ORIGINAL RESEARCH PAPER

Medicine

HISTOLOGICAL STUDY OF RABBIT LACRIMAL GLANDS UNDER EFFECT OF PILOCARPINE

KEY WORDS: Rabbits, lacrimal glands, histology, Pilocarpine, vacuolation

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ABSTRACT

The rabbit has been a popular animal used for lacrimal gland (LG) and dry eye studies. Lacrimal gland is the major source of tears that are essential for the health and function of the ocular surface. Dysfunction of the LG can result in decreased tear production. Pilocarpine is a parasympathomimetic agent with mild □-adrenergic. It was proposed to increase tear production in patients with dry eye consequent to Sjögren's syndrome (SS). This study was carried out to elucidate the light morphology of rabbit lacrimal glands under effect of Pilocarpine drug. Eighteen male rabbits were used in this experiment to show the effect of Pilocarpine. Different doses of drug 3mg/kg and 5mg/kg were given to treated groups (B and C) within the therapeutic limits. The experimental animals were injected intraperitoneally twice daily, for two and six weeks. Samples of the lacrimal glands were processed for light microscopy. Sections of the lacrimal glands were stained with H&E stain. 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 basophilic reaction in the acinar cells was transformed to eosinophilic reaction 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 therapeutic agents in patients suffering from dry eye (xerophthalmia).

INTRODUCTION:

The lacrimal gland (LG) is an exocrine gland whose main function is to produce the aqueous component of the tear film consisting of proteins, water and electrolytes (Dartt, 2009).

The LG fluid not only helps to lubricate the eye, but also aids in bringing nutrients and oxygen to the cornea and removing waste products and preventing infection. Tearing is critical to the maintenance of the homeostasis of the ocular surface. Tear film consists of aqueous phase, mucins, and a lipid layer. Traditionally, the mucin layer was felt to be derived from goblet cells of the conjunctiva, the aqueous component from the lacrimal gland, and the lipid layer from the meibomian glands, (Bron and Tiffany,2004). Recent advancements in proteomics have slightly altered this view of the tear film by identifying mucin as a product of the goblet cells but the lacrimal gland as well (Tsai et al., 2006). Tears contain water, proteins, vitamins, and other materials (Tsubota, 1998).

Lgs are comprised of acinar and ductal epithelia, myoepithelial cells, nerves, plasma cells, vascular and stromal cells, which are necessary to produce and secrete tear film components (Batista et al., 2012). Acinar cells, which comprise about 80% of the gland, form acini comprised of pyramidal shaped cells that lead into the duct system. Acinar cells secrete the majority of proteins, water, and electrolytes produced by the gland. The primary fluid from acini is then secreted into the ducts where it is modified by ductal cells before being released onto the surface of the eye. Myoepithelial cells surround the acinar cells on the basal side and because they contain α-smooth muscle actin (α SMA), it is believed that they contract to help expel the secretory products, as in the salivary and mammary glands (Ohtomo et al., 2011). More recently demonstrate that a population of myoepithelial cells could serve as stem/progenitor cells for the LG (Shatos et al., 2012). The importance of the tear film and its role as an indispensable integral component of the ocular surface system is well established (Pflugfelder etal., 1998).

Pilocarpine was proposed to increase tear and saliva production in patients with dry eye and dry mouth. It was shown, by binding the muscarinic receptors M3 and M1, to stimulate the watery secretions of lacrimal and salivary glands (Fisher, 2000). The use of oral pilocarpine as an agonist of the muscarinic receptor for the treatment of the oral and ocular dryness was recently approved by the US Food and Drug Administration (Fox, 2001). Pilocarpine is a parasympathomimetic agent with mild β-adrenergic stimulating properties, it can significantly improve symptoms of dry eyes and increase tears output (Vivino, 1999; Cifuentes, 2018).

In humans, pilocarpine eye drop is mainly employed in the treatment of glaucoma and it causes pupil constriction. The eye drop causes temporary blur vision that last for 2 to 3 hours following topical instillation (Michael Emina,2010).

The present study was undertaken to investigate the participation of Pilocarpine drug in the histological structural changes in rabbit lacrimal glands.

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, they 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 and D). Each group was further subdivided into two subgroups according to duration of administration of the drug and saline. The Institute Ethics Committee for animal Studies gave consent to the study.

