EFFECT OF DIAZEPAM AND GREEN TEA ON SALIVARY ALPHA-AMYLASE SECRETION AND STRUCTURE OF THE SALIVARY GLANDS IN MALE ALBINO RATS

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ABSTRACT

Background: Human saliva is a complex oral fluid that serves several functions including food digestion, swallowing, preservation of tooth integrity as well as antiviral and antibacterial activities. Salivary alpha amylase (SAA) is a salivary enzyme that is thought to play a role in response to psychological and physical stress. Diazepam is a sedative drug that most commonly causes xerostomia. The frequent intake of green tea causes the stimulation of salivary secretion thus counteracting the xerostomic effect of several drugs besides its antioxidant ability.

Objective: The objective of the current study is to describe the effect of diazepam and green tea on salivary alpha-amylase secretion and structure of the salivary glands in male Albino rats and correlate the results with Ki67 tissue expression as a marker for proliferation to evaluate the condition of the salivary tissue before and after their admission.

Material and Methods: sixty male albino rats of local strain were used for studying the effect of diazepam and green tea on the secretion of salivary alpha amylase and the structure of the salivary glands. Saliva samples were collected after 30 days of treatment to measure the salivary alpha amylase activity. Immunohistochemical study was performed on specimens retrieved from both submandibular and parotid glands to evaluate the expression of Ki67 before and after treatment. The area fraction expressed by Ki67 was calculated histomorphometrically.

Results: A marked decrease in salivary alpha amylase activity was found in rats taking Diazepam. This decrease was also noticed in rats that take a combination of green tea and diazepam. A marked decrease in the expression of Ki67 was noticed after administration of the drug followed by a gradual increase observed in the specimens treated with the combination of both the drug and green tea.

Conclusion: The present study revealed that rats receiving diazepam show marked decrease in salivary alpha amylase activity. Administration of green tea lead to further inhibition of amylase activity with correction of salivary secretion caused by the drug as well as an increase in the proliferation of acinar cells indicating the role of green tea in tissue repair and restoration of the proliferative power of the cells.

KEYWORDS: Salivary alpha-amylase, salivary glands, green tea, diazepam, Ki67.
INTRODUCTION

Human saliva is a clear, slightly acidic (pH=6.0-7.0) biological fluid containing a mixture of secretions from multiple salivary glands, including the parotid, submandibular, sublingual and other minor glands beneath the oral mucosa as well as gingival crevice fluid. This complex oral fluid serves the execution of multiple physiological functions such as oral digestion, food swallowing and tasting, tissue lubrication, maintenance of tooth integrity, antibacterial and antiviral protection. In addition to the important role of maintaining the homeostasis of the oral cavity system, the oral fluid is a perfect medium to be explored for health and disease surveillance. (Mandel D., 1987).

Salivary alpha amylase is one of the most important enzymes in saliva which is synthesized in the acinar cells of the salivary glands and stored in secretory granules inside the cells (Abraham M. et al., 2011). Its release from the salivary cells is greatly increased in response to taste or chewing motions of the jaw but it is present in lower levels in non-stimulated saliva between meals due to its important enzyme activity in the oral cavity carrying out several functions (Nater M. et al., 2009). Alpha-amylase production in the salivary glands increases in response to psychological and physical stress through interactions with the autonomic nervous system, and it has been found to be a useful as a marker of activity in the autonomic nervous system (Abraham M. et al., 2011). SAA has been investigated during the past thirty years as a biomarker of stress, although there still remains certain doubt regarding its utility. There has been a lot of debate whether SAA reflects sympathetic activity solely; however, previous data suggested that this is unlikely due to the parasympathetic activity which innervates salivary glands whose product is SAA (Bosch A. et al., 2011).

The benzodiazepines (Diazepam) were first introduced in 1960. After their introduction into the market, they quickly substituted the barbiturates, becoming the most used medicine with sedative properties. Drugs are the most common cause of reduced salivation (Kaplan I. et al., 1994). Diazepam appears to act on areas of the limbic system, thalamus and hypothalamus, inducing anxiolytic effects. Its actions are due to the enhancement of GABA activity. Benzodiazepine drugs including Diazepam increase the inhibitory processes in the cerebral cortex (Jakson W. et al., 1986). Diazepam is indicated for the management of anxiety disorders and tension associated with the stress of everyday life. In acute alcohol withdrawal, diazepam may be useful in the symptomatic relief of acute agitation, tremor, impending or acute delirium tremens and hallucinosis. Diazepam is a useful adjunct for the relief of skeletal muscle spasm due to reflex spasm to local pathology; spasticity caused by upper motor neuron disorders, athetosis, and stiff-man syndrome (Jakson W. et al., 1986).

