EFFECT OF NEOSTIGMINE ADMINISTRATION ON THE ULTRASTRUCTURE OF THE PAROTID SALIVARY GLAND IN RATS WITH INDUCED DIABETES

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ABSTRACT

INTRODUCTION: Neostigmine is parasympathomimetic drug. It is commonly used for treatment of myasthenia gravis, glaucoma, urinary retention, xerostomia, and post-operative ileus. Parasympathomimetic drugs increase the rate of salivation. Dry mouth is a frequent clinical complain among diabetic patients.

OBJECTIVES: The present study is designed to investigate the effect of neostigmine administration on the ultrastructure of the parotid salivary gland in rats with induced diabetes.

MATERIALS AND METHODS: Twenty one adult male rats were divided into three equal groups (seven rats each) Group I: Control group, Group II: Induced diabetes group (with no treatment), Group III: Induced diabetes group (with Therapeutic doses of Neostigmine which were administered intramuscularly every other day for one month. Diabetes was induced by a single intravenous injection of Streptozotocin. After one month all rats were sacrificed and the parotid glands were excised and processed for scanning electron microscopy. Histomorphometric analysis of the number of the intra-cellular vacuoles was done, and the data obtained were statistically analyzed.

RESULTS There was a statistically significant difference between the studied groups regarding the diameter of secretory granules, as it was increased in neostigmine treated group and decreased in diabetic group. Also there was restoration of the density and organization of the nerve distribution adjacent to secretory acini in neostigmine treated group.

CONCLUSIONS: The ultrastructural findings noticed in this study substantiate the use of Neostigmine in cases of diabetes associated xerostomia.

KEYWORDS: Neostigmine, Parotid Gland, Diabetes, Rats.

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INTRODUCTION

Saliva has a major role in maintaining a healthy oral cavity. It is produced by major salivary glands (parotid, sub-mandibular and sub-lingual) and numerous minor salivary glands distributed in the oral cavity. Salivary dysfunction has been reported in patients with diabetes (1, 2).

Diabetes mellitus is the most common endocrine disorder. It is the result of a malfunction of insulin and lipid metabolism. There are two major forms of diabetes mellitus: Type 1 is caused by the destruction of pancreatic beta cells that produce insulin. It is considered an absolute deficiency of insulin and is commonly diagnosed at young age. In type 2 diabetes, the target tissues do not respond to insulin. It is associated with both insulin resistance and deficiency and is commonly diagnosed later in life (3). The chronic hyperglycemia of DM is associated with long-term damage, dysfunction, and failure of various organs (4).

In well-controlled diabetic patients, salivary gland function does not seem to be significantly affected (5). However, several studies have reported subjective complaint of dry mouth among diabetic patients in general (1, 6 and 7).

A cross sectional epidemiological study was conducted on type 1 diabetic patients and control subjects without diabetes. The investigators found that symptoms of reduced salivary flow rate and xerostomia were more frequently reported by patients with diabetes than the controls, especially by those diabetics who had developed neuropathy (8). Other studies conducted in type 2 diabetics also confirmed that xerostomia and hyposalivation were more prevalent in this group of patients (9). It has been shown that poorly controlled type 2 diabetics have a lower stimulated parotid gland flow rate compared to well-controlled patients and patients without diabetes (10).

Patients with diabetes usually complain of xerostomia and the need to drink very often (polydypsia and polyuria). The constant dryness of the mouth would irritate the oral soft tissues, which in turn will cause inflammation and pain. Diabetic patients with xerostomia are more predisposed to periodontal infection and tooth decay. The cause of this could not yet be fully understood, but may be related to polydypsia and polyuria or alternation in the basement membrane of the salivary glands. It is known that diabetes mellitus is associated with chronic complications such as neuropathy, micro-vascular abnormalities and endothelial dysfunction that lead to deterioration of microcirculation and this may play a role in reduction of the salivary flow rate and composition (11).

A significant reduction in resting salivary flow rates was reported in Patients with type 1 DM taking parasympatholytic drugs in association with elevated fasting blood sugar levels (1). A similar association was reported between parasympatholytic drugs and autonomic neuropathy in patients with type 2 DM (12).

Many drugs of medical applications either directly or indirectly cause volume increase of exocrine gland secretions. Among these drugs is the neostigmine, which is a parasympathomimetic drug. It is commonly used as anticholinestrase of carbamate ester type for treatment of

myasthenia gravis, glaucoma, urinary retention, xerostomia, and post-operative ileus (13, 14).

