EFFECTS OF MORINGA OLEIFERA AQUEOUS LEAF EXTRACT ON SUBMANDIBULAR SALIVARY GLANDS OF DIABETIC ALBINO RATS

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ABSTRACT

Moringa Oleifera (MO), also known as the ‘drumstick tree,’ is recognized as a nutritious and cheap source of phytochemicals, that have a prominent anti-oxidative effect. Salivary gland dysfunction has been frequently reported in diabetic patients. Diabetes is a chronic metabolic disease that has complications mainly resulting from persistent hyperglycemia.

Aim of the study: To Assess the effect of MO aqueous leaf extract on blood glucose levels in diabetic albino rats and its effect on submandibular salivary glands of diabetic albino rats.

Materials and methods: the study comprised three groups; control, diabetic and MO treated groups. The experiment was terminated after fourteen days. The evaluation was performed by measuring the blood glucose levels and weight. Histological evaluation was done by H&E, PAS and IHC for COX-2.

Results: the blood glucose levels and histological signs of diabetic complications were significantly lowered in the MO treated group.

Conclusion: MO is a promising anti-diabetic treatment and may even reverse some of the diabetic complications.

KEYWORDS: COX-2, Diabetes, Moringa Oleifera, Reactive oxygen species, Submandibular salivary gland.

INTRODUCTION

Moringa Oleifera (MO), also known as the ‘drumstick tree,’ is documented as a nutritious and cheap source of phytochemicals. MO is rich in proteins, β-carotene, vitamins (principally C and E-tocopherol), calcium, iron and potassium. It is also a source bioactive compounds, such as phenolic acids, flavonoids, glucosinolates and isothiocyanates. It also been proved to stimulate both cellular and humoral immune responses (Saini Sivanesan et al. 2016). Not just that, Polyphenols and flavonoids from MO are described as natural antioxidants as they can directly react with superoxide anions and...
lipid peroxyl radicals. Therefore, this plant has numerous medicinal applications and is used as a traditional medicine for the treatment of various illnesses such as skin diseases, ear and dental infections, hypertension, anemia, diabetes and cancer (Al-Asmari Albalawi et al. 2015).

The seromucous submandibular gland is made up of secretory cells of both serous and mucous types. The submandibular gland is a mixed gland, though most of the cells are serous. It has long striated ducts and short intercalated ducts. Extensive cellular degeneration and structural and functional abnormalities have been reported in the submandibular glands of diabetic rats. The mechanisms responsible for these abnormalities are not clear yet (Iro & Zenk 2014).

The incidence of diabetes is increasing in the developing world (Little Gillen et al. 2011). For the majority of healthy individuals, normal blood sugar level is up to 200 mg/dL, when randomly measured. A person is considered to have diabetes when the blood glucose levels are higher. Age, genetics, environment, and lifestyle influence the development of Diabetes. The relative importance of these factors and their combined effects are not yet fully understood. Two types of DM are commonly recognized: type-1 DM (DM1) which results from autoimmune destruction of pancreatic β cells and type-2 DM (DM2) which is characterized by high blood sugar, insulin resistance (Tang Martin et al. 2012).

Hyperglycemia associated with diabetes plays a key role in the pathogenesis of diabetic complications. These complications include stomatitis, geographic tongue, angular cheilitis, delayed mucosal wound healing, mucosal neuro-sensory disorders, dental caries and tooth loss. Periodontal diseases (periodontitis and gingivitis), changes in saliva composition, salivary dysfunction and taste dysfunction leading to a reduction in salivary flow, have also been reported frequently in diabetic patients (Al-Maskari Al-Maskari et al. 2011).

With chronic hyperglycemia, glucose enters the mitochondria and releases reactive oxygen species (ROS), and since beta cells have low number of antioxidants, this in turn causes apoptosis of the beta cells (Tangvarasittichal 2015). Over production of ROS damages all important cellular components, such as DNA, lipids, and proteins, as well as, the disruption of cellular metabolism. Elevated glucose levels causes altered gene expression, signal transduction, cell growth and apoptosis (Stadiman & Levine 2003; Kołodziej Maciejczyk et al. 2017). Hyperglycemia associated with diabetes may also result in de novo accumulation of glycogen, in diabetic salivary glands (Kumar & Pandey 2013) as well as, significant morphological and functional changes including altered secretory protein expression and accumulation of basement membrane material (Mednieks Szczepanski et al. 2009).