Green tea is one of the most popular beverages consumed worldwide. Tea, from the plant Camellia sinensis, is consumed in different parts of the world as green, black, or Oolong tea. Among all those, however, the most significant effect on human health have been observed with the consumption of green tea (Cabrera C. et al., 2006). Green tea contains polyphenols, which include flavanols, flavandiols, flavonoids, and phenolic acids; these compounds may account for up to 30% of its dry weight. Most of the green tea polyphenols (GTPs) are flavanols, commonly known as catechins. The major flavonoids of green tea are various catechins, which are found in greater amounts in green tea than in black or Oolong tea (Vinson A., 2002). There are four kinds of catechins mainly found in green tea: epicatechin, epigallocatechin, epicatechin-3-galate, and EGCG. The preparation methods influence the catechins both quantitatively and qualitatively; the amount of catechins also varies in the original tea leaves due to differences in variety, origin and growing conditions (Khokhar S. and Magnusdot-tir S., 2002).
Salivary glands can be stimulated with green tea. The active ingredients in green tea can provide relief to bouts of dry mouth. The use of green tea once to twice daily causes a noticeable decrease in dry mouth symptoms. Powerful antioxidants in green tea, called polyphenols, reduce the damage to the salivary gland. With green tea polyphenols, we have an agent that is helping to correct the salivary glands abnormal behavior. Green tea consumption has been reported to increase the acid resistance of teeth to damage by cariogenic bacteria by inhibiting the causative bacteria, which contribute to the formation of dental plaque and caries (Gutman L. and Ryu H., 1996).

Ki67 has been widely used as a proliferation marker for human cells. The Ki67 antigen encodes two protein isoforms originally identified by Scholzer and Gerdes in the early 1980s (Scholzen T and Gerdes J., 2000). Ki67 protein has a half-life of approximately 1-1.5 hours. It is present during all active phases of the cell cycle (G1, S, G2 and M) playing different roles, but is absent in resting cells (G0) (Hooghe B. et al, 2008, Shirendeb U. et al 2009). Previous studies showed a sharp decrease in Ki67 levels during the later phases of mitosis (during anaphase and telophase) (Modlin M. et al, 2008). Expression of the Ki67 protein (pKi67) was found to be associated with the proliferative activity of cells (Lian L. et al, 2015). Recent studies revealed multiple molecular functions of this protein. Its cellular distribution changes continuously during cell cycle progression. These distributions correlate with distinct functions before and after chromosomal division during mitosis (Xiaoming S. and Paul D., 2018).

During the interphase, Ki67 antigen can be found within the cell nucleus. During mitosis, most of the protein is shifted to the surface. The fact that the protein is present during the active phases of the cell cycle (G1, S, G2 and M) but is absent in the G (0) phase of normal resting cells, makes it a promising marker to determine the growth fraction of a cell population (Scholzen T and Gerdes J., 2000).

As aforementioned, the division activity measured by Ki-67 has been reported in previous studies, and was found to be of great prognostic importance in several types of malignancies as a protein linked with cell proliferation and function (Yerushalmi R. et al, 2010). It has been reported that Ki-67 has a prognostic character for many types of malignant tumors, such as lymphomas, breast, prostate and colorectal cancers as it defines the ability of the cells to proliferate as well as restore functional activities (Tretiakova S. et al, 2016).

The present study was undertaken to assess the effect of diazepam and green tea on secretion of SAA from both submandibular and parotid salivary glands and observe whether the use of green tea will restore normal histologic and physiologic activity of these glands.

**MATERIAL AND METHODS**

Sixty male albino rats, 10 weeks of age and weighing from 130 to 250 g, were obtained from the Animal Facility, Faculty of Medicine, Misr International University Cairo, Egypt. They were housed in the Animal facility, Faculty of Dentistry, Misr International University, Cairo, Egypt.

The animals were divided into three groups (twenty each) and were housed and caged separately in plastic cages in an air conditioned room at 22±2°C and 55±10% humidity. The experimental procedure was conducted in compliance with ethical principles for animals’ research as reviewed and approved by institutional guidelines of Misr International University.

**Experimental design:**

The experimental diets were prepared in the form of corn starch and water. The rats were divided into three groups as follows:
Group C (control group): control group received 0.2 mg/Kg of physiological saline solution by intraperitoneal (i.p.) route for 30 days.

Group D: received 0.2 mg/Kg of Diazepam by i.p. route for 30 days.

Group (D+T): received 0.2 mg/Kg of Diazepam by i.p. route for 30 days and were given green tea in their water. The green tea extract used in this study was in the form of veggie capsules purchased from Amazon.com as a dietary supplement from Nature’s Nutrition (green tea extract 98% standardized epigallocatechin gallate). The capsules were dissolved in boiling distilled water and time was given for the mixture to cool down. The mixture was then filtered and the animals received a dose of 1mg/kg/day by i.p. route for 30 days.

Saliva samples were collected after 30 days of treatment. The salivary flow was stimulated by hydrochloride 2% (Sigma, 5 mg/kg BW, IP). Two drops of 4% pilocarpine hydrochloride eye drops were briefly instilled in the rats’ mouths. After 2 minutes, the saliva samples were collected with the animals gently positioned in ventral decubitus on the operator hands. The whole saliva dropped from their mouths was collected in a pre-weighed sterile universal collection vial for further assessment of SAA activity.

Ideally, samples are assayed fresh. When stored frozen, α-amylase is stable for one month. EDTA, EGTA and citrate are α-amylase inhibitors and should be avoided in sample preparation.

A simple, direct kinetic method for determination of SAA (1,4, alpha-D-glucan 4-glucanohydrolase, EC 3.2.1.1) in which the assay makes use of a well-defined substrate, p-nitrophenyl alpha-maltoside, which is hydrolyzed by alpha-amylase to a chromogenic product, p-nitrophenol (Bao L. et al, 1999).

