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COMPARING THE EFFECT OF MORINGA OLEIFERA AQUEOUS LEAVES EXTRACT TO INSULIN ON SUBMANDIBULAR SALIVARY GLANDS OF STREPTOZOTOCIN INDUCED DIABETIC ALBINO RATS (HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY)

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ABSTRACT

Introduction: Diabetes Mellitus Type I occurs due to autoimmune degeneration of beta cells of islets of Langerhans and can be treated by insulin administration. Moringa Oleifera also has been traditionally used for treatment of diabetes due to its hypoglycemic activity.

Purpose: is to compare the antidiabetic effect of MO leaves extract with insulin on the submandibular salivary glands of diabetic Albino rats.

Materials & Methods: Twenty-eight adult male Albino rats were used: control, diabetic, insulin-treated and Moringa-treated groups. Rats of control group were injected intraperitonial with citrate buffer, while rats in other groups were injected intraperitonial with Streptozotocin . Rats of both insulin & Moringa-treated groups received subcutaneous injection of insulin of 5 IU/kg or a daily oral dose of leaves extract of 250 mg/kg respectively for twenty-eight days.

Results: Increased inter-acinar spaces with many cytoplasmic vacuolations were detected in the diabetic group. Intercalated duct (ID) cells showed decreased cellular height. Striated duct (SD) and Excretory duct (ED) cells revealed some degenerated cells. Serous acini and IDs in treated groups were almost similar to control. SDs showed few areas with cellular degeneration in insulin-treated group. EDs were also comparable to those of the control. In both insulin and Moringa-treated groups, few localized areas of Proliferating cell nuclear antigen (PCNA) cytoplasmic and nuclear reactions were detected in some acini, SDs, GCTs. EDs showed few localized areas of nuclear reaction.

Conclusion: Moringa Oleifera leaves extract showed a comparable effect to insulin on decreasing signs of degeneration and cellular damage caused by diabetes.

KEYWORDS: Moringa, Insulin, Diabetes, Submandibular.

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INTRODUCTION

Diabetes Mellitus is classified by WHO into type I (insulin dependent or Juvenile type) and type II (non-insulin dependent or adult type) [1]. Type I is due to autoimmune degeneration of beta (β) cells of islets of Langerhans in the pancreas [2, 3] and can be treated by insulin administration. Type II is due to deficient insulin secretion, or the defect of insulin receptors and it can be treated by diet and oral hypoglycemics [4-6].

Streptozotocin (STZ) is a nitrosamide extracted from Streptomyces Acromogenes and it has been commonly used to induce diabetes type I in animal models [7-9].

STZ can destroy β-cells [10] by generating ROS that result in oxidative stress and cell death and subsequently leads to changes in blood insulin and glucose concentration [11].

Previous studies observed few small sized lipid droplets, autophagic structures in acini and degeneration of acinar cells in submandibular glands of rats with induced diabetes [12, 13]. Mitochondrial degeneration and lipid droplets were observed in the cytoplasm of acinar cells in submandibular glands of diabetic New Zealand rabbits[14].

Insulin, which is produced in β-cells, is a critical regulator of metabolism. Insulin is secreted primarily in response to glucose, while other nutrients such as free fatty acids and amino acids can augment glucose-induced insulin secretion. In addition, various hormones, such as melatonin, estrogen, leptin, growth hormone, and glucagon like peptide-1 also regulate insulin secretion [15]. Islet glucose metabolism is strongly activated following meal intake and leads to both increased β-cell insulin secretion and suppressed α-cell glucagon secretion to return blood glucose levels back to baseline [16].

Moringa Oleifera (MO) is a member of the Moringacea family and is known as drumstick tree, horse radish tree or Ben tree. It is considered as a good source of amino acids, vitamins and phenolics [17-19].

Moringa has been traditionally used for treatment of diabetes[20]. It was reported that MO leaves are a powerful source of polyphenols including quercetin-3- glycoside, rutin and kaempferol glycosides which may play a role in its hypoglycemic activity[21]. In addition, terpenoids in MO leaves might have been able to stimulate β-cells resulting in secretion of insulin [22]. Flavonoids are postulated to act as insulin secretagogues or insulin mimetics[23]. Flavonoids are also known to possess a hypoglycemic action[24].