In addition, increase in blood glucose levels causes degenerative changes such as vacuolization and the accumulation of lipid droplets in acinar cells and ducts of the salivary glands (Saravanan Ponmurugan et al. 2014). Hyperglycemia also causes expression changes of microRNAs (miRNAs), TGF-β genes, TGF-β proteins and their receptors. Activated TGF-β leads to fibrosis, through SMAD-dependent and independent pathways (Wang Lyu et al. 2018).

Not just that, hyperglycemia and elevated free fatty acids associated with diabetes promote inflammation by stimulating glucose use, along with changes in oxidative phosphorylation. This metabolic dysregulation has been shown to promote a pro-inflammatory state in macrophages (Pollack Donath et al. 2016). This causes up-regulation of the inflammatory response including; iNOS, caspase 1, COX-2, VEGF and NF-κB, as well as, increase capillary permeability and leukostasis (Kiritoshi Nishikawa et al. 2003; Du Tang et al. 2010). This inflammatory response has detrimental effects on the salivary glands of diabetic patients (Yagihashi Mizukami et al. 2011).
MO inhibits elevation of mRNA and protein levels of IL-1, TNF-alpha, iNOS synthase, and COX-2. The suppressive effect was mediated partly by inhibiting phosphorylation of inhibitor kappa B protein and mitogen-activated protein kinases (MAPK). These results indicate that the anti-inflammatory activity from bioactive compounds present in the MO constituents may contribute to reverse the pathogenesis of inflammatory-associated diabetic complications (Muangnoi Chingsuwanrote et al. 2012). Flavonoids like quercetin and phenolics in MO scavenge the ROS released from mitochondria in diabetes, thereby protecting the beta cells and in turn keeping hyperglycemia under control (Gopalakrishnan Doriya et al. 2016). MO decreases blood glucose levels, which is associated with decreased intestinal glucose uptake and slowing gastric emptying time by the fibers in MO leaf. In addition, MO has been proved to increase insulin secretion in healthy subjects without any adverse effects on liver and kidney function (Jaiswal Rai et al. 2013; Fard Arulsevan et al. 2015).

**AIM OF THE STUDY**

To assess the effect of MO leaves on blood glucose level in diabetic albino rats and its effect on submandibular salivary gland of diabetic albino rats.

**MATERIALS AND METHODS**

**MO aqueous extract preparation**

MO aqueous extract was prepared by mixing 10 gm of dried and powdered MO leaves with 100 mL of distilled water for 24 h and then stored at 4°C. Afterwards, the mixture was filtered twice through a2-μm pore filter paper. The aqueous extract stock solution (100 mg/mL) was stored at 4°C for up to 5 days, or freshly prepared for each set of experiment (Tuorkey 2016).

**Animals care and treatments**

Twenty-one male albino rats (200-250 gm) were adapted in the laboratory for two weeks under the same natural environmental conditions of temperature and photoperiod. This was done in the animal house of the medical research center in Future University in Egypt. All the procedures were in accordance with the protocol of the National Animal Care and Use Committee and Guidelines for the Care and Use of Experimental Animals. Rats were randomly divided into three groups (7 rats each) as follows: control group (Group I), diabetic untreated group (Group II), and diabetic group treated with 100 mg/kg of MO aqueous extract given by an oral gavage (Group III). The experiment was terminated after fourteen days of diabetes induction (Tuorkey 2016).

Diabetes was induced with two intraperitoneal injections of alloxan (Sigma-Aldrich), previously dissolved in ice-cold ultra pure water, pH 6.8 (Millipore system). The first dose was 150 mg/kg, and the second dose was 100 mg/kg given two days after the first dose, to ensure the induction of diabetes throughout the experimental duration, as recommended by Bromme et al. 2000 (Brömme Mörke et al. 2000). Diabetes threshold was blood glucose level >250 mg/dL (one touch ultra 2, China).

