HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY ON THE POSSIBLE AMELIORATING EFFECTS OF ANTOX ON THE PAROTID GLAND OF RATS WITH STREPTOZOTOCIN-INDUCED DIABETES MELLITUS TYPE 1

BY

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ABSTRACT

Background: Hyperglycemia associated with the diabetes mellitus (DM) commonly results in structural abnormalities and hyposalivation in the parotid glands. An antioxidant action in a potent multivitamin called Antox (ANX) aids in reducing oxidative stress. **Aim of study:** The current study examined the role of ANX in preventing complications from diabetes in the parotid gland, as well as its potential mechanisms. **Material and methods:** 36 rats were randomly divided into six groups; each group contained six rats. **Group 1** (Negative (-ve) control): the rats received only regular food and water. **Group 2** (vehicle group): rats received a single i.p. dose of citrate buffer as a vehicle. **Group 3**: rats were given ANX by oral gavage with a polyethylene canula 0.5 mm in a volume not to exceed 0.3 ml/100 gm at a dose of 10 mg/kg/day. **Group 4** (Diabetic/ DM): rats received a single i.p. dose of 50 mg/kg of STZ dissolved in citrate buffer. **Group 5** (DM+ Insulin): rats received a single i.p. dose of 50 mg/kg of STZ dissolved in citrate buffer and insulin (Mixtard 30/70; Novo Nordisk) 1 U/100 gm once daily subcutaneous (S.C.). **Group 6** (DM+ Insulin + ANX): rats received a single i.p. dose of 50 mg/kg of STZ and insulin 1 U/100 gm /day/S.C. then received ANX orally in a dose of 10 mg/kg/day. All medications were given for 4 weeks. Rats were anaesthetized, and the parotid tissues were obtained for biochemical analysis to measure Malondialdehyde (MDA), superoxide dismutase (SOD), reduced glutathione (GSH), and nitric oxide (NO), for Histopathological examination (Hematoxylin and Eosin staining, Masson trichrome stain) and immunohistochemistry using (8-OHdG, α-SMA and BAX). **Results:** Remarkably, co-administration of ANX with insulin significantly reduced malondialdehyde (MDA) levels and increased expression of 8-hydroxy-2'-deoxyguanosine (8-OHdG) while enhancing the activities of superoxide dismutase (SOD), glutathione peroxidase (GPX), and total antioxidant capacity (TAC). Additionally, compared to the diabetic rat group and the diabetic group receiving insulin, the combination of ANX and insulin resulted in a considerably decreased expression of Bcl-2, Associated X-protein (Bax) and Smooth muscle alpha-actin (α-SMA), with significant reduction in interleukin-1beta (IL1β) level in relation to diabetic group. **Conclusion:** In comparison to insulin administration alone, co-delivery of ANX with insulin throughout diabetes treatment was more effective in preserving the structure and function of the parotid gland.

Key words: *Diabetes mellites; rats; parotid glands; Antox; oxidative stress; inflammation; apoptosis*.

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INTRODUCTION

As a system of exocrine glands, the salivary glands are anatomically situated in the head, in and around the buccal cavity. Normally, a system of ducts allows saliva to be secreted into the mouth *(Contreras-Aguilar and Gómez-García 2020).* The salivary glands are the basis of good dental health because they create saliva, which serves as a buffer to prevent enamel decalcification as well as lubrication, hydration, and protection against bacteria, fungi, and viruses *(Amerongen and Veerman 2002).* The size of the glands and the type of saliva they generate are used to classify them *(Basset et al., 2020)*.

The parotid gland, the largest of the three salivary glands, is composed of serous follicles and ducts. The serous follicles account for roughly 25–30% of the entire daily salivary output *(Holmberg and Hoffman 2014; Kessler and Bhatt 2018).*

Diabetes mellitus (DM), a metabolic disorder brought on by the pancreas' failure to generate enough insulin, or insensitivity of the body's cells to the effect of insulin, resulting in extremely high blood glucose levels

(hyperglycaemia) *(Wold et al., 2005; Kowluru and Chan, 2007).* Its onset is linked to poor protein, lipid, and carbohydrate metabolism *(Chamberlain et al., 2016; Kharroubi and Darwish 2015).*

Various long-term complications and harmful effects are caused by chronic hyperglycemia and insulin resistance *(Rahimi et al. 2005; Wold et al., 2005).* For example, the serious impairment of salivary gland function and several problems with oral health. DM is thought to promote xerostomia, which is the absence of both qualitative and/or quantitative saliva in the oral cavity *(Gandara et al., 2011; Sreebny et al., 1992).*

Dental caries and periodontal disorders were found in diabetic patients and linked to reduced saliva due to change in the structure of salivary glands caused by DM *(Mata et al., 2004).* Such a decrease in saliva results in taste disturbances *(Neyraud and Dransfield 2004),* difficulties in swallowing *(Sreebny and Vissink 2010).*

The oral cavity is susceptible to a number of problems because of DM effects on the parotid gland's structure and the decreased salivation that results, including several oral deteriorations, a change in the flavour of food, halitosis, and severe caries *(Stewart et al., 2016).* Members of the Bcl-2 family, which includes cell death inducers and inhibitors, control the mitochondrial pathway of apoptosis *(Bjelaković et al., 2005).*

B-cell lymphoma protein 2 (Bcl-2) and (Bax) are members of this family. When Bax predominates, programmed cell death is expedited *(Oltval et al., 1993).*

It has been suggested that ROS cause membrane lipid peroxidation and that the harmful fatty acid peroxides that arise are major causes of cellular failure *(Sanocka and Kurpisz 2004).* The promutagenic DNA 8-hydroxy deoxyguanosine (8-OHdG) is produced by oxygen radicals and is employed as a marker to evaluate DNA damage brought on by oxidative stress *(Laudat et al., 2002)*. According to evidence, diabetes is more likely to experience oxidative stress because it produces more reactive oxygen species (ROS) and has a weaker antioxidant defense system *(West, 2000; Laudat et al., 2002).* Because it is plentiful in DNA and continuously detectable, 8-OHdG is one of the most popular oxidative stress indicators *(Karihtala and Soini 2007)*.