Amylase activity in rat mixed saliva is (163.8 U/mL ± 14.1) in (U/mL of saliva) (Gracieli P. et al, 2006).

Generally, if the calculated activity was higher than 300 U/L, the sample was diluted in water and the assay was repeated. The results were multiplied by the dilution factor (n).

Unit definition: one unit of enzyme catalyzes the production of 1 μmole of product per minute under the assay conditions (pH 7.0).

Histological examination:

All submandibular and parotid salivary glands specimens were fixed with 10% formalin, treated with alcohol and then embedded in paraffin wax. Sections of 4µm thickness were made for routine histopathological examination with hematoxylin and eosin and examined under light microscope.

Immunohistochemical examination:

Paraffin embedded tissue sections were dewaxed and rehydrated through grade ethanol to distilled water. Endogenous peroxidase was blocked by incubation with 3% H2O2 in methanol for 10 minutes. The antigen retrieval was achieved by microwave in citrate buffer solution (2.1 g citric acid/L D.H2O; 0.37g EDTA/L D.H2O; 0.2g Trypsin) (pH 6.0) for 10 minutes, followed by washing step with Tris-buffered saline (8g NaCl; 0.605g Tris) (pH 7.6). The tissue sections were then incubated with power BlockTM reagent (BioGenex, San Ramon, CA, USA), universal proteinaceous blocking reagent, for 15 minutes at room temperature to block non-specific binding sites. The tissue sections were then incubated with the primary anti-Ki-67 antibodies (Santa Cruz Biotechnology catalogue # sc-23900) overnight at 4°C. The bound primary antibody was detected by incubation with the secondary antibody conjugated with horseradish peroxidase (BioGenex, San Ramon, CA, USA) for 30 minutes at room temperature.

After rinsing with Tris-buffered saline, the antigen-antibody complex was detected using 3, 3'-diaminobenzidine, the substrate of horseradish
peroxidase. When acceptable color intensity was reached, the slides were washed, counter stained with hematoxylin and covered with a mounting medium. Each slide was microscopically analyzed and enumerated.

**Statistical analysis**

The area fraction expressed by ki67 was calculated histomorphometrically. Data were coded and entered using the statistical package for the Social Sciences (SPSS) version 28 (IBM Corp., Armonk, NY, USA). Data was summarized using median and interquartile range. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis and Mann-Whitney tests (Chan H., 2003). P-values less than 0.05 were considered as statistically significant.

**RESULTS**

**Salivary alpha Amylase levels:**

Assessment of SAA revealed that the control group recorded the greatest mean value (160.964±9.29) U/ml. The mean value decreased in the group receiving Diazepam alone (94.669±5.48), while the group receiving Diazepam and green tea had the lowest SAA mean value (25.638±26.26), (Table 1, fig. 1-3).

**TABLE (1)** Summary of the values of α-amylase levels in all groups

<table>
<thead>
<tr>
<th></th>
<th>Group (C)</th>
<th>Group (D)</th>
<th>Group (D+T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>160.964</td>
<td>94.669</td>
<td>25.6385</td>
</tr>
<tr>
<td>SD</td>
<td>9.2978002</td>
<td>5.4872438</td>
<td>26.2694846</td>
</tr>
<tr>
<td>Maximum</td>
<td>176.03</td>
<td>105.1</td>
<td>70.9</td>
</tr>
<tr>
<td>Minimum</td>
<td>149.05</td>
<td>87.08</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*SD= standard deviation*

**Statistical Analysis**

Using the ANOVA test, a highly significant difference was detected between the three groups (the control group, the group receiving Diazepam and the group receiving Diazepam and green tea).
TABLE (2) Descriptive statistics of the difference in α-amylase levels in all groups

<table>
<thead>
<tr>
<th>Sum of squares</th>
<th>d.f.</th>
<th>Variance</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>183154.8527</td>
<td>57</td>
<td>91577.4264</td>
<td>340.5864</td>
</tr>
<tr>
<td>Within groups</td>
<td>15326.2541</td>
<td>2</td>
<td>268.8817</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>198481.1068</td>
<td>59</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*d.f. = Degree of freedom, ** highly significant difference

Using unpaired t-test for pairwise comparison between groups, a highly significant difference was noted between the control group and each of the experimental groups. Moreover, the difference between the two experimental groups (the group receiving Diazepam and the group receiving Diazepam and green tea) was highly statistically significant (table 3).

TABLE (3) Pairwise comparison between groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>df</th>
<th>Standard error of difference</th>
<th>t value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vs Diazepam</td>
<td>38</td>
<td>2.414</td>
<td>27.4614</td>
<td>&lt;0.0001**</td>
</tr>
<tr>
<td>Control vs Diazepam &amp; green tea</td>
<td>38</td>
<td>6.231</td>
<td>21.7177</td>
<td>&lt;0.0001**</td>
</tr>
<tr>
<td>Diazepam vs Diazepam &amp; green tea</td>
<td>38</td>
<td>6.001</td>
<td>11.5035</td>
<td>&lt;0.0001**</td>
</tr>
</tbody>
</table>

*d.f. = degree of freedom, ** highly significant

Percent decrease in salivary α amylase levels:

Calculating the percent decrease in relation to control, the Diazepam group showed 41.43% decrease, while the Diazepam & green tea group showed 84.07% decrease. Moreover, the Diazepam & green tea group showed 72.91% decrease in relation to the Diazepam group.