**Blood glucose and weight measurements**

Random Blood glucose levels and weight were measured after two, seven and fourteen days of the second Alloxan injection (One Touch Ultra 2, China & Balance Shimadzu Unibio TX423L, Japan).

**Histological preparation**

At the end of the experimental period, the rats were sacrificed by an overdose of anesthesia (chloroform, HPLC Sigma Aldrich), the submandibular salivary glands were immediately removed, labeled and fixed in 10% formaldehyde and prepared for:
**Histochemistry:**

a- H&E: The sections were stained with Hematoxylin and Eosin stain, for the detection of any structural changes of submandibular salivary gland between the groups.

b- Peraiodic acid Schiff (PAS) to detect polysaccharides such as glycogen, and mucosubstances such as glycoproteins, glycolipids and mucins in tissues.

**Immunohistochemistry:**

Monoclonal COX-2 antibody (Cat. No. 240R-18, key code: CMC 24031022, Cell Marque, Rocklin, CA 95677, USA) was used according to the manufactures’ instructions. In this study, avidin-biotin complex peroxidase (ABC-P) method was used for immunohistochemistry.

For negative control, primary antibody was not added to the sections.

**Microscopic evaluation:**

The histochemically and immunohistochemically stained sections, were examined under the light microscope. For the immunohistochemical evaluation, the percentage of immunopositive cells, 100 cells calculated in 10 different microscopic high-powered fields of each slide were examined under the 40x objective of a trinocular microscope Olympus, BX46, Japan and evaluation was carried out using the image analysis software (image J,1.41a, NIH, USA).

**Statistical analysis:**

In the statistical evaluations, Student t test was used. Calculations were made using the SPSS 13.0 program pack. P≤0.05 was accepted as statistically significant.

**RESULTS**

**Blood glucose and body weight measurements**

Hyperglycemia was initially observed in rats in group II and III (445.2 mg/dl and 458.2 mg/dl, respectively) after the 2nd injection of Alloxan. After fourteen days, the blood glucose level rose in group II and was reduced drastically in group III (593.4 mg/dl and 92 mg/dl, respectively) while the body weight did not vary much (236.6 grams and 248.6 grams, respectively). The blood glucose and the body weight measurements remained more or less steady in group I throughout the experiment. The blood glucose and body weight measurements are summarized in (Table 1) (fig. 1).

<table>
<thead>
<tr>
<th></th>
<th>2 days after the 2nd Alloxan injection</th>
<th>After 7 days</th>
<th>after 14 days</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Glucose Level g/Dl</td>
<td>Weight grams</td>
<td>Glucose Level mg/dl</td>
</tr>
<tr>
<td>Control (group I)</td>
<td>86.5</td>
<td>248</td>
<td>84.5</td>
</tr>
<tr>
<td>Diabetes (group II)</td>
<td>445.2</td>
<td>246.2</td>
<td>486.8</td>
</tr>
<tr>
<td>MO (group III)</td>
<td>458.2</td>
<td>244.8</td>
<td>343</td>
</tr>
</tbody>
</table>
**Histological evaluation**

**Histochemistry**

**H&E**

**Group I**

Histological examination of the submandibular salivary glands of Group I showed the normal architecture and morphology of the major salivary glands. The glands consisted of a spherical single type of secretory portion; serous acini surrounding a narrow lumen. The acinar cells appeared pyramidal in shape with its base resting on basement membrane and the apex toward the lumen. The cells had basophilic cytoplasm with spherical basally situated nuclei. The intercalated ducts were seen between the acini, they are small and rounded, lined by cuboidal epithelium with central rounded nuclei and eosinophilic cytoplasm. (fig. 2- I)

The striated ducts were columnar in shape with a centrally placed nucleus and were arranged in a single layer. The cytoplasm was eosinophilic with basal striations perpendicular to the basement membrane. The granular convoluted tubules appeared kidney shaped lined by columnar epithelium with eosinophilic cytoplasm. The excretory ducts were found in the connective tissue septa between lobes and lobules. They had large lumen and lined with pseudostratified columnar epithelium with goblet cells. The gland appeared to be highly vascularized, the blood vessels located in the connective tissues around these ducts, also the connective tissue septa separating the lobules appeared with normal thickness. (fig. 2- II)