ANX, a potent non-enzymatic antioxidant, also contains ascorbic acid, vitamin A acetate, and vitamin E *(Abdel-Ghaffar et al., 2010).* Free radicals produced by oxygen can all be eliminated by these vitamins *(Fadupin et al., 2007).*

It possesses potent antioxidant properties that can guard against free radical damage to mitochondrial membranes and DNA, preventing the development of diabetic oxidative stress *(Amal and Mona 2009).*

It is generally known that vitamins C and E have favorable effects on glycated haemoglobin (HbA1c), serum lipids, and blood sugar *(Matough et al., 2012)*. People with Type 2 diabetes can also lower their blood lipid and blood sugar levels by taking a daily vitamin C supplement, which lowers their risk of complications *(Prajapat et al., 2017a).*

AIM OF THE WORK

The objective of this study was to investigate the possible protective effects of ANX against streptozotocin (STZ)-induced changes in the parotid gland's histological structure in a rat model of diabetes mellitus.

MATERIAL AND METHODS

- **Animals**

In this study, thirty-six adult male albino rats were obtained from the animal house at Zagazig University, weighing between 210 and 250 gm. The rats were kept individually in cages with regular daily light and dark cycles, as well as free access to regular food and water, for a period of two weeks prior to the start of the experiment. After that, the rats were randomly divided into six groups with each one containing 6 rats. The procedure was created in compliance with the standards for the moral use of animals in research, and the Zagazig University Faculty of Medicine's Research Ethics Committee approved it.

- **Induction of Type 1 DM:**
  To mimic Type 1 DM, each rat received a single intraperitoneal (i.p.) injection of STZ dissolved in citrate buffer at a concentration of 50 mg/kg *(Nazıroğlu et al., 2004).* Blood samples obtained from the rats' tail veins were used for measurement of blood glucose levels using a glucometer. The samples were taken starting from the second day after STZ injection. When the measured blood glucose level reached 200 mg/dl or higher, the diagnosis of diabetes mellitus was assured.

- **Experimental protocol and drug dosage**
  Rats were randomly divided into six groups;
  Group 1 (Negative (-ve) control): the rats received only regular food and water.
  Group 2 (vehicle group): rats received a single i.p. dose of citrate buffer as a vehicle (0.1 M, PH =4.5).
  Group 3: For four weeks, rats were given ANX by oral gavage with a polyethylene canula 0.5 mm in a volume not to exceed 0.3 ml/100 gm at a dose of 10 mg/kg/day *(Nazıroğlu et al., 2004).*
  Group 4 (Diabetic/ DM): rats received a single i.p. dose of 50 mg/kg of STZ dissolved in citrate *buffer (Amal and Mona 2009) (Zhou et al., 2010).*
  Group 5 (DM+ Insulin): rats received a single i.p. dose of 50 mg/kg of STZ dissolved in citrate buffer and insulin (Mixtard 30/70; Novo Nordisk) 1 U/100 gmonce daily subcutaneous (S.C**.**) *(Zhou et al., 2010).*
  Group 6 (DM+ Insulin + ANX): rats received a single i.p. dose of 50 mg/kg of STZ and insulin 1 U/100gm /day/S.C. then received ANX orally in a dose of 10 mg/kg/day *(Amal and Mona 2009).* All medications were given for 4 weeks.

(1)Tissue preparations and histological examination:
Rats were thiopental-anesthetized at the end of the experimental period and then, the parotid glands were removed. According to Bancroft and Layton, parotid specimens were preserved in 10% neutral-buffered formalin and embedded in paraffin hematoxylin and eosin (H&E) to reveal the histological structurs of the parotid gland *(Bancroft and Layton, 2018),*

and Masson's trichrome (MT) stains to reveal the collagen fiber *(Zalewska et al., 2015).* The LEICA DM500 with LEICA ICC50 W camera Module microscopy was used to examin the stained slides at Zagazig University Image Analysis Unit of the Anatomy and Embryology Department.

(2) Biochemical markers:

- **Glutathione peroxidase (GPx) assay**:
  Glutathione peroxidase (Biodiagnostic, CAT. No. GP 25-24) was assayed according to the method of *(Paglia and Valentine 1967).*

- **Superoxide dismutase (SOD) assay**:
  Superoxide dismutase was assayed colorimetrically according to the method of *(Nishikimi et al., 1972)* according to the manufacturer's instructions (Biodiagnostic, CAT. No. SD 2521).

- **Lipid peroxidation (MDA) assay**:
  Malondialdehyde (Biodiagnostic, CAT. No. MD 25-29) was assayed according to the method of *Ohkawa et al. (1979).*

- **Total antioxidant capacity (TAC) assay**:
  Total antioxidant capacity (Biodiagnostic, CAT. No. TA 25 13) was assayed according to the method of *(Koracevic et al., 2001).*

- **Measurement of IL-1β level**:
  Interleukin-1 levels were assessed in the tissue using the Infinite 200 Pro plate reader and the rat IL-1 beta/IL-1F2 Quantikine ELISA Kit from R&D System, Minneapolis, MN, USA (Tecan 226 Trading AG, Switzerland).