Statistical analysis of immunohistochemical results:

For ki67, the nuclear staining was considered to be positive. Using the light microscopy, ki67-positive nuclei were evaluated on each slide. A minimum of 200 cells per field in five different fields, the ki67-positive nuclei were counted and the area fraction was then calculated. Regardless of the intensity, any nuclear staining was considered immunopositive for ki67 (Cowen D. et al, 2002).

Upon examination of the gene expression in the three groups of both glands, statistical findings indicated a significantly reduced expression of ki67 in group 2 (Diazepam treated group) than in group 1 (control group) followed by slight elevation in the value of gene expression in group 3 (treated with both the drug and green tea). By calculating the area fraction, the least expression was measured in group 2 followed by group 3. The highest expression was measured in group1 (table 4 and 5).

TABLE (4) The median of the area fraction of the three groups (Group 1: control group, Group 2: Diazepam, Group 3: Diazepam and green tea) in the parotid salivary gland.

<table>
<thead>
<tr>
<th>Ki-67 tissue expression</th>
<th>G1 (control)</th>
<th>G2</th>
<th>G3</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>1.22</td>
<td>0.19</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>1* quartile</td>
<td>0.67</td>
<td>0.15</td>
<td>0.45</td>
<td>0.004</td>
</tr>
<tr>
<td>3* quartile</td>
<td>1.61</td>
<td>0.26</td>
<td>0.62</td>
<td></td>
</tr>
</tbody>
</table>

*P-values less than 0.05 were considered as statistically significant.*
TABLE (5) The median of the area fraction of the three groups (Group 1: control group, Group 2: Diazepam, Group 3: Diazepam and green tea) in the submandibular salivary gland.

<table>
<thead>
<tr>
<th></th>
<th>G1 SM (control)</th>
<th>G2 SM</th>
<th>G3 SM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67 tissue expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>1.12</td>
<td>0.27</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>1st quartile</td>
<td>0.84</td>
<td>0.22</td>
<td>0.47</td>
<td>0.006</td>
</tr>
<tr>
<td>3rd quartile</td>
<td>1.39</td>
<td>0.36</td>
<td>0.88</td>
<td></td>
</tr>
</tbody>
</table>

P-values less than 0.05 were considered as statistically significant.

As for mentioned, comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis and Mann-Whitney tests confirming the results presented in tables 4 and 5 (figure 4 A and B).

![Fig. (4) Box plot illustrating median and interquartile range of Ki67 area fraction immune expression of the three groups (Group 1: control group, Group 2: Diazepam, Group 3: Diazepam and green tea) in (A) the parotid salivary gland and (B) the submandibular salivary gland.](image)

P-values less than 0.05 were considered as statistically significant.

According to Post hoc pairwise comparison (P value between each 2 groups), there was a significant difference between group 1 (the control group) and group 2 (Diazepam alone) and group 3 (Diazepam and green tea) in the groups studying the parotid salivary glands, with P-value < 0.001 and 0.004 respectively (table 6).

TABLE (6) The Post hoc pairwise comparison of the three groups (Group 1: control group, Group 2: Diazepam, Group 3: Diazepam and green tea) in the parotid salivary gland.

<table>
<thead>
<tr>
<th>Comparisons between groups</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2 PART VS G3 PART</td>
<td>0.214</td>
</tr>
<tr>
<td>G2 PART VS G1 PART (control)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>G3 PART VS G1 PART (control)</td>
<td>0.040</td>
</tr>
</tbody>
</table>

P-values less than 0.05 were considered as statistically significant.
Concerning the groups studying the submandibular glands, there was a significant difference between group 1 (the control group) and group 2 (Diazepam alone). Similarly, there was a significant difference between group 2 (Diazepam alone) and group 3 (Diazepam and green tea), with $P$-value = 0.002 and 0.023 respectively (table 7).

<table>
<thead>
<tr>
<th>Comparisons between groups</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2 SM VS G3 SM</td>
<td>0.023</td>
</tr>
<tr>
<td>G2 SM VS G1 SM (control)</td>
<td>0.002</td>
</tr>
<tr>
<td>G3 SM VS G1 SM (control)</td>
<td>0.417</td>
</tr>
</tbody>
</table>

$P$-values less than 0.05 were considered as statistically significant.

**TABLE (7) The Post hoc pairwise comparison (P value between each 2 groups) of the three groups (Group 1: control group, Group 2: Diazepam, Group 3: Diazepam and green tea) in the submandibular salivary gland.**

**Histological Results:**

Careful macroscopic examination of the parotid and submandibular salivary glands of the albino rats of studied groups revealed that the glands are well capsulated with thin fibrous tissue capsule.

1- **Parotid salivary glands in Group I (Control Group):**

The histological structure of the parotid salivary glands of albino rats showed the lobules composed of pure serous acini, which were small and arranged close to each other. The serous acini were lined by pyramidal secretory cells with well-defined cell boundaries and granular basophilic cytoplasm surrounding narrow lumens. Their nuclei appeared large, rounded, deeply stained and located mostly at the basal third of the cells (fig. 5).