**Group II**

The diabetic submandibular glands of Group II revealed loss of the normal architecture which was appeared in both the parenchyma and connective tissue stroma. The acinar cells were hypertrophied and compacted together and showing indistinct boundaries. They allowed space for connective tissues to separate them and showed areas of extravasated blood. Some of the intercalated and striated ducts appeared distorted with no lumen and some appeared with narrow lumen. The excretory ducts surrounded by thick and dense concentric bands of fibrous tissue, some of their lumens appeared with stagnant secretions. The cells of the excretory duct showed flattening and some cytoplasmic vacuolations, their nuclei appeared with variable forms and density. The blood vessels wall also showed fibrosis, they appeared dilated and engorged with RBCs. (fig. 2-III & IV)

**Group III**

The submandibular glands of the MO treated rats revealed the normal general features appeared
in the glands of the control group. The acini had basophilic cytoplasm with spherical basally situated nuclei, they had normal size and lumen, intact cell boundaries and were not separated by connective tissues.

The intralobular ducts exhibited normal appearance and lumen size, no areas of extravasated blood were noticed. The excretory duct appeared normal as well however some areas showed some flattening without goblet cells. The connective tissue stroma surrounding the duct appeared with normal thickness, containing numerous blood vessels of regular wall thickness. (fig.2- V & VI)

PAS

Group I

Histochemical examination of the submandibular gland of the control group showed few reactions to the PAS- stain, these reactions were seen in the lumina of some acinar cells, the cells of striated and intercalated ducts. (fig.3-I)

Group II

The submandibular gland of the diabetic group revealed a positive reaction to the PAS stain that was in the lumina of some acinar cells, the cells of striated and intercalated ducts. (fig. 3-II)

Fig 2: (I) Photomicroscopic picture of Group I showing the serous acinar cells and the striated ducts with clear basal striations. (II) Photomicroscopic picture of Group I showing the excretory duct (A), connective tissue septa and blood vessels (B). (III) Photomicroscopic picture of diabetic group, group II, showing hypertrophied acinar cells (A), distorted intercalated and striated ducts (B), extravasated blood (C). (IV) Photomicroscopic picture of group II showing flattening of nuclei of the excretory ducts (A), thick bands of fibrous tissues (B) and thick walled blood vessels engorged with RBCs (C). (V) Photomicroscopic picture of group III showing, normal striated and intercalated ducts (A) surrounded by serous acini with intact boundaries and normal features (B). (VI) Photomicroscopic picture group III showing the excretory duct surrounded by connective tissue stroma and blood vessels. (H&Ex200)
**Group III**

Histochemical examination of the submandibular gland of the treated group showed a positive reaction to the PAS-stain. (fig. 3- III)

**Immunohistochemistry**

**Group I**

Immuno-positivity with COX-2 antibody was seen in the control group in the intercalated and striated ducts while the acinar cells were immune-negative. The immune-positive cells exhibited the positive reaction in the cytoplasm while the nuclei were immune-negative. (fig. 4- I)

**Group II**

The overall Immuno-positivity with COX-2 antibody was relatively higher in this group than in the control group. The intercalated and striated ducts as well as some of the acinar cells were immune-positive. The immune-positive cells exhibited the positive reaction in the cytoplasm while the nuclei were immune-negative. (fig. 4- II)

Fig. (3) (I) Photomicroscopic picture of group I, showing reactions to PAS stain in the acinar cells, striated and intercalated ducts. (II) Photomicroscopic picture of group II, showing positive reaction to PAS stain. (III) Photomicroscopic picture of group III, showing reactions to PAS stain in the acinar cells and the ductal elements of the submandibular gland. Note that the reaction to PAS stain was almost similar in the three studied groups (PAS x200).