(3) Immunohistochemical study:

(8-OHdG), (α-SMA) and Bax Immuno histochemistry expression in parotid gland: Streptavidin-biotin immunoperoxidase stain Dako- Cytomation, Glostrup, Denmark) was used in immunohistochemistry. Formalin-fixed, paraffin-embedded blocks were cut into sections of 3-5 mm thickness, then mounted on slides that were positively charged, deparaffinized in xylene, and rehydrated in graded alcohol. The sections were boiled for 20 minutes in a citrate buffer (pH 6.0), and then rinsed in PBS (pH 7.3). To inhibit the activity of endogenous peroxidase, sections were treated with hydrogen peroxide at a 3 percent concentration. The primary antibody was then

used in treatment of the sections. **- For 8-OHdG (a marker for oxidative stress)** Slides were incubated with the following monoclonal antibodies overnight: Glypican-3 (GPC-3); 8-Hydroxydeoxyguanosine (8-OHdG): Monoclonal antibody, dilution 1:100 (Clone15A3, SC-66036, Santa Cruz, California, USA; mouse monoclonal antibody, dilution 1:100 (CM396, A, B, clone IG12, Biocare Medical LLC, Concord, USA) *(El-Alfy et al., 2017).*

- **For α-SMA (a marker for myoepithelial cells).** The primary antibody (mouse monoclonal antibody to α-SMA, 1/500 dilution, clone 1A4, ab7817, Abcam) was used to treat the sections overnight in a humid chamber, then PBS rinsed three times *(Ramos-Vara et al., 2008).*

- For Bax (an apoptotic protein marker). Sections were treated with a monoclonal antibody against the Bax protein after being first incubated with 1 percent preimmune rabbit serum to reduce nonspecific staining**.** (1:200 dilution; Dako, Carpinteria, California, USA) *(Singh et al., 2015).*

Product visualization and secondary antibody incubation were carried out (Dako-Cytomation): In order to stain 8-OHDG and identify the site of immunological reactions, diaminobenzidine substrate (Research Genetics, Huntsville, Alabama, USA) was employed as the chromogen *(El-Alfy et al., 2017).* For α-SMA 3, 3'diaminobenzidine (DAB)-hydrogen peroxide was utilized as a chromogen *(Ramos-Vara et al., 2008).* Bax was detected using 3-amino-9-ethyl carbazole (AEC) diaminobenzidine substrates; following this, slices that had been immune-stained were counterstained using Mayer's haematoxylin.

Morphometrical assessment:

At a magnification of 400, the image analysis was done using the Leica Q 500 MC program at the Anatomy and Embryology Department of Zagazig University's Faculty of Medicine to measure the pursuing parameters "in 10 separate fields from each specimen":
1-The Acinar diameter [μm]
2-The Acinar epithelial height [μm]
3-Striated duct diameter [μm]
4-Striated duct epithelial height [μm]
5-(%) of collagen fibers
6-(%) of α-smooth muscle actin
7-The optical density of 8OHdG
8-The optical density of α-SMA
9-The optical density of Bax.

STATISTICAL ANALYSIS:

All data was presented as mean±standard deviation (S.D.). Version 5 of the Graphpad Prism program was used for the statistical analysis (GraphPad Software, Inc. La Jolla, CA, USA). Following a one-way analysis of variance (ANOVA), Tukey's test was used to determine the intergroup variation. P-values (probabilities values) <0.05 are considered significant results.

RESULTS

- **Biochemical Results:** This study showed that there were no statistically significant differences in mean values of tissue MDA, GPx, TAC, SOD and tissue IL-1β in the vehicle and ANX group in comparison to the control group (P>0.05).

The results shown in Table 1 revealed that DM significantly increased MDA that is indicative of increased oxidative stress. Alternatively, GPx, TAC and SOD were significantly decreased as compared to the resultant values in the control. The insulin administration had protective effects against these changes as it significantly reduced the levels of MDA and significantly elevated the catalase activity and GPx in the DM group. However, the difference was more significant in DM+insulin+ANX group. As shown in **Table (1)**, TNF-α and IL-1β levels were significantly elevated in the DM group as compared to the control group. The insulin and ANX co-administration resulted in significantly decreased IL-1β when compared to the DM group.

- **ANX with insulin repaired histopathological disturbances of the parotid gland in diabetic rat.** Histological examination of -ve control, vehicle group and ANX group showed nearly similar histological results. Therefore, -ve control was used as the control group.

The H&E-stained section from the -ve control group showed normal architecture of the parotid parenchyma composed of uniform closely packed pure serous acini in addition to the duct system. These acini were characterized by secretory dark stained pyramidal cells that had spherical basal nuclei and basophilic cytoplasm. The intralobular ducts were including intercalated ducts and striated ducts; however, the striated ducts were more prominent than intercalated ducts that were seen with difficulty as being compressed by neighboring acini. The striated ducts were easy recognized as they are lined by simple columnar cells that have rounded nuclei and exhibit basal striations **(Fig. 1A).**
The H&E-stained sections of the DM group revealed structural changes of parotid parenchyma. The acini were separated from each other by connective tissue containing some mononuclear infiltrating cells. The acini revealed abnormal architecture as some acini were amalgamated and coalesced however others were shrunken and atrophied. Most acinar cells showed dark stained nuclei of different size and shape. Moreover, a lot of acinar cells exhibited irregular displaced, dark stained, compressed nuclei by multiple variable-sized and shaped intra cytoplasmic vacuolations. The intralobular ducts could not be differentiated into intercalated ducts (IDs) and striated ducts (SDs). The ducts demonstrated structural changes; some were dilated with irregularity of their lumina, some revealed disruption and disorganization of their epithelial lining and some showed retained secretions. The ductal cells showed flattened darkly stained nuclei with cytoplasmic vacuolations. Abundant mononuclear cellular infiltration was observed around the ducts. Also congested blood vessels were detected **Fig. 1 (B, C, D, and E).**
Stained Sections with H&E of (DM+ insulin) group demonstrated apparently semi normal histological appearance except for the presence of some pyknotic nuclei and some vacuolations **Fig. 1 (F).**

