were minimized with Pilocarpine drug, it produced a clinically significant increase of salivary flow from the palatal glands before and 7 months after radiation [67].

The movement of water through the plasma membrane of secretory and absorptive cells regulated by channels proteins to be called Aquaporins (AQPs), AQP5 is the major aquaporin expressed on the apical membrane of acinar cells [68].

Pilocarpine markedly increased the amount of AQP5 in the apical plasma membrane of parotid cells isolated from normal but not irradiated rats. Pilocarpine induces parotid salivary secretion mainly via the M_3 receptor subtype in both irradiated and normal rats [69].

The muscarinic agonists like Pilocarpine act on or stimulate a mixture of glandular M_1 and M_3 , muscarinic acetylcholine receptors (mAChRs) lead to trigger salivary secretion in vivo [70]. The central nervous system, particularly the anteroventral third ventricle (AV3V) region, is important for the effect of Pilocarpine on salivary secretion in rats. The use of peripheral Pilocarpine caused activation of central pathways, and play an important part in the salivary secretion [71].

In a study conducted on70 Wistar rats to illustrate the influence of the autonomous nervous system on the ultrastructure of the salivary acini and the terminal neuraxon, under the following experimental conditions: sympathetic stimulation (Aludrin 2 mg/100 gm of body weight daily), parasympathetic stimulation (Pilocarpine 0.2 mg), where after sympathetic stimulation occurred a rapid secretion and stimulation of the protein resynthesis after secretion results (nuclear swelling, enlargement of the nucleolus, multiplication of the granular endoplasmatic reticulum and the formation of light secretory granula) [(72)]. Secretion increase also results after parasympathetic stimulation although to a lesser extent than after sympathetic stimulation (light as well as dark secretory granula are present). Sympathetic stimulation results in the multiplication of granular vesicles. Morphometric measurements show a significant enlargement of the acini, particularly with sympathetic and parasympathetic stimulation[(72)].

In study performed on rat. The mimetic pilocarpine was administered via an intraperitoneal injection, the autonomic nervous system innervate the salivary glands of the rat, where the acini and granular convoluted tubules were surrounded by both an adrenergic and a cholinergic plexus. The mimetic pilocarpine was able to stimulate the exocytotic secretion from the acinar cells either via the beta-adrenoceptor or via the cholinergic muscarinic receptor. Strongest effect was observed on serous and mucous cells are reported after stimulating with Pilocarpine , where it shows effect on the mucous cells with formation of intracytoplasmic vacuoles and granular release from the acinar cells [(73)].

In study was evaluated the effect of 0.1% pilocarpine mouthwash in xerostomic patients. The short- and long-term effects of pilocarpine were investigated by measuring, minor salivary flow rates and unstimulated whole salivary flow rate at predetermined times. Until 1 h after mouthwashing, 0.1% pilocarpine mouthwash increased minor salivary and unstimulated whole salivary secretions, in the experimental group exhibited increased labial and palatal secretions, but not buccal secretion [(74)].

In other study was designed to assess quantitatively changes in size of secretory acini of the salivary glands of pigs after stimulation by pilocarpine, The experiment 10 pigs weighing 40-50 lb were used. A group of 6 animals were given daily i.m. injections of a sterile aqueous solution containing 30 mg pilocarpine nitrate for 6 consecutive days, and a group of 4 control animals received 6 daily i.m. injections of 5 ml sterile 0-9% saline, the Pilocarpine pigs and controls were killed 24 h after the 6th injection. The clinical effect observed after 5-15 min from injection, all pigs salivated profusely and all vomited several times. The respiratory and the pulse rate were increased slightly. These signs persisted for 30 min after the injection and the pigs then rapidly returned to normal. In histological findings of all comparisons groups, there were very highly significant increases in areas of the acini caused by pilocarpine treatment, which mimics cholinergic stimulation, led to a considerable increase in parotid and submaxillary glands weight [(75)].

Benzalkonium chloride (BKC):

It is preservative used to maintain the sterility of a variety of prescription and over-the-counter products, such as cosmetics, infant care products, and pharmaceutical nasal sprays, ophthalmic solutions, and otic drops. It has been considered one of the safest synthetic biocides known and has a long history of efficacious use [(76)].

It is a mixture of alkylbenzyldimethylammonium chlorides of various even numbered alkyl chain lengths. It is a quaternary ammonium compound and has three main categories of use; as a biocide, a cationic surfactant and phase transfer agent in the chemical industry, that has been in clinical use since 1935 as an antimicrobial additive. Its applications are extremely wide ranging, from disinfectant formulations to microbial corrosion inhibition in the oilfield sector. As reported in the Journal of American College of Toxicology, the Cosmetic Ingredient Review panel concluded that BKC can be safely used as an antimicrobial agent at concentrations up to 0.1% [(77)].

We done this study to complement previous studies performed on salivary glands, as well as to clarify information on the effect of pilocarpine on minor salivary glands.

Chapter 3

AIM OF THE WORK

AIM OF THE WORK

To determine histological changes in minor salivary glands due to the administration of Pilocarpine in rabbits .

Chapter 4

MATERIALS AND METHODS

MATERIALS AND METHODS

Experimental animals:

The present experiment was conducted on eighteen healthy male rabbits, 4-5 months old, of local mixed breed, weighing between 1.5 – 2.5 kg .

The experimental animals were kept under controlled laboratory conditions for two weeks for acclimatization of animals to the laboratory environment. The animals were allowed unrestricted access to food and water.

According to the dosage of the drug used, and the duration of administration of the drug, the rabbits were divided into four groups (A,B,C,D) . Each group was further subdivided into two subgroups according to duration of administration of the drug and saline .

Drug used :

- Pilocarpine HCL was used for groups (A,B) , it is a cholinergic agonist (parasympathomimetic) agent. This drug was available as sterile eye drops, each 1ml contains 20mg of Pilocarpine HCL . Drug was stored at room temperature and protected from light.
- Benzalkornium chloride(0.2 mg) which is present as preservative in the drug. This preservative was given to group (C), and it was used in this study to determine if it had any effect on minor salivary glands or not.
- Saline was used as placebo for control group (D)

Calculation of the drug dose:

The drug dose used in this experiment was calculated according to Pagat and Barnas formula(1964) [78].

Human dose of Pilocarpine is 20mg twice a day. According to this formula, the dose for rabbit weighing 2kg = 20× 0.07=1.4 ×2=2.8.

So the therapeutic dose used in this study was 3mg of Pilocarpine twice a day. Beside using a double dose of approximately 5mg of the drug.

Sectioning:

Serial sections 5µm thickness were cut and mounted on glass slide.

Stain :

1- Hematoxylin and eosin (H and E) stains were used for general examination.

2- Periodic acid Schiff's (PAS)reaction was used as a general stain for presence of mucopolysaccharide substances .

Methods:

The present study was conducted on 18 male rabbits; the experimental animals were injected intraperitoneally twice daily for each group and divided into the following groups :

Group -A includes 6 rabbits. They were given the drug twice daily.

This group was divided into the following sub-groups:

1- $Group_{a1}$ 3 rabbits were given 3 mg/kg Pilocarpine. < for 2 weeks>

2- $Group_{a2}$ 3 rabbits were given 3 mg/kg Pilocarpine. <for 6 weeks>

Specimens were taken immediately at the end of the experiment period from the minor salivary glands (Weber's, buccal and soft palatine glands).

Group –B includes 6 rabbits. They were given the drug twice daily.

This group was divided into the following sub-groups:

1 - $Group_{b1}$ 3 rabbits were given 5mg/kg Pilocarpine. < for 2 weeks>

2 - $Group_{b2}$ 3 rabbits were given 5mg/kg Pilocarpine. <for 6 weeks>

Specimens were taken immediately at the end of the experiment period from the minor salivary glands(Weber's, buccal and soft palatine glands).

Group -C includes 2 rabbits. They will be given the preservative < benzalkornium chloride 0.2mg> twice daily. This group was divided into the following sub-groups:

1- $Group_{c1}$ 1 rabbit was given benzalkornium chloride < for 2 weeks>
2- $Group_{c2}$ 1 rabbit was given benzalkornium chloride <for 6 weeks>

Specimens were taken after that from the minor salivary glands (Weber's, buccal and soft palatine glands).

Group -D includes 4 rabbits serves as a control and were given saline injection to simulates the effect of injection. This group was divided into the following sub-groups:

1- $Group_{d1}$ 2 rabbits were given saline < for 2 weeks>
2- $Group_{d2}$ 2 rabbits were given saline <for 6 weeks>

Specimens were taken after that from the minor salivary glands (Weber's, buccal and soft palatine glands).

The collected samples of the minor salivary glands, included in this study, were ran through paraffin embedding technique to get paraffin blocks.

Histological serial sections were cut from the minor salivary gland of each group:

Collection of samples :

At the end of the period allocated for each group, the animals were sacrificed and the samples collected were immediately fixated in 10% buffered neutral formalin (pH7.0) (figure1) [79].

Processing of samples [79]:

The tissues collected were processed for light microscopy using automatic tissue processing machine (figure2). A traditional technique of paraffin embedding was followed.

1- Washing in water for fifteen minutes to remove the traces of formalin which might be left on the tissues of the glands.

2- Dehydration: Ascending grades of alcohol were used for dehydration as following :

a - 70% ethanol 15 min , b- 90% ethanol 15 min , c- 100% ethanol 15 min,

d- 100% ethanol 15 min , e- 100% ethanol 30 min , f- 100% ethanol 45 min.

3- Clearing : Xylol is considered as intermediate solvent that is fully miscible with both ethanol and paraffin wax. This solvent will displace the ethanol in the tissue, then this in turn will be displaced by molten paraffin wax. Another important role of the clearing agent is to remove a substantial amount of fat from the tissue which otherwise presents a barrier to wax infiltration [79]. Three changes of xylol were used for clearing of the samples to be miscible with paraffin.

4- Paraffin infiltration : Low melting point paraffin was used to ensure proper impregnation and infiltration of paraffin to the inside of the tissue.

5- Embedding: High melting point paraffin was used for making tissue blocks out of the samples to be ready for microtomy.

Microtomy and tissue sectioning [79]:

Rotary microtome was used for preparation of paraffin sections. Five µm thick serial sections were cut from the paraffin blocks. Four sections were mounted on each slide. Numbering for each slides was done in order to have serial records for the sections of each block (Figure 3).

Figure 1: the samples collected immediately then fixed with 10% buffered formalin (pH7.0).

Figure2: histological processing machine for paraffin tissue processing steps.

Figure 3: rotary microtome

Staining procedure:

For general histology and histometric evaluation of diameters of secretory endpieces, the slides were stained with Harris`s hematoxylin and aqueous eosin.

Staining technique for hematoxylin and eosin (H and E) stain [79]:

1- Removal of wax : sections were placed in two changes of xylene for 10-15 minutes to dissolve wax.

2- Hydration : through 100% alcohol, 95% alcohol, 70% alcohol and lastly distilled water, 2 minutes each.

3- Staining :

   a- Sections were stained with Harris`s hematoxylin for 2 minute.

   b- Differentiate the sections using acid alcohol for few seconds. (Acid alcohol: 1%conc. Hydrochloric acid in 70% alcohol).

   c- Wash in running tap water for 10 minutes for bluing of the sections.

   d- Slides were transferred to 1% aqueous eosin for 5 minutes for counter staining, differentiated in running tap water for 3 minutes.

4- Dehydration: through 70% alcohol, 95% alcohol, 2 changes of absolute alcohol, 1 minute each (with agitation).

5- Clearing: using 2 changes of xylene, 1 minute each.

6- Mounting: finally the sections were mounted using DPX medium.

Results: Nuclei : Blue

Cytoplasm : Shades of pink

Zymogen granules : Bright orange to red

Staining technique for periodic acid Schiff's (PAS) [79]:

1- Removal of wax: sections are placed in xylene for 3-5 minutes to dissolve wax.
2- Hydration: through 100% alcohol, 95% alcohol, 70% alcohol and lastly distilled water 1minute for each.
3- The slides were left in distilled water for 35 minutes.
4- Oxidize for 5 minutes in 1% aqueous periodic acid.
5- Wash in running tap water for 5 minutes and rinse in distilled water.
6- Treat with Schiff's reagent for 15 minutes.
7- Wash for 10 minutes in running tap water.
8- Counter stain with Mayer's hematoxylin for 5-10 minutes.
9- Dehydrate, clear, and mount with synthetic resin medium.

Result: PAS positive substances----------Red to magenta.

Nuclei ----------Blue.

Statistical analyses:

Histometric evaluations of diameter of secretory end pieces were conducted on slides stained by hematoxylin and eosin stain.

For the measurement we used Mùller USB Microscope Camera with TUCSEN Digital Imaging Technique (figure 4).

The readings obtained randomly were in micrometer with scale 10.00 . The number of readings were 144 readings ,18 for each group representing the glands included in the present study (Weber's, palatine and buccal) (figure 5).

Data was checked and fed to personal computer using statistical package for social sciences "SPSS" version 22 [(80)].

Descriptive statistics was used in the form of minimum, maximum, mean and standard deviation.

To compare between the mean of diameters of salivary glands of different groups,

Analysis of Variance (ANOVA) test was used. P value was considered significant if it is less than 0.05 and highly significant if P value is less than 0.01 .

To decide which group is different from other groups (ANOVA)a post HOC test was followed.

Figure 4 : Mϋller USB Microscope Camera.

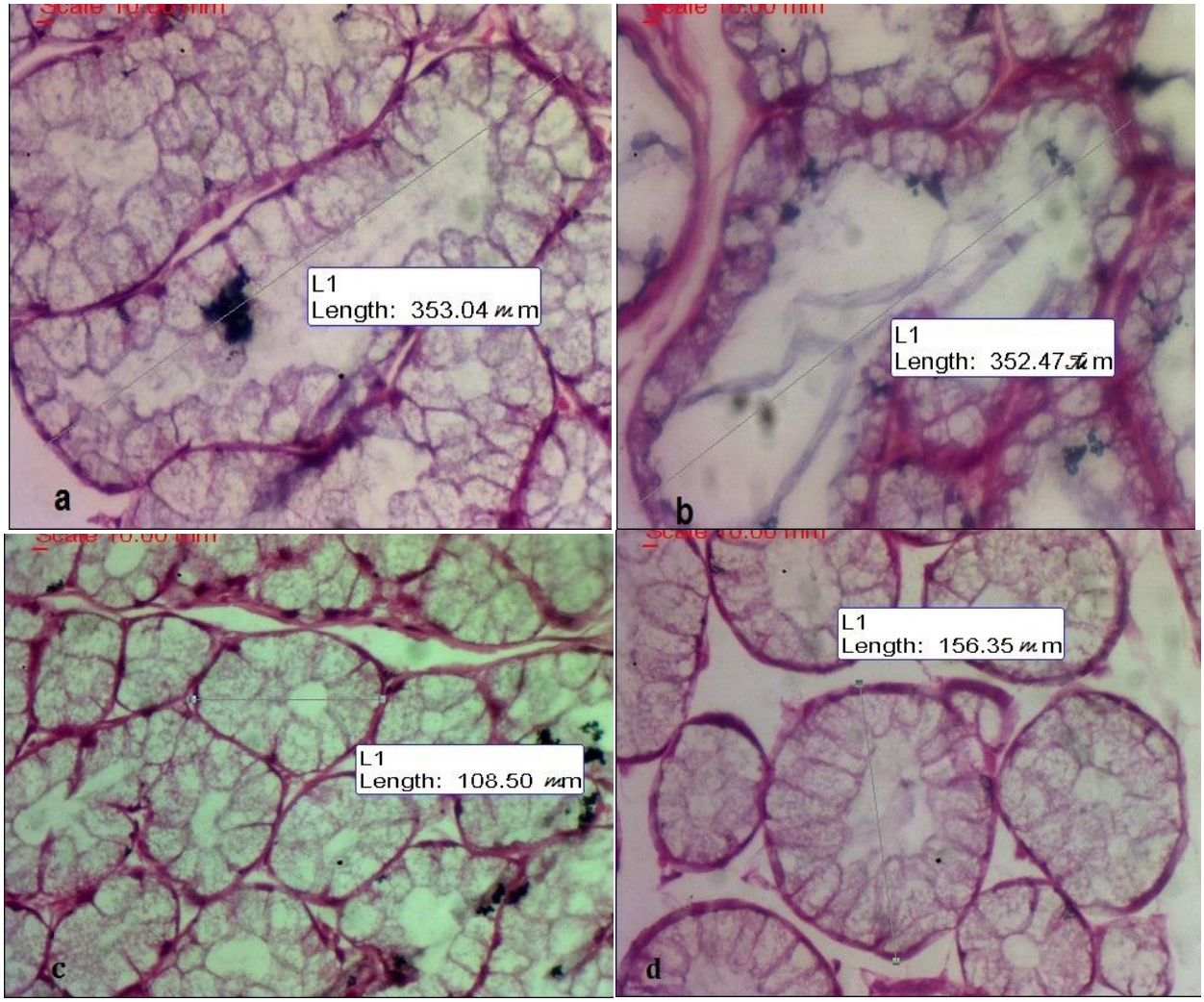


Figure 5: photomicrographs of H&E stain with magnification X400 have taken by Mùller USB Microscope Camera, with different measurement of minor salivary glands diameters, which are taken with micrometer - scale 10.00 .

Chapter 5

RESULTS

RESULTS

Clinical observation:

The following observations were noticed 20 minutes after injection in the rabbits of group A and group B which were injected with therapeutic dose of Pilocarpine:

- Increase in activity of rabbits and they became more aggressive.
- Increase in body temperature. -Increase in water consumption.
- Excessive salivation (drooling of saliva).

Histological findings :

In sections stained with the H and E stain, the minor salivary glands in both treated and untreated groups were located beneath the epithelium in submucosa throughout most of oral cavity . These glands were consisting of several small groups of secretory units, usually open via short ducts directly into the mouth cavity . They lack a distinct capsule, instead they were mixed with the connective tissue of the submucosa or muscle fibers of the tongue or cheek . The minor salivary glands secretory units in the rabbits of both control and treated groups were composed of mostly mucus acini. The most prominent feature of mucous cells was the accumulation in the apical cytoplasm of large amounts of secretory product (mucus) , which compressed the nuclei towards the basal side of the cells, thus the nuclei appeared flattened (figure 6). The secretory products ,which were accumulated on the apical side of the cell, appears pale and poorly stained giving an empty appearance to the supranuclear cytoplasm (figuer7).