Proliferating cell nuclear antigen (PCNA) is involved in a wide range of functions in the nucleus. However, a substantial amount of PCNA is also present in the cytoplasm, although their function is unknown [25]. PCNA is a critical eukaryotic replication accessory factor that supports DNA binding in DNA processing, such as DNA replication, repair, and recombination [26].

So, the aim of the present study is to compare the antidiabetic effect of MO leaves extract with insulin as a traditionally used medication on the submandibular salivary glands of STZ-induced diabetic Albino rats.

MATERIALS AND METHODS

Twenty-eight adult male Albino rats weighing between 200-250 grams were used in this study. The rats were housed in the Animal House of "The Medical Research Center" in Ain Shams University. Rats were kept under good ventilation and adequate stable diet consisting of fresh vegetables, dried bread and tap water throughout the experimental period.

All animal experimental procedures used were approved by institution guidelines of Ain Shams Ethical Committee (approval n= FDASU-RECIR 061903).

Materials:

1- Streptozotocin: (purchased from Sigma Chemical Co., St. Louis, USA) in powder form and was dissolved in citrate buffer immediately before use.

2- Insulin: (purchased from Novo Nordisk Pharmaceuticals Ltd.) in the form of suspension ready for injection.

3- Moringa Oleifera leaves: (obtained from the National Research Center in Dokki, Giza) in the form of powder and an aqueous extract was prepared.

Citrate buffer preparation: Citric acid and sodium citrate were prepared in the National Research Center. 28 ml of citric acid and 22 ml of sodium citrate were mixed with 50 ml of distilled water before dissolving STZ in the buffer. The Citrate buffer solution had pH 4.5.

Moringa Oleifera aqueous extract Preparation:

This was done by adding 1g of dried powdered leaves of MO to 10 ml of boiled distilled water for 5 minutes. The mixture was then filtered twice using a sterile filter paper. The stock solution was 100 mg/ml [30]. Rats received 250mg/0.25 ml/kg.

Experimental Design:

The rats were randomly divided into 4 groups, seven rats each. After an overnight fasting, rats were subjected to intraperitonial (i.p.) injection either with citrate buffer or STZ for one time at the beginning of the study.

1) Control Group (group I):

Rats were injected i.p. with citrate buffer (1 ml/kg of body weight) [27].

2) Diabetic group (group II):

Rats were injected i.p. with STZ (40mg/kg of body weight) dissolved in 1ml of citrate buffer[28]. After two days from injection, rats with blood glucose level above 250mg/dl were considered diabetic [23].

3) Insulin-treated group (group III):

Rats were first treated as in group II then, once diagnosed diabetic; they received a daily dose of subcutaneous injection of insulin of 5 IU/kg of body weight [29]. The treatment was continued for twenty eight successive days.

4) Moringa Oleifera-treated group (group IV):

Rats were first treated as in group II then, once diagnosed diabetic; they received an oral daily dose of MO extract of 250 mg/kg of body weight[22], through an oral feeding tube. The treatment was continued for twenty eight successive days.

At the end of the experiment, rats were terminated separately by overdose of anesthesia and dissected to obtain the submandibular salivary glands. The specimens were fixed immediately in 10% formalin solution for two days and then washed under running tap water to remove all fixative residues. Specimens were then dehydrated by being transferred in increasing concentrations of alcohol (50%, 60%, 80%, 90%, 96% and then absolute alcohol), then cleared by xylol. The dehydrated samples were then embedded in the center of paraffin wax blocks to be sectioned by microtome to a thickness of four to five microns.

Sections were transferred in decreasing concentrations of alcohol (96%, 80%, 70%, 60%, 50% and then distilled water) to be stained by:

1- H&E stains [31] for routine histological examination.