- Parotid glands of (DM+ insulin+ ANX) group showed a picture very similar comparable to control group as majority of acini and ducts have normal histological appearance **(Fig. 1E).** **Co-administration of ANX with insulin averted Masson's trichrome changes of Parotid gland in diabetic rat.** Few collagen fibers stained with green color were seen in the control group around the intralobular ducts and blood vessels (**Fig. 2A**).
On the opposite side, Masson-stained sections of diabetic rat's parotid gland revealed abundant collagen fibers, especially surrounding ducts and blood vessels (**Fig. 2B**). An obvious reduction of collagen fibers was observed in diabetic rats treated with insulin (**Fig. 2C**). Moreover, Masson-stained sections from diabetic rats concomitantly treated with insulin and Antox showed scanty amounts of collagen fibers (**Fig. 2D**). According to area % collagen fibers, slides from DM group parotid gland showed significantly elevation of collagen fibers compared to study groups (p<0.0001). Also, with insulin treatment to diabetic animals display significantly elevation in collagen fibers in relation to other study groups (p<0.0001). But with administration of ANX plus insulin for treatment showed no significant difference to -ve control group (p=0.9) **(Fig. 2E).**

- **Examination using (8-OHdG) immunohistochemistry:** In parotid gland sections 8-OHdG immuno-stained sections of the negative control group demonstrated negative immunoreaction in the cytoplasm of the cells of the acini and ducts **(Fig. 3A),** whereas in DM group, intense positive cytoplasmic reaction was noticed as dark brown color in the secretory acini with Positive cytoplasmic immune reaction appeared in striated ducts, moreover the reaction was more obvious and intense in the acinar cells rather than duct cells **(Fig. 3B)**.
In the insulin-treated group, weak positive cytoplasmic immuneoreactivity was observed in both ductal and acinar cells **(Fig. 3C)**. DM +insulin+ ANX group, displayed minimal or even negative cytoplasmic immunoreactivity to

8-OHDG (**Fig. 3D**). Slides from the DM group demonstrated significantly higher 8-OHdG expression (p<0.0001) in PG when compared to the study groups. Additionally, the diabetic rats treated with insulin have noticeably higher 8-OHdG expressions in comparison to other study groups (p<0.0001). However, treatment with ANX and insulin didn't significantly vary from the -ve control group (p=0.9) **(Fig 3E).**

- **Examination using (α-SMA) immuno histochemistry:** In respect to α-SMA immunohistochemical results, **the parotid** control group exhibited minimal positive reaction in the periphery of the cytoplasm of the acini (**Fig. 4A**). In contrast the diabetic group demonstrated an abundant increase of immunohistochemical reaction to α-SMA that were observed surrounding both acini and intercalated ducts (**Fig. 4B**). The diabetic and insulin group, most acini appeared with minimal immunoreaction to α-SMA; however, some acini exhibited a strong reaction to α-SMA (**Fig. 4C**). Co-administration of insulin and Antox to diabetic rats showed faint immunoreaction to α-SMA in a manner very close to the control **(Fig. 4D).** DM group slides showed considerably larger area % in and OD of α-SMA when compared to study groups (p<0.0001). Whatever diabetic rats treated with insulin alone or ANX and insulin for 4 weeks, there were no appreciable difference from the negative control group or each other (p>0.05) **(Fig. 4E and 4F).**

- **Examination using (Bax) immuno histochemistry:** The control group of parotid glands showed in negative cytoplasmic immunoreactivity to Bax all acini and ducts (**Fig. 5A**). The diabetic group revealed abundant strong positive cytoplasmic expression to Bax in a diffuse pattern (**Fig. 5B**). In group DM + insulin group weak cytoplasmic expression of Bax was displayed in most of the acini, but other acini still retained abundant

cytoplasmic immunoreactivity to Bax **(Fig. 5C).**

The serous acinar cells together with duct cells of diabetic rat treated concomitantly with both insulin and Antox (group VI) showed very weak or even negative expression of Bax (**Fig. 5D**).

In relation to OD of BAX, comparing slides from DM group to the study groups, they significantly elevated (p<0.0001). Also, with insulin treatment to diabetic animals, significant elevation in OD of BAX in relation to other study groups was displayed (p<0.0001 and p=0.0008 compared to ANX plus insulin group). But with administration of ANX plus insulin for treatment, there was no significant difference to -ve control group (p=0.4) **(Fig. 5E).**

- **Effects of co-administration of ANX with insulin on quantitative morphometric changes of parotid gland in diabetic rat. Morphometric analysis:** non-significant differences were found between the -ve control group, vehicle group, and ANX group for all morphometric parameters. In the DM group compared to the control groups, the acinar diameter, acinar epithelial height, and the diameter of the striated ducts all showed a substantial increase. When compared to the diabetic group, the DM+ Insulin+ ANX group demonstrated a considerable reduction of the prior diameter **Table (2).** When compared to the control group, the diabetic group displayed a discernible drop in epithelial height of the striated duct of the parotid gland. This difference was highly significant. Insulin administration in groups (5&6) and the simultaneous administration of ANX and insulin, respectively, caused a notable rise in duct height **Table (2).**

Table (1): The biochemical parameters in the different studied groups.