Acetylcholinestrase (AChE) is an intrinsic membrane-bound enzyme that is essential for nerve tissue. This enzyme is responsible for the rapid hydrolysis of the cationic neurotransmitter acetylcholine after its release at cholinergic synapse. Acetylcholine reacts with a specific residue at the active site of AChE to form a covalent acetyl-enzyme intermediate, and choline is released to prevent re-excitation (15).

Neostigmine reversibly inhibit the acetylcholinestrase enzyme by acting as a substrate, so inhibiting the breakdown of acetylcholine in synapses with widespread muscarinic receptor activation due to enhancement of acetylcholine activity at parasympathetic postganglionic synapses leading to increased secretions of salivary, lacrimal, bronchial, and gastrointestinal glands, increased peristaltic activity, pupillary constriction, bronchoconstricion, bradycardia and hypotension, fixation of accommodation for near vision and fall in intraocular pressure (14, 16, 17).

In 1995, Wiseman et al, (18) stated that Sialogogues are also used to treat the symptoms of xerostomia. They increase the flow of saliva and therefore require functional salivary gland parenchyma in order to be effective. While a significant proportion of the salivary glands may be damaged by radiation therapy, it is rare for all the minor and major glands to be totally compromised. The residual function of the salivary glands can be evaluated by measuring salivary gland flow rate and salivary gland scintigraphy (salivary gland scan). A number of substances have been previously used as a sialagogue (e.g. neostigmine, nicotinic acid, potassium iodide, bromhexine), but pilocarpine which was proved to be the most effective substance and approved for the treatment of radiation-induced xerostomia in several European countries and in the USA.

In 2007, Davies et al, (19) encouraged the use of parasympathomimetic drugs for the treatment of salivary gland dysfunction due to radiotherapy. There was limited evidence to support the use of pilocarpine hydrochloride in the treatment of radiation-induced salivary gland dysfunction. It would seem appropriate to offer patients a trial of the drug, assuming that there are no contraindications to the use of the drug. However, many patients fail to respond to pilocarpine hydrochloride. Currently, there is little evidence to support the use of other parasympathomimetic drugs in the treatment of radiation-induced salivary gland dysfunction.

In 2013, El-Ramli et al, (20) studied the structural histological changes in the parotid salivary gland of rabbits treated with neostigmine. The structural histological alterations noticed in this study substantiate the use of this drug in cases of xerostomia.

Neostigmine as a parasympathomimetic drug has been used to overcome the problem of compliance of hyposalivation associated with certain diseased conditions, and medication (13). The effect of parathympathomimetic drugs on the salivary gland have been investigated in many studies, but studies on the effect of neostigmine on the parotid gland in diabetic subjects are limited, especially the ultrastructure alterations have been found to be insufficient and that is why the current study has been conducted.

MATERIALS AND METHODS

This study was conducted after the approval of the Research Ethics Committee, Faculty of Dentistry Alexandria University.

Twenty one adult male rats weighing about 200-250 grams (approximately six months of age) were used. These animals were obtained from and caged in the Institute of Medical Research, Alexandria University. Animals were caged in specially designed wire mesh cages. The animals were supplied a regular diet throughout the whole experimental period which lasted for one month.

The animals were divided into three equal groups (7 rats each) as follows:

Group I: Control group.

Group II: Induced diabetes group (with no treatment).

Group III: Induced diabetes group (with intramuscular Neostigmine administration at a dose of 0.1mg every-other day for one month).

For group I (control), the rats were injected with vehicle equivalent of isotonic saline to induce the same effect of injection stress.

Experimental procedures:

Induction of diabetes:

For groups II, III the rats were kept fasted overnight and diabetes was induced by a single intravenous injection of Streptozotocin 40 mg/kg body weight in 0.1 M citrate buffer (21).

Measurement of Blood Glucose Level:

Blood glucose level of the animals in group II and III was measured 48 hours after the administration of Streptozotocin then every week throughout the time of experiment. Blood sample was obtained from the tail vein of the animals and their blood glucose level was determined in mg/dl using a digital glucometer (22).

Administration of the drug and duration of treatment:

Neostigmine (Amriya company, Alexandria, Egypt), is a synthetic quaternary ammonium parasympathomimetic agent. This drug was available as 0.5 mg vial or ampoules served for an injection. Neostigmine was stored at room temperature and protected from light.

Calculation of the drug dose:

The drug dose used in this experiment was calculated according to Lethal Dose 50 (LD50) (23). So the therapeutic dose used in this study was 0.1 mg intramuscular injection every other day for one month given to group III animals.