The intercalated ducts appeared small in size. They were lined by cuboidal cells with rounded centrally placed and deeply basophilic nuclei, which were surrounded by thin rim of cytoplasm. The striated ducts were lined by a single layer of columnar cells with rounded basally placed and deeply stained nuclei. The basal part of the cell showed a well-defined acidophilic striated border (fig. 5).

The excretory ducts in between the lobules of this gland were lined by pseudo stratified columnar epithelial cells with goblet cells. Their nuclei were rounded to oval deeply stained and arranged in different levels. The cytoplasm was moderately eosinophilic and homogenous. The lumens of these ducts contained few stagnated secretions.

2- **Parotid salivary glands in Group II (experimental group):**

Serous secretory cells showed the appearance of clear vacuoles in their cytoplasm. Few nuclei became pyknotic and degenerated. The epithelial lining of the intralobular ducts showed disruption and discontinuity. Some ductal epithelial cells were degenerated. The blood vessels around the ducts were dilated and engorged with many red blood cells and a few inflammatory cells appeared around the ducts. (fig. 6 and 7)

Fig. (5) Photomicrograph of group I (parotid gland) showing serous acini, which are condensed, and with rounded nuclei (H&E, x100).
EFFECT OF DIAZEPAM AND GREEN TEA ON SALIVARY ALPHA-AMYLASE SECRETION

Fig. (6) Photomicrograph of group II (parotid gland) revealed rupture and discontinuity of lining epithelium of striated ducts. The blood vessels are dilated and engorged with red blood cells (H&E, x100).

Fig. (7) Photomicrograph of group II (parotid gland) showing many dilated blood vessels which are filled with large number of red blood cells. The secretory cells showing many vacuoles (H&E, x100).

3- Parotid salivary glands in Group III (experimental group):

The secretory cells demonstrate less number of degenerative vacuoles. The nuclei appear deeply stained. The acini at the periphery of lobes show fewer pyknotic nuclei. The intralobular duct cells appear to be normal. There are no inflammatory cells around both acini and ducts. Some blood vessels are dilated (Fig. 8, 9).

Fig. (8) Photomicrograph of group III (parotid gland) showing less number and size of degenerative vacuoles. The blood vessels are small and contain less number of red blood cells. No inflammatory cells are noticed (H&E, x100).

Fig. (9) Photomicrograph of group III (parotid gland) III showing some blood vessels is dilated. No inflammatory cells are noticed. (H&E, x100).

4- Submandibular salivary glands in Group I (Control Group):

Histological structure of the submandibular salivary gland of albino rats showed that the lobules are composed mainly of serous acini, while the mucous part are in the form of few pure mucous.

The serous acini of the submandibular salivary gland are composed of pyramidal cells with granular basophilic cytoplasm surrounded the narrow lumen. Their nuclei appear rounded and large (fig. 10).

The mucous terminal portions of submandibular salivary gland were composed of cuboidal cells that surround wide lumen. Their nuclei appeared angular, darkly stained and are compressed basally. The secretory cells have trabecular basophilic cytoplasm, which contain dissolved mucigen granules.
The intercalated ducts are hardly to be seen, as they are small in the size. They are lined by cuboidal cells with rounded and large centrally placed and deeply stained nuclei surrounded by thin rim of cytoplasm. While the striated ducts are large, branched and are lined by single layer of columnar cells with rounded basally situated and deeply basophilic nuclei and acidophilic cytoplasm.

The granular convoluted tubules (GCT) are long, numerous and branched tubules. They are lined by columnar cells characterized by the presence of acidophilic cytoplasmic granules and small darkly stained basophilic nuclei.

Excretory ducts are distributed between the lobules in the connective tissue septa and lined by pseudostratified columnar epithelial cells with goblet cells. Their nuclei are rounded to oval darkly stained nuclei arranged in different levels (fig. 10).

5- Submandibular salivary glands in Group II (ex-perimental Group):

The serous acini are noticeably decreased in size and number in comparison to control group (fig.11). The secretory cells present loss of their cell outline. Some acini have lost all its cell boundaries and appear to be degenerated and filled with many vacuoles. The cytoplasm become homogenous and deeply stained acidophilic. Also, the cytoplasm of the secretory cells may show large cytoplasmic vacuolization.

The striated ducts decrease in the size and loss their basal striations. The GCT is also decrease in number and size. Their lining epithelium show signs of degeneration, also some GCT are seen to be completely degenerated.

3. Submandibular salivary glands in Group III (Experimental Group):

The submandibular salivary gland appears with slightly atrophied secretory portion and GCT (fig.12). Some lobules show more atrophy in their acini and GCT. The cytoplasm of serous secretory cells may present some cytoplasmic vacuolization. Also, the lining epithelium of the GCT shows the same cytoplasmic vacuolization (fig.12). The striated ducts are widely dilated and have thickened wall.
**Immunohistological Results:**

The immunoeexpression pattern of Ki67 in the parotid and submandibular salivary glands of all groups are shown in (fig. 13-18). **Group (1)** showed normal parotid and submandibular salivary glands with immunopositively labeled nuclear staining in the acini, striated and excretory ducts (fig.13, 16).