	Negative Control	Vehicle group	ANX group	DM group	DM+ insulin group	DM +insulin+ ANX
Tissue MDA (nmol/g)	89.01±2.72	87.51±3.51	89.19±1.37	135.22±3.74[abc]	97.54 ±1.65[abcd]	100.47±0.41[abcd]
Tissue GPx(ng/mg)	22.07±1.30	23.22±0.46	20.88±2.42	12.43±1.33[abc]	16.54±1.44[abcd]	17.31±1.62[abcd]
Tissue TAC mmol/100 g tissue)	0.94 ± 0.03	0.92 ± 0.01	0.94 ± 0.01	0.75 ± 0.02[abc]	0.82 ± 0.01[abcd]	0.85 ± 0.05[abcd]
Tissue SOD (ng/mg)	0.281±0.006	0.282±0.004	0.277±0.006	0.154±0.007[abc]	0.241±0.037[abcd]	0.262±0.007[abcd e]
Serum IL-1β (pg/ml)	25.64±1.83	25.67±2.26	23.47±1.01	53.86±3.59[abc]	28.40± 1.10[abcd]	31.59±2.26[abcde]

When P-value <0.05, the difference is significant. Values are all presented as mean±SD, n=6. [a] versus negative control group; [b] versus vehicle group; [c] versus ANX group; [d] versus DM group and [e] versus insulin treated group.
Abbreviations*: MDA, Malondialdehyde; GPx, Glutathione peroxidase; IL-1β, Interleukin-1β; TAC, Total antioxidant capacity; SOD, Superoxide dismutase; ANX, antox; DM, diabetes mellitus.*

Table (2): Morphometric analysis of Parotid acinar diameter (AD) (µm), acinar epithelial height (AEH) (µm), diameter of straited duct (SDD) (µm), epithelial height of striated duct (DEH) (µm), In the different studied groups via using ANOVA (analysis of variance) test. Data represented as Mean ± SD.

	Negative Control	Vehicle group	ANX group	DM group	DM+ insulin group	DM +insulin+ ANX
Acinar diameter (µm)	30.67± 4.24	30.49±4.72	30.52±4.41	44.66± 8.67[abc]	36.98±8.65[d]	31.59± 3.25[d]
Acinar epithelial height (µm)	9.63± 1.76	9.53±1.69	9.67±1.76	15.51±4.71[abc]	13.56±3.84	9.94±2.67[d]
Diameter of straited duct (µm)	37.4±7.12	38.58±7.22	36.9±6.74	57.93±13.9[abc]	42.87±9.84	38.40±8.486[d]
Epithelial height of striated duct (µm)	10.85± 1.95	10.81±1.28	10.83±2.13	4.88±1.66[abc]	7.57±1.78[abc]	9.69±1.84[d]