Tissue processing:

After 30 days, all rats were sacrificed and the parotid salivary glands were excised, divided into two portions, fixed in 2.5% glutaraldehyde in phosphate buffer (PH 7.3), and then processed for scanning electron microscopic examination. Histomorphometric analysis of the diameter of the acinar secretory granules was performed using Image J* software and data were tabulated. All data were represented as mean and standard deviation (mean - S.D.). Statistical analysis of the obtained data was done using ANOVA test (24) to compare between the control and study groups. A result was considered statistically significant at $p < 0.05$.

RESULTS

Scanning Electron microscopic Results:

The SEM examination was focused on evaluating the diameter of the secretory granules in the serrous acinar cells and the neural organization adjacent to the acini of the gland.

Diameter of the secretory granules in the acinar cells:

Control group, (group I):

In the acinar cells of this group, an appropriate diameter of the secretory granules was noted as well as their number, (fig. 1.A).

Diabetic group, (group II):

Noticeable decrease in the diameter of the secretory vesicles was evident. Also their number seemed less than that seen in the control group, (fig. 1.B).

Neostigmine treated group, (group III):

An outstanding increase in the size and number of the secretory granules was an important feature in this group. These vesicles could be traced at the different levels of the field observation, (fig. 1.C).

Configuration of the neural organization on the surface of the acini:

Control group, (group I):

Profound aggregates of nerve fibers of adjacent calipers could be traced adjacent to the acini basal lamina. The minute nerves prevailed and were greater in density than the large ones. Also some small vessels could be seen in association of these nerves, (fig. 1.D).

Diabetic group, (group II):

The individual nerves showed aggregation in groups with corrugated outline giving an impression of decreased neural supply, (fig. 1.E).

Neostigmine treated group, (group III)*:*

A noticeable restoration of the configuration and density of the neural supply was obvious change from that seen in diabetic group. Profound minute capillaries were traced at different levels of the field, (fig. 1.F).

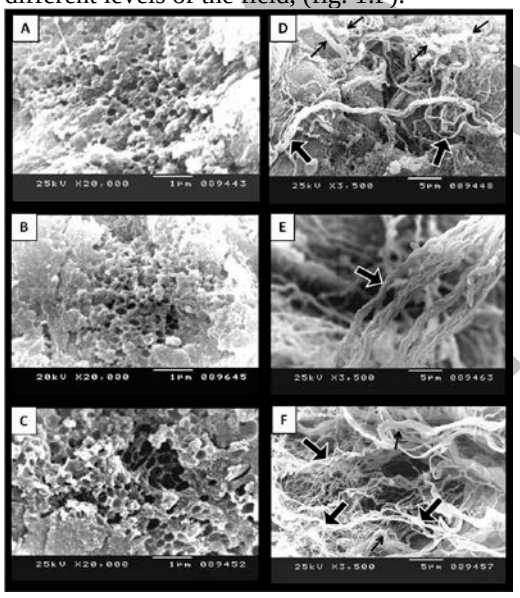

Fig. 1.A: Scanning electron micrograph (SEM) [Control group, (group I)}: showing an appropriate diameter and density of the secretory vesicles in the cytoplasmic acinar cell. (X20, 000)

Fig. 1.B: SEM [Diabetic group, (group II)]: showing diminished diameter and density of the secretory vesicles in the cytoplasmic acinar cell. (X20, 000)

Fig. 1.C: SEM [Neostigmine treated group, (group III)]: showing restoration of the normal diameter of cytoplasmic secretory vesicles in the acinar cells. Note their prevalence at the different depth of the field. (X20, 000)

Fig. 1.D: SEM [Control group, (group I)}: showing sufficient neural network adjacent to the acini in the parotid gland of rat (thick arrows). Note the accompanying blood capillaries could be seen in association of these nerves (thin arrows). (X3, 500)

Fig. 1.E: SEM [Diabetic group, (group II)]: showing diminished and collapsed neural network (arrow) adjacent to the outer surface of the parotid gland acini. (X3, 500)

Fig. 1.F: SEM [Neostigmine treated group, (group III)]: showing restoration of the density of the neural elements (thick arrows) adjacent to the outer surface of the secretory acini. Some blood capillaries are seen in association with these nerves (thin arrows). (X3, 500)

Histomorphometric Results:

The morphometric results confirmed the ultrastructural findings of the increased diameter of the acinar secretory granules in neostigmine treated group than in diabetic group without treatment, as demonstrated at table (1).