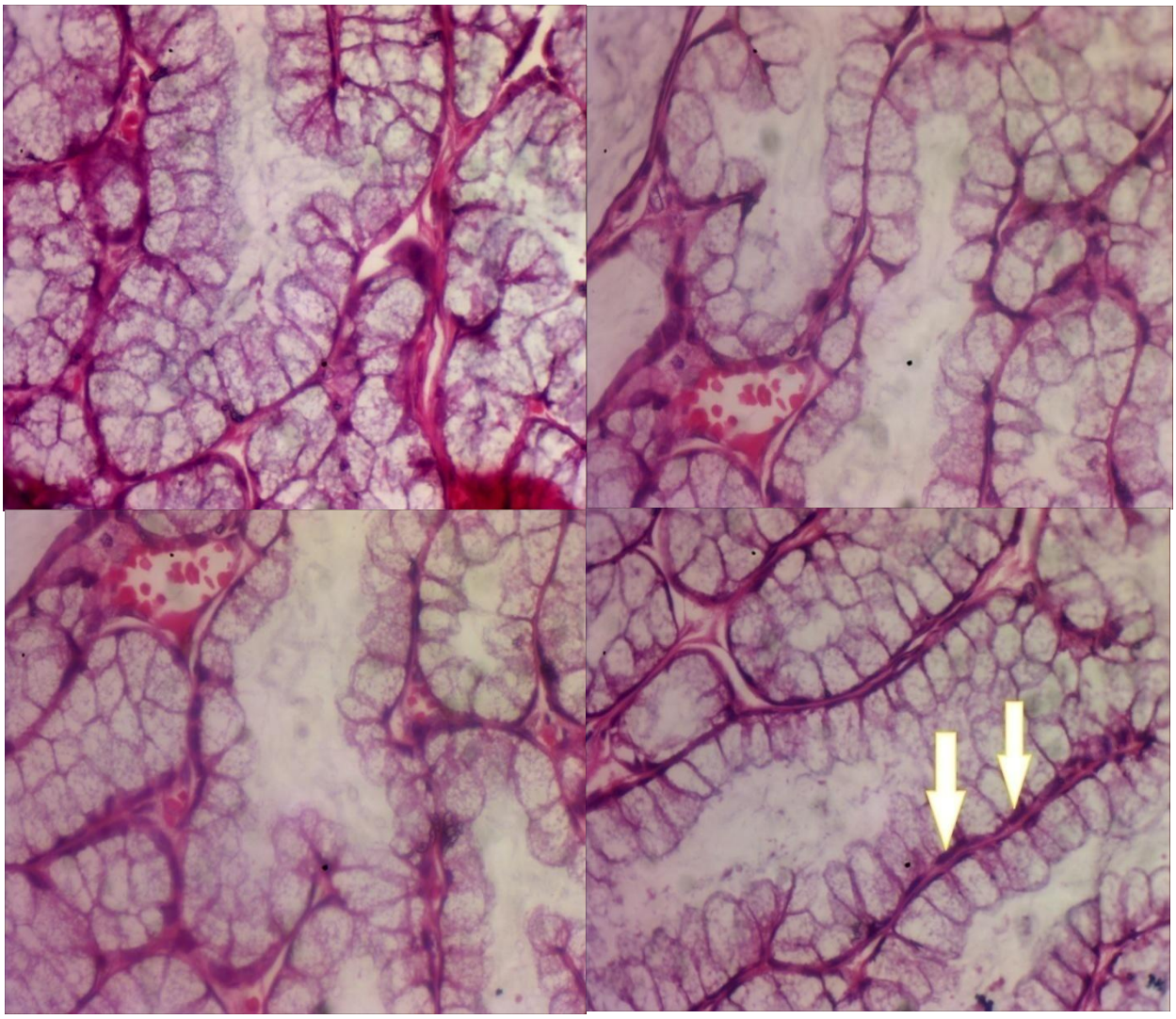


Figure 6: mucous cell in tubular secretory end pieces of soft palatine minor salivary gland randomly selected from treated and untreated groups stained by hematoxylin and eosin. Poorly stained mucous secretory granules fill the cytoplasm, and the nuclei are flattened and compressed against the basal surfaces of the cells <arrows>. X400.

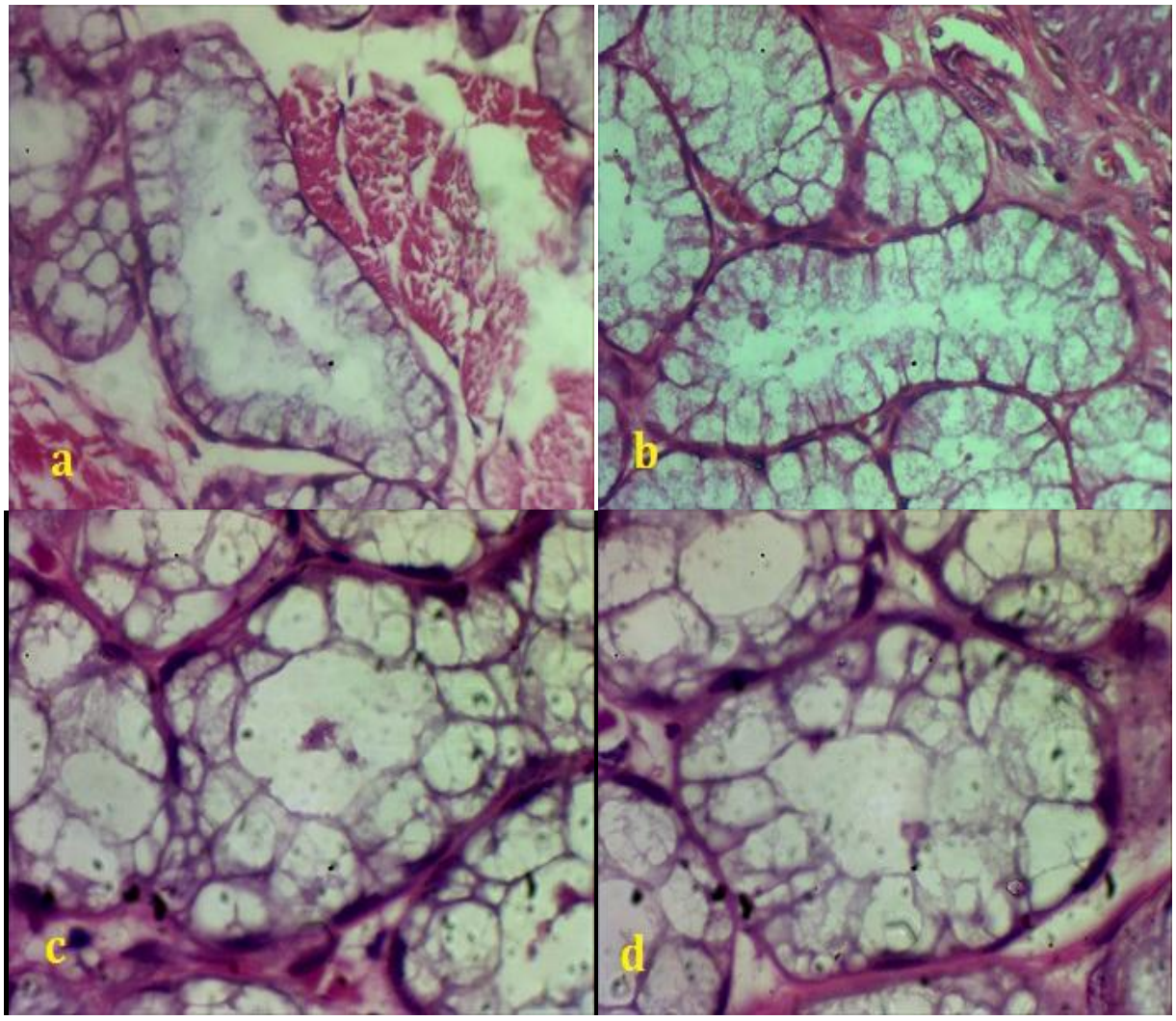


Figure 7: photomicrographs of the minor salivary glands: < in picture (a) soft palatine gland in group b1, in picture (b) weber gland in group b1, in pictures (c,d) buccal gland in group a2 > showing pale staining tubuloacinar mucous cells giving an empty appearance to the supranuclear cytoplasm. Hematoxylin and eosin (H&E) stain. X400 (a, b) and X1000(c, d).

Posterior lingual glands (Weber's glands) :

The glands were located in submucosa of the root of the tongue lateral and posterior to circumvallate papillae. Their ducts open in crypts at dorsum of tongue. They were appeared with general specification in both treated and untreated groups as uncapsulated small aggregations of discrete lobules of glandular tissues consisted of secretory acini which were arranged in tubuloalveolar units and excretory system (intercalated, striated and excretory ducts) for example Weber's glands in groups (a1,a2,b1,b2 figure8).

There were well defined connective tissue separating the glandular lobules which were merged with the epimysium of the underlying muscles, for example Weber's glands in groups (d2,b2 figure 9).

Weber's salivary gland of rabbit appeared as mixed gland with predominant mucous cells, serous cells were few and mostly appeared as serous demilunes, for example Weber's glands in groups (b1,d2,c2 figure 10).

The mucous cells were arranged in form of tubules where they were surrounding a central large lumen. The cells were tall columnar with flattened basally located nuclei and pale appearance cytoplasm in H and E stain, for example Weber's glands in groups (b1,b2 figure 11).

The serous cells were few and mostly appeared as serous demilunes with discrete acini arranged in a form of round structure with a narrow lumen lined by pyramidal cells .Their nuclei were spherical in shape located in the basal region, eosinophilic secretory granules can be seen at the apical part, the cytoplasm stained intensely with hematoxylin and eosin (H&E) stain gave the cells their acidophilic appearance (figure 11).

The duct system started as intercalated ducts, these were narrow thin walled ducts lined by simple cuboidal epithelium, with central nuclei. Secretory striated ducts appeared as wider ducts, lined by tall columnar epithelial cells, their nuclei were large spherical, and centrally placed, with eosinophilic cytoplasm. Large excretory ducts also seen in between secretory tubules, they were lined by pseudo-stratified columnar epithelium, for example Weber's glands in groups (c2,d2,b1 Figure 12).

The Weber's gland of treated groups was the most affected one by Pilocarpine treatment. The most obvious change was the increase in the diameters of acini that give the highest readings among them (Table 1).

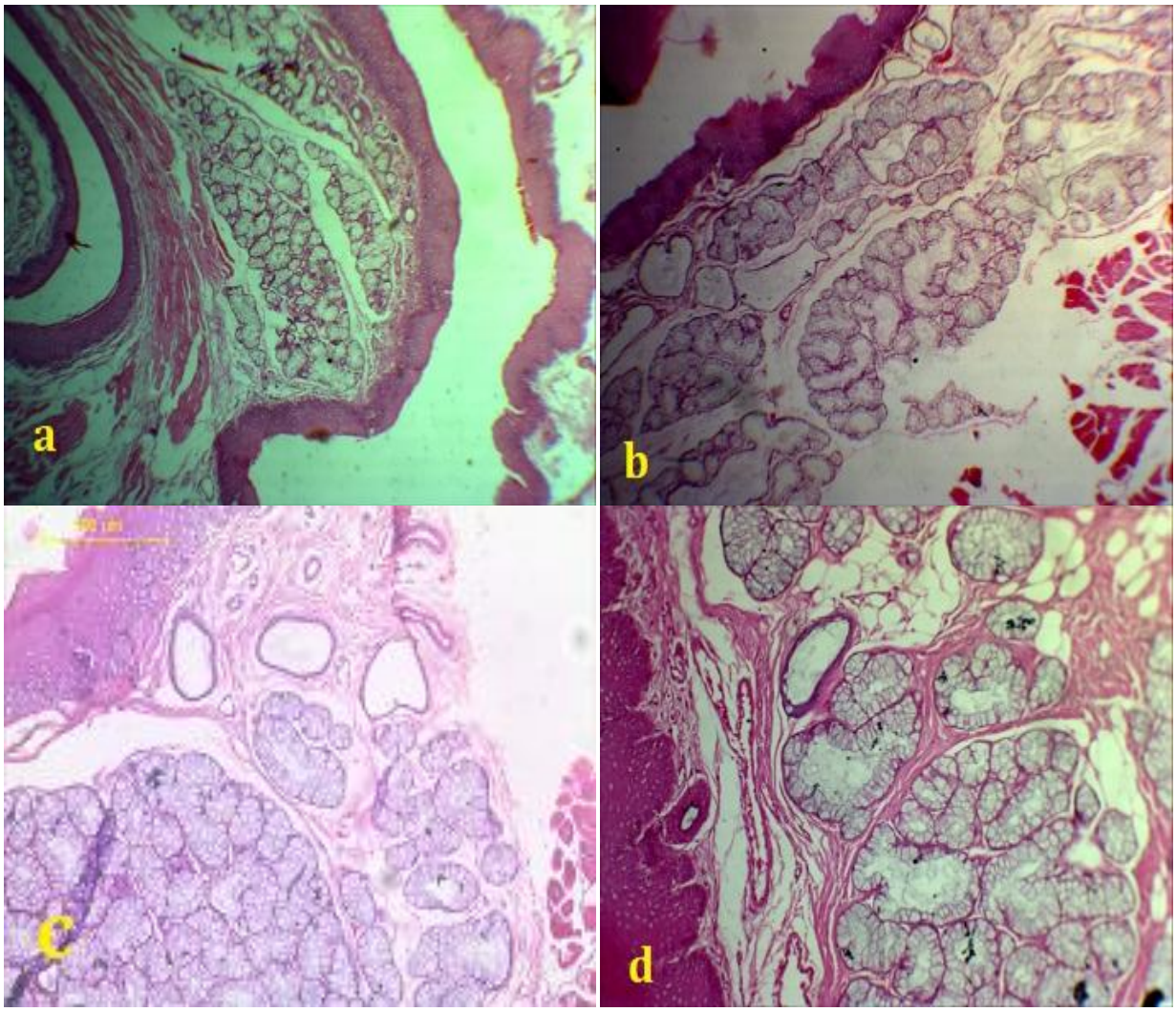


Figure 8: photomicrographs showing uncapsulated small aggregations of discrete lobules of glandular tissues of the Weber's glands : in group b1 in picture (a),in group a1in picture(b), in group a2 in picture (c), in group b2 in picture (d). Hematoxylin and eosin (H&E) stain. X40 (a, b) and X100(c, d).

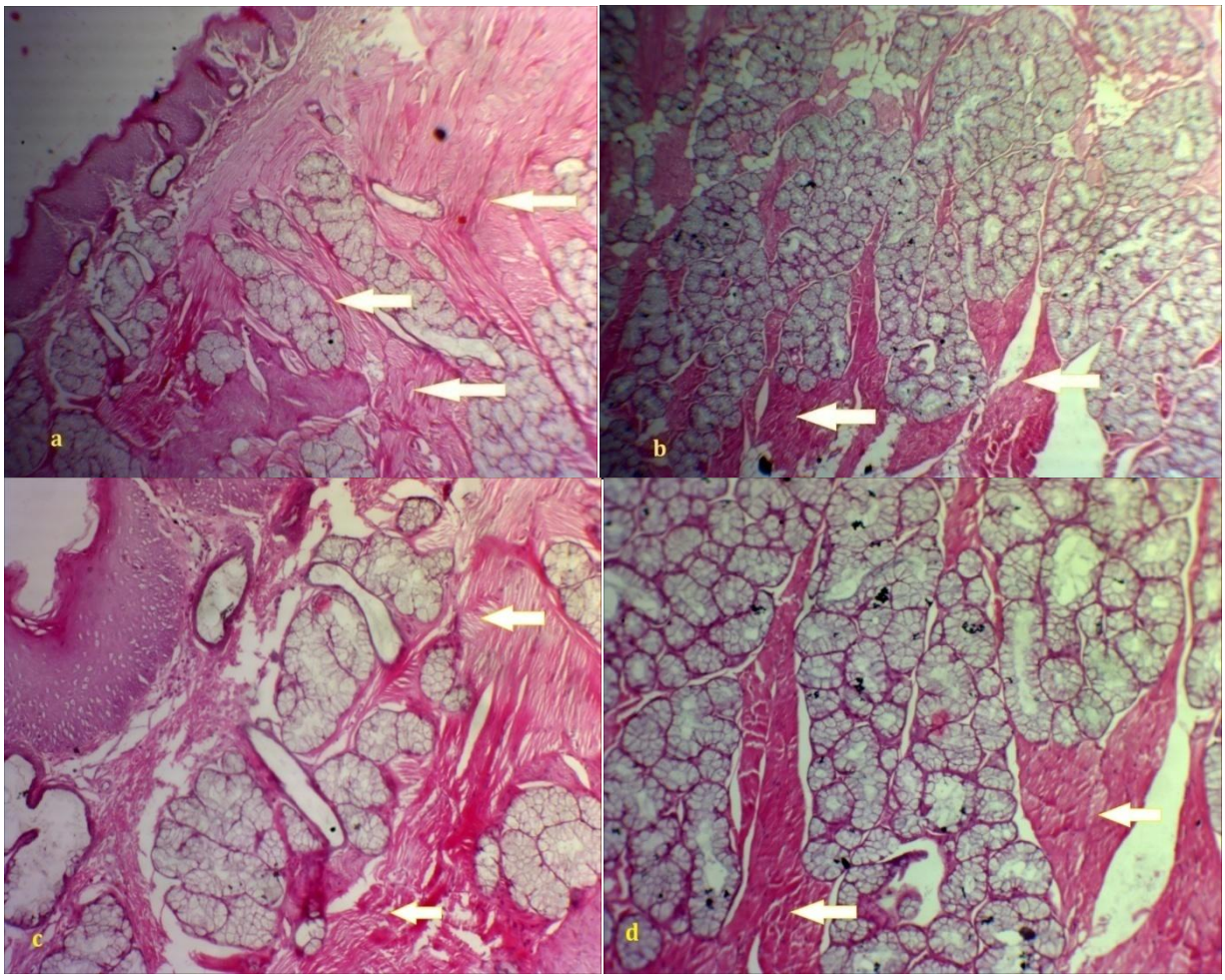


Figure 9: photomicrographs for predominance mucous cells in Weber's glands : in group b2 in pictures (b,d), in group d2 in pictures (a,c), showing well defined connective tissue separating the glandular lobules which were merged with the epimysium of the underlying muscles <arrows>. Hematoxylin and eosin (H&E) stain. X 40 (a, b) and X100(c, d) .