2- Immunohistochemical staining using PCNA antibody to evaluate DNA damage. Sections were subjected to antigen retrieval in citrate buffer (10 mM, pH 6.0) at 98°C for 20 min. Then, the sections were incubated in 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase. The sections were incubated with primary mouse monoclonal antibody anti-PCNA

(FL-261; Santa Cruz Biotechnology, CA, U.S.A., diluted 1:100) overnight at 4°C. After washing in PBS, the antigen-antibody interaction for PCNA was detected using peroxidase conjugated polymer for 45 min. The immunohistochemical reactions were revealed in DAB chromogen. The sections were counterstained with hematoxylin [32].

RESULTS

I-H&E results

The acini of control group were lined by pyramidal cells with basophilic cytoplasm and basally located nuclei. ID cells appeared cuboidal in shape with large, rounded nuclei. The cells lining GCTs showed basal rounded nuclei and esinophilic cytoplasm. SD cells were columnar with rounded nuclei and basal striations and the ducts were surrounded by normal sized blood vessels **(figure 1A)**. The lining of EDs was pseudo stratified columnar epithelium with goblet cells. EDs were surrounded by fibrous connective tissue **(figure 1B)**.

Increased inter-acinar spaces were detected in the diabetic group with some desquamated cells between acini. Acinar cells showed many cytoplasmic vacuolations. ID cells showed decreased cellular height. GCT cells showed some vacuolations with decreased acidophilic appearance of the cytoplasm **(figure 1C)**. SD and ED cells revealed some degenerated cells and the EDs showed stagnant secretion in their lumens **(figure 1D)**.