On the other hand, **Group (2)** presented clear negative to minimal nuclear and cytoplasmic expression in both epithelial and connective tissue cells (fig.14, 17). However, **Group (3)** showed positive immunostaining expression of ki67 in the nuclei of the acini as well as the epithelial cells of the striated and excretory ducts of both types of glands (fig.15, 18).

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**Fig. (13)** Photomicrograph of group I (parotid gland) showing serous acini, which are showing immunopositivity to ki67 (H&E, x100).

**Fig. (14)** Photomicrograph of group II (parotid gland) revealed minimal staining of the nuclei of the serous acini and the epithelium of the engorged ducts (H&E, x100).

**Fig. (15)** Photomicrograph of group III (parotid gland) showing scattered immunopositivity to ki67 (H&E, x100).

**Fig. (16)** Photomicrograph of group I (submandibular salivary gland) showing serous acini, which are showing immunopositivity to ki67 (H&E, x100).

**Fig. (17)** Photomicrograph of group II (submandibular salivary gland) revealed minimal staining of the nuclei of the serous acini and the epithelium of the engorged ducts (H&E, x100).

**Fig. (18)** Photomicrograph of group III (submandibular salivary gland) showing scattered immunopositivity to ki67 (H&E, x100).
DISCUSSION

In our study, we chose a commonly used group of drugs that is benzodiazepines (Diazepam) which are used clinically as anxiolytic, anticonvulsant, and hypnotic drugs. Their effects are mediated by specific BDZ receptors which are classified into a central-type linked to the GABA receptor-chloride channel complex and a peripheral-type, not linked to the GABA receptor. They block the actions of the parasympathetic system by inhibiting the effects of acetylcholine on the salivary gland receptors. This results in a dry mouth sensation, probably because the sympathetic portion of the independent nervous system predominates over the “blocked” parasympathetic system (Wynn R. and Meiller, 2001). According to Schubert M. and Izutsu K., the drugs may affect the salivary flow and its composition by interferences in the acinar and duct functions, and by means of alterations in the blood flow of the salivary glands. Besides that, the high serum half-life of Diazepam makes them detectable in saliva for long periods of time probably enhancing their anticholinergic effect on the salivary glands (Schubert M. and Izutsu K., 1987 and Phuu H. et al, 2015).

Previous studies demonstrated that both central and peripheral-type benzodiazepine receptors exist in the rat salivary glands as well as in the brain (Yamagishi H. and Kawaguchi M., 1998). According to Douglas C., diminishment of the salivary flow is due to the reduction in the blood flow of the gland, produced by adrenergic sympathetic vasoconstriction. Therefore, when there is sympathetic hyperactivity the mouth presents dryness (Douglas C., 2002).

Data of the present study showed marked decrease in SAA by drug induced xerostomia and adding green tea caused further decrease in SSA. BDZs suppressed the release of amylase from rat acinar cells. These findings indicate that BDZs not only suppress the central nervous system but also act directly on the salivary glands and that BDZ receptors in the salivary glands are linked to the inhibitory responses of BDZs. These results agree with study of Ambudkar S.; who found that stimulation of the m3-muscarinic and a1-adrenoceptors in the salivary glands led to the hydrolysis of a plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2), via the activation of GTP-binding regulatory protein (G protein; the Gq family) coupled to these receptors and a membrane-bound phosphoinositide-specific phospholipase C (PLC). This results in the generation of the second messenger molecules, inositol 1,4,5-trisphosphate (IP3) and diacylglycero- cerol. IP3 gates the release of Ca2+ from intracellular Ca2+stores, resulting in Ca2+ entry from the external medium and a sustained elevation of the intracellular Ca2+ concentration. The resulting increase in Ca2+ regulates, directly or indirectly, a number of Ca2+ dependent ion channels, for example, the Ca2+-activated potassium channel in the basolateral membrane and the Ca2+-activated chloride channel in the luminal membrane, which lead to diminishing the salivary flow and protein content of the secretions (Ambudkar S., 2000).

Salivary alpha amylase is secreted from the acinar cells but whether the secretion could occur by destruction of the plasma membrane remains to be elucidated. To investigate the mechanisms of amylase secretion, secretory vesicles and secretory granules were fractionated. Granules and vesicles were clearly separated while secretory granules were included in fractions. Secretory vesicles were in the lighter fractions. In this study, we distinguished amylase from the secretary vesicles from the secretary granules (fig.19).

One possible model for amylase secretion from parotid acinar cells is illustrated in figure 19. The vesicles, including the newly-synthesized amylase, are formed from tGN or condensing vacuoles before the formation of immature secretory granules (route b), and then transported quickly to the plasma membrane independent of microtubules. The transportation mechanisms of the secretary
vesicles and the direction of the secretory vesicles, apical or basolateral, remain to be elucidated. Here, we have demonstrated the vesicular secretion of the newly-synthesized amylase distinguished from the amylase accumulated in the secretory granules. This unstimulated amylase secretion might be constitutive secretion, but the formation of the secretory vesicles required proteoglycan similar to the secretory granules. The factors that affect the vesicular secretion may be important to keep continuous secretion of amylase. Further investigation is required (Nashida T. et al, 2008).