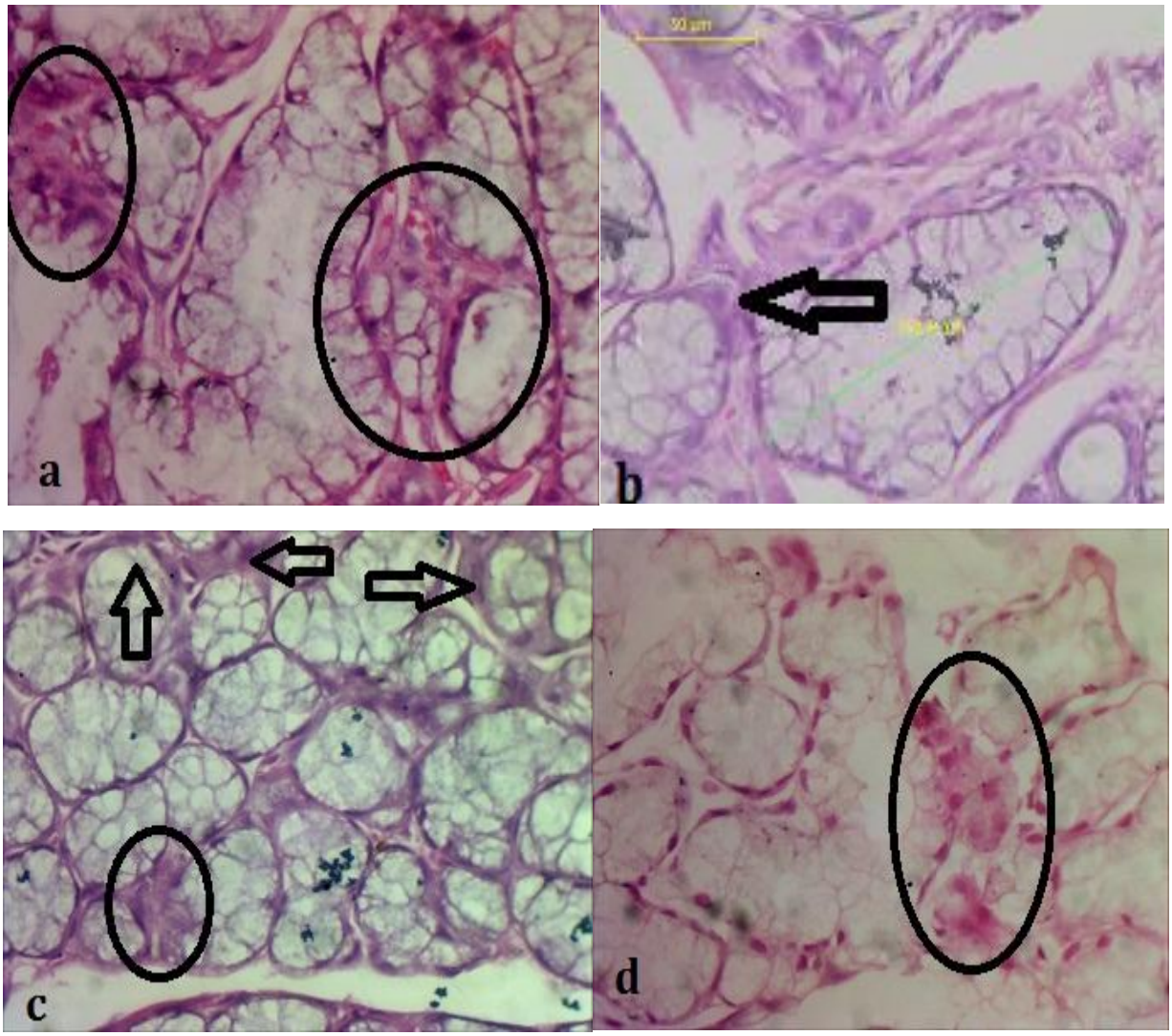


Figure 10: photomicrographs for Weber's salivary gland of rabbit: in group b1in pictures (a,b), in group c2 in picture (c), in group d2 in picture (d), appeared as mixed gland with predominant mucous cells, serous cells were few <within the circles > and mostly appeared as serous demilunes <arrows>. Hematoxylin and eosin (H&E) stain. X400.

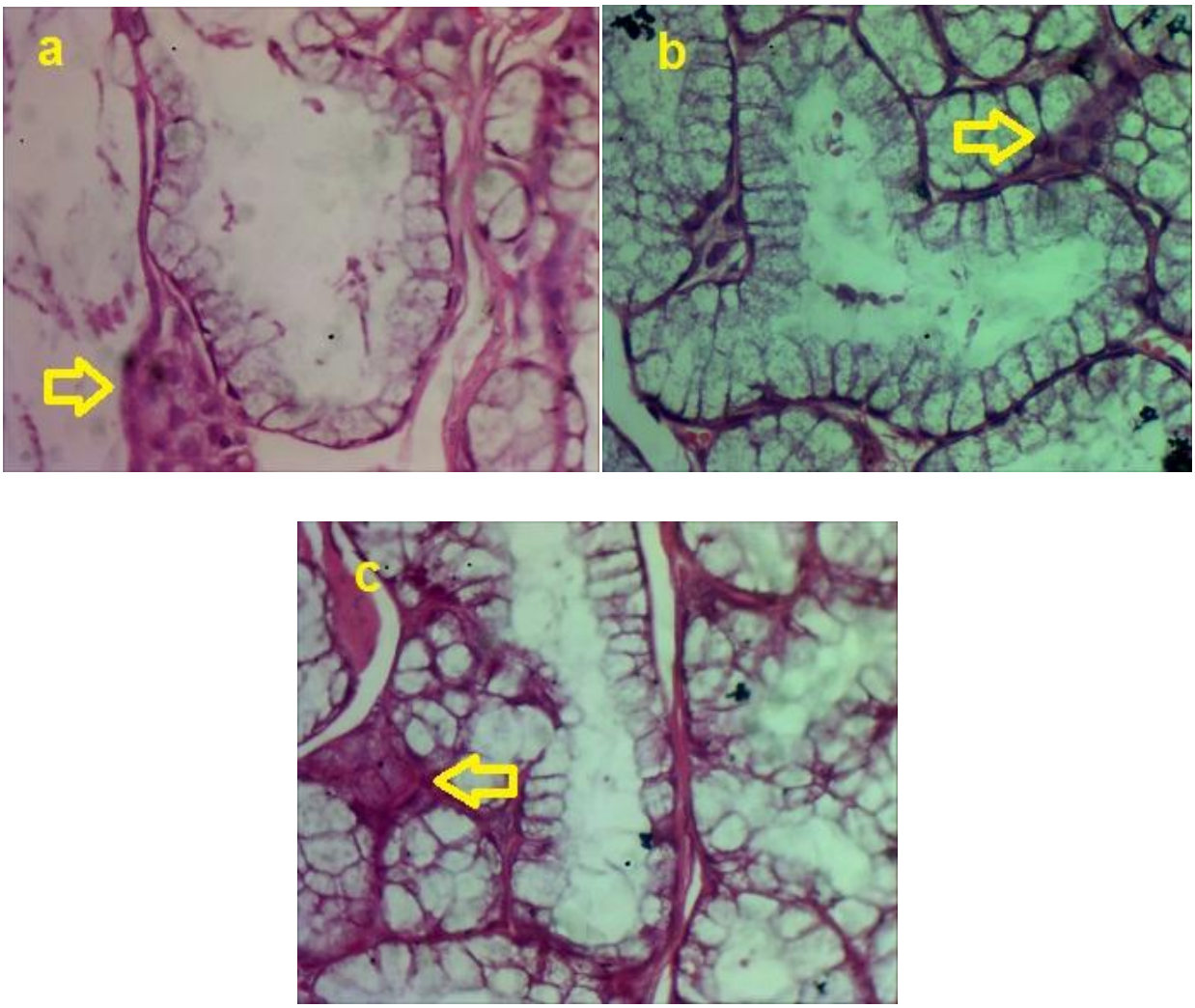


Figure 11: photomicrographs of Weber's glands: in group b1 in pictures (a,b), in groupb2 in picture (c), showing mucous cells were arranged in form of tubules where they were surrounding a central large lumen. While the serous cells were arranged in a form of round structure with a narrow lumen lined by pyramidal cells <arrows>. Hematoxylin and eosin (H&E) stain. X400(a,b,c) .

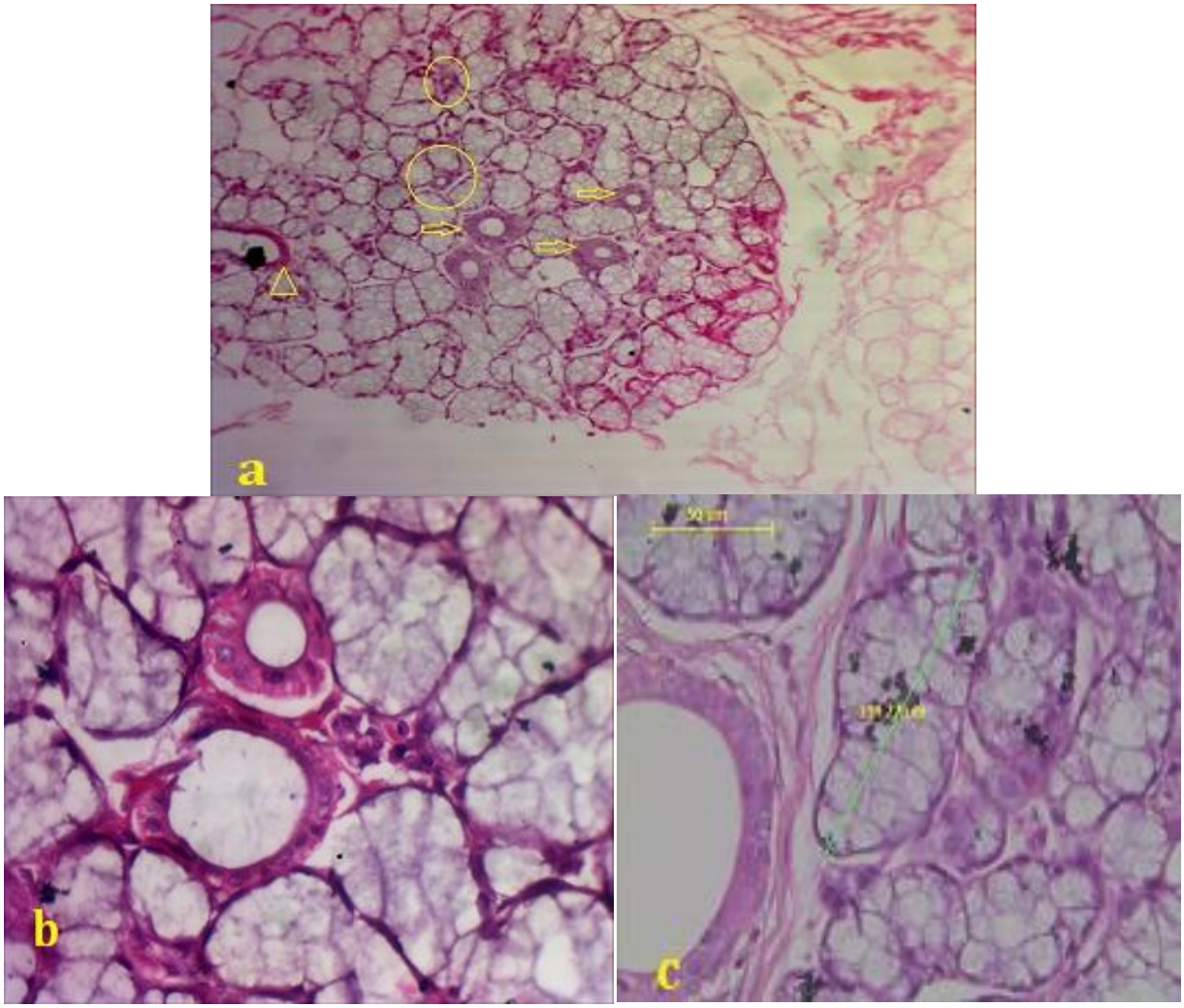


Figure 12: photomicrographs of the duct system in minor salivary glands started as intercalated ducts in group c2, appeared as smallest one within the <circles>, striated ducts appeared as wider ducts <arrows >, Large excretory ducts also seen interposed in between secretory units <triangle> at picture (a). The striated ducts in group d2 at picture (b), Large excretory ducts in group b1at picture (c). Hematoxylin and eosin (H&E) stain. X100(a) and X400 (b,c).

Table 1: demonstrates the mean of acini diameters for the three different glands in treated groups were examined in the present study. The most affected glands were the weber's glands, where obvious change was in the increase in the diameters of acini, and gave the highest readings among them. While the least affected one were the buccal glands, where the change in the diameters of acini were less than the other.

The treated groups	Mean of Buccal glands diameters	Mean of Palatal glands diameters	Mean of Weber glands diameters
a1	343 µm	430 µm	456 µm
a2	384 µm	473 µm	457 µm
b1	532 µm	532 µm	584 µm
b2	671 µm	677 µm	712 µm

Buccal glands:

Located in submucosa of cheek in between the muscle strands of the buccinators muscle .They appeared in both groups treated and untreated as classical tubuloacinar mixed gland, with both mucous cells (the predominant cell types) and serous cells. for example, buccal glands in groups (b2, c1,c2 Figure 13). Some of mucous tubules were capped by serous demilunes. The serous cells could be seen also as discrete acini embedded in between the mucous tubules, for example, buccal glands in groups (d1,b2,b1,a2 Figure14).

Histological structure were similar to that appeared in the Weber's glands, with duct system is formed by confluence of small intercalated ducts, lined by single layer of low cuboidal cells, that connect the terminal secretory unit to the next larger striated ducts in the interlobular connective tissue, that were wider ducts, lined by tall columnar epithelial cells with large spherical, and centrally placed nuclei , the duct continue to join each other, increasing in size to form main excretory duct, the epithelium here becomes pseudostratified with increasing number of smaller basal cells between tall columnar cells on longest duct, for example, buccal glands in groups (a2 Figure 15).

In the treated groups the lowest diameters measurements were registered among the buccal glands, which were less affected one by Pilocarpine treatment (Table 1).

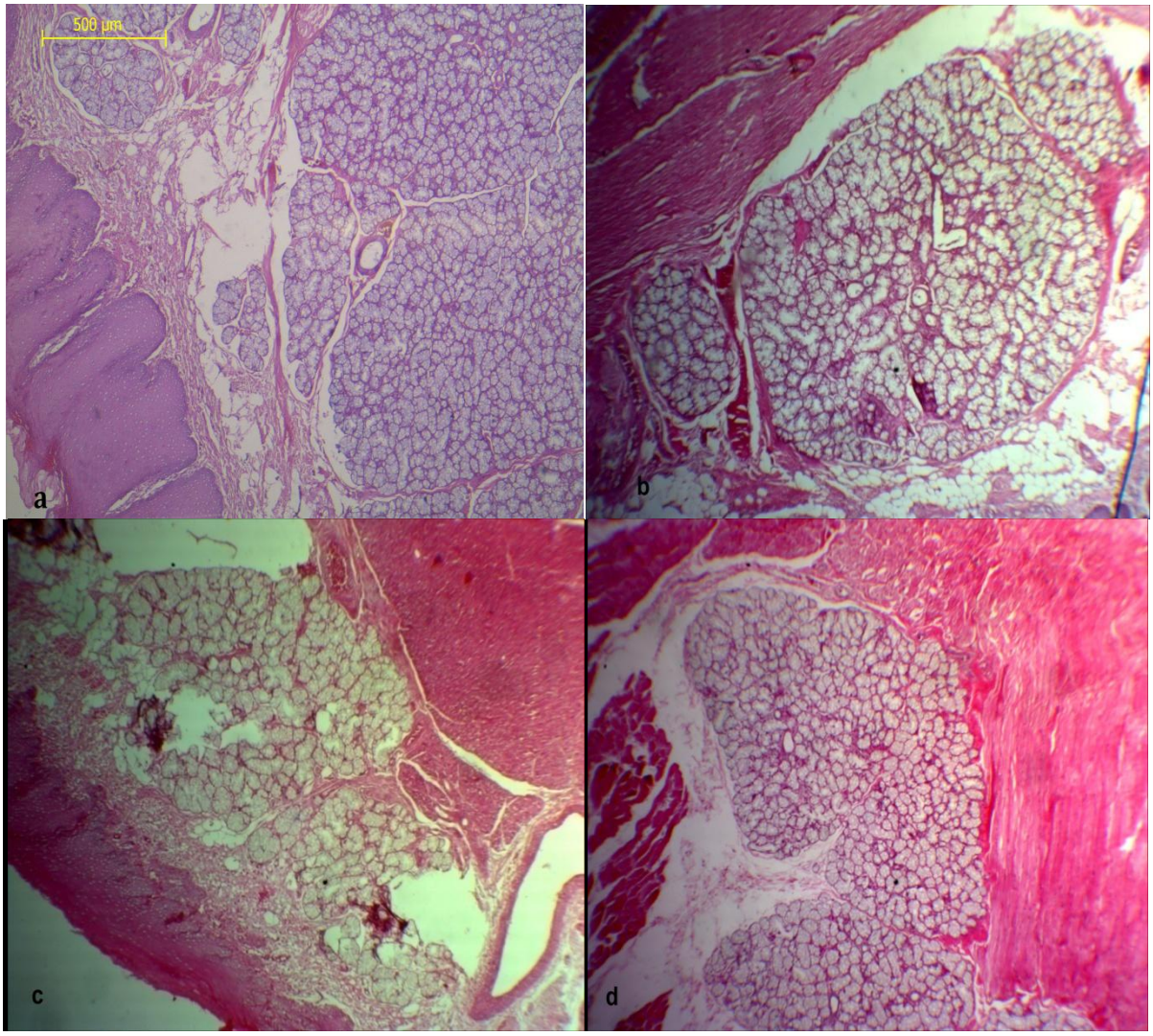


Figure 13: photomicrographs hematoxylin and eosin (H&E) stained section of the buccal glands: in group b2 in pictures (a,b), in group c1 in picture (c), in group c2 in picture (d), where located in submucosa of cheek and found between the muscle strands of the buccinators muscle .They appeared as classical tubuloacinar. X40.

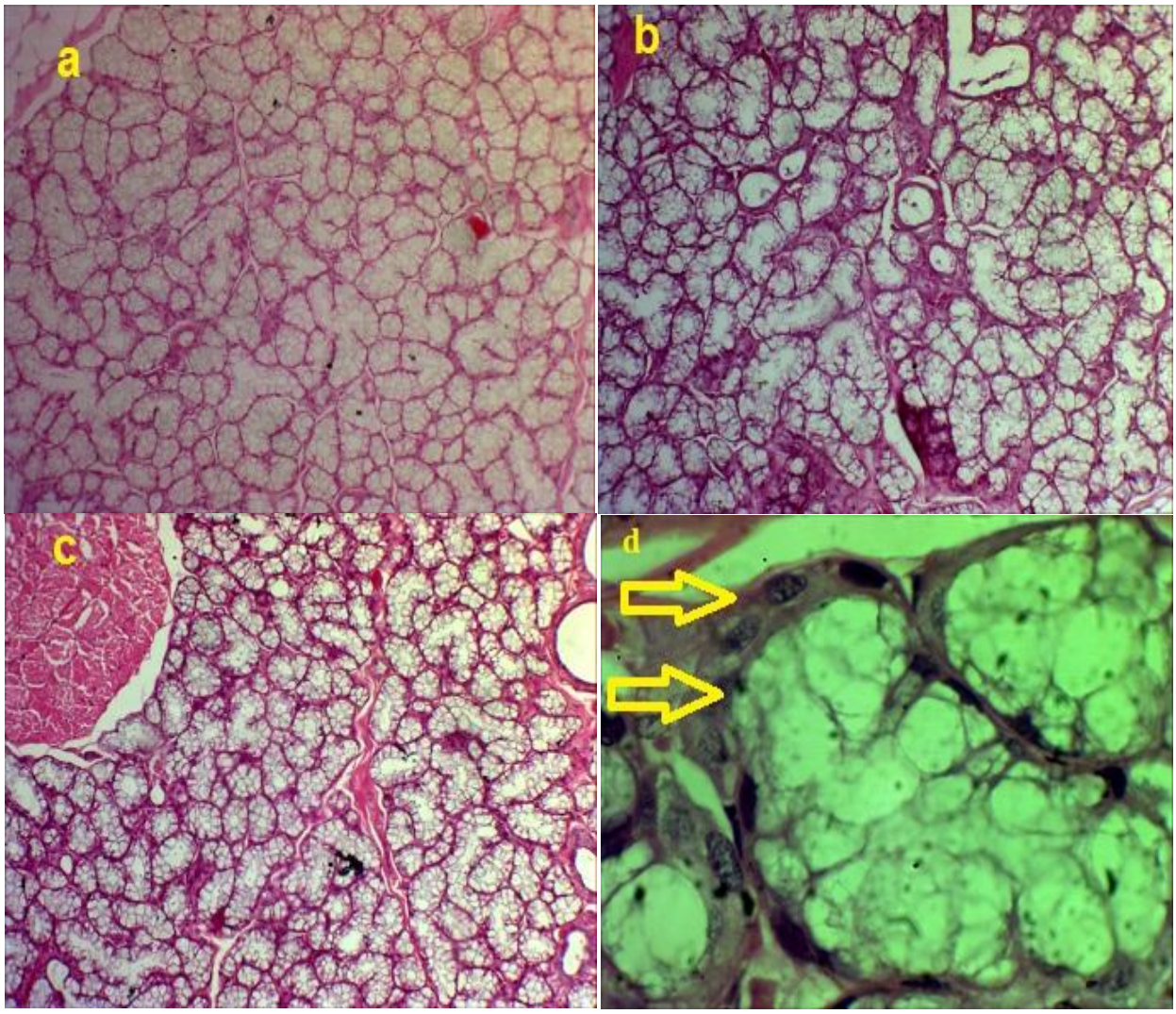


Figure 14: photomicrographs hematoxylin and eosin (H&E) stained section of the buccal glands: in group d1in picture (a), in group b2 in picture (b), in group b1 in picture (c), in group a2 in picture (d), showing the predominance of mucous secretory acini (tubuloalveolar) with serous cells and excretory duct system. X100(a,b,c). Some of mucous tubules were capped by serous demilunes (arrows in d). X1000.