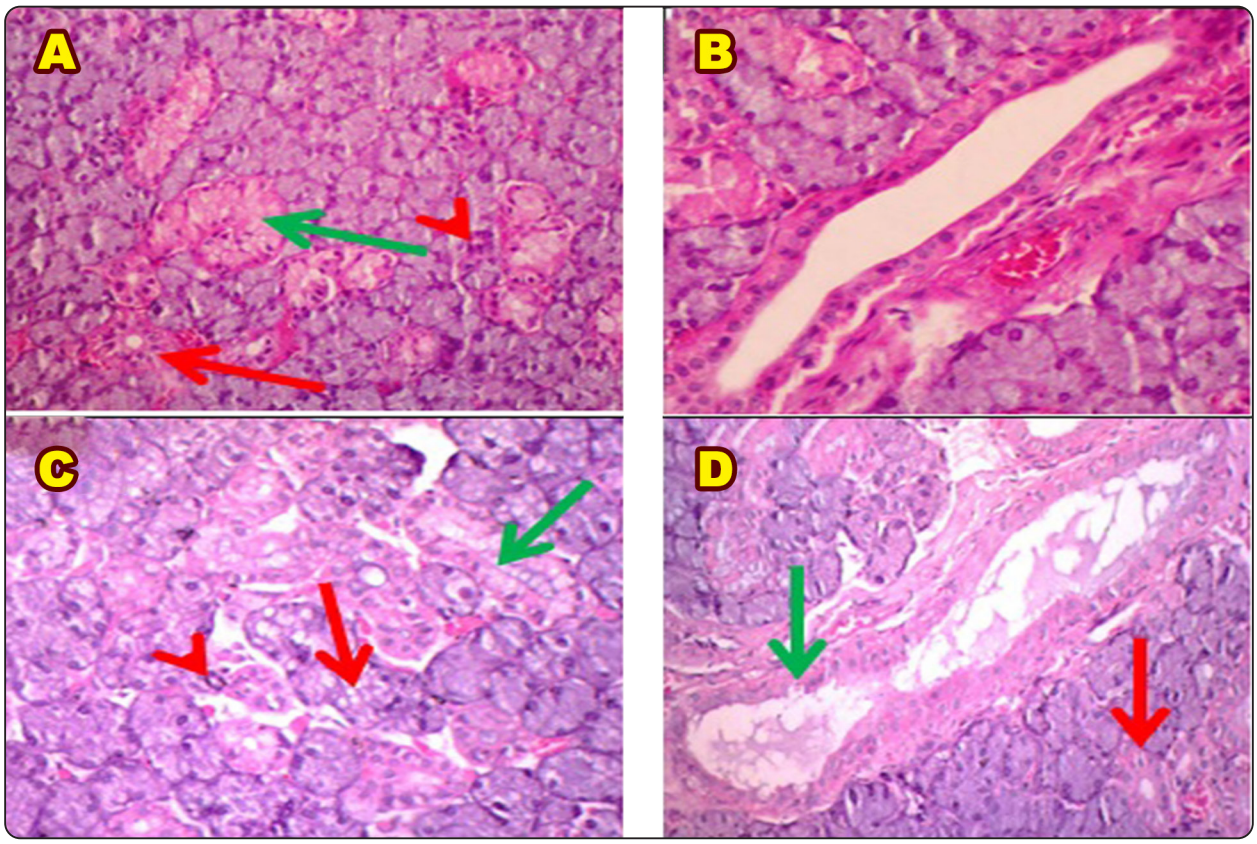

Fig. (1) (A): Submandibular gland of control group showing normal appearance of serous acini, ID (arrow head), GCT (green arrow) and SD (red arrow) surrounded by normal sized blood vessel. (B): ED is lined by pseudo stratified columnar epithelium with goblet cells. (C): Acinar cells of diabetic group showing many cytoplasmic vacuolations (red arrow), ID cells with decreased cell height (arrow head). GCT cells have vacuolations with decreased acidophilic appearance of the cytoplasm (green arrow). (D): SD (red arrow) and ED (green arrow) cells showing some degenerated cells with stagnant secretion in the ED lumen (H&Ex200).

Serous acini and IDs in insulin-treated group were almost similar to control group. SDs appeared comparable to control except for few areas with cellular degeneration. GCT cells showed some degeneration with decreased acidophilic appearance of the cytoplasm in some samples **(figure 2A)**, while others appeared normal. EDs showed decreased cellular height **(figure 2B).**

Serous acini in Moringa-treated group were almost similar to the control except for few cytoplasmic vacuolations. IDs, SDs and GCTs showed normal cellular lining **(figure 2C).** EDs were also comparable to those of the control **(figure 2D).**

II- Immunohistochemical results:

The gland in the control group showed few localized cytoplasmic and nuclear reactions in some serous acini and GCTs. Positive nuclear reaction was detected in SDs, **(figure 3A)** and EDs **(figure 3B)**.

In the diabetic group, diffuse areas of cytoplasmic and nuclear reactions were seen in acini, SDs, GCTs **(figure 3C)** and EDs **(figure 3D)**.

In both Insulin and Moringa-treated groups, few localized areas of cytoplasmic and nuclear reactions were detected in some acini, SDs, GCTs **(figure 4A and B).** EDs showed few localized areas of nuclear reaction **(figure 4C and D)**.