When P-value <0.05, the difference is significant. Values are all presented as mean±SD, n=6. [a] versus negative control group; [b] versus vehicle group; [c] versus ANX group; [d] versus DM group and [e] versus insulin treated group.
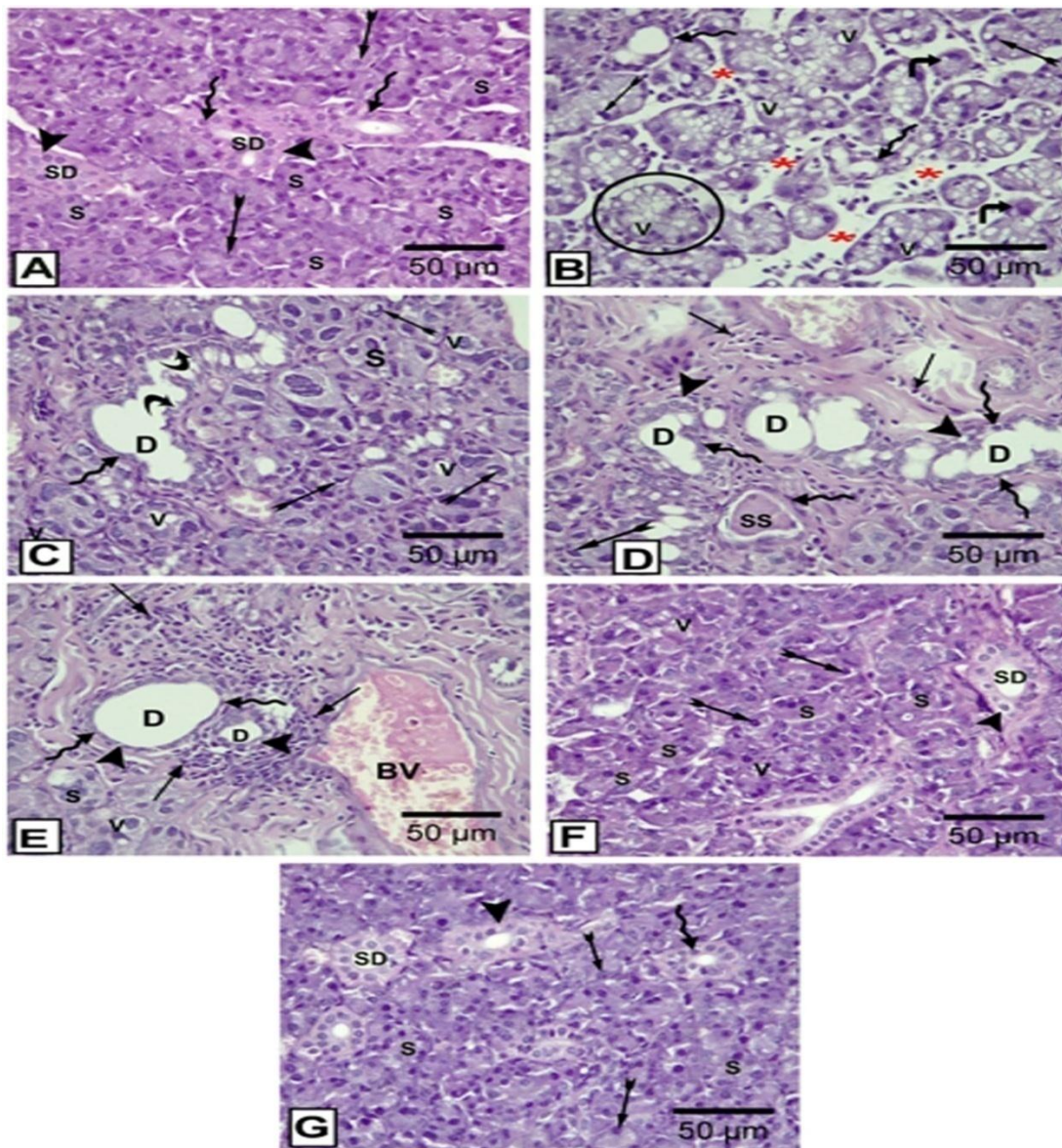
Fig. (1): *Photomicrographs of sections of parotid gland from the different studied groups;* **(A):** *control group showing uniform closely packed serous acini (S) beside the striated ducts (SD). The acini compose of pyramidal cells with spherical basal nuclei and basophilic cytoplasm (bifid arrow). The striated ducts (SD) are lined by simple columnar cells that have rounded nuclei (wavey arrow) and exhibit basal striations (arrowhead).* **(B-E):** *Diabetic group the acini appear widely separated (*) and exhibit disturbed architecture; some acini are amalgamated and coalesced (circle) and others are shrunken and atrophied (angled arrow). A lot of acinar cells exhibit irregular displaced, dark stained, compressed nuclei (bifid arrow) with multiple variable-sized and shaped intra cytoplasmic vacuolations (V). The ducts show disrupted and disorganized epithelial lining (curved arrow) and luminal dilatation (D) with retained secretions (SS). The ductal cells show flattened darkly stained nuclei (wavey arrow) with cytoplasmic vacuolations (arrowhead) within serous acini (S). Abundant mononuclear cellular infiltration (arrow) around the ducts and congested blood vessels (BV) are detected* **(F):** *Diabetic + insulin group demonstrates apparent improvement in the histological appearance of the serous acini (S) and striated ducts (SD) except for the presence of some pyknotic nuclei (bifid arrow) and vacuolations (V).* **(G):** *Diabetic +insulin + antox (ANX) group shows normal histological picture comparable to control group as the acini (S) have normal pyramidal cell with spherical nuclei and basophilic cytoplasm (bifid arrow). The striated ducts (SD) exhibit basal striation (arrowhead) with round nuclei (zigzag arrow)* **(H & E, X400).**