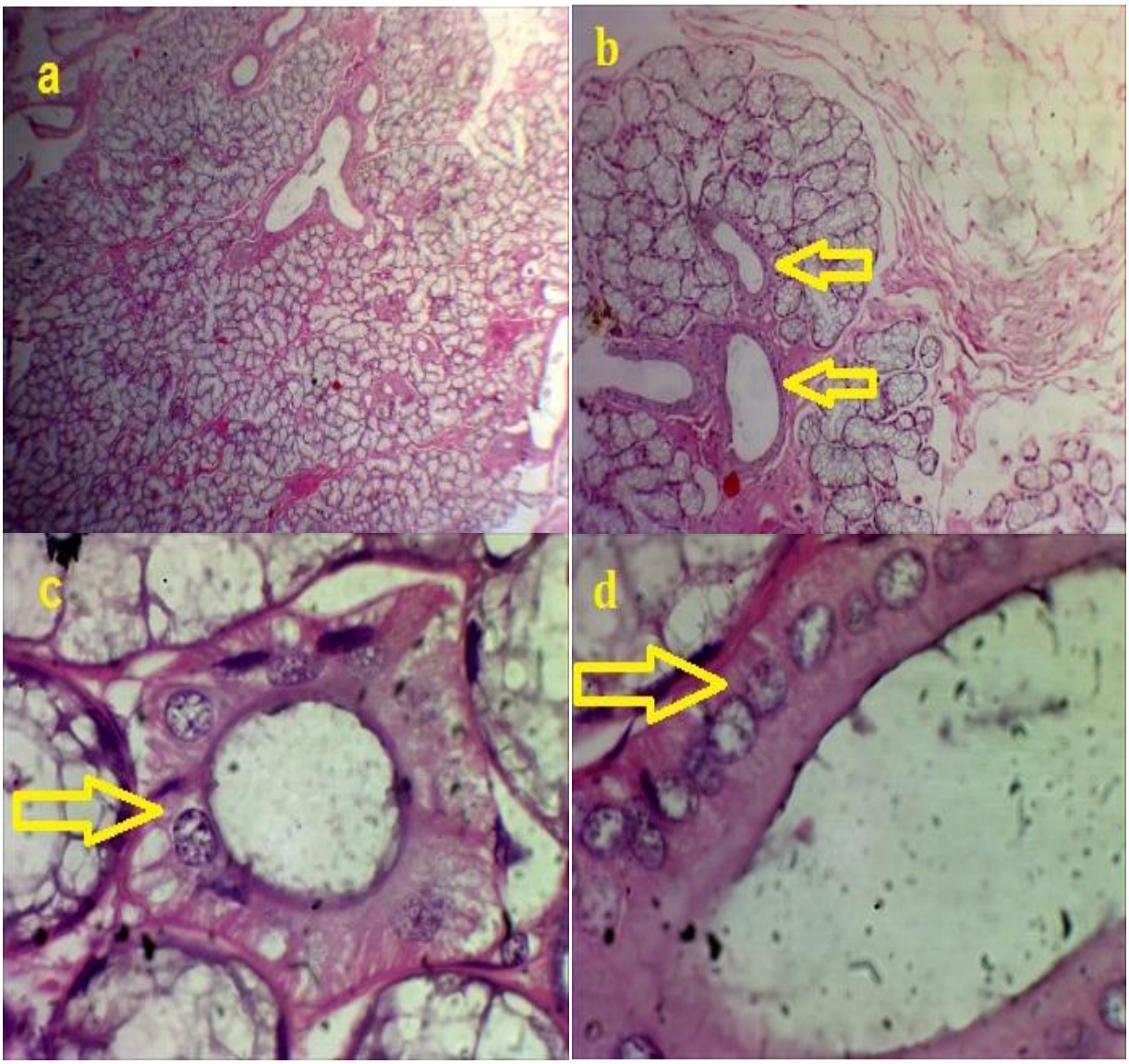


Figure 15 : photomicrographs hematoxylin and eosin (H&E) stain of the buccal glands in group a2, showing the duct system in picture (a): X40. Excretory duct in picture (b): X100. Intercalated duct in picture (c): X1000. And secretory striated ducts in picture (d):X1000.

Soft palatine glands:

The H&E staining has revealed that these glands in both treated and untreated groups were purely mucous glands. The lobules of the secretory units were aggregating in the submucosa between the mucosa and palatal musculature .Their excretory ducts course through the lamina propria then open across the palatal mucosal epithelium towards the mouth cavity.

Histologically they appeared in treated and untreated groups as tubular configuration with wide lumen, the cells were tall columnar with flattened basal nuclei, the cytoplasm appeared pale clear lightly-stained and vacuolated in H and E stain.

Separated and independent duct system was draining each lobule. It was comprised of intercalated, excretory and main duct, for example, palatine glands in groups (b2,c1figure 16).

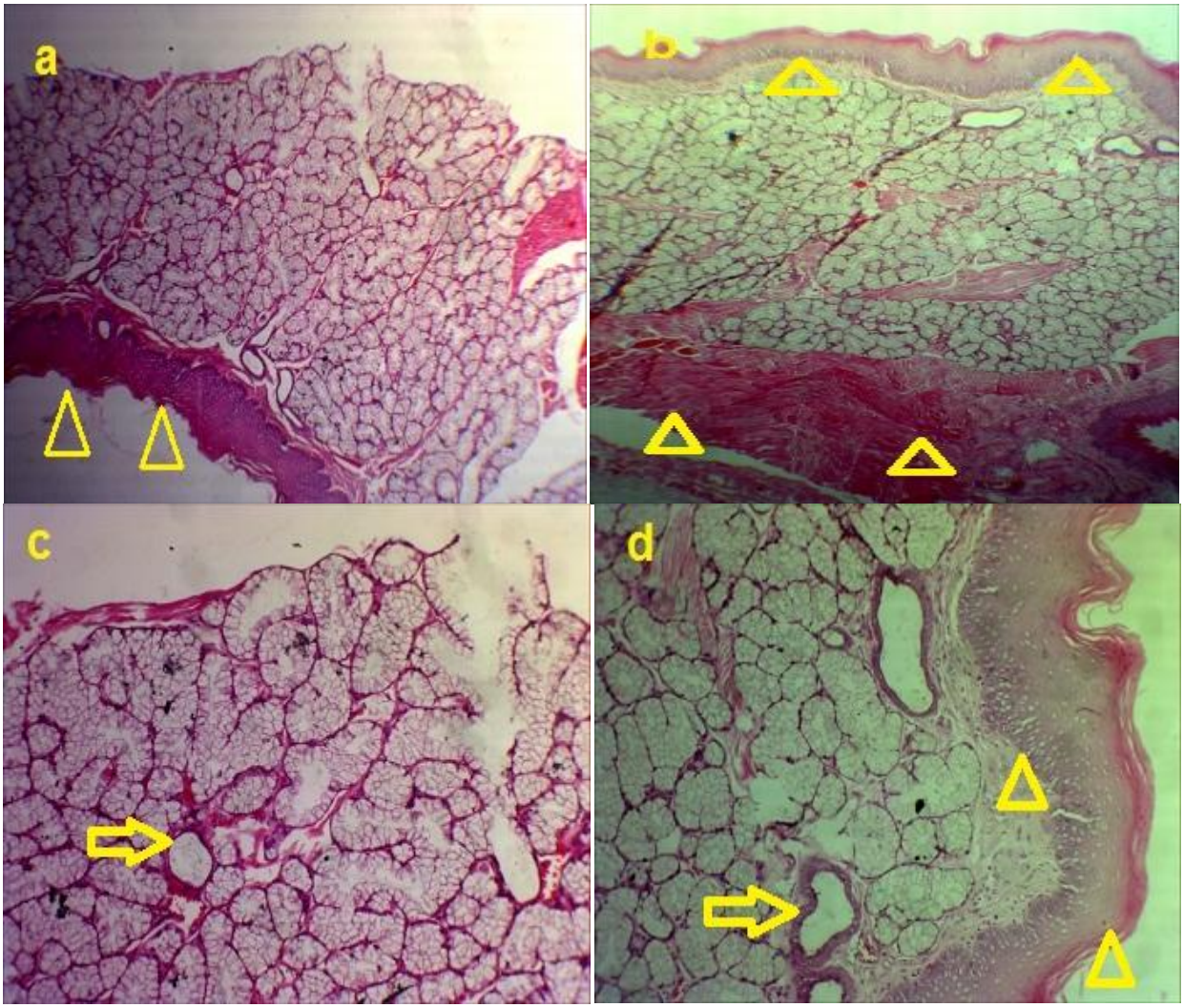


Figure 16: photomicrographs hematoxylin and eosin (H&E) stained section of the soft palatine glands: in group b2 in pictures (a,c), in group c1 in pictures (b,d), showing the purely mucous acini between the mucosa and palatal musculature < triangles>, and separated independent duct system was draining each lobule <arrows>. X40 (a, b), X100(c, d).

Histological effect of Pilocarpine :

In the treated groups (GA ,GB) the most noticeable changes in the minor salivary glands were the vacuolation and foamy appearance of the cells of the secretory acini (figure 17 ,18) . And increasing size of acini with wide lumens (figure 19).

Other obvious change was an increase of the diameter of the acini in treated groups compared with acini in the control groups (Figure20 and table 2).

In treated groups (GA ,GB) show transformations, spacing present between acini, increase in diameters and sizes of acini (Figure 21).

The effect of the drug and its transformation was more obvious in group B with the increasing of the dose of the drug , than that occur in group A (graph 1). This transformation and diameter increasing became more with time increasing.

No difference was seen between preservative groups and control groups. The measurements of the diameters of the acini of both groups were approaching each other (table 2) . Also there was no effect of used preservative on minor salivary glands .

For the PAS stained sections, positive reactions were identified in all groups of the minor salivary glands. The most obvious reaction obtained was in treated groups (GA ,GB), there were abundant mucosubstance secretions (Figure 22, 23).

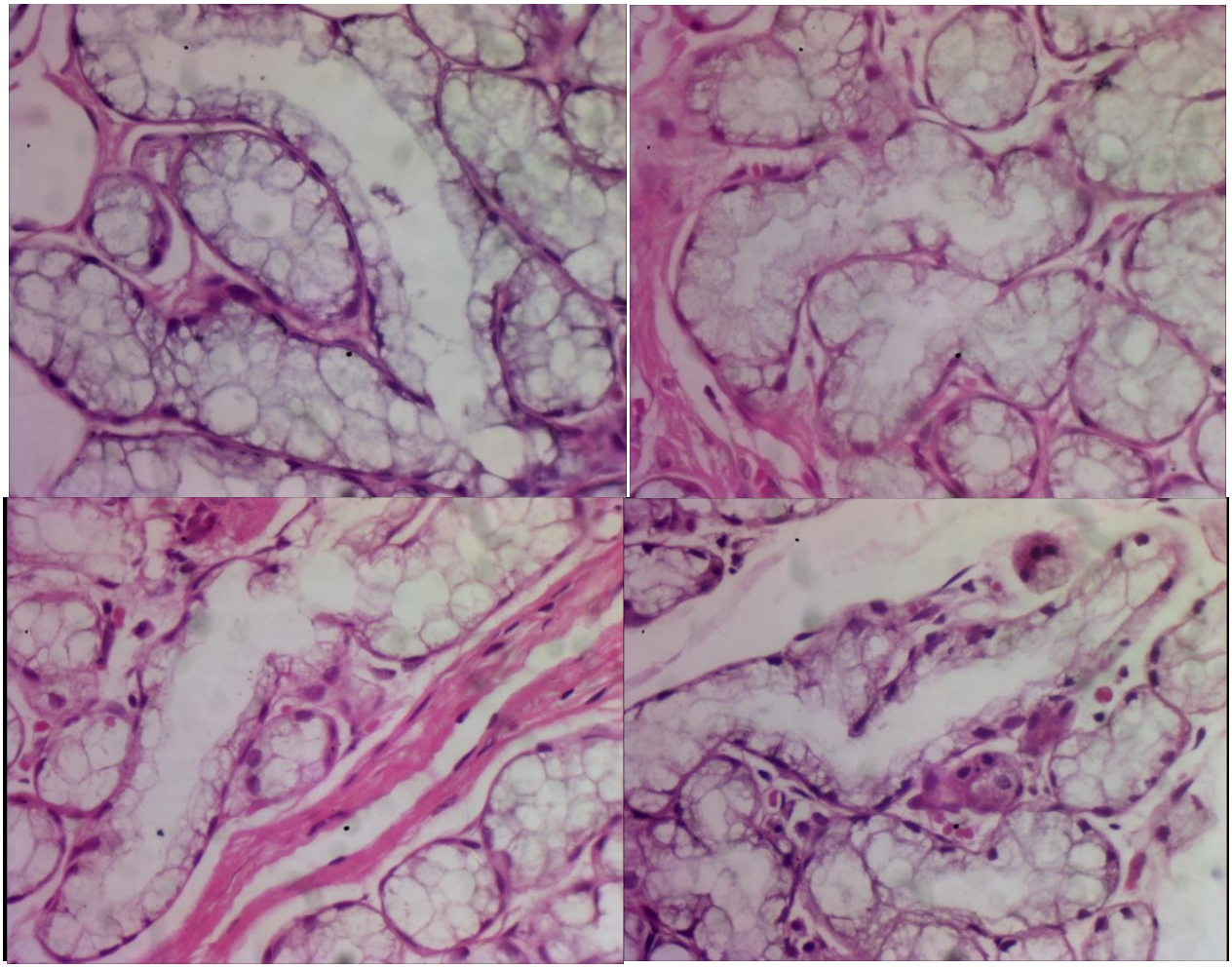


Figure 17: photomicrographs showing the vacuolations and foamy appearance of the cells of secretory acini in group a2 of buccal glands. Hematoxylin and eosin (H&E) stain. X 400.

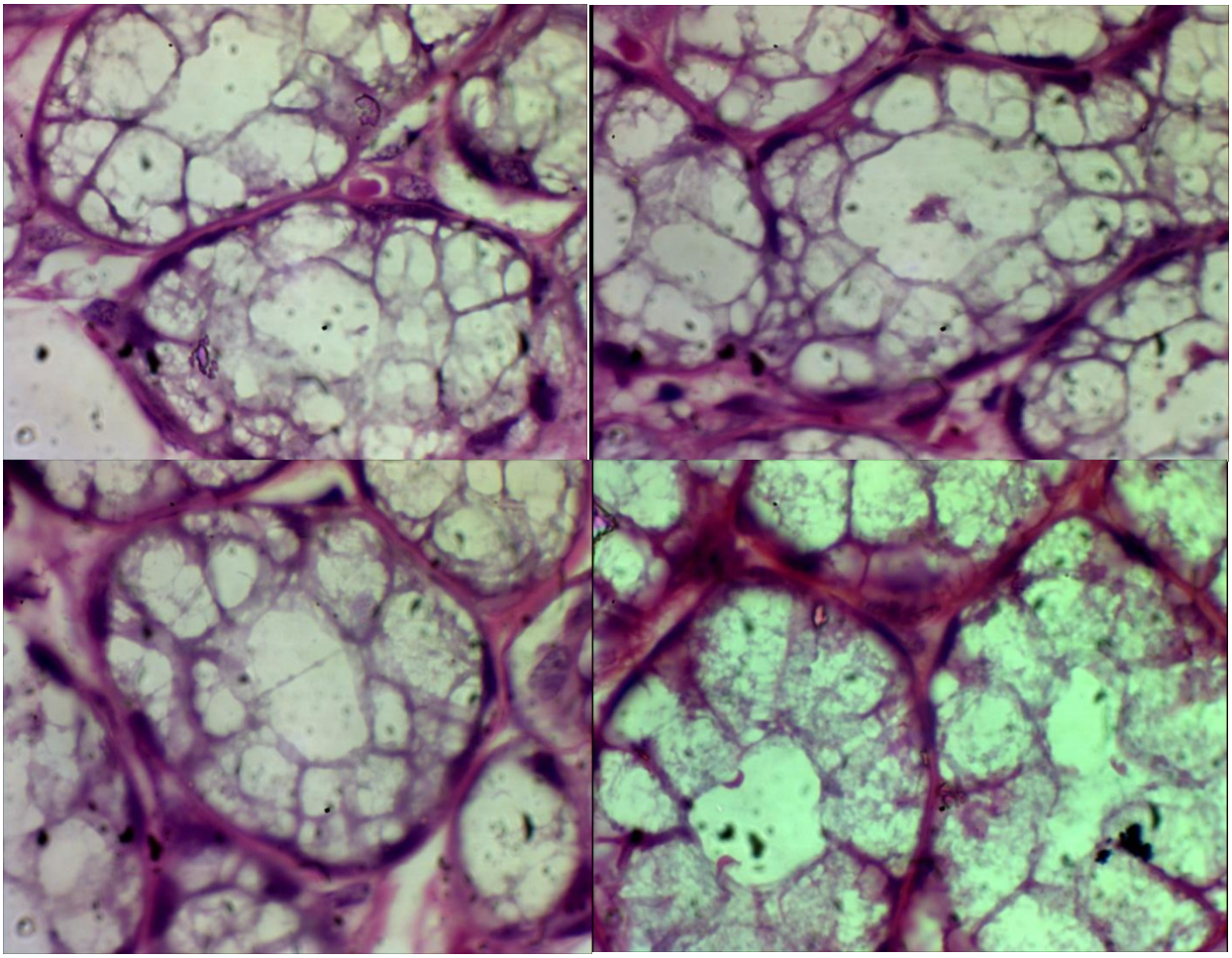


Figure 18: photomicrographs of buccal glands in treated groups (a2 , b1) show the vacuolation and foamy appearance of cells in secretory acini. Hematoxylin and eosin (H&E) stain. X1000.