Drug used: Saline was used as placebo for control group (A). Pilocarpine HCL was used for groups (B and C). It is a cholinergic agonist (parasympathomimetic) agent. This drug was available as sterile eye drops, each 1ml contains 20 mg 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 (D), and it was used in this study to determine if it had any effect on lacrimal glands or not.

Calculation of the drug dose: The drug dose used in this experiment was calculated according to Pagat and Barnas formula (Laurance and Bachanch 1964). Human dose of Pilocarpine is 20 mg twice a day. According to this formula, the dose for rabbit weighing 2 kg = 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 5 mg of the drug.

METHODS:

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

Group -A includes four 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:

- Group A1 two rabbits were given saline for two weeks.
- Group A2 two rabbits were given saline for six weeks. Specimens were taken after that from the lacrimal glands.

Group -B includes six rabbits. They were given the drug twice daily. This group was divided into sub-groups:

- Group B1 three rabbits were given 3 mg/kg Pilocarpine for two weeks.
- Group B2 three rabbits were given 3mg/kg Pilocarpine for six weeks.

Specimens were taken immediately at the end of the experiment period from the lacrimal glands.

Group –C includes six rabbits. They were given the drug twice daily. This group was divided into the following sub-groups:

- Group C1 three rabbits were given 5mg/kg Pilocarpine for two weeks.
- Group C2 three rabbits were given 5mg/kg Pilocarpine for six weeks.

Specimens were taken immediately at the end of the experiment period from the lacrimal glands.

Group -D includes two rabbits. They were given the preservative Benzalkornium chloride 0.2mg twice daily. This group was divided into the following sub-groups:

- Group D1 one rabbit was given Benzalkornium chloride for two weeks.
- Group D2 one rabbit was given Benzalkornium chloride for six weeks. Specimens were taken after that from the lacrimal glands.

The collected samples of the lacrimal glands, included in this study, were ran through paraffin embedding technique to get paraffin blocks. Histological serial sections were cut from the lacrimal gland of each group. Serial sections 5 μm thickness were cut and mounted on glass slide, and then stained with ordinary Hematoxylin and Eosin (H and E) stain, which was used for general examination.

RESULTS:

The lacrimal glands (LGs) in both treated and control groups were situated in the glandular lacrimal fossa under the zygomatic process of the frontal bone dorsolaterally to the eye ball surface.

In sections stained with the H and E stain, the normal LGs of control group (A) are composed of several large lobes separated into lobules by the connective tissue septae rich in blood vessels and possesses excretory ducts. Each lobule composed of numerous secretory branched end pieces, the acini. The duct system is also highly branched. The secretory units of the lacrimal gland explained that it is a mixed gland consisting of a tubulo-acinar units. The secretory acini are of two types; serous and mucous, where most of the acinar cells are serous cells (Figure. 1a).

The cells in serous acini are cuboidal to low columnar whose spherical nuclei are located near the cell's basal region. The cytoplasm of these cells displays a basophilic reaction (Figure. 1b).

The mucous cells have a vacuolar cytoplasm and their flattened elongated nuclei are situated in the basal surface of the cell. The serous and mucous parts are mixed together, but the serous acini were dominant while the mucous ones were interspersed between them (Figure 1c).

Histological effect of Pilocarpine: In the treated groups (B and C), the most noticeable changes in the lacrimal glands were the vacuolation and foamy appearance of the cells of the secretory acini (Figure 2a) and massive condensation of acini with wide lumens. The cytoplasm of the secretory cells change their basophilic reaction gradually into vacuolated cytoplasm (Figure 2b).

Other obvious change was an increase of the diameter of the acini in treated groups compared with acini in the control groups (Figure 3).

The effect of the drug and its transformation was more obvious in group C with the increasing of the dose of the drug than that occur in group B.

They were appeared as uncapsulated small aggregations of discrete lobules of glandular tissues consisted of secretory acini which were arranged in tubuloacinar units and excretory system.

There were well defined connective tissue separating the glandular lobules.

Under effect of Pilocarpine, the gland of rabbit appeared as mixed gland with predominant mucous cells, serous cells were few and mostly appeared as serous demilunes (Figure 4). 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 (Figure 5). While 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 6).

The duct system of the rabbit lacrimal glands in both treated and control groups were divided into intralobular, interlobular, intralobar, interlobar, and main excretory ducts.

Intercalated ducts represents the smallest branches of the intralobular duct to which the secretory end pieces are attached and emerge from the acini as a distinctive group of cells. These ducts are lined with a single layer of cuboidal epithelial cells that stain red, in contrast to the pyramid-shaped acinar cells that typically show a foamy and pale appearance (Figure 7).