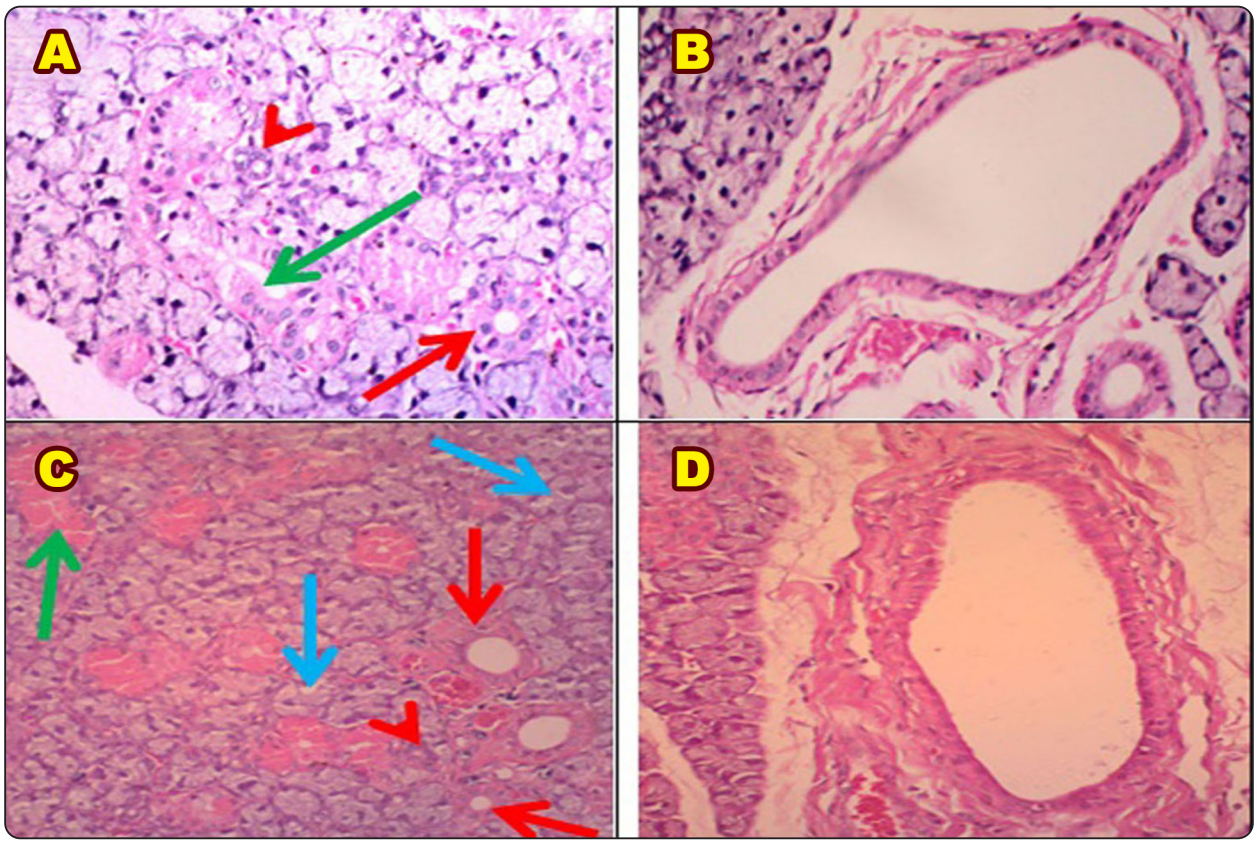

Fig. (2): (A): Serous acini and ID (arrow head) in insulin-treated group appear similar to control. SDs show few areas of cellular degeneration (red arrow). GCT cells show some degeneration with decreased acidophilic appearance of the cytoplasm (green arrow). (B): ED has decreased cellular height. (C): Serous acini of Moringa-treated group with normal appearance except for few vacuoles (blue arrows). Normal ID (arrow head), SDs (red arrows), and GCT (green arrow). (D): ED similar to control group (H & E x 200).