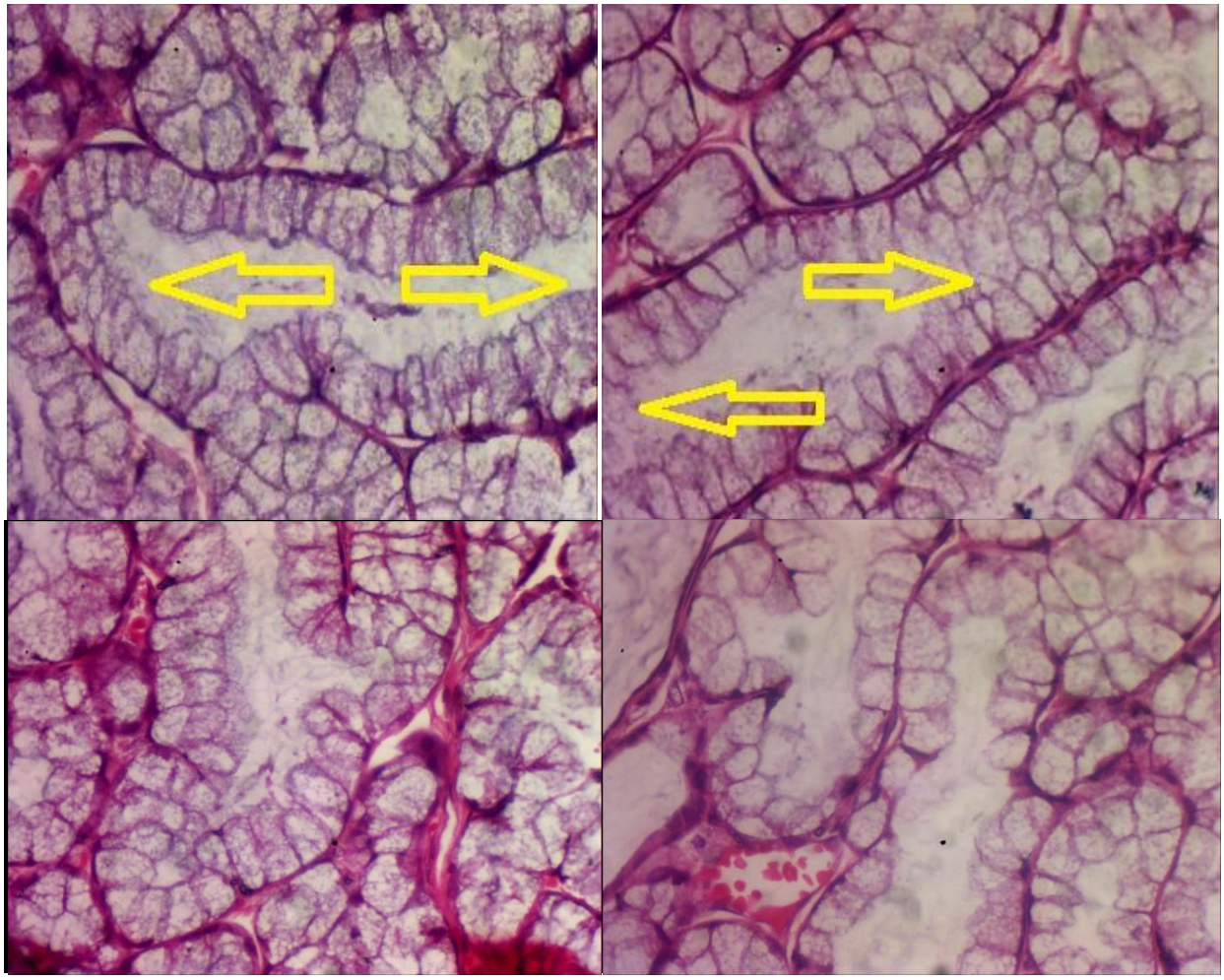


Figure 19: photomicrographs of sections taken from group a2 (soft palatine glands) showing increasing size of acini with wide lumens <arrows>. Hematoxylin and eosin (H&E) stain. X 400.

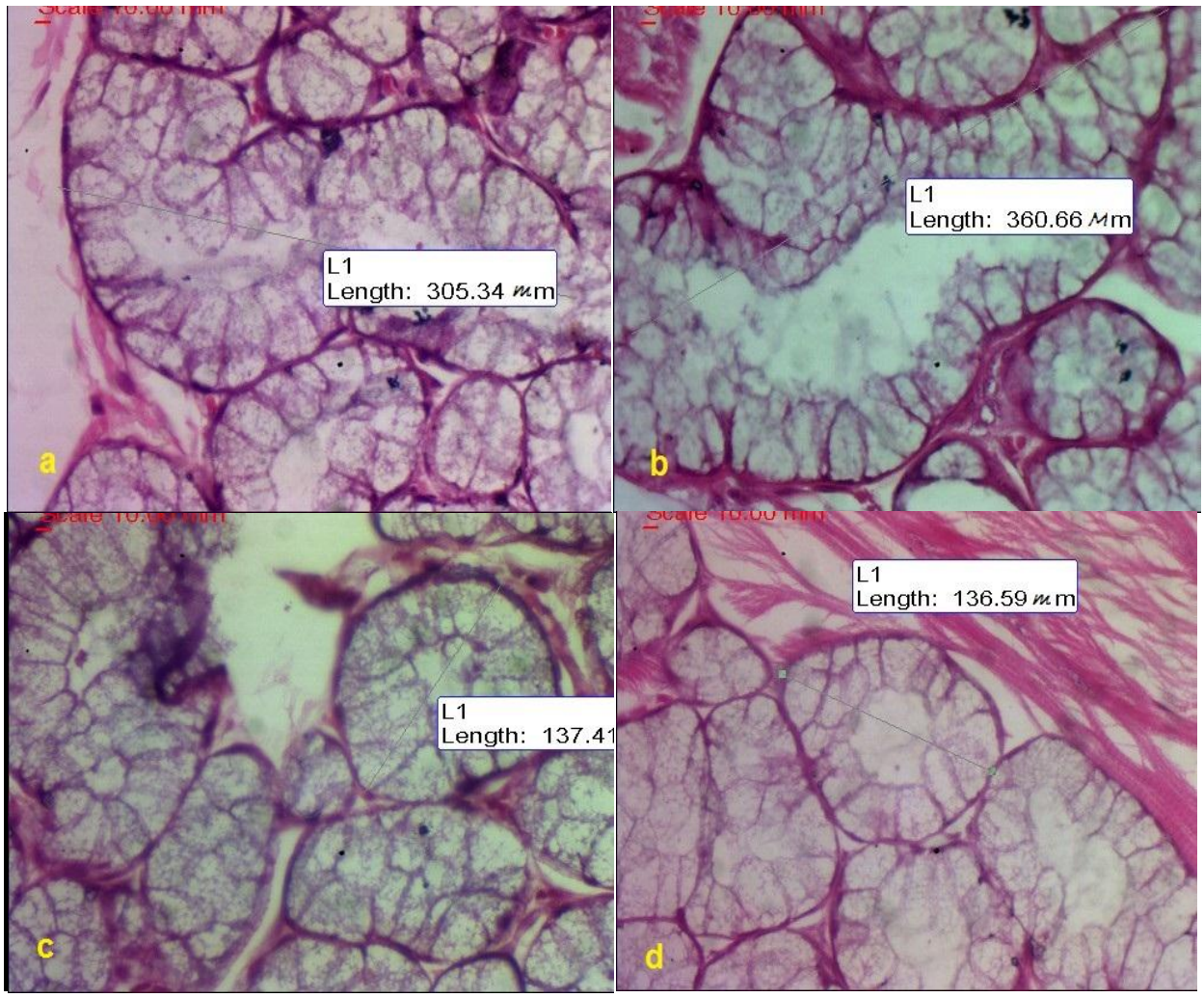


Figure 20 : photomicrographs H&E stain. X400 of weber's glands showing the variation of diameters between treated groups Ga2,Gb2 in pictures (a,b) and preservative Gc2 in picture (c) , control groups Gd2 in picture (d).

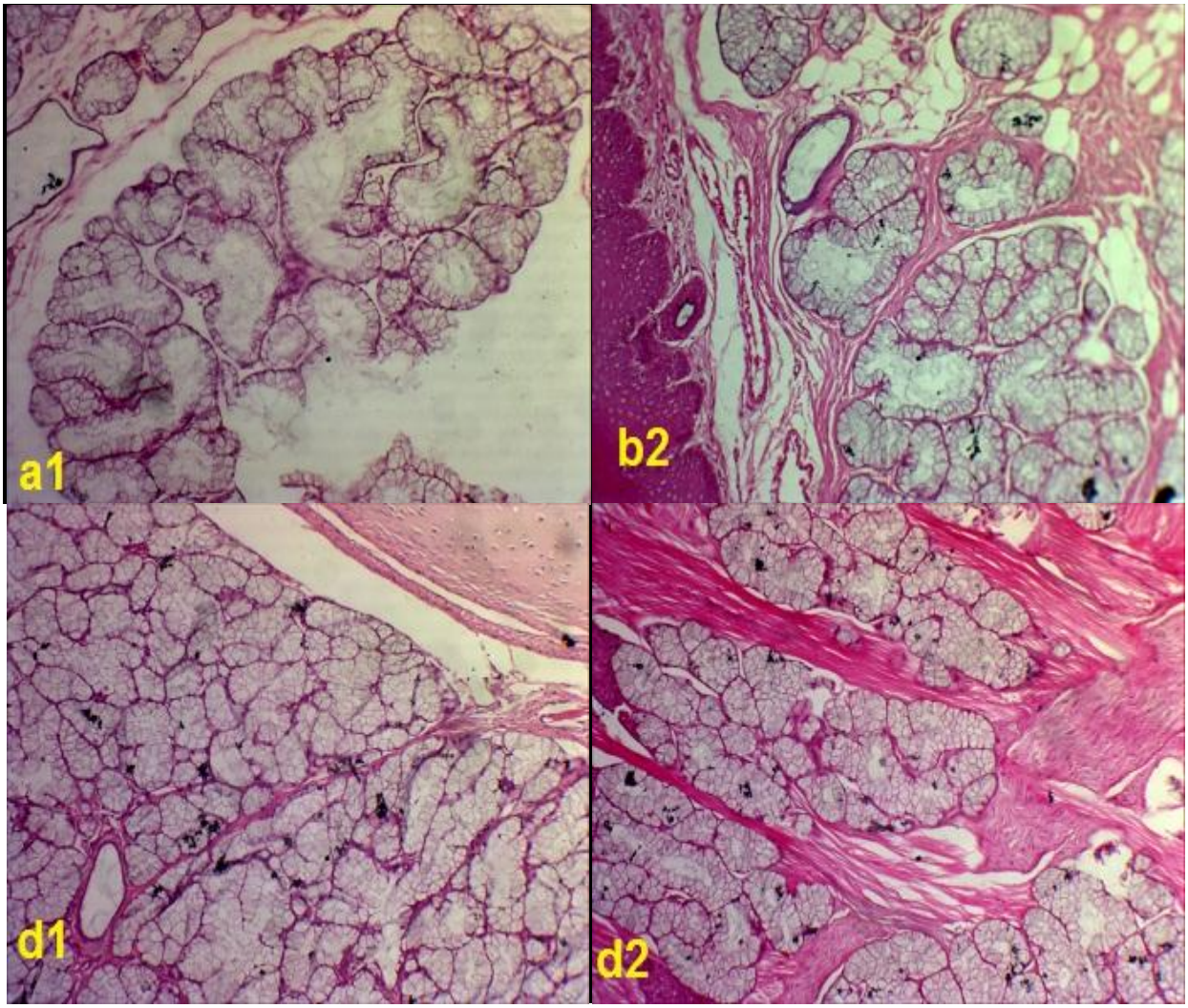


Figure 21: photomicrographs of weber's glands showing spacing ,transformations and increase in diameters of acini in groups(a1,b2) compared with acini in groups(d1,d2). H&E stain. X 100.

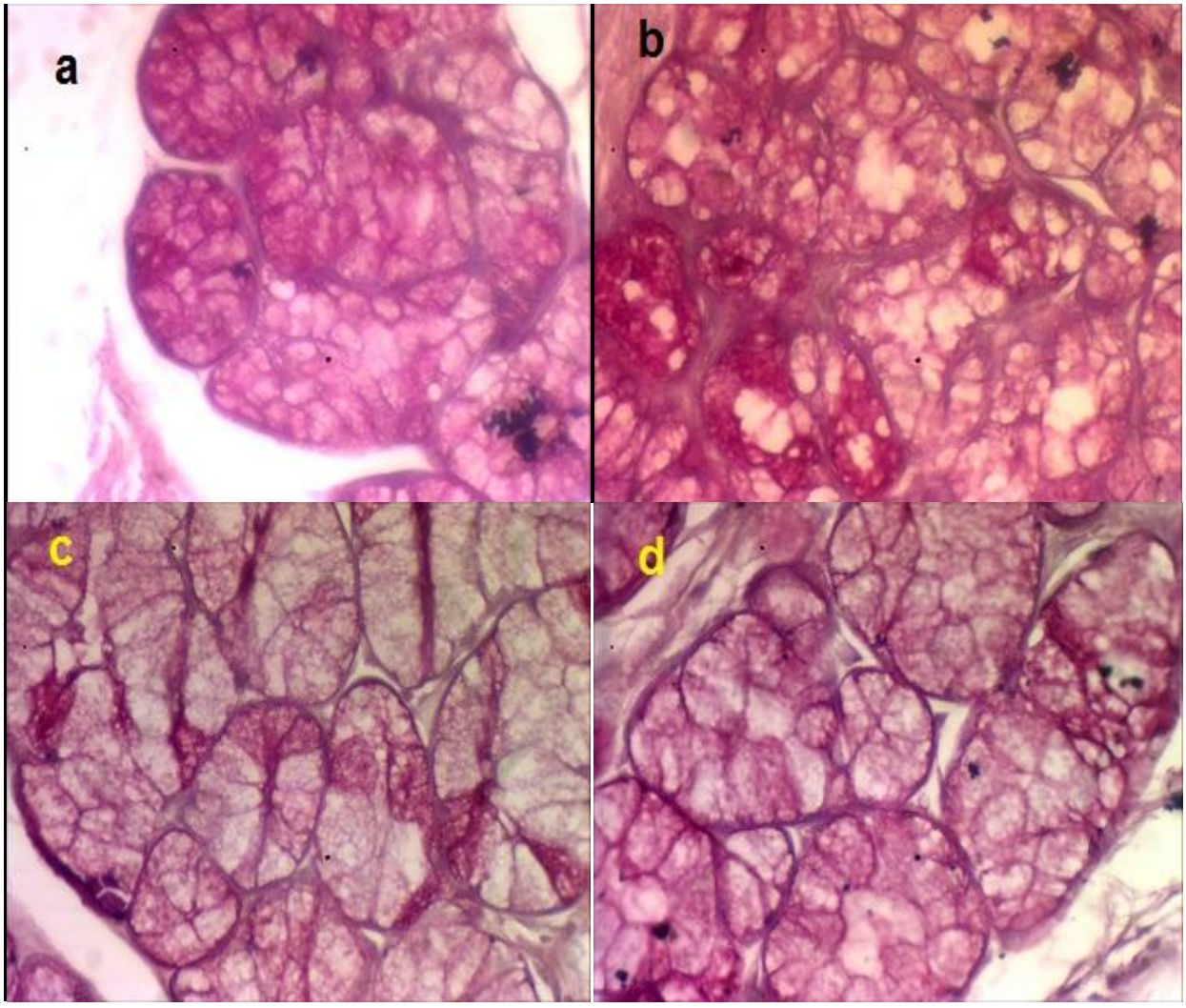


Figure 22: photomicrographs showing positive reactions of PAS stain were identified in all groups of the minor salivary glands, and more obvious in treated groups in pictures (a, b) than preservative in picture (c), control in picture (d) groups . X 400.

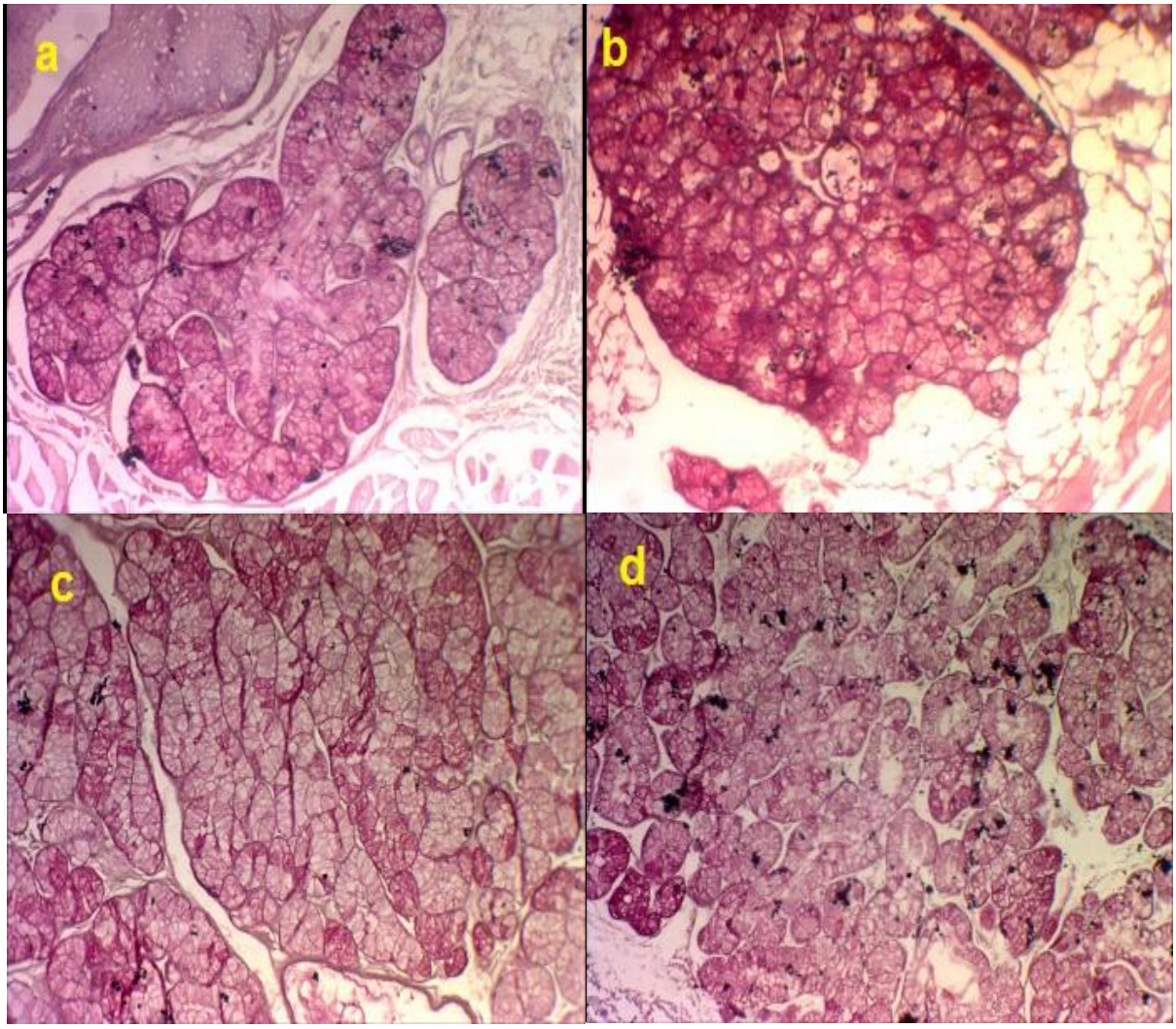


Figure 23: photomicrographs showing strongly stained sections with PAS in treated groups in pictures (a, b) in comparison with less stained sections in preservative groups in picture (c) and control in picture (d). X 100.

The statistical analysis has revealed a change in the diameter of the acini of minor salivary glands (see table 2 and graphs 1) . There was a difference between the treated and the control groups with P value ≤ .000 (table 3).

ANOVA test applied on the diameters of salivary glands of the groups treated with Pilocarpine & control groups with degree of significance ≤ .000, that indicate to big difference between them, this difference to be direct proportional to increase of Pilocarpine dose and duration of treatment,(table 4,5).