Table (1): Histomorphometric data of the secretory granules diameter among the control and study groups:

Groups	Control group	Diabetic group	Neostigmine group
Diameter of Secretory Granules	0.217391 0.23913 0.217391 0.195652 0.206522 0.217391 0.23913 0.206522 0.198345 0.203408	0.163043 0.141304 0.130435 0.173913 0.152174 0.130435 0.180124 0.13913 0.14567 0.1789	0.5 0.371739 0.456522 0.25 0.293478 0.271739 0.423913 0.36677 0.29567 0.39876
Mean	0.214088	0.153513	0.362859
Standard Deviation	0.015287	0.019276	0.083705

Statistical analysis:

There was a statistically significant difference among the studied groups regarding the diameter of the secretory granules between the control and diabetic groups and also between the diabetic and neostigmine treated groups, as indicated in table (2).

Table (2): Comparative tabulation of the median secretory granules diameter among the studied groups using ANOVA test:

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.232	2	0.611	45.737	.000
Within Groups	.069	27	.003		
Total	.301	29			

*p value considered significant (<0.05) using one-way ANOVA

DISCUSSION

This study has demonstrated that there was pronounced increase in the diameter of the acinus secretory granules in neostigmine treated group than that observed in diabetic rats and was comparable to that seen in the control group.

Sandberg et al, (8) conducted a cross sectional epidemiological study on type 1 diabetic patients and control subjects without diabetes to investigate the prevalence of hyposalivation and xerostomia and to determine the relationship between salivary dysfunction and diabetes complications. This study was conducted on type 1 diabetics and control subjects without diabetes. The investigators found that symptoms of reduced salivary flow rate and xerostomia were more frequently reported by patients with diabetes than the controls, especially by those diabetics who had developed neuropathy and these findings are in accordance with our results.

Neostigmine as a parasympathomimetic drug has been used to overcome the problem of compliance of hyposalivation associated with certain diseased conditions (25), and medication (7). The evaluation of neostigmine as sialogogues drug to simulate the effect of parasympathetic nervous system is very important (7).

In fact, neostigmine and other parasympathomimetic drugs have been used in many pharmacological approaches as prophylactic agents in patients receiving radiotherapy at head and neck malignancies, who were suffering from diminished salivary gland output (25, 26).

In 2013, El-Ramli et al, (20) studied the structural histological changes in the parotid salivary gland of rabbits treated with neostigmine. They found a significant increase in the diameter of the secretory acini and vacuolation with foamy appearance of the cell of the acini in triple dose neostigmine treated group and these results support our findings.

Hakim et al, (25) have noticed an improvement in compliance of the patients receiving radiotherapy after the use of the drug. In fact neostigmine like other parasympathomimetic drugs was important to induce compound exocytosis to the secretory granules (27), a process that was considered radioprotective in other study (28, 29). Compound exocytosis is an essential process in production of excessive amount of saliva by the parotid salivary gland (30). Similar results were obtained for amifostine in experimental studies on rat and rabbit models and clinical trials which provided encouraging results (31).

In the current study, the most striking features were the increase in number of the secretory granules and the vacuolation of the acinar cells in addition to restoration of the neural organization in neostigmine treated rats.

In parasympathetic denervated gland in rabbit there was an extensive cellular change in the glands. The acini were reduced in size and the degree of vacuolation has decreased (32). On contrary, our study has demonstrated an increase in the incidence of the density of secretory granules in acinar cells of rat parotid gland, so that the present results and these presented by Cope et al, (33) clearly proved that neostigmine induced supersensitization of the acinar cells. This is in accordance with the findings of Watanabi et al, (34-36) on the effect of the parasympathomimetic drugs on exocrine serous cells of the trachea and pancreas.

Early in vitro studies on rat parotid gland showed that stimulation with muscarinic and adrenergic agonists caused movement of water and vacuole formation (37). It has been sometimes considered that vacuole formation is an essential part of water secretion (38).

In rat parotid prolonged strong parasympathetic stimulation has increased the tendency for acinar vacuolation (39), representing of secretory granules. This was quite evident in neostigmine treated rats of the present work.

The production of endogenous saliva is of greatest benefit to patient both for its convenience and the importance of natural saliva to oral function. The artificial saliva does not replace the many macromolecules critical to protective and other functions of saliva. Stimulation of gland function also may help prevent any infection of salivary glands and retard the formation of mucous plug (40). The structural histological findings noticed in this study support the use of this drug in cases of xerostomia.

CONCLUSION

The ultrastructural findings noticed in this study substantiate the use of Neostigmine in cases of diabetes associated xerostomia.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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