Many intercalated ducts combine together to form a larger intralobular ducts. Both intercalated and intralobular ducts are closely attached with the acini and are surrounded by some loose connective tissue. Intralobular ducts are fused together to form interlobular ducts, which drain several lobules of the gland. Interlobular duct epithelial cells are simple cuboidal to low columnar.They stain deeply with eosin without distinctive intercellular plasma membranes between them (Figure 8).

Interlobular ducts are fused together to form intralobar ducts, which drain every individual lobe. Epithelial cells that lines the intralobar ducts are simple to pseudostratified columnar (Figure 9). Interlobar ducts are of variable dimensions due to compression by the surrounding tissues . Their epithelial lining range from simple to pseudostratified to stratified columnar as they approach the main excretory duct.

There was no difference seen between preservative groups (group D) and control groups (group A). Also there was no effect of used preservative on lacrimal glands (Figure 10).

DISCUSSION:

A lot of studies performed on clinical effect of pilocarpine drug, but there had been no cross- sectional and histological studies available of the lacrimal glands of the rabbit response to different dosages of pilocarpine drug for two different periods followed in the present work.

Pilocarpine is a natural alkaloid first used for the treatment of dry mouth induced by radiation and for treatment of dry eye in Sjögren's syndrome (SS) (Wiseman and Faulds, 1995). Tsifetaki, 2003, revealed recently oral pilocarpine was shown to be effective in improving symptoms and signs of dry eye in Sjögren's syndrome (SS) patients. It was shown, by binding the muscarinic receptors M3 and M1, to stimulate the watery secretions of lacrimal and to prevent the acinar apoptosis (Fox etal., 2001). In preceding study by Coulson and Fryer 2003, shown that pilocarpine, at 20 mg/day, significantly improved the patients' ability to expectorate mucus.

The results of our investigation support the conclusions of numerous workers that Pilocarpine produces an increase in secretion by the lacrimal gland.

Seunghee Cha etal., 2017, showed that highly water-soluble pilocarpine hydrochloride administered through IMDDS resulted in sustained effects with increased tear volume in normal rabbits.

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 (Johnson et al., 1993). Cholinergic agonists also have a complex effect on secretory component synthesis and secretion.

Dartt, 2009 revealed that Stimulation of β-adrenergic receptors causes regulated secretion by producing cAMP. The cAMP activates protein kinase A to stimulate secretion. The neural response consists of the activation of the afferent sensory nerves in the cornea and conjunctiva to stimulate efferent parasympathetic and sympathetic nerves that innervate the lacrimal gland.

Neurotransmitters are released from the stimulated parasympathetic and sympathetic nerves that cause secretion of water, electrolytes, and proteins from the lacrimal gland and onto the ocular surface.

Another study performed on rat parotid gland, emphasized that stimulation with muscarinic and adrenergic agonists causing movement of water and vacuole formation (Schramm and Selinger 1974).

In view of the fact that vacuole formation is an essential part of water secretion, the vacuolation occurred to a variable degree in certain cells as a normal part of reflex secretion, and frequently found experimentally after strong stimulation (Garrett, 1989). Like other exocrine secretions, LG fluid secretion is an osmotic process driven by the transepithelial secretion of electrolytes and water that is mediated by Aquaporin (AQP) and ion transporters (Selvamm etal,. 2007; Ding, 2010). Raina etal,.1995 and Delporte, et al.,1997 were stated that the AQP is a group of water channel proteins that are responsible for rapid water transport across plasma membranes in many organisms, and NKA are responsible for providing a Na^+ gradient that can be used by other carrier processes (Russell, 2000). In the rabbit LG, recently found that AQP4 was expressed in the basolateral sides of acinar and duct cells, while AQP5 was present in both apical and basolateral sides of acinar cells (Ding, 2010).

Mucins, as secreted forms, are a family of high molecular weight, heavily glycosylated proteins produced by epithelial tissues that are vital for the maintenance of a healthy moist ocular surface (Gipson, 2004). Paulsen et al., 2004 were indicated that mucins in LG may also play a role in signaling events and may be involved in the pathological events that occur at the ocular surface such as dry eye. However, other studies have also indicated that the majority of acinar cells in rabbit LG were mucous producing but were based on structural characteristics rather than PAS staining (Ubels and Harkema,1994).Pan, et al., 2009 were suggested these mucins are secreted from the LG epithelial cells that are also rich in AQP5. LG fluids produced in response to different agonists have different protein compositions, supporting the notion of differential secretion.