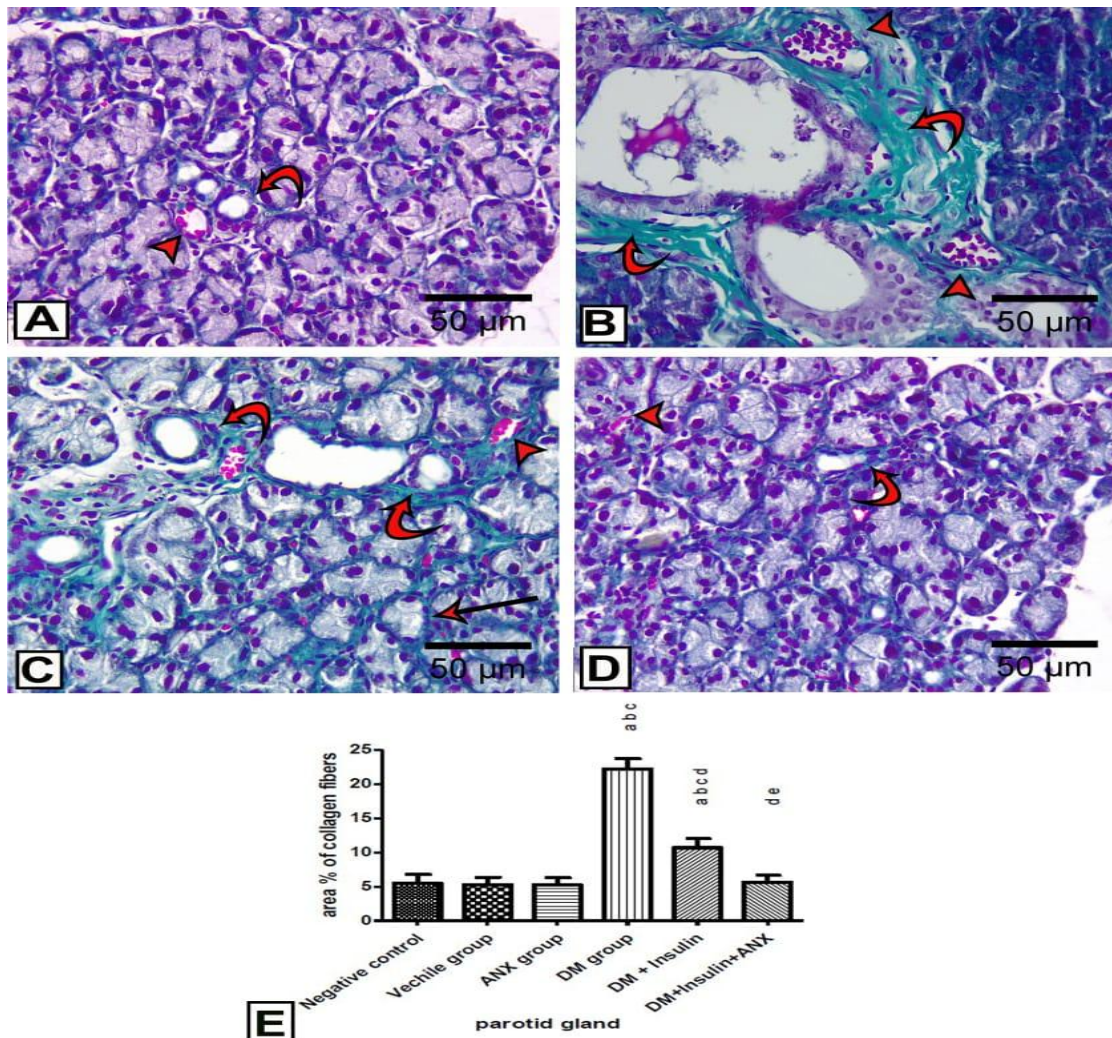
Fig. (2): *Photomicrographs of parotid gland sections from different studied groups; **(A):** control group shows few collagen fibers surrounding blood vessels (arrowhead) and striated ducts (curved arrow). **(B):** Diabetic group shows abundant collagen fibers surrounding ducts (curved arrow) and blood vessels (arrowhead). **(C):** Diabetic + insulin group shows still deposition of moderate amounts of collagen fibers around acini (arrow), striated ducts (curved arrow) and blood vessels (arrowhead). **(D):** Diabetic +insulin + ANX group show minute amount of collagen fibers around the blood vessel (arrowhead) and striated ducts (curved arrow) **(Masson trichrome, X 400). (E):** Effect of ANX plus insulin on the percentage of collage fibers in parotid gland of streptozotocin-induced diabetic rats is shown in a Bar graph. When P-value <0.05, the difference is significant. [a] versus negative control group; [b] versus vehicle group; [c] versus ANX group; [d] versus DM group and [e] versus insulin treated group.*
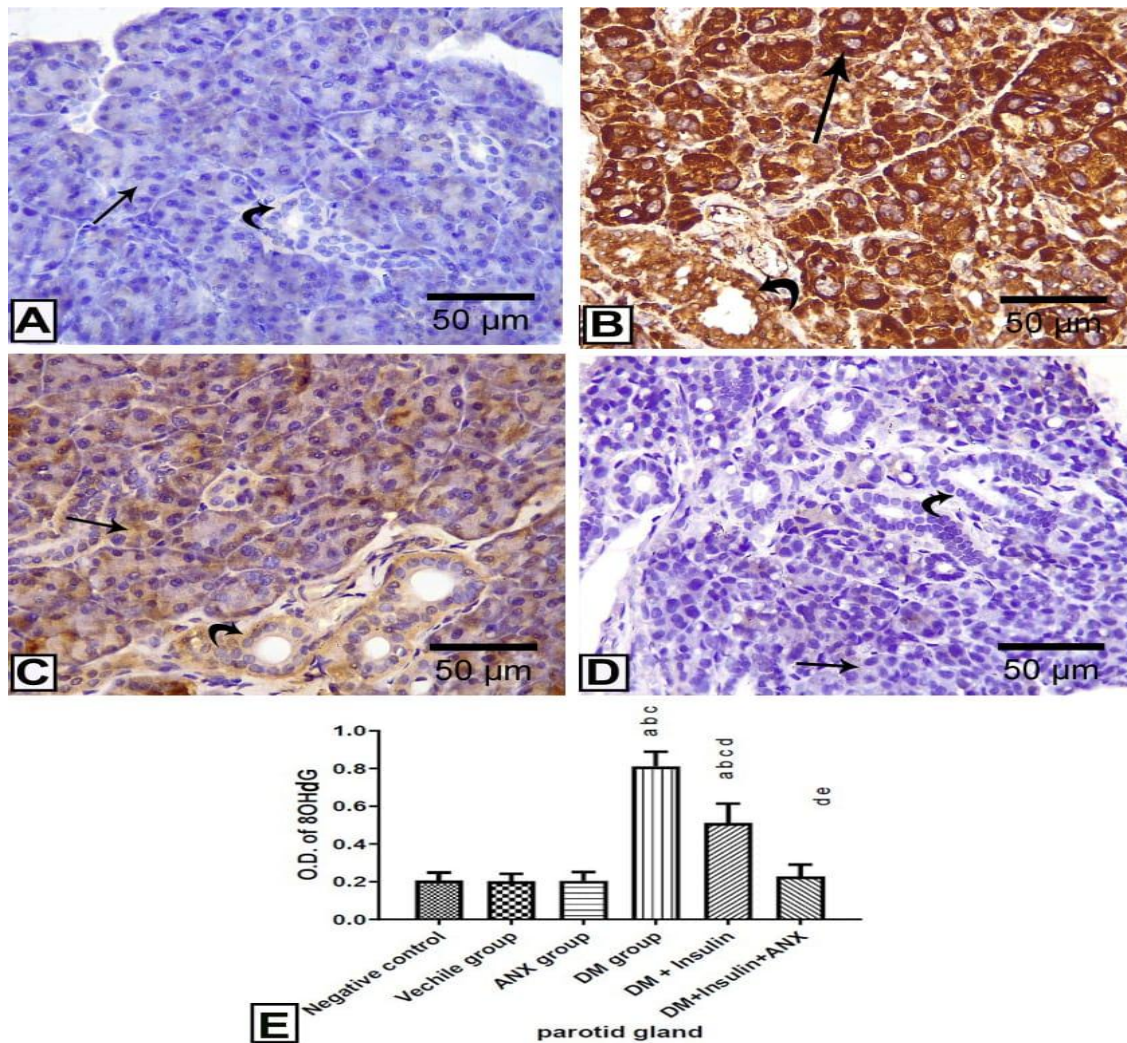
Fig. (3): *photomicrographs of parotid gland sections from different studied groups; (A): control group showing negative immune reaction for 8-OHdG in cytoplasm of acini (arrow) and duct (curved arrow). (B): Diabetic group showing intense positive cytoplasmic dark brown immune-expression for 8-OHdG in the secretory acini (arrow) and in striated ducts (curved arrow); notice the acinar cells show more intense reaction than ductal cells. (C): Diabetic + insulin group