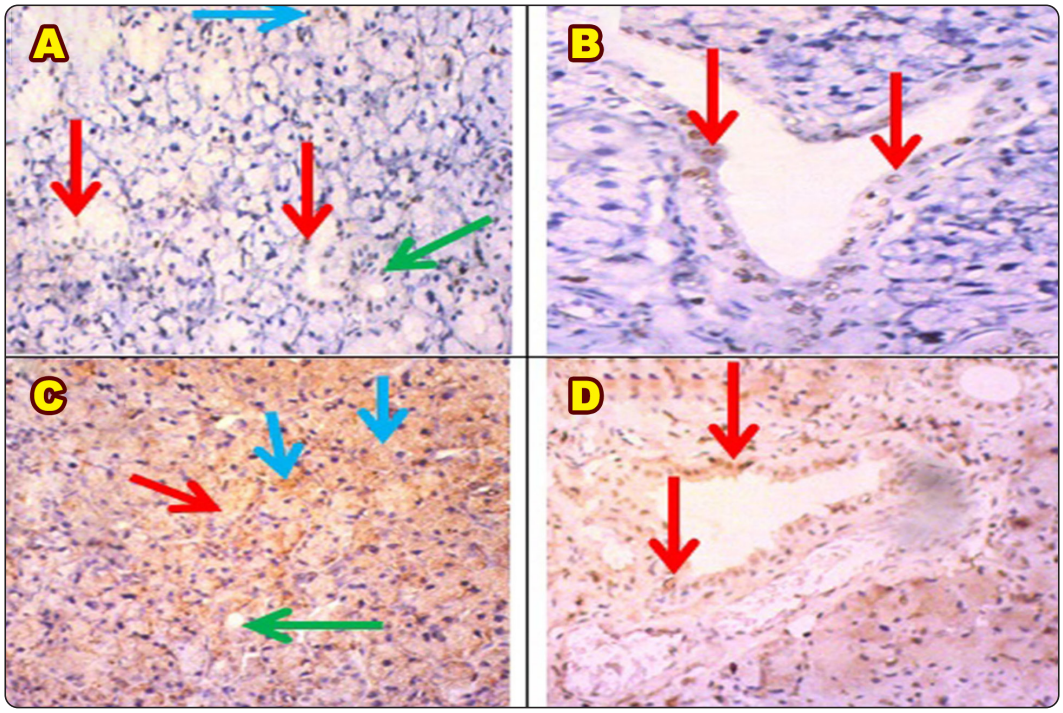

Fig. (3) (A): Some serous acini of control group (blue arrow) and GCT (red arrows) show few localized cytoplasmic and nuclear reactions. SD (green arrow) shows positive nuclear reaction. (B): ED shows few positive nuclear reactions (red arrows). (C): Acini of the diabetic group (blue arrows), GCT (red arrow) and SD (green arrow) show diffuse areas of cytoplasmic and nuclear reactions. (D): ED has diffuse areas of cytoplasmic and nuclear reactions (red arrows) (anti-PCNA x 200).

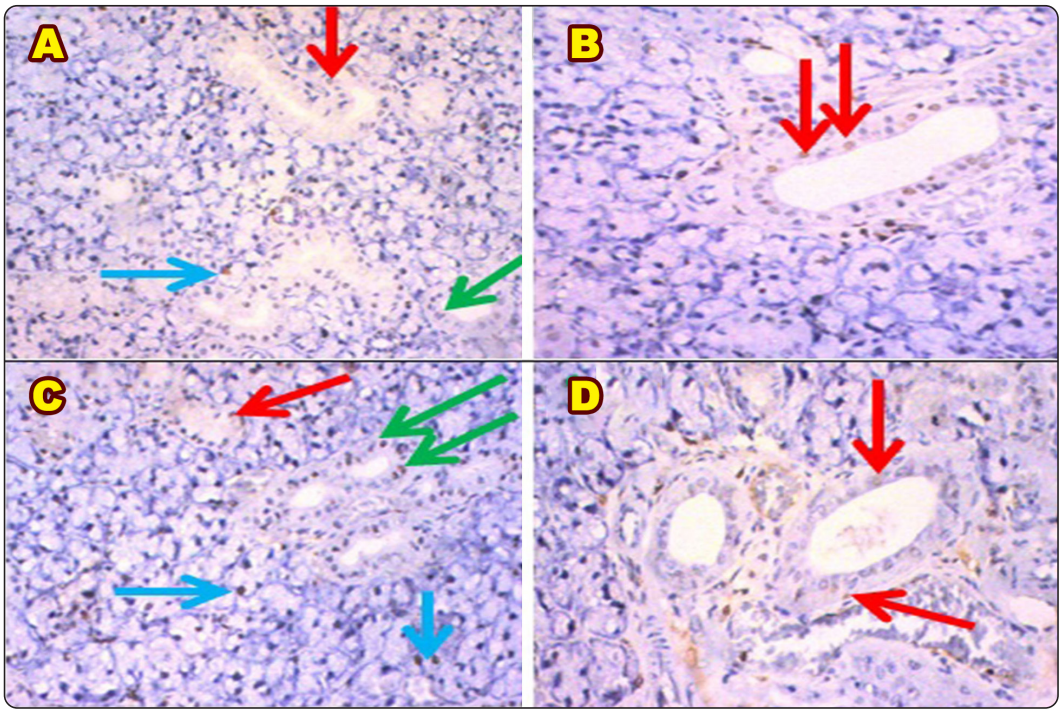

Fig. (4) (A): Some acini in Insulin-treated group (blue arrow), GCT (red arrow) and SD (green arrow) reveal few localized areas of cytoplasmic and nuclear reactions. (B): ED has few localized areas of nuclear reactions (red arrows). (C): Some acini of Moringa-treated group (blue arrows), GCT (red arrow) and SD (green arrows) reveal few localized areas of cytoplasmic and nuclear reactions. (D): ED has few localized areas of nuclear reactions (red arrows) (anti-PCNA x 200).