These previous data were generally in agreement with our findings. In the present work, there was a pronounced increase in diameter of lacrimal acini with obvious vacuolation in the cytoplasm of treated groups, which were accompanied by an increase in acinar area, that more obvious in group C with the increasing of the dose of the drug than that occur in group B. Thus, the end results ofwater movement and protein secreation might be the main explanation to the increase in size of acini noticed in the Pilocarpine treated rabbits of our study.

The rabbit has been a popular animal used for LG and dry eye studies. However, some controversies exist regarding whether and how many mucous cells are present in Lgs.

Kühnel et al 1968 first reported very little PAS positive material within ductal cells of the rabbit LGs, while others reported that PAS positive acinar cells were found in most lobules (Oikawa and Okano, 1978) and the majority of acinar cells in rabbit LG were mucous producing (Ubels and Harkema1994). Millar et al.,1996 reported that some epithelial cells in the rabbit LG were mucous cells, forming demilune in some acini, along with some individual acinar cells in serous acini.

Al-Murshidi M, 2015 indicated that the secretory units of the lacrimal gland are mixed gland consisting of two types serous and mucous.

In summary, during the inspection to the sections of present

work indicates that the rabbit LG is a heterogeneous exocrine gland that is composed of both serous and mucin-secreting cells. Some acini and individual acinar cells in serous acini are also mucin-secreting cells. Mucins produced by the LG are secreted and transported onto the ocular surface. The pictorial evidence presented for normal untreated and treated rabbit glands were shown us the difference between the acini of Pilocarpine treated groups and that of normal untreated rabbits.

CONCLUSION:

The lacrimal gland (LG) is an exocrine gland whose main function is to produce the aqueous component of the tear film consisting of proteins, water and electrolytes (Dartt, 2009).

The LG fluid not only helps to lubricate the eye, but also aids in bringing nutrients and oxygen to the cornea and removing waste products and preventing infection. Tearing is critical to the maintenance of the homeostasis of the ocular surface.

Pilocarpine was proposed to increase tear production in patients with dry eye. It was shown, by binding the muscarinic receptors M3 and M1, to stimulate the watery secretions of lacrimal glands (Fisher, 2000). Pilocarpine is a parasympathomimetic agent with mild β-adrenergic stimulating properties, it can significantly improve symptoms of dry eyes and increase tears output (Cifuentes, 2018).

On the basis of alterations of histological structure which noticed in this study substantiate the use of the Pilocarpine drug in cases of dry eye (xerophthalmia), and as one of most important choice for treatment of autoimmune diseases like Sjogren's syndrome.

RECOMMENDATION:

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

Suggestion of further work done to see the effect of the drug on other exocrine glands, such as pancreas.

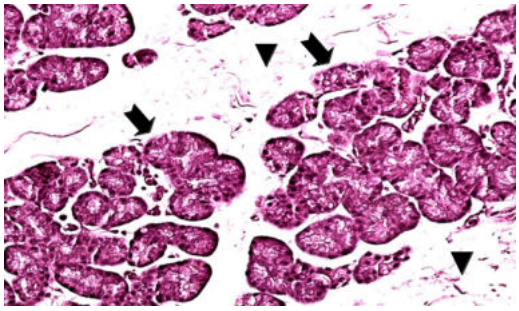

Figure.1a: Photomicrographs of LGs showing serous acini (arrow) and C.T. septum (arrow head) in control group A. Hematoxylin and eosin (H&E) stain. X 10.

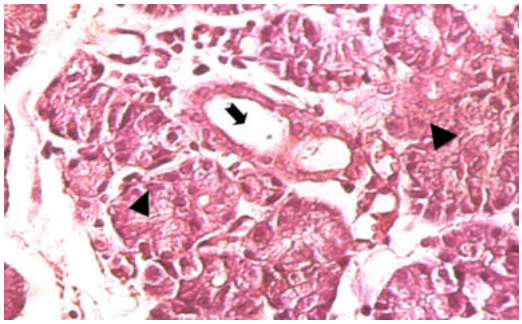

Figure.1b: Photomicrograph shows a high magnification of a basophilic reaction of the serous acini (arrow head). Intralobular duct (arrow). Hematoxylin and eosin (H&E) stain. X 40.