Fig (4) (I) Photomicrograph of COX-2 expression in submandibular gland of group I. Cytoplasmic immune-positivity was noted in the ductal cells (A) while the acinar cells were immune-negative. (B). (II) Photomicrograph of COX-2 expression in submandibular gland of group II. Cytoplasmic immune-positivity was noted in the ductal cells (A) as well as the acinar cells (B). (III) Photomicrograph of COX-2 expression in submandibular gland of group III. Cytoplasmic immune-positivity was noted in the ductal cells (A) while the acinar cells were immune-negative (B). (COX-2x200)
**Group III**

Immuno-positivity with COX-2 antibody in group III treated with MO was similar in pattern and intensity to this seen in the control group. The immune-positive reaction in the intercalated and striated ducts while most of the acinar cells were immune-negative. The immune-positive cells exhibited the positive reaction in the cytoplasm while the nuclei were immune-negative. *(fig. 4-III)*

**Statistical results**

The results showed that the least value of mean area fraction of COX-2 immunopositivity was noted in the group I (1.05514), followed by the group III (1.10186), while the highest reaction was noted in the group II (13.80343). *(Table 2) (fig. 14).* Anova test showed that there are significant difference in the mean area fraction of immunopositivity of COX-2 between the studied groups. *(Table 3).*

On performing the post hoc test it was observed that there was no statistically significant difference between the immunopositivity for COX-2 in group I and group II (p = 1.00), but the difference was highly significant between both of group I and group III and group II (p = 0.001). *(Table 4)*

**TABLE (2) descriptive analysis of the mean area fraction of immunopositivity with COX-2 among the studied groups**

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Std. Deviation</th>
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<tbody>
<tr>
<td>Group I</td>
<td>1.05514</td>
<td>1.020961</td>
</tr>
<tr>
<td>Group II</td>
<td>13.80343</td>
<td>.620055</td>
</tr>
<tr>
<td>Group III</td>
<td>1.10186</td>
<td>9.172436</td>
</tr>
</tbody>
</table>

**TABLE (3) ANOVA for the mean area fraction of immunopositivity with COX-2 among the studied groups**

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>13.248</td>
<td>.000</td>
</tr>
<tr>
<td>Within Groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
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</tbody>
</table>

**TABLE (4) Post Hoc Test for the mean area fraction of immunopositivity with COX-2 among the studied groups**

<table>
<thead>
<tr>
<th>(I) Groups</th>
<th>(J) Groups</th>
<th>Mean Difference (I-J)</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Group III</td>
<td>-0.046714</td>
<td>1.000</td>
</tr>
<tr>
<td>Group I</td>
<td>Group II</td>
<td>-12.748286*</td>
<td>.001</td>
</tr>
<tr>
<td>Group III</td>
<td>Group II</td>
<td>-12.701571*</td>
<td>.001</td>
</tr>
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</table>

*p≤0.05 significant*
DISCUSSION

MO has been studied intensely over the past decade. It is considered to be an affordable and easily grown source of phytochemicals. MO has been proved to improve the blood glucose levels in diabetic patients. Several studies report the effect of MO on different tissues of diabetic patients, especially, the retina and the kidney. However, few reports are available for the effect of MO extracts on salivary glands of diabetic patients. For this reason, this study evaluated the effect of MO on salivary gland tissue of diabetic rats (Tuorkey 2016).

Diabetes mellitus is the fifth most common chronic condition and the sixth most frequent cause of death among the elderly (Latti Birajdar et al. 2015). The main drawback of currently available anti-diabetic drugs is their potential toxicity on the long run and their lack of efficiency (Little Gillen et al. 2011). The most favorable anti-diabetic drug should combine both hypoglycemic and antioxidant properties. For this reason, there is a growing interest in evaluating herbal remedies, which are rich in anti-oxidants and have negligible side effects (Matsushita Mahmoodi et al. 2012).

The etiology of the complications associated with diabetes are mainly due to oxidative stress, perhaps as a result of hyperglycemia which leads to protein oxidation and glycation. Antioxidants like vitamins E and C, have been considered as treatments to counter act this oxidative stress. Vitamin E has been found to decrease the susceptibility of lipid peroxidation. MO is a rich source of these vitamins (Jaiswal Rai et al. 2013).