TABLE (1) Mean, standard deviation and P values of PCNA positive area percentage in submandibular glands of all groups.

PCNA area%	Control group *(n=7)*	Diabetic group *(n=7)*	Insulin treated group *(n=7)*	Moringa treated group *(n=7)*	ANOVA	p-value
Mean±SD	1.30±0.59	24.07±2.64a	16.06±2.58ab	15.26±2.76ab	115.914	<0.001**
Range	0.7-2.1	20.4-27.3	12.4-19.4	10.85-19.2		

***p-value <0.001* *a: Significant difference with control group.* *b: Significant difference with diabetic group.*

III- Statistical results

PCNA positive area percentage

By using ANOVA test, a comparison between PCNA positive area percentages of all groups was done. The positive area percentage in diabetic group was significantly higher than the control group. Both insulin and Moringa-treated groups, showed significant increase in PCNA positive area percentage when compared to the control group and showed significant decrease when compared to diabetic group. However, insulin and Moringa-treated groups presented statistically insignificant difference to each other **(Fig.5, Table 1)**.

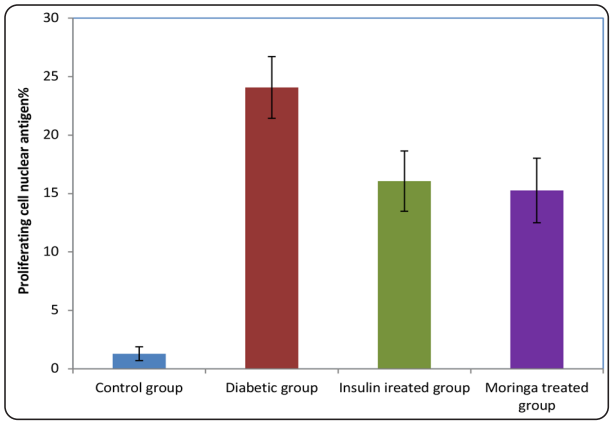

Fig. (5): Bar chart of PCNA area percentage in submandibular glands between groups.

DISCUSSION

It has been documented that STZ reaches its peak in destructing β-cells at three to four weeks[33]. Hyperglycemia resulting from STZ injection has been used by many authors as a common method to study the effect of different antidiabetic drugs [34].

In the present study, increased inter-acinar spaces were seen in diabetic group. This agrees with an earlier study that documented a decrease in acinar size after diabetes induction by Alloxan in rats [35]. This might be due to the decrease of released secretions from acinar cells as a result of growth failure, degeneration of cells, nuclear and cytoplasmic atrophy and disorganization of cell membrane after diabetes induction [35].

Desquamated cells could be seen between acini which could be explained by ***Adams and watt*** [36] who suggested that when an epithelial cell loses its contact with extracellular matrix, it sheds and is replaced by another cell later.

Cytoplasmic vacuolations in acinar cells in the current study diabetic group was previously explained by the accumulation of lipid droplets due to a decrease in the amount of lipid used for limiting membrane formation resulting in the storage of excess lipids as lipid droplets in the cytoplasm[37]. Furthermore, it has been reported that the accumulation of secretory granules occurs to form larger vacuolated structures and several autophagic vacuoles in submandibular acinar cells in diabetic rats [38]. ID cells showed decreased cellular height and

GCT cells showed some vacuolations. SD and ED cells revealed some degenerated cells. This agrees with ***AbuBakr et al.,*** [39] who found degeneration in the lining cells of duct system in submandibular gland of Alloxan induced diabetic rats.

In the current research, GCTs of diabetic group showed decreased acidophilic appearance of the cytoplasm. This has been previously documented as a significant reduction in density of secretory granules of the granular ducts in STZ-induced diabetes [40]. The authors explained this by impairment in Golgi complex function. This also agrees with a recent study who reported a decrease in acidophilic appearance of cytoplasm in GCTs in submandibular gland of Alloxan induced diabetic rats [39].