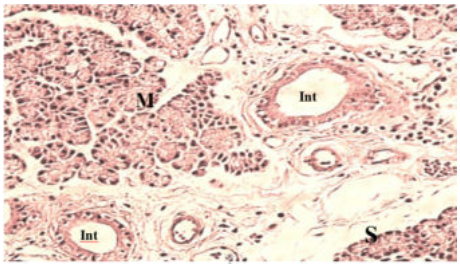

Figure 1c: Photomicrograph showing a mixed gland containing both mucous secretory portions (M) and serous secretory portions (S) and interlobular ducts (Int) in control group A. Hematoxylin and eosin (H&E) stain. X 20.

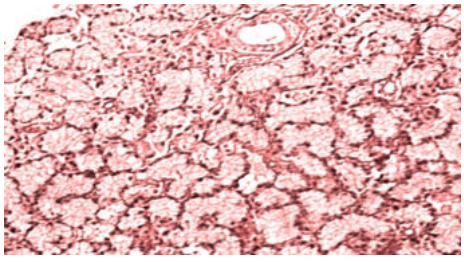

Figure 2a: Photomicrograph showing foamy appearance of the cells of the secretory acini with massive condensation of acini in treated groups (B & C). Hematoxylin and eosin (H&E) stain. X 10.

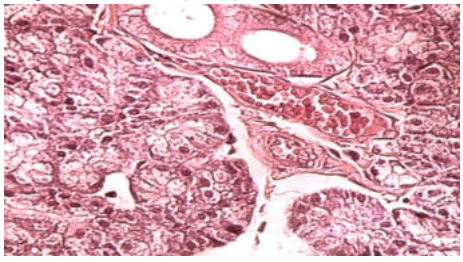

Figure 2b: Photomicrograph showing in treated groups (B&C), The cytoplasm of the secretory cells change their basophilic reaction gradually into vacuolar cytoplasm .Hematoxylin and eosin (H&E) stain. X 40.

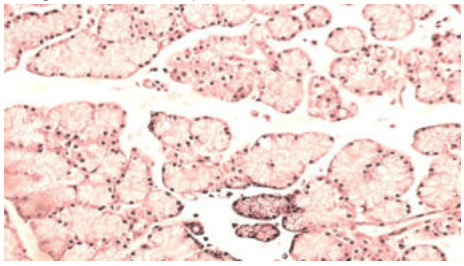

Figure 3: Photomicrograph showing an increase of the diameter of the acini in treated groups compared with acini in the control groups. Hematoxylin and eosin (H&E) stain. X 40.

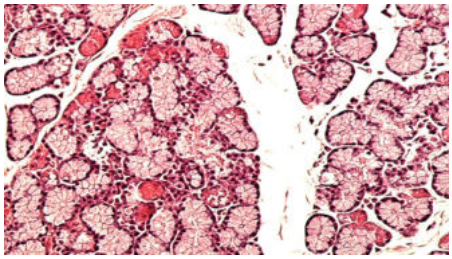

Figure 4: Photomicrograph showing in treated groups (B&C) predominant mucous cells, serous cells were few and mostly appeared as serous demilunes. Hematoxylin and eosin (H&E) stain. X 20.

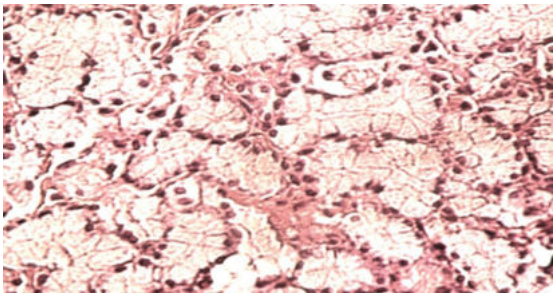

Figure 5: Photomicrograph shows a high magnification of the mucous cells in treated groups (B&C), which have a vacuolar cytoplasm and their flattened elongated nuclei are situated in the basal surface of the cell. Hematoxylin and eosin (H&E) stain. X 40.