Through previous animal studies and human sample testing, Georgia Health Sciences University GHSU researchers found that xerostomia involves salivary gland inflammation, reduced antioxidants and elevated markers for abnormal growth and DNA damage caused by free radicals. Polyphenols, which are powerful antioxidants in green tea, reduce that damage to the salivary gland, the GHSU researchers noted. “With green tea polyphenols, we have an agent that’s helping to correct the salivary gland’s abnormal behavior,” said Douglas Dickinson (Douglas C., 2002).

Green tea is a popular drink throughout the world, and it contains various components, including the green tea polyphenol, epigallocatechin gallate (EGCG). Tea interacts with saliva upon entering the mouth, so the interaction between saliva and EGCG interested us, especially with respect to EGCG–protein binding. SDS-PAGE revealed that several salivary proteins were precipitated after adding EGCG to saliva. The major proteins precipitated by EGCG were alpha-amylase and cystatins. In addition, EGCG inhibited the activity of alpha-amylase by non-competitive inhibition, indicating that EGCG is effective at inhibiting the formation of fermentable carbohydrates involved in caries formation. Interestingly, alpha-amylase reduced the antimicrobial activity of EGCG against the periodontal bacterium. Therefore, we considered that EGCG–salivary protein interactions might have both protective and detrimental effects with respect to oral health (Hara K. et al, 2012).

Green tea is known to inhibit SAA and reduce the oral concentration of fermentable sugars after eating starchy foods; the nature of the inhibition remains under study. Paolino J. et al. found that tea beverage inhibited salivary amylase under conditions existing in the mouth during and shortly after ingestion of green tea (Paolino J. et al., 1980). Furthermore, Zhang and Kashket have shown that green tea inhibited salivary amylase (Zhang J. and Kashket S., 1998).

The effect of green tea on salivary amylase may contribute significantly to reducing the carcinogenicity of starch-containing foods. The ability of a tea extract to inhibit carbohydrate absorption has potential clinical utility for weight control and the treatment of diabetes. Assuming that the tea extract causes malabsorption of 25% of
ingested carbohydrate, striking weight loss would be expected providing that caloric intake was not commensurately increased and the caloric content of malabsorbed carbohydrate was unavailable to the host. This hypoglycemic effect generally has been attributed to alterations of the intermediary metabolism of glucose. The carbohydrate malabsorption induced by tea extracts could also influence blood glucose concentrations (Carmen C. et al, 2006).

The inhibition of salivary amylase activity by extracts of green teas was dependent on the tea: saliva ratio so that, in vivo, conditions may permit optimal inhibition during and immediately after tea ingestion. Zhang and Kashket also demonstrated that the fluoride content of the tested tea brews did not correlate with amylase inhibition (Zhang J. and Kashket S., 1998).

Koukiekolo R. et al. investigated the effects of alpha-amylase inhibitors on the digestion of starch; they potentially improving postprandial carbohydrate tolerance in people with low glucose tolerance. As excess dietary carbohydrate is metabolized to fat, inhibition of carbohydrate digestion may help in weight management as well; however, they are indirectly helpful in weight loss due to inhibition of sugar assimilation, through inhibiting starch breakdown. With reduced amount of amylase available for break down, the complex carbohydrare has a better chance of traveling through the body without being assimilated, and is eventually excreted from the body instead of being converted into storage fat. An animal model study revealed that amylase inhibitors alter the amount and pattern of food intake and reduce weight gain probably through inducing satiety and increasing carbohydrate delivery to the distal (farthest) part of the small intestine in rats (Koukiekolo R. et al., 2001).

Furthermore, they found that the inhibition takes place only when the enzyme and inhibitor are preincubated together, before the substrate is added. This shows that the inhibitor and enzyme complex is formed during preincubation period. This model differs from those previously reported for acarbose. The mechanism of action is thus reported to be “mixed non-competitive inhibition (Koukiekolo R. et al, 2001).

In our study, the histomorphometric results revealed that, after the long-term treatment with Diazepam, the parotid glands exhibited a disorganized parenchyma with loss of inter-lobular limits and appearance of clear vacuoles in their cytoplasm. They also showed some pyknotic and degenerated nuclei while the epithelial lining of the intralobular ducts showed disruption and discontinuity. Some ductal epithelial cells were degenerated. The blood vessels around the ducts were dilated and engorged with many red blood cells with few inflammatory cells around these ducts. These findings implied a reduction in cellular activity and proliferation which was confirmed by the absence of an immune reaction after immunohistochemical staining with ki67. The immune negativity of the cells indicated a reduction in cell proliferation and activity.

Submandibular and sublingual salivary glands in Group II treated with Diazepam showed serous acini noticeably decreased in size and number in comparison to control group. The secretory cells presented loss of their cellular outline. Some acini lost all their cell boundaries and appeared to be degenerated and filled with many vacuoles. The cytoplasm became homogenous and deeply stained acidophilic. Also, the cytoplasm of the secretory cells appeared with large vacuolization. The striated ducts decreased in the size and lost their basal striations. The GCT also decreased in number and size. Their lining epithelium showed signs of degeneration. Loss of the normal cellular architecture was accompanied with reduced cellular proliferation detected by the absence of immune reactivity to ki67 in the cells of the salivary glands.