But there is no degree of significance and noticeable difference between preservative groups and control groups with P value ≤ .227 in 2week and with P value ≤ .172 in 6 week, (table 4,5).

Table 2 : descriptive statistics of groups treated with Pilocarpine & their controls

The readings obtained were in micrometer.

Groups	Number of readings	Mean	Standard deviation	Minimum	Maximum
b1 5 mg 2 weeks	18	549.67	62.37	464.80	699.50
a1 3mg 2 weeks	18	401.09	64.86	307.01	487.00
d 1 control 2 weeks	18	267.95	18.05	232.60	298.41
b 2 5 mg 6 weeks	18	687.30	68.79	577.88	820.70
a2 3 mg 6 weeks	18	437.89	65.70	339.00	556.00
d 2 control 6 weeks	18	250.65	22.34	215.13	289.31
c1 preservative 2 weeks	18	247.56	28.96	201.91	295.80
c2 preservative 6 weeks	18	227.59	38.37	142.06	290.77
Total	144	383.71	164.94	142.06	820.70

Table 3: : shows results of ANOVA test applied on the acini diameters of minor salivary glands of the treated and control groups with degree of significance less than O.001

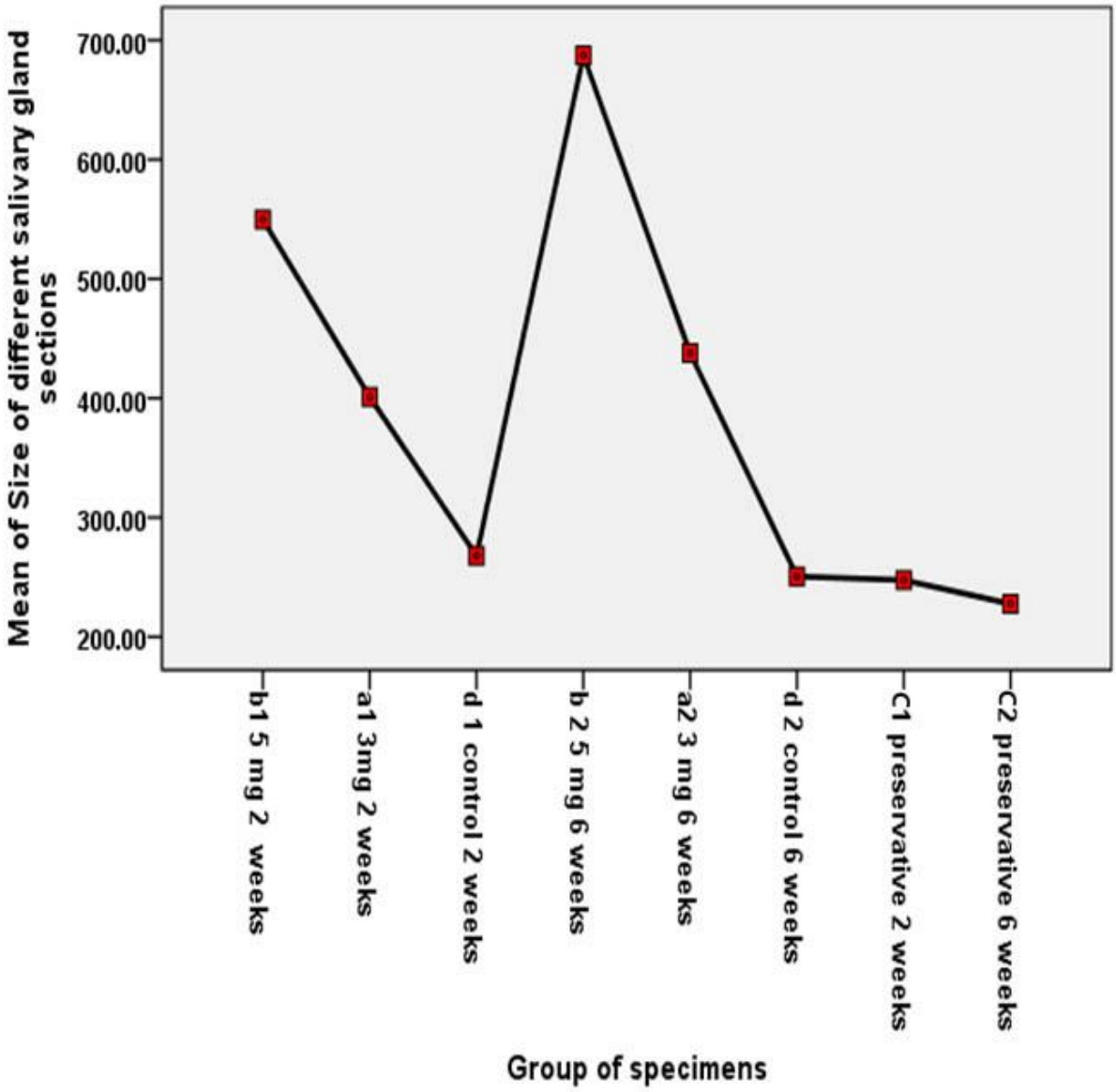
	Sum of Squares	Mean Square	F	Sig.
Between Groups	3545414.09	506487.73	199.77	.000
Within Groups	344810.39	2535.37		
Total	3890224.48			

Table 4: demonstrates the results of ANOVA a post HOC test, applied on the acini diameters of all groups in 2week.

(I) Group of specimens	(J) Group of specimens	Mean Difference (I-J)	Std. Error	Sig.
d 1 control 2 weeks	b1 5 mg 2 weeks	-281.72611*	16.78415	.000
	a1 3mg 2 weeks	-133.14000*	16.78415	.000
	c1 preservative 2 weeks	20.38833	16.78415	.227

Table 5: demonstrates the results of ANOVA a post HOC test applied on the acini diameters of all groups in 6 week.

(I) Group of specimens	(J) Group of specimens	Mean Difference (I-J)	Std. Error	Sig.
d 2 control 6 weeks	b 2 5 mg 6 weeks	-436.65667*	16.78415	.000
	a2 3 mg 6 weeks	-187.25056*	16.78415	.000
	c2 preservative 6 weeks	23.05333	16.78415	.172



Graph 1: relationship between the dose of pilocarpine and the mean of the acini diameters of the minor salivary glands

Chapter 6

DISCUSSION

DISCUSSION

A lot of studies performed on clinical effect of Pilocarpine drug, but few studies involved both sides: clinical observation and histological findings. This study has demonstrated the correlation between the histological changes of the minor salivary glands and clinical observations of the rabbits response to different dosages of Pilocarpine drug for two different periods followed in the present work.

As previously mentioned, Pilocarpine is a parasympathomimetic agent as "cholinergic" drug, that functions primarily as a muscarinic agonist with mild beta-adrenergic activity, its effect causes pharmacologic stimulation of exocrine glands in humans, lead to diaphoresis, salivation, lacrimation, and gastric and pancreatic secretion [(81)].

Among sialagoguse drugs, the Pilocarpine is an effective and safe treatment for salivary gland hypofunction and xerostomia in selected patients [(82)].

Oral Pilocarpine formulations are more economical and can be used in lower doses, and it is very soluble and stable in water solution and the effect lasts for up to 3 hours [(83)].

Our choice to the minor salivary glands were based on their specific characteristic, as they exhibit a continuous slow secretory activity, especially at night when the major salivary glands are mostly inactive, this continuously in salivary flow manifest the important role of minor salivary glands in protecting and moistening the oral mucosa[(11)]. In spite of they produce less than 10% of the total volume of saliva, but contribute more than 70 % of the total mucin content [(6)].

Also the minor salivary glands have displayed more sensitivity to Pilocarpine, this is confirmed in a previous study performed on Sjögren's syndrome patients, demonstrated a significant increase in labial salivary gland flow, as well as whole salivary flow as stimulated by Pilocarpine drug [84].

In preceding study by Niedermeier et al 1998, the findings illustrated the greater resistance and recoverability of the mucous secreting minor palatal glands in contrast with the serous secreting parotid glands. They also show the significant post radiation ability of the mucous secreting glands to be stimulated by Pilocarpine [67].

In a further study by Horiot J.C. et al 2000, showed that oral Pilocarpine acts primarily by stimulating minor salivary glands and can be of benefit to patients suffering of severe xerostomia regardless of radiotherapy dose/volume parameters [85].

These examples of some studies that support the usage and treatment by Pilocarpine drug, so during evaluating the effect of oral Pilocarpine treatment on conjunctival epithelium of patients with Sjögren's syndrome, beside improvement of ocular dryness, also had been recognized improvement in dry mouth [86].

Pilocarpine as a parasympathomimetic drug, improves saliva production and relieved symptoms of xerostomia after irradiation for cancer of the head and neck, with minor side effects that were predominantly limited to sweating [81].

In earlier study performed by Davies A.N. et al, 1998 comparing a mucin-based artificial saliva and Pilocarpine hydrochloride in the management of xerostomia in patients with advanced cancer, the patients preferred using of Pilocarpine, and reported that it had helped their xerostomia. The Pilocarpine was found to be more effective than the artificial saliva [87]. However, the artificial saliva or salivary substitutes do not contain the digestive and antibacterial enzymes and other proteins present in real saliva [88].

Salivary substitutes assist some patients but muscarinic, cholinergic sialogogues provide a more physiological replacement in those patients who have reckonable residual salivary function [89].

The literature clearly demonstrates that Pilocarpine evokes constituents of saliva such as mucins, proteins, glycoproteins, and electrolytes, recognized increases in salivary amylase, protein-bound carbohydrate, lysozyme, total protein, and calcium after Pilocarpine stimulation in normal subjects [81].

In addition, the use of the Pilocarpine drug pretreatment was protective against irradiation, that illustrated in the past study which performed on the submandibular gland of rat, displaying much less damage after irradiation ,with complete recovery after 4 weeks [90] . This protection does not involve tumor cells or tumors, therefore use of this drug may lead to therapeutic gain in the treatment of head and neck cancer [91].

Thus, Pilocarpine drug should be regularly used in treatment of xerostomia [92]. It could be used for post irradiation-induced xerostomia with fewer adverse effects [93].

The radiations are most serious factor that produce dysfunction of the salivary glands and cause an impairment of exocytosis and a reduction of water secretion. This dysfunction as a result of loss of aquaporin 5 (AQP5) and possibly other membrane-fusion proteins in acinar cells [94]. Whereas (AQP5) is the major pathway for regulating the water permeability in acinar cells [68].

The results of study by Li J. et al, 2006 suggest that Pilocarpine induce salivary secretions in human by activating K^+ channels, increasing Ca^{2+} via phospholipase C dependent pathway, and increasing AQP-5 protein expression in the apical membrane of Submandibular gland acinar cells [95].

Although cholinergic agonists Pilocarpine injected peripherally, it can act directly on salivary glands to induce salivation. In previous study performed on the rats, by Inenaga K.et al 2008, has illustrated that peripherally applied Pilocarpine does not act only on the salivary glands as a sialogogue, but also it can be stimulate the thirst sensation by acting on the centre controlling body fluid balance in the central nervous system [96].

As well, systemically injected Pilocarpine also enters the brain and acts on central muscarinic receptors, activating autonomic efferent fibers to induce a central stimulation for salivation [97].

A previous study by Sato N. et al. 2006 performed on conscious rats , the researchers concluded that peripherally injected Pilocarpine affects the parotid glands and the thirst centre in the central nervous system, while it may provoke salivary secretion mainly via peripheral responses, but water intake mainly via the central nervous system [(98)].

The experimental animals of the present work, which were injected intraperitoneally twice daily by Pilocarpine, as liquid ophthalmic solution, showed similar action in salivary glands, that has been noticed by Rosas J.et al 2002 study [(66)].

The clinical observations of our study regarding Group A and group B who were showed an increase in salivary flow, injected with therapeutic dose of Pilocarpine, and within 20 minutes after injection, the following events were noticed :

Increase in activity of rabbits and they became more aggressive, rise in body temperature of rabbits, increase in water intake and excessive salivation .

Since the salivary glands are supplied by parasympathetic and sympathetic efferent nerves, which were inducing morphological changes in the parenchymal cells after nerve stimulations or denervation, that in fact explain the important roles of the autonomic innervations and their functions.

In previous study, has detected at least four types of influence can be exerted on salivary parenchymal cells by the nerves: hydrokinetic (water mobilizing), proteokinetic (protein secreting), synthetic (inducing synthesis), and trophic (maintaining normal functional size and state) [(99)].

Morphometric measurements of the present work have shown a significant enlargement of the acini at treated groups, similar conclusion has been reached by K. Donath K. ,et al.1974, particularly with sympathetic and parasympathetic stimulation [(72)]. while absence of nerve impulses causes variable atrophic and other metabolic effects on the parenchymal cells [(100)].

A past study by Baskerville A. et al. 1976 performed on pigs which were given 6 daily injections of isoprenaline or Pilocarpine, showed that both drugs produced a significant increase in the weight of the parotid and submaxillary glands, which was accompanied by an increase in acinar area [(75)].

The vasodilatation is resulting occur as part of secretion, in response to parasympathetic impulses, that usually provides the main stimulus for fluid formation by parenchymal cells, whereas sympathetic nerves have a tendency to increase the output of pre-formed components from certain cells.

related conclusion has been reached by Mills J.W. and Quinton P.M. ,1981[(101)] who pointed that the swelling seen in serous cell of the tracheal serous gland of the rat was related to the accumulation of fluid during intense parasympathetic stimulation.

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Another study by Schramm M. and Selinger Z. ,1974 performed on rat parotid gland, it emphasized that stimulation with muscarinic and adrenergic agonists caused movement of water and vacuole formation [102].

Thus, the end results of water movement might be the main explanation to the increase in size noticed in the Pilocarpine treated rabbits of this study .

In view of the fact that vacuole formation is an essential part of water secretion, also the vacuolation occurs to a variable degree in certain cells as a normal part of reflex secretion, and frequently found experimentally after strong stimulation [100].

In the present work, there was pronounced increase in diameter of salivary acini with obvious vacuolation in the cytoplasm of treated groups, which were accompanied by an increase in acinar area, that more obvious on Weber's glands. Soft palatine glands were less affected and buccal glands were the least to be affected by the drug.

In agreement with study of Lundquist P. G., Lena Norberg ,1989 [73] on rat's salivary glands , Pilocarpine showed its effect on the mucous cells with formation of intracytoplasmic vacuoles.

In this study the positive results of PAS stained sections have shown that there was accumulation of mucopolysaccharides substance, especially in acini of treated groups, that agrees with the study aimed to investigate histomorphological and histochemical structures of three major salivary glands in the adult local rabbits Al-Saffar F. J. 2014 [36]. The author has pointed out that the parotid gland acini were reacted positively with PAS stain. Similarly submandibular and sublingual glands showed PAS positive reaction characterized by magenta color formation of its mucinous contents.

Significance mentioning at this stage, there are limitations of usage Pilocarpine drug specially when there is destruction of salivary gland tissue, the influence of Pilocarpine drug is effective if there are functional salivary glands or even residual functional salivary gland tissue. This conception confirmed by several previous studies like, Fox PC, et al,1991 [82] study who proposed that Pilocarpine will not work for a patient whose salivary glands are completely nonfunctional. Another study by Rosas J. et al 2002, hypothesizes that the response of salivary glands to Pilocarpine requires residual functional salivary gland tissue [66].

Furthermore study by Davies AN, Shorthose K ,2007 [103], that suggested a limited evidence to support the use of Pilocarpine hydrochloride in the treatment of radiation-induced salivary gland dysfunction.

Other shortage with Pilocarpine treatment is effectively supportive, but not curative. That confirmed with study of Alajbeg et al 2005, which they justify the reason of that, there is no residual effect in salivary gland stimulation once the drug is discontinued and eliminated [104].

Few other studies go to e xtremes, and controvert the benefit and role of Pilocarpine in treatment of xerostomia, and make suspicious of its effect, like study of Warde P, et al, 2002 [105] considered no difference was observed between the Pilocarpine-treated patients and the placebo group in the severity of xerostomia and they were unable to detect a beneficial effect of Pilocarpine on radiotherapy-induced xerostomia when administered during radiotherapy for head-and-neck cancer. Probably the reasons for the lack of Pilocarpine effect, may referred to possibility of salivary glands dysfunction, destruction of glandular tissue, or other surroundings factors.

During inspection to the sections of present work, and the pictorial evidence presented for normal untreated and treated rabbit glands showing us the difference between the acini of Pilocarpine treated groups and that of normal untreated rabbits.

The production of endogenous saliva has the greatest benefit to patient both for its convenience and importance of natural saliva to oral function, so the clinical observations and histological structure alterations noticed in this study, substantiate the use of Pilocarpine drug in treatment of xerostomia and cases of pre and post radiation therapy in head and neck .

Chapter 7

SUMMARY AND CONCLUSION

SUMMARY

Saliva is the familiar fluid present in the mouths of humans and animals, which serves principally to moisten and lubricate food. In addition, it contains enzymes that begin the process of digestion, it aids our sense of taste, and it helps cleanse and protect the teeth, gums, and other tissues inside the mouth, and speech is well-appreciated, especially in people suffering from xerostomia. Salivary glands are susceptible to a variety of medication, as well as to a number of pathological conditions.

The intention of the present work was to describe the effects of Pilocarpine drug which is used as a drug with parasympathomimetic effect and the histological changes during treatment with it.

Pilocarpine is a parasympathomimetic agent with mild β-adrenergic stimulating properties. It has been proposed as a treatment for dry mouth for over 100 years. And in treatment of patients with Sjögren's syndrome and post radiation salivary gland hypofunction.

To illuminate its histological profiles, the usage of drug was compared in two group of rabbits with two control groups, with regard to possible histological structural changes which might occur in the secretory endpieces of the minor salivary glands.

Eighteen male rabbits were selected for the purpose of this study to show the effect of Pilocarpine as parasympathomimetic drug, the dose used was formulated according to the Pagaet and Barnas formula. Different doses of drug 3mg/kg ,5mg/kg were given within the therapeutic limits.

The drug was injected intraperitoneally for two and six weeks, in group A and B, At the end of the experimental period of time, the samples were collected and fixated with buffered formalin.

The minor salivary glands of each group were dissected and examined histologically .Sections were stained with H and E stain and PAS stain.

Histometric evaluations of diameter of secretory pieces were conducted on slides stained by H and E stain .

Data was checked and fed to personal computer using statistical package for social sciences “SPSS” version 22.

Descriptive statistics was used in the form of minimum, maximum, mean and standard deviation.

To decide which group is different than other groups (ANOVA) post HOC test was used .

Analysis of variance (ANOVA) test was used, P value was considered significant if it is less than 0.05 and highly significant if P value is less than 0.01.

There was a difference between the treated groups and the control groups with P value $\leq .000$

In experimental treated groups (GA,GB), significant increase in the diameter of the acini as the dose of Pilocarpine injected has increased.

In addition to that, one of the mainly prominent observations in groups A,B were the appearance of vacuolations in the cytoplasm of the acinar cells. It has been considered that vacuole formation is an essential part of water secretion.

Within twenty minutes after injection of the drug, during clinical observations we recognized Increase in activity of rabbits and they became more aggressive, increase in body temperature and increasing water intake, with excessive salivation.

For the PAS stained sections, positive reaction was identified in the acinar cells, and more obvious in treated groups.

Relying on present study, the supersensetivity of the acinar cells of major and minor salivary glands of rabbits was manifested by increase rate of salivation within 20 minute after injection.

Pilocarpine could be used as prophylactic and treated agents in patients suffering from xerostomia , patients receiving radiotherapy at head and neck malignancies, and some diseases like Sjögren's syndrome .