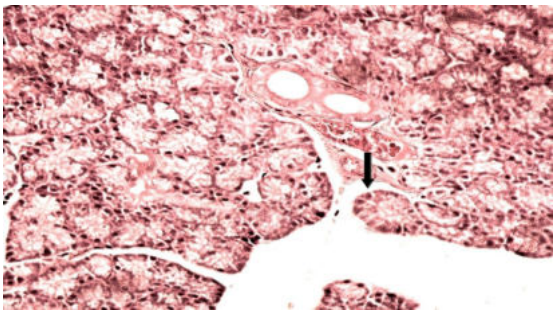

Figure 6: Photomicrograph showing in treated groups (B&C), the cells in serous acini were few and mostly appeared as serous demilunes (arrow), while most of secretory acini were contained eosinophilic secretory granules at the apical part of the cytoplasm. Hematoxylin and eosin (H&E) stain. X 20.

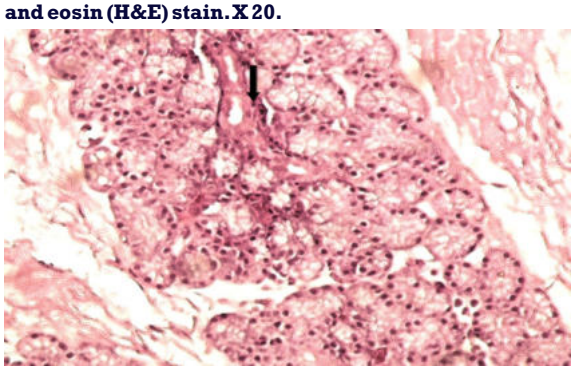

Figure 7: Photomicrograph showing in treated groups (B&C), intralobular duct (arrow) was stained bright red (eosinophilic) in a smooth pattern. Hematoxylin and eosin (H&E) stain. X 20.

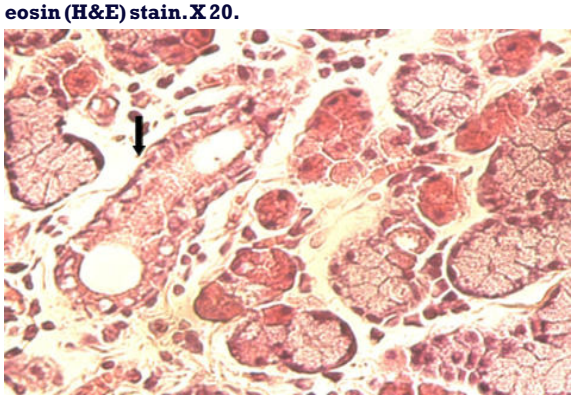

Figure 8: Photomicrograph showing in treated groups (B&C), interlobular duct (arrow)was stained deeply with eosin without distinctive intercellular plasma membranes between them. Hematoxylin and eosin (H&E) stain. X

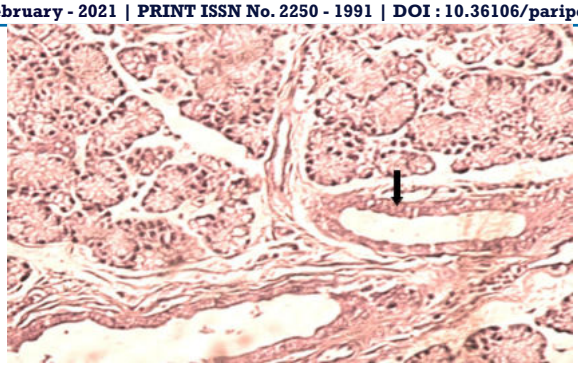

Figure 9: Photomicrograph showing in treated groups (B&C), interlobar duct (arrow) was lined with simple to pseudostratified columnar. Hematoxylin and eosin (H&E) stain. X 20

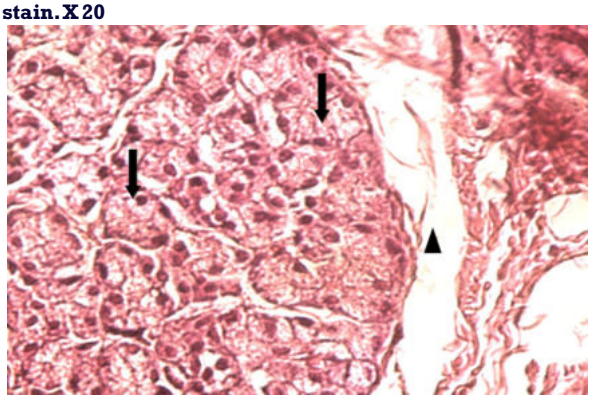

Figure.10: Photomicrographs of LGs showing most of the acinar cells are serous cells (arrow) separated by C.T. septum(arrow nead) in group D. Hematoxylin and eosin (H&E) stain. X 20.

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