In the present study, some EDs of the diabetic group showed stagnated secretion in their lumen. This agrees with ***Ali and Mubarak*** [41] who noticed stagnated secretion in the lumens of some SDs and EDs of submandibular gland of diabetic mothers' rats offsprings.

This was previously explained as stagnant secretion might be due to mitochondrial impairment that results in ATP depletion with failure of biosynthesis and membrane pumps. This leads to absence of energy needed by cells for the process of secretion[42].

Insulin-treated group in the present work showed serous acini and IDs almost comparable to control with apparent decrease in cellular degeneration of SDs and GCTs than diabetic group. Cytoplasmic vacuolations were also decreased. These results were in accordance with a previous study [43] who reported normal lipid profile of STZ-induced diabetic rats' salivary glands after one week of insulin treatment. Moreover, insulin demonstrated a regulating effect on expression of salivary secretory proteins in diabetic rats' parotid glands after one week of its administration [44]. It has been furtherly suggested that insulin might have a corrective effect on hyperglycemia-related oxidative stress and related histological alterations in lacrimal glands of diabetic Wistar rats [45]. Furthermore, ***Jamshidi*** [46] showed the potency of insulin to attenuate hyperglycemia, oxidative stress, and inflammation in diabetes, with less severe microvascular steatosis and fatty degeneration in livers of Wistar rats.

In the present study, the glands of Moringa-treated group were comparable to those of the control groups except for few cytoplasmic vacuolations. This agrees with ***Gupta et al.,*** [23] who reported renewal of pancreatic islets after 21 days of treatment for diabetic rats with methanolic extract of MO pods. They concluded that flavonoids could act as insulin secretagogues or insulin mimetics. Furthermore, it has been reported that treating diabetic rats with 250 mg/kg of MO aqueous leaf extract for 18 days resulted in regenerated hepatocytes and islets of Langerhans cells with little congestion. The researchers explained the regenerative effect of MO extract on β-cells by its flavonoid, terpenoids, quercetin, and kaempferol contents which could regenerate destructed β-cells resulting in insulin secretion by their antioxidant activities [47].

Flavonoids can scavenge free radicals, chelate metal ions, or inhibit enzymes responsible for free radical generation [48]. Treating diabetic rats with MO leaves or its ethanolic extract significantly increased the antioxidant enzyme superoxide dismutase [49].

In the current study, PCNA-positive area percentage in diabetic group was significantly higher than the control group. This agrees with a study carried by ***Gillespie et al.,*** [50] who documented that the submandibular glands of non-obese diabetic mice showed an intense rise in PCNA-positive cells. They also reported that the majority of the PCNA-positive cells are localized to ductal lining cells where, most of those cells showed cytoplasmic staining and significant minority showed nuclear positive reaction. The authors explained this increase in PCNA reaction as stem cell and fibroblasts proliferation to replace lost cells of the gland. It has been also suggested that gland cells

are converted from terminally differentiated cells to less differentiated proliferating cells. In addition, the same authors [50] documented that PCNA is expressed during S phase of cell cycle and during repair thus, cytoplasmic localization of PCNA suggests inappropriate intracellular trafficking.

Naryzhny and Lee [51] showed that PCNA has three isoforms with different subcellular localization, where two isoforms are found in the nucleus and the third is present in the cytoplasm. This may explain PCNA positive nuclear and cytoplasmic reactions. It has been also suggested that cytoplasmic PCNA may have a role in oncogenesis regulation, the glycolysis pathway and cytoskeleton integrity [25].

In the present study, insulin and Moringa-treated groups, showed significant increase in PCNA positive area percentage when compared to the control group and significant decrease when compared to diabetic group. Both insulin and Moringa-treated groups were statistically insignificant to each other

CONCLUSION

MO was able to minimize signs of degeneration and cellular damage resulting from STZ-induced diabetes on submandibular glands when compared to insulin as a traditional antidiabetic treatment.

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