CONCLUSION

- Saliva plays a significant role in the protection of the intraoral structures against injuries caused by various pathogenic microbes, mechanical or chemical irritants, so the maintenance of natural saliva production is very significant role

- Although cholinergic agonists Pilocarpine injected peripherally, it can act directly on salivary glands to induce salivation and acts on central muscarinic receptors, activating autonomic efferent fibers to induce a central stimulation for salivation, also affects the thirst centre in the central nervous system.

- Pilocarpine as cholinergic agonist (parasympathomimetic) could be used as prophylactic agents, and we also support the usage of the drug in treatment of symptoms of xerostomia from salivary gland hypofunction , xerostomia caused by post radiotherapy treatment of head and neck cancer, and as one of most important choice for treatment of autoimmune diseases like Sjogren's syndrome.

Chapter 8

RECOMMENDATIONS

RECOMMENDATION

1. Further researches to be done to see the effect of the drug on the major salivary glands and oral mucosa

2. Suggestion of further work, the researcher suggests usage the drug for long term, to see the exact structural modifications and other histological changes.

3. Further works will be needed to exhibit if the Piliocarpine drug has far reaching effect, or if there is still any histological changes in minor salivary glands after end of treatment.

4. Further works will be needed to know why the Weber's gland of treated groups was the most affected one by Pilocarpine treatment.

Chapter 9

REFERENCES

REFERENCES

1. Dawes C. How much saliva is enough for avoidance of Xerostomia? . Caries Research. 2004; 38: 236–240.
2. Tenovuo J. Salivary parameters of relevance for assessing caries activity in individuals and populations. Community Dentistry and Oral Epidemiology. 1997 Feb;25(1):82-6.
3. Dodds M.W, Johnson D.A, Yeh C.K.. Health benefits of saliva: a review. journal of dentistry. 2005Mar ; 33(3): 223-33.
4. De Almeida PDV, Grégio AMT, Machado MÂN, de Lima AAS, Azevedo LR. Saliva Composition and Functions: A Comprehensive Review . The Journal of Contemporary Dental Practice. 2008 Mar ; 9 (3) .
5. Proctor G.B, Carpenter G.H. Regulation of salivary gland function by autonomic nerves . Autonomic Neuroscience: Basic and Clinical .2007; 133 (1) :3-18.
6. Junqueira L. C. ,Carneiro J. Basic Histology.11th edition. America : McGraw-Hill; 2005. salivary gland: p 317-321.
7. Eliasson L, Carlen,A. An update on minor salivary glandsecretions. Eur J Oral Sci. 2010 ; 118: 435–442.
8. Turner R.J, Sugiya H. Understanding salivary Fluid and protein secretion . Oral Diseases. 2002; 8: 3-11 .
9. Kontis T.C., Johns M.E. Anatomy and Physiology of the Salivary Glands. In: Bailey B.J. (Ed.). Head and Neck Surgery-Otolaryngology.3rd edition. Philadelphia :Lippincott Williams & Wilkins; 2001. P 308-312.

10. Liquidato B.M, Barros M.D,FilhoI.B, Pereira C.S. B, Macea M.I.M. Minor salivary glands morphology in xerostomia patients. Int. J. Morphology. 2006; 24 (3):489-493.
11. Nanci A. Ten cate's Oral histology- Development, Structure and Function. 7th edition. Missouri: Mosby Elsevier; 2008.salivary gland : p 290 -317 .
12. Avery J.K. Essentials of Oral Histology and Embryology. 2nd edition. Philadelphia: Mosby;2000. Salivary gland:p 189-190.
13. Rao R.S, Patil S, Amrutha N, Sanketh D.S, Agarwal A.The ambiguous salivary myoepithelial cells.The Journal of Contemporary Dental Practice. 2014 Aug ; 15(4):523-528.
14. Shah,A.A.K., Mulla,A.F, Mayank M. Pathophysiology of myoepithelial cells in salivary glands. J Oral MaxillofacPathol. 2016 Sep; 20(3): 480–490.
15. Holsinger F.C. , Bui D.T. Anatomy, Function, and Evaluation of the Salivary Glands. In: Myers E.N, Ferris R.L. (Eds.). Salivary Gland Disorders. 1st edition. Berlin: Springer Berlin Heidelberg; 2007. P:1-16 .
16. Langdon J.D. Surgical Anatomy, Embryology, andPhysiology of the Salivary Glands. In: Carlson E.R. , Ord R.A. (Eds). Textbook and color atlas of salivary gland pathology diagnosis and management. 1ST edition. Iowa USA :Wiley-Blackwell; 2008. p 3-18 .
17. Fritsch H. andKuehnel W. Color Atlas Of Human Anatomy Internal Organs. 5th edition. Stuttgart, New York :Thieme; 2011.
18. Hand A. R, Pathmanathan D, Field R.B. Morphological features Of the minor salivary glands. Archives of Oral Biology. 1999 May;44(1): S3-S10.

19. Schroeder H.E. Oral Structural Biology. 3rd edition. Georg Thiemeverlag Stuttgart .New York:Thieme Medical publishers ;1991.immunologicdefense system :392-393

20. Eroschenko V.P. difiore`s Atlas of Histology with functional correlations. 10th edition. Baltimore; Philadelphia : Lippincott Williams &Wilkins; 2005.salivary glands : P:219-226.
21. Humphreys J.V.A. The role of saliva. Health Watch News .2005 June; 1(8).
22. Llena-Puy C. The rôle of saliva in maintaining oral health and as an aid to diagnosis. Med Oral Patol Oral Cir Bucal .2006; 11:E449-55.
23. Dawes C. Salivary flow patterns and the health of hard and soft oral tissues. American Dental Association. 2008; 139(2): 18S-24S.
24. Yoichi I. Remineralization and Function of Saliva. Cell (Tokyo).2005;.37(3): 92-95.
25. Carpenter G.H. The Secretion, Components, and Properties of Saliva. Food Science and Technology. 2013 Feb ; 4: 267-276 .
26. Villa A, Wolff A, Narayana N, Dawes C, Aframian D.J, LyngePedersenA.M, et al. World Workshop on Oral Medicine VI: a systematicreview of medication-induced salivary gland dysfunction. Oral Diseases. 2016; 22: 365–382.
27. Zhang GH,Castro R.Role of Oral Mucosal Fluid and Electrolyte Absorption and Secretion in Dry Mouth. The Chinese Journal of Dental Research.2015 sep ;18(3):135-54.
28. Guggenheimer J, MOORE P. A. Xerostomia,Etiology, recognition and treatment . American Dental Association. 2003;134(1): 61-69.

29. Berk L.Systemic pilocarpine for treatment of xerostomia. Informa healthcare. 2008; 4(10):1333-1340.
30. Freudenreich O.Drug-induced sialorrhea. Drugs of Today. 2005; 41(6): 411.
31. Fox P.C. Salivary Enhancement Therapies. Caries Research. 2004; 38:241–246.
32. Actis A.B. A hypothesis to relate salivary tumors with mammary and prostate neoplasias. Bioinformation. 2005;1(1) : 12-13.
33. Berkovitz, B.K.B, Holland G.R, Maxham B.J.Oral Anatomy, Histology and Embryology. 3rd edition. Edinburgh; New York: mosby ; 2002. salivary glands : p 267-8 .
34. Crawford JM, Taubman MA., Smith DJ.Minor salivary glands as a major source of secretory immunoglobin A in the human oral cavity. *Science*Magazine. 1975Dec; 190(4220): 1206-1209.
35. Eversole L.R.The histochemistry of mucosubstances in human minor salivary glands. Archives of Oral Biology.1972;17(8): 1225-1238,IN11-IN12,1239.
36. AL-Saffar F.J. Histomorphological and histochemical study of the major salivary glands of adult local rabbits. International Journal of Advanced Research . 2014 Nov ; 2(11): 378-402.
37. Hand A.R. salivary glands. In: Bhaskar S.N.(Ed).Orban's .Oral histology and Embryology. 11th edition. Missouri: Mosby Year Book; 1991.P 361.
38. Yasear A.Y. and Ali I.I. Weber's salivary glands of Rabbit: Histological and Histochemical studies. Sebha Medical Journal. 2008; 7(1) .
39. YasearA.Y, Sultan A.A, Gaballah K.Y. Histological And Histochemical Studies On The vonEbner's Glands Of The Rabbit.Cairo dental Journal. 2004 ;20(1_s): 175-178.

40. Watanabe I,Heyn R, Motta PM. Scanning electron microscopy study of the palatine salivary glands of rabbits. Ital J AnatEmbryol .1995;100 (1) :375-83.
41. Malfertheiner P, Kemmer T. Neural regulation of salivary gland secretion .Zeitschriftfur Gastroenterologic. 1987Apr ; 25(1): 15-20.
42. Kyriacou K., Garrett J.R, Gjöstrup P. Structural and functional studies of the effects of parasympathetic nerve stimulation on rabbit submandibular salivary glands. Archives of Oral Biology. 1986; 31(4): 235-44.
43. Kyriacou K., GarrettJ.R.,Gjörstrup P.Structural and functional studies of the effects of sympathetic nerve stimulation on rabbit submandibular salivary glands. Archives of Oral Biology. 1988; 33(4) :271-280 .
44. Ekström J. Autonomic control of salivary secretion. proceedings of the finnish dental society. 1989; 85(4-5):323-31.
45. Som P.M. and Brandwein M.S. Salivary Glands: Anatomy and Pathology. In: Somp.m, Curtin H.D(Eds). Head and neck imaging. 4th edition. Philadelphia: Mosby; 2003. p 2005-2011.
46. Kyriacou K., Garrett J.R. Morphological changes in the rabbit submandibular gland after parasympathetic or sympathetic denervation .Archives of Oral Biology. 1988; 33(4): 281–290 .
47. Johnson D.A, Alvares O.F, Etzel K.R, Kalu D.N. Regulation of Salivary Proteins. Journal of Dental Research. 1987 Feb ;66 (2) : 576-582 .
48. Liu,Frank T. Y, Lofgren, Robert, Hoots, Iris. Effect of Steroid Hormones on Dental Caries and Salivary Glands in Female Rats. Journal of Dental Research. 1966 Jul ; 45(4) : 1236 .

49. Uyanıkgil Y., Türkkozan N. Y., Balcıoğlu H. A., Ateş U., Özel S. The effects of ovariectomy on the submandibular gland in young female adult rats. Ege Journal of Medicine. 2011; 50 (1) : 007-011 .

50. Clark P.G, Shafer W.G, Muhler J.C. Effect of Hormones on Structure and Proteolytic Activity of Salivary Glands. Journal of Dental Research. 1957 Jun ; 36(3) : 403-408.

51. Laine M, Leimola-Virtanen R. Effect of hormone replacement therapy on salivary flow rate, buffer effect and pH on perimenopausal and postmenopausal women. Arch Oral Biol.1996Jan ; 41(1):91-6.

52. Ekström J, Månsson B, Tobin G. Vasoactive intestinal peptide evoked secretion of fluid and protein from rat salivary glands and the development of supersensitivity. Acta Physiologica Scandinavica. 1983; 119 (2): 169 – 175 .

53. Syrjänen S. Age-related changes in structure of labial minor salivary glands. Oxford JournalsMedicineAge and Ageing.1984 ;13(3):159-165.

54. Drummond J.R, Chisholm D.M. A qualitative and quantitative study of the ageing human labial salivary glands.Archives of Oral Biology. 1984; 29(2):151-155.

55. Vered M, Buchner A, Boldon P, Dayan D.Age-related histomorphometricchanges in labial salivaryglands with special reference to the Acinar component. Experimental Gerontology . 2000 Oct. ; 35(8) : 1075–1084 .

56. Scott J. Qualitative and quantitative observations on the histology of human labial salivary glands obtained post mortem. Journal de BiologieBuccale. 1980; 8(3):187-200.

57. Taylor L. The Healing Power of Rainforest . USA: Square One; Nov,2005. Tropical plant database:316-320.
58. Bernardi R , Perin C, Becker F.L, Ramos, G.Z, Gheno G.Z, Lopes L.R,eatl. Effect of pilocarpine mouthwash on salivary flow. Brazilian Journal of Medical and Biological Research. 2002; 35:105-110.
59. Frydrych A.M., Davies G.R, Slack-Smith L.M, J Heywood J.An investigation into the use of pilocarpine as a sialagogue in patients with radiation induced xerostomia , Australian Dental Journal. 2002 ;47:(3):249-253.
60. Lopez-Solis R, Puente M, Duran V, Morales-Bozo I, Kemmerling U, Pardo R, etal. Characterization of mouse salivary polypeptide secretion after oral administration of pilocarpine. Revistachilena de historianatural . 2001; 74 (1).
61. Bartels C.L. Xerostomia, Helping patients with dry mouth. The Oral Cancer Foundation.. 2014.
62. Pimentel M.J, Filho M.M, Araújo M, Gomes D.Q, DA Costa L.J. Evaluation of radioprotective effect of pilocarpine ingestion on salivary glands. Anticancer Research. 2014 Apr ; 34(4):1993-9.
63. Abbasi F, Farhadi S, Esmaili M.Efficacy of Pilocarpine and Bromhexine in Improving Radiotherapy-induced Xerostomia. J Dent Res Dent Clin Dent Prospects. 2013 May; 7(2):86-90.
64. Brimhall J, Jhaveri MA, Yepes JF. Efficacy of cevimeline vs. pilocarpine in the secretion of saliva: a pilot study. Spec Care Dentist.2013 May ; 33(3):123-7.

65. Tomiita M, Takei S, Kuwada N, Nonaka Y, Saito K, Shimojo N, et al. Efficacy and safety of orally administered pilocarpine hydrochloride for patients with juvenile-onset Sjögren's syndrome. Modern Rheumatology. 2010 Oct ;20(5):486-90.

66. Rosas J, Ramos-Casals M, Ena J, García-Carrasco M, Verdu J, Cervera R, et al. Usefulness of basal and pilocarpine-stimulated salivary flow in primary Sjögren's syndrome. Correlation with clinical, immunological and histological features. Rheumatology (Oxford).2002 Jun ;41 (6):670-675.

67. Niedermeier, Matthaeus W, Meyer C, Staar C, Muller S, Schulze R.P,et al.Radiation-inducedhyposalivation and its treatment with oral pilocarpine. Oral-Surg-Oral-Med-Oral-Pathol-Oral-Radiol-Endod.1998 Nov ; 86(5): 541-9 .

68. Krane CM, Melvin JE, Nguyen HV, Richardson L, Towne JE, Doetschman T,et al.Salivary acinar cells from aquaporin 5-deficient mice have decreased membrane water permeability and altered cell volume regulation. The Journal of Biological chemistry .2001 Jun ;29;276(26):23413-20.

69. AsariT., Maruyama K. and Kusama H. Salivation triggered by pilocarpine involves aquaporin-5 in normal rats but not in irradiated rats. Clinical and Experimental Pharmacology and Physiology. 2008 Oct; 36 (5-6**)**:531 – 538.

70. Gautam D, Heard T.S, Cui Y, Miller G, Bloodworth L, and Wess J. Cholinergic Stimulation of Salivary Secretion Studied with M_1 and M_3 Muscarinic Receptor Single- and Double-Knockout Mice. Molecular Pharmacology. 2004Aug ; 66 (2): 260-267.

71. Renzi A,Colombari E,MattosFilho T.R ,Silveira J.E.N, Saad W.A, Camargo L.A.A, et al. Involvement of the Central Nervous System in the Salivary Secretion induced by Pilocarpine in Rats. JDR Journal of dental Research 1993 Nov.; 72(11): 1481-1484 .
72. DonathK, SpillnerM. and Seifert G. The influence of the autonomic nervous system on the ultrastructure of the parotid acinar cells. VirchowsArchiv. 1974 Mar ;364(1): 15-33.
73. Per G. Lundquist, Norberg L. Morphological Changes in Salivary Glands after Treatment with Sialagogues An Electronmicroscopical Study. Acta Oto-Laryngologica.1989 ; 107(5 & 6) : 434 – 440.
74. Kim JH, Ahn HJ, Choi JH, Jung DW, Kwon JS. Effect of 0.1% pilocarpine mouthwash on xerostomia: Double-blind, randomised controlled trial .Journal of Oral Rehabilitation. 2014March ;41(3) :226-35.
75. Baskerville A, Stirrup JA, Cox CS. Effects of isoprenaline and pilocarpine on salivary glands as assessed by gravimetric and image analysis techniques.British Journal of experimental pathology.1976 Aug ;57(4):404-10.
76. Mehta J, Patidar K. and Vyas N. Development and Validation of a Precise Method for Determination of Benzalkonium Chloride (BKC) Preservative, in Pharmaceutical Formulation of Latanoprost Eye Drops. E-Journal of Chemistry. 2010 ; 7(1): 11-20.
77. Marple B, Roland P. and Benninger M. Safety review of benzalkonium chloride used as a preservative in intranasal solutions: An overview of conflicting data and opinions. Otolaryngology–Head and Neck Surgery. 2004 ;130 (1): 131-41.

78. Laurance D. R. andBachanch A.L. Evaluation of drug activities: Pharmacometrics. Academic press.1964.
79. Drury R.A.B. and Wallington E.A. Carleton's histological technique.4th edition. New York: Oxford university press; 1973. P: 41,48-58,68-69,114-130 and 201-207.
80. IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.
81. Johnson J.T, Ferretti G.A, Nethery W.J, Valdez I.H, Fox P.C, David N.G, et al. Oral Pilocarpine for Post-Irradiation Xerostomia in Patients with Head and Neck Cancer.The New England journal of medicine. 1993 Aug; 5,329 (6) :390-395 .
82. Fox PC, Atkinson JC, Macynski AA, Wolff A, Kung DS, Valdez IH, et al. Pilocarpine treatment of salivary gland hypofunction and dry mouth (xerostomia). Archives of Internal Medicine. 1991Jun ; 151 (6) : 1149-52 .
83. Nikles J, Mitchell G.K, Hardy J, Agar M,Senior H,Carmont S.A, et al. Do pilocarpine drops help dry mouth in palliative care patients: a protocol for an aggregated series of n-of-1 trials. Bio Medicl Central Palliative Care. 2013 Oct ; 12(39) .
84. Rhodus NL. Oral pilocarpine HCl stimulates labial (minor) salivary gland flow in patients with Sjögren's syndrome. Oral Disease.1997 Jun ;3 (2) : 93-8.
85. Horiot J.C, Lipinski F, Schraub S, Maulard-Durdux C, Bensadoun R.J, Ardiet J.M, et al.Post-radiation severe xerostomia relieved by pilocarpine: a prospective French cooperative study. Radiotherapy& Oncology. 2000 Jun ; 55 (3) :233-239 .