The results of this study show that there was a significant reduction in the blood glucose levels in the MO treated group (group III) when compared to the untreated diabetic group (group II). The histopathological sections assessment results correlated with the blood glucose levels, in terms of fibrosis, vaculation and vascular changes, that may be attributed to the hyperglycemia of the diabetic group. However, the PAS positive granules in the acini and ducts of the salivary glands of the untreated diabetic group (group II), were relatively similar to those noted in both the control group (group I) and MO treated group (group III). These results were contradictory to those of (McCubrey et al., 2014 and Maciejczyk Kossakowska et al. 2017), who stated that there was glycogen accumulation in oral epithelial cells due to the decrease in Glycogen Synthase Kinase-3 (GSK-3) enzyme phosphorylation (McCubrey Steelman et al. 2014). This may be due to the difference in the studied specimens where in this study the tissue studied was that of the submandibular gland and not lining mucosa.

(Maciejczyk Kossakowska et al. 2017), reported massive accumulation of lipids in the glandular cells, glandular tissue hypertrophy, as well as the accumulation of cells typical of chronic inflammation (lymphocytes, macrophages, and neutrophils) in the salivary glands of diabetic rats (Maciejczyk Kossakowska et al. 2017). Similar changes were noted in the histological sections of this study. There was evident intra-cytoplasmic vacuolations histopathologically in group II of this study. Despite the fact that, the present study did not confirm the nature of these vacuolations, these vacuoles, however, appeared to be a lipid nature since they were removed during fixation and processing of the samples for H&E staining.

Added to that, there was increased deposition of collagen fibers around the salivary gland parenchyma in group II of this study. This may be attributed to the fact that high glucose increases TGF-beta expression, and the elevated TGF-β increases the deposition of collagen fibers. Several studies reported that hypertrophic and matrix-stimulatory effects of high glucose are prevented by anti-TGF-beta therapy (Chen Jim et al. 2003).

Hyperglycemia can also cause diabetic vascular complications by triggering oxidative
stress, forming advanced glycation end products (AGEs), increasing the flux of glucose to sorbitol, activating protein kinase C (PKC), and provoking inflammation. Diabetic microvascular disease is pathologically characterized by excessive vessel growth and increased vessel permeability (Singh Bali et al. 2014). Also, it has been proved that, hyperosmotic stress always accompanies hyperglycemia, and may therefore contribute to some aspects of hyperglycemia-induced vascular injury.

Hyperglycemia has been reported to increase human β-cell production of interleukin-1β and that this cytokine can induce COX-2 expression. COX-2 participates in inflammation and angiogenesis. COX-2 regulates the expression and activity of matrix metalloproteinase (MMP-9), which degrades extracellular matrix and promotes endothelial cell migration and vessel sprouting (Chen Jim et al. 2003). These findings may explain why the histopathological results of group II in this study showed evident abnormalities in the blood vessels, from vasodilatation to extravasation of RBCs, as well as, a significant increase in COX-2 expression.

Group III in this study showed a significant reduction in the blood glucose levels on treatment of the diabetic rats with MO extract. This may be due to the effect of MO fibers on the digestion and uptake of glucose from the intestine. The results of this study confirmed that, on treatment of diabetic rats with MO, the fibrosis and vacuolizations of the salivary glands were reduced. This may be due to effects of tocopherols and anti TNF-alfa of the MO extract (Fard Arulselvan et al. 2015).

Researches showed that, MO extract inhibited mRNA of IL-6 and COX-2. The suppressive effect was mediated partly by inhibiting phosphorylation of inhibitor kappa B protein and MAPK (Muangnoi Chingsuwanrote et al. 2012). As IL-6 and COX-2 are inflammatory mediators their reduction by administration of MO results in the reduction in their detrimental effects on the salivary gland tissues. These results were consistent with the results of this study where group III showed a significant reduction in the COX-2 expression.

CONCLUSIONS

MO Aqueous extract administration can significantly reduce hyperglycemia of diabetic rats. MO Aqueous extract administration can reverse some of the inflammatory effects of hyperglycemia associated with diabetes.

Conflict of interest

The authors have declared that there is no conflict of interest.

REFERENCES