On the contrary, upon examining the parotid salivary glands in Group III, we found that the
secretory cells demonstrated less number of degenerative vacuoles. The nuclei appeared deeply stained. The acini at the periphery of lobes showed fewer pyknotic nuclei. The intralobular duct cells appeared to be normal. There was no inflammatory cells around both acini and ducts. Some blood vessels were dilated. A slight restoration of the tissue repair mechanism was indicated by the presence of immunopositive cells to ki67 in some acinar cells as well as few ductal epithelial cell, marking cellular proliferation in an attempt to restore the damage caused by the drug. This may be attributed to the healing effect of the green tea.

The submandibular salivary gland of the same group presented with slightly atrophied secretory portion and GCT. Some lobules showed more atrophy in their acini and GCT. The cytoplasm of serous secretory cells presented some cytoplasmic vacuolization. Also, the lining epithelium of the GCT showed the same cytoplasmic vacuolization. The striated ducts were widely dilated and had thickened walls. Similarly, this attempt to tissue healing was obvious by the slight elevation in the number of immunopositively stained cells to ki67.

In general, the expression of ki67 was highest in the control group and lowest in the group treated with diazepam. A slight elevation in the values of gene expression in the group treated with both the drug and green tea confirmed the possible role of green tea in the attempt to restore the normal behavioral functions of the salivary gland cells. The degenerative effect of diazepam on the salivary glands ducts and acini which consequently causes the alteration in the salivary secretion and eventually leads to “dry mouth” which passively affects the integrity of the oral structures collectively, might actually be reversed by the proliferation of the acinar and ductal cells in response to the intake of green tea. The findings of this study confirmed that the toxic effect of diazepam on the salivary glands can be reduced by the simultaneous use of green tea.

These findings also clarified the cytotoxic effect of psychotropic drugs on the salivary glands. Grégio et al., studied the effects of chronic administration of a benzodiazepine (diazepam) and an antidepressant (amitryptilne) on the parotid glands of rats and observed hyposalivation and hypertrophy of the serous cells. These findings suggested a possible inhibition of the activity of the myoepithelial cells (originating from nervous stimulation), a decrease in the number of myoepithelial cells following chronic administration of psychotropic drugs, or an alteration in the number of acinar and ductal cells (Grégio M. et al., 2007).

As for mentioned, green tea contains polyphenols, which are strong antioxidants and reduce that damage to the salivary gland. “With green tea polyphenols, we have an agent that’s helping to correct the salivary gland’s abnormal behavior,” stated Douglas Dickinson (Douglas C., 2002).

In a study published in the 2007 issue of “Autoimmunity”, researchers studied the effect of green tea on patients with Sjogren’s syndrome. The team commented that the research has shown that a component of tea called EGCG helped to suppress inflammation. The team found that the group treated with green tea had fewer white blood cells specially lymphocytes, that gather at sites of inflammation, and also lower levels of autoantibodies. The authors concluded that green tea helped to decrease the damage to the salivary glands and therefore helped to reduce the symptoms of Sjogren’s syndrome.

Korany and Ezzat found that in a rat model the administration of natural antioxidants could be of beneficial effect on prevention of cytotoxicity induced by organophosphorous compounds. However, green tea showed more promising results than that of Nigella sativa (Korany S. and Ezzat A., 2011).

Furthermore, studies investigated other beneficial roles of green tea green tea and green tea polyphenols. They were found to have a preventive effect against chronic diseases including heart disease, diabetes, neurodegenerative disease and
cancer (Yang S. et al, 2002 and Higdon V. et al, 2003). Other mechanisms have been proposed to account for the cancer preventive effects of green tea in laboratory animal models. These mechanisms include the inhibition of growth factor signaling and key cellular enzymes. Other studies discussed the possible role of green tea in inhibition of gene transcription and induction of tumor suppressor genes (Chen D. et al, 2004, Yang S. et al, 2006 and Khan N. et al, 2006). The antioxidant activity of green tea polyphenols has been investigated and more recently, the potential mechanism for cancer prevention of these compounds has also been suggested (Tachibana H., 2009 and Butt S. and Sultan T., 2009).

SUMMARY

The results of the present study showed decrease of salivary α amylase activity in rats taking Diazepam. This decrease was marked in rats that take green tea and diazepam. The histomorphometric results revealed the toxic effect of diazepam on salivary gland. The result of this study proves that intake of diazepam led to reduction of salivary secretion and alpha amylase activity and had a cytotoxic effect on salivary glands which can be reduced by using green tea. Green tea lead to marked decrease in alpha amylase activity with correction of xerostomia induced by diazepam, as well as restoration of cellular proliferation and tissue healing. It is important to make future studies on the importance of the inhibition of salivary alpha amylase on the general health of individuals.

RECOMMENDATION

1. Routine use of salivary alpha amylase as stress marker cannot be done in patients with drug induced xerostomia.
2. Use of green tea in treatment of xerostomia has many benefits over other drugs that increase salivary secretions as pilocarbine which lead to stimulation of SAA.
3. Inhibition of SAA has many benefits for diabetic and obese patient as it leads to inhibition of carbohydrate digestion and absorption.
4. Dose of antidiabetic drugs must be re-evaluated in patient who receive diazepam or green tea.

REFERENCES

• Grégio M., Almeida V., Brancher A., Ignáicio A., Machado A., de Lima A. and Luciana A.: Effects of antidepressants and benzodiazepines on stimulated salivary flow rate and