86. Aragona P, Di Pietro R, Spinella R, Mobrici M. Conjunctival epithelium improvement after systemic pilocarpine in patients with Sjögren's syndrome. British Journal of Ophthalmology. 2006 ;90 (2) :166-170 .
87. Davies A.N, Daniels C, Pugh R, Sharma K. A comparison of artificial saliva and pilocarpine in the management of xerostomia in patients with advanced cancer. Palliative Medicine. 1998; 12 (2) : 105-111 .
88. Silva M.P, Junior J.C, Jorjão A.L, Da Silva Machado A.K , De Oliveira L.D, Junqueira J.C, et al. Influence of artificial saliva in biofilm formation of Candida albicans in vitro. Brazilian Oral Research. 2012 Feb ; 26 (1) .
89. Taylor S.E. Efficacy and economic evaluation of pilocarpine in treating radiation-induced xerostomia. Expert Opinion on Pharmacotherapy. 2003 Sep ; 4 (9) :1489-1497 .
90. Kim K. H, KimJ. Y, Sung M.W, Kim C. W. The Effect of Pilocarpine and Atropine Administration on Radiation-induced Injury of Rat Submandibular Glands. Acta Oto-laryngologica.1991 ; 111(3) : 967-973 .
91. Licht R, Kampinga HH, Coppes RP. Salivary gland-sparing prophylactic pilocarpine treatment has no effect on tumor regrowth after irradiation. Radiation Research Society. 2002 may ;157 (5) : 596-598 .
92. Hladki A, Alajbeg I, CekiE-Arambaπin A,Alajbeg I.Pilocarpine-Hydrocloride Has a Short-Time Effect on Reducing Xerostomia.ActaStomatologicaCroatica. 2003 Sep ; 37 (3) :330-331.

93. Omori Y, Asari T, Maruyama K., Kusama H, Kojima M, Shibata N. Effects of pilocarpine hydrochloride and cevimeline on submandibular/ sublingual salivation in rat xerostomia model produced by X-ray irradiation. Arzneimittel -forschung. 2003; 53(5): 342-350.
94. Takagi K, Yamaguchi K, Sakurai T, Asari T, Hashimoto K, and Terakawa S. Secretion of Saliva in X-Irradiated Rat Submandibular Glands. Radiation Research. 2003 Mar ; 159(3):351-360.
95. Li J, Lee S, Choi SY, Lee SJ, Oh SB, Lee JH,et al. Effects of pilocarpine on the secretory acinar cells in human submandibular glands. Life Sciences. 2006 Nov ;79 (26) : 2441-2447.
96. InenagaK,Wakasugi-Sato N, Ono K, Hirase M and Honda E. Intraperitoneal injection of pilocarpine activates neurons in the circumventricular organs and hypothalamus in rats. Brain Research. 2008; 1200 : 51-57.
97. Takakura A.C.T, Moreira T.S, Laitano S.C, De Luca L.A, Jr, A. Renzi A, et al. Central Muscarinic Receptors Signal Pilocarpine-induced Salivation. Journal of Dental Research. 2003 Jun; 82(12): 993-997 .
98. Sato N, Ono K, Honda E, Haga K, Yokota M and Inenaga K. Pilocarpine-induced Salivation and Thirst in Conscious Rats. Journal of Dental Research. 2006; 85 (1) : 64-68 .
99. Garrett J.R, Kidd A. The innervation of salivary glands as revealed by morphological methods. Microscopy Resaerch and Technique. 1993 Sep 1;26(1):75-91 .
100. Garrett J.R.The proper role of nerves in salivary secretion: a review. Journals of Dental Research. 1987 Feb; 66 (2) : 387-97.

101. Mills J.W. and Quinton P.M. Formation of stimulus-induced vacuoles in serous cells of tracheal submucosal glands. The American Journal of physiology. 1981 Jul ; 241 (1) :C18-24.

102. Schramm M and Selinger Z. The function of α and β-adrenergic receptors and a cholinergic receptor in the secretory cells of rat parotid gland. In Advances in Cytopharmacology. 1974 ;2:29-32.

103. Davies AN, Shorthose K. Parasympathomimetic drugs for the treatment of salivary gland dysfunction due to radiotherapy. The Cochrane Database of Systematic Reviews. 2007 Jul ; 18 (3) : CD003782.

104. Alajbeg, Ivan, Hladki, Nina, Alajbeg, Iva Ž. et al. Absence of Long term Effects of Pilocarpine in Patients with Xerostomia. 1st International Congress of Oral Medicine. 2005 ; 85-86 .

105. Warde P, O'Sullivan B, Aslanidis J, Kroll B, Lockwood G, Waldron J, et al. A Phase III placebo-controlled trial of oral pilocarpine in patients undergoing radiotherapy for head-and-neck cancer. International Journal of Radiation Oncology, Biology and Physics. 2002 Sep ; 54 (1) :9-13 .

THESIS PROPOSAL

EFFECT OF PILOCARPINE ON MINOR SALIVARY GLANDS OF RABBITS: A HISTOLOGICAL STUDY

تأثير البايلوكاربين على الغدد اللعابية الصغرى للأرانب

(دراسة نسيجية)

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Thesis Proposal

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INTRODUCTION

Salivary gland

The minor salivary glands are found scattered as small discrete aggregates of secretory tissue present in the submucosa throughout most of oral cavity, with concentrations in the lips and soft palate mucosa. They are predominantly mucous glands, which volume of produced secretion corresponds to approximately 10% of the total Saliva. Saliva takes part in tasting, mastication, deglutition, and speech processes performing functions such as digestion of amide and lipids, mucosa defense through lubricating action, and enzyme antimicrobial activity, such as lysozyme, lactoferrin, and sialoperoxidase. The decrease in the volume of secretion leads to deglutition and speech impairments, adding to complications including erythematous candidiasis and caries [(1)].

The minor salivary gland estimated to number between 600 and 1000, and only places they are not found are the gingiva and the anterior part of the hard palate. The salivary glands are innervated by postganglionic nerve fibres of the sympathetic and parasympathetic divisions of the autonomic nervous system. There are three types of cells in the secretory units: serous cells, mucous cells and myoepithelial cells. Secretory end pieces that are composed of serous cells are typically spherical and consist of 8 to12 cells surrounding a central lumen, the cells are pyramidal in shape. Secretory end pieces that are composed of mucous cells typically have a tubular configuration, mucous cells surrounding a central large lumen [(2)].

Myoepithelial cells are contractile cells associated with the secretory end pieces and intercalated ducts of the salivary glands [3].

Salivary gland dysfunction is a predictable side effect of radiotherapy to head and neck region. Also due to several diseases like Sjôgren's syndrome in which the salivary glands do not function properly. Pilocarpine hydrochloride is the one of many drugs licensed in many countries for the treatment of xerostomia [4] .

Species variation:

There are number of differences existed between various mammalian species, such as variations in the proportion of serous to mucous cells or the extent of innervations .Other variations in the structure and biochemistry of the parenchymal cells [5] .

Pilocarpine HCL:

Pilocarpine is a cholinergic agonist (parasympathomimetic) used in treatment of symptoms of xerostomia from salivary gland hypofunction caused by radiotherapy for cancer of the head and neck and Sjogren's syndrome [6] .

Effect of Pilocarpine on salivary gland:

Pilocarpine is a parasympathomimetic agent that functions primarily as a muscarinic agonist with mild beta-adrenergic activity; this alkaloid causes pharmacologic stimulation of exocrine glands in humans resulting in diaphoresis, salivation, lacrimation and gastric and pancreatic secretion. Constituents of saliva such as mucins, proteins, glycoproteins, and electrolytes are stimulated by Pilocarpine. Documented increases in salivary amylase, protein bound carbohydrate, lysozyme, total protein and calcium after Pilocarpine stimulation in normal subjects are existed [(7)].

Pilocarpine produced a significant increase in the weight of the parotid and submandibular glands which was accompanied by an increase in acinar area of these glands, however these results have not been measured in minor salivary glands [(8)].

Also intercellular canaliculi and luminal ducts are widened during stimulation[(9)]. Following the administration of Pilocarpine the diameter of the myoepithelial cells from submandibular gland is of the same order as in the controls groups of experimental animals [(10)]. About 180% increase in both labial salivary gland flow as well as whole salivary flow in the Sjôgren's syndrome-1 and Sjôgren's syndrome-2 subjects [(11)].

Oral Pilocarpine hydrochloride acts primarily by stimulating minor salivary gland can be of benefit to patients suffering of severe xerostomia regardless of radiotherapy dose/volume parameters and all responders are identified at 12 weeks [(12)] . Pilocarpine pre-treatment was protective against irradiation, displaying much less damage after irradiation and with complete recovery after 4 weeks [(13)] . Administration of Pilocarpine before irradiation can improve radiation-induced hyposalivation[(14)] . Significant post radiation ability of the mucous secreting glands to be stimulated by Pilocarpine [(15)].

AIM OF THE WORK

To determine histological changes in minor salivary glands due to the administration of Pilocarpine in rabbits .

MATERIALS AND METHODS

Material:

Experimental animals:

-RABBITS : - Number →→18 - Sex →→male

Drug: - Pilocarpine HCL

- Two Dose →→ 3mg /kg, and 5 mg/kg. - Twice daily.

- Saline: as placebo for control group.

- Benzalkornium chloride(0.2 mg) which is present as preservative in the used drug, and it was used in this study to determine if it had any effect on minor salivary glands.

Sectioning: serial sections 5µm thickness will be cut and manipulated in glass slide.

Stain: hematoxylin and eosin stain for general examination.

Periodic acid Schiff's (PAS)reaction was used as a general stain for presence of mucopolysaccharide substances .

Methods:

The present study will be conducted on 18 male rabbit; the experimental animals will be divided into the following group:

Group -A includes 6 rabbits. They will be given the drug twice daily. This group will be divided into the following subgroups:

- Groupa_1 3 rabbits will be given 3 mg/kg Pilocarpine. < for 2 week >
- Groupa_2 3 rabbits will be given 3 mg/kg Pilocarpine. <for 6 week >

Specimens will be taken immediately at the end of period from the minor salivary glands (Weber's, buccal and soft palatine glands).

Group -B includes 6 rabbits. This will be given the drug twice daily. This group will be divided into the following subgroups:

- Groupb_1 3 rabbits will be given 5mg/kg Pilocarpine. < for 2 week >
- Groupb_2 3 rabbits will be given 5mg/kg Pilocarpine. <for 6 week >

Specimens will be taken immediately at the end of period from the minor salivary glands (Weber's, buccal and soft palatine glands).

Group -C includes 2 rabbits. They will be given the preservative < benzalkornium chloride 0.2mg > twice daily. This group was divided into the following subgroups:

- $Group_{C1}$ 1 rabbits was given benzalkornium chloride < for 2 week >
- $Group_{C2}$ 1 rabbits was given benzalkornium chloride <for 6 week >

Specimens were taken after that from the minor salivary glands
(Weber's, buccal and soft palatine glands).

Group -D includes 4 rabbits serves as a control and will be given saline injection to simulates the effect of injection. This group will be divided into the following subgroups:

- $Groupd_1$ 2 rabbits will be given saline < for 2 week >
- $Groupd_2$ 2 rabbits will be given saline <for 6 week >

Specimens will be taken after that from the minor salivary glands
(Weber's, buccal and soft palatine glands).

Histological sections will be prepared from the minor salivary gland ((Weber's, buccal and soft palatine glands)), for each group: Serial sections will be taken; statistical analysis will be conducted to measure the diameter of secretory acini in each experimental group and compared with that of control group.

Data analysis: The results will be tabulated and statistically analysed.

REFERENCES

(1) Liquidato B.M, Barros M.D, Filho I.B, Pereira C.S. B, Macea M.I.M. Minor salivary glands morphology in xerostomia patients. Int. J. Morphology. 2006; 24 (3):489-493.

(2) Nanci , A. Ten cate's Oral histology, Development, Structure and Function. 6th edition. Missouri: Mosby Elsevier; 2003. Salivary gland : P 307-309,326,303.

(3) Avery J.K. Essentials of Oral Histology and Embryology. 2nd edition. Philadelphia: Mosby; 2000. Salivary gland: p 189-190.

(4) Berk L. Systemic Pilocarpine for treatment of xerostomia. Informa healthcare. 2008Oct ; 4(10):1333-1340.

(5) Hand A.R. salivary glands. In: Bhaskar S.N.(Ed). Orban's .Oral histology and Embryology. 11th edition. Missouri: Mosby Year Book; 1991. P 361.

(6) Gage T.W, Pickett F.A. Mosby's Dental Drug Reference. 7th edition. St Louis, United States: Mosby Elsevier - Health Sciences Division; 2005 Sep: P 624-625.

(7) Johnson J.T, Ferretti G.A, Nethery W.J, Valdez I.H, Fox P.C, David N.G, et al. Oral Pilocarpine for Post-Irradiation Xerostomia in Patients with Head and Neck Cancer. The New England journal of medicine. 1993 Aug; 5,329 (6) :390-395 .

(8) Baskerville A, Stirrup JA, Cox CS. Effects of isoprenaline and Pilocarpine on salivary glands as assessed by gravimetric and image analysis techniques. British Journal of experimental pathology.1976 Aug ; 57(4):404-10.

(9) Takagi K, Yamaguchi K, Sakurai T, Asari T, Hashimoto K, and Terakawa S. Secretion of Saliva in X-Irradiated Rat Submandibular Glands. Radiation Research. 2003 Mar ; 159(3):351-360.

(10) Travill A.A, Hill M.F. Histochemical demonstration of myoepithelial cell activity. Experimental Physiology translation and integration.1963 Jun; 48: 423-426.

(11) Rhodus NL. Oral Pilocarpine HCl stimulates labial (minor) salivary gland flow in patients with Sjögren's syndrome. Oral Disease.1997 Jun ;3 (2) : 93-8.

(12) Horiot J.C, Lipinski F, Schraub S, Maulard-Durdux C, Bensadoun R.J, Ardiet J.M, et al. Post-radiation severe xerostomia relieved by Pilocarpine: a prospective French cooperative study. Radiotherapy& Oncology. 2000 Jun ; 55 (3) :233-239 .

(13) Kim K H, Kim JY, sung MW, Kim CW. The effect of Pilocarpine and atropine administration on radiation-induced injury of rat submandibular glands. Acta oto-laryngologica.1991; 111(5):967-73.

(14) Burlage F.R, Faber H, Kampinq H.H, Lanqendijka J.A. ,Vissink A. , Coppes R.P. Enhanced proliferation of progenitor cells by prophylactic Pilocarpine treatment. Radiotherapy and oncology. 2009 Feb; 90 (2) : 253-256 .

(15) Niedermeier, Matthaeus W, Meyer C, Staar C,Muller S, Schulze R.P,et al. Radiation-induced hyposalivation and its treatment with oral Pilocarpine. Oral-Surg-Oral-Med-Oral-Pathol-Oral-Radiol-Endod.1998 Nov; 86(5) : 541-9.

ARABIC SUMMURY

الملخص العربي

تأثير البايلوكاربين على الغدد اللعابية الصغرى للأرانب

(دراسة نسيجية)

اللعاب وهو السائل المألوف الموجود في الفم لدى الإنسان كما عند أغلبية الحيوانات، والذي يعمل على ترطيب الفم والمساعدة في مضغ وترطيب الطعام ،إلي جانب كونه مادة تحتوي على كثير من الأنزيمات والمواد الفعالة للهضم والجسم .

كما أن للعاب دورا فعالا في عملية الإحساس ، التذوق والنطق. كذلك يساعد في تنظيف الأسنان وحماية الفم بأكمله وأهمية اللعاب ودوره يكون واضحا وجليا خاصة عند أولائك الذين يعانون من جفاف الفم. غالبا ما تكون الغدد اللعابية عرضة لمختلف الأدوية وتأثيرها وكذلك عرضة لعدة حالات مرضية مختلفة.

ومن ضمن الأدوية المؤثرة , دواء البايلوكاربين, وهو ما قمنا بدراسة تأثيره في هذا البحث, كونه دواء يستخدم مشابها للتأثير العصبي للباراسيمباثتيك على الخلايا وخاصيته التحفيزية للإفراز. وأيضا استخدم على مدى أكثر من 100عام لعلاج جفاف الفم الناتج من عدة أمراض , و بعد استخدام الأشعة لعلاج سرطانات الرأس والرقبة .

والغاية من هذه الدراسة هي توضيح تأثير البايلوكاربين على الغدد اللعابية الصغرى، ومعرفة التغيرات الحاصلة من الناحية الإكلينيكية, ولتوضيح الناحية الهيستولوجية والتغيرات التي قد تحدث في النهايات الإفرازية للغدد اللعابية الصغرى ,من بعد مدة زمنية محددة.

ولتوضيح الناحية الهيستولوجية والتغيرات التي قد تحدث , اختيرت لهذه الدراسة 18 أرنباً لتوضيح تأثير هذا الدواء على الغدد اللعابية الصغرى. تم احتساب الجرعة المعطاة لكل أرنب حسب طريقة باجت و بارنس 1964 , ومن ثم تم اختيار جرعات مختلفة من الدواء وهي : 3 و 5 ملي جرام / كيلوجرام وتم حقن الجرعات المختلفة من الدواء كل على حسب مجموعته لمدة أسبوعين , ولمدة ستة أسابيع .

و عند نهاية الفترة المحددة الخاصة بكل مجموعة. أخذت الغدد اللعابية الصغرى من كل أرنب وتم تثبيتها بمادة الفورمالين المتعادلة وفحصت من الناحية النسيجية بعد تحضير العينات وصبغها بصبغة الهيماتوكسيلين والإيوسين التي استخدمت في هذه الدراسة. بالإضافة إلي استخدام صبغة خاصة لتمييز وتوضيح وجود المواد المخاطية داخل الخلايا وهي صبغة حمض شيف الدوري والتي تفاعلت ايجابيا وظهرت بوضوح في العينات المستقطعة وخاصة في المجموعات الأكثر حقنا بالدواء .

تم حساب وتقدير قطر العنبات الإفرازية للغدد اللعابية الصغرى على الشرائح المصبوغة وذلك باستخدام كاميرا < مولر > الميكروسكوبية مع استخدام التقنية الرقمية < تيسوون > للتصوير ومن خلال هذه الأقطار وحسب المتوسط الحسابي و الانحراف المعياري , تم تحليل البيانات باستخدام اختبار تحليل المتغيرات و اختبار المقارنة

المتعدد وتعتبر النتيجة ايجابية إذا كان الاحتمال أقل من 0.05, ومن خلال الحسابات الإحصائية ظهرت النتيجة ايجابية وبمقدار : (000. ≤ درجة الأهمية).

لوحظ من خلال هذه البيانات، الزيادة الواضحة في قطر العنبات الإفرازية للغدد اللعابية الصغرى كلما زادت جرعة البايلوكاربين وهذا الفرق يبدو واضحا بين المجموعات التجريبية مقارنة مع المجموعة المعيارية.

بالإضافة إلى ذلك فإن أكثر الملاحظات المميزة في المجموعتين اللاتي حقنت بالبيلوكاربين كانت ظهور الفراغات داخل سيتوبلازم الخلايا , وهذا ظهر واضحا في المجموعة التي حقنت بالجرعة الدوائية الأكبر للبيلوكاربين, يعزى ذلك إلى زيادة التأثير العصبي الباراسيمباثاوي نتيجة لتأثير البايلوكاربين وهذا بدوره يؤدي إلى زيادة إفراز اللعاب والذي حصل في هذه الدراسة.

خلاصة لذلك, يمكننا القول بأننا نؤيد وندعم استعمال البايلوكاربين واستخدامه لعلاج و وقاية المرضى الذين يعالجون بالأشعة العلاجية ضد سرطانات الوجه و الرقبة , وكذلك بعض الحالات المرضية مثل (متلازمة شغرن) .

وقد أوصت الدراسة : بالمزيد من الأبحاث لمعرفة تأثير الدواء علي الغدد اللعابية الكبرى , ومعرفة تأثيره على الأنسجة وملائمتها في المدى الطويل لاستخدام الدواء , ومزيد من الأبحاث ومعرفة تأثيره بعد الاستخدام وانتهاء مدة العلاج به.

هذا والله أعلم .....


الملخص
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