showing moderate positive cytoplasmic immunoreactivity in both ductal (curved arrow) and acinar (arrow) cells. (D): Diabetic +insulin + ANX group showing minimal or even negative cytoplasmic immunoreactivity to 8OHdG in acini (arrow) and duct (curved arrow). (8-OHdG immunostaining, X 400) (E): Effect of ANX plus insulin on the OD of 8 OHdG in parotid gland streptozotocin-induced diabetic rats shown in a Bar graph. When P-value <0.05, the difference is significant. [a] versus negative control group; [b] versus vehicle group; [c] versus ANX group; [d] versus DM group and [e] versus insulin treated group.*
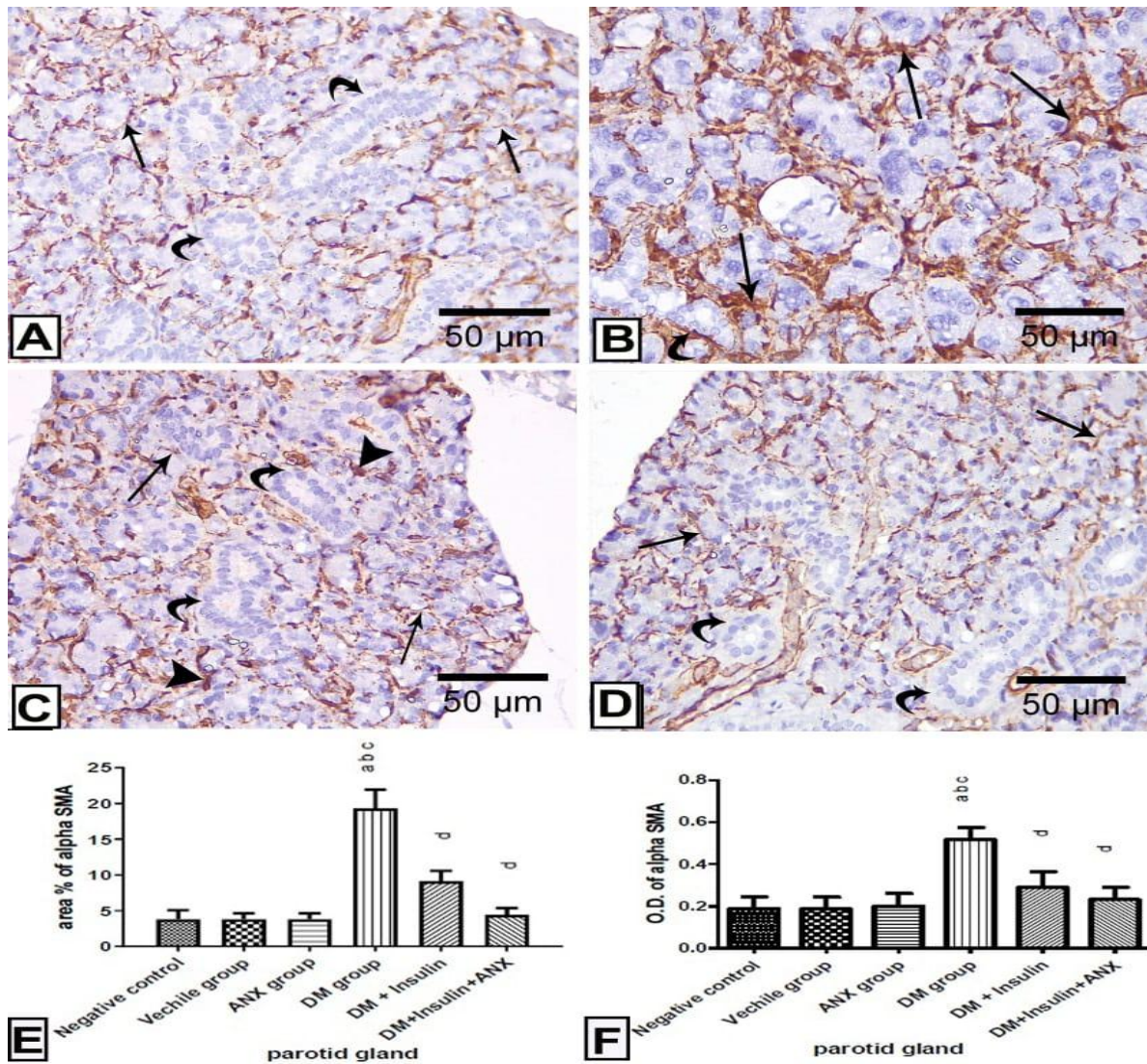
Fig.(4): *Photomicrographs of parotid gland from different studied group; **(A):** control group shows minimal positive reaction for α SMA at the periphery of the acini (arrow) with no reaction at periphery of the duct (curved arrow). **(B):** diabetic group exhibited abundant increase of α-SMA reaction (arrow) around the acini and intercalated duct (curved arrow) **(C):** The diabetic and insulin group shows some acini with minimal immune reaction to α-SMA (arrow) and while other acini appear with strong reaction to α-SMA (arrowhead) and no reaction to α-SMA around the striated duct (curved arrow). **(D):** The diabetic + insulin + ANX group shows faint immune reaction to α-SMA surrounding the acini (arrow) with negative reaction at periphery of the duct (curved arrow). **(Immunostaining for α-SMA X 400), (E, F).** Effect of ANX plus insulin on the area % of α SMA and OD of α SMA in parotid gland streptozotocin-induced diabetic rats is shown in Bar graph When P-value <0.05, the difference is significant. [a] versus negative control group; [b] versus vehicle group; [c] versus ANX group; [d] versus DM group and [e] versus insulin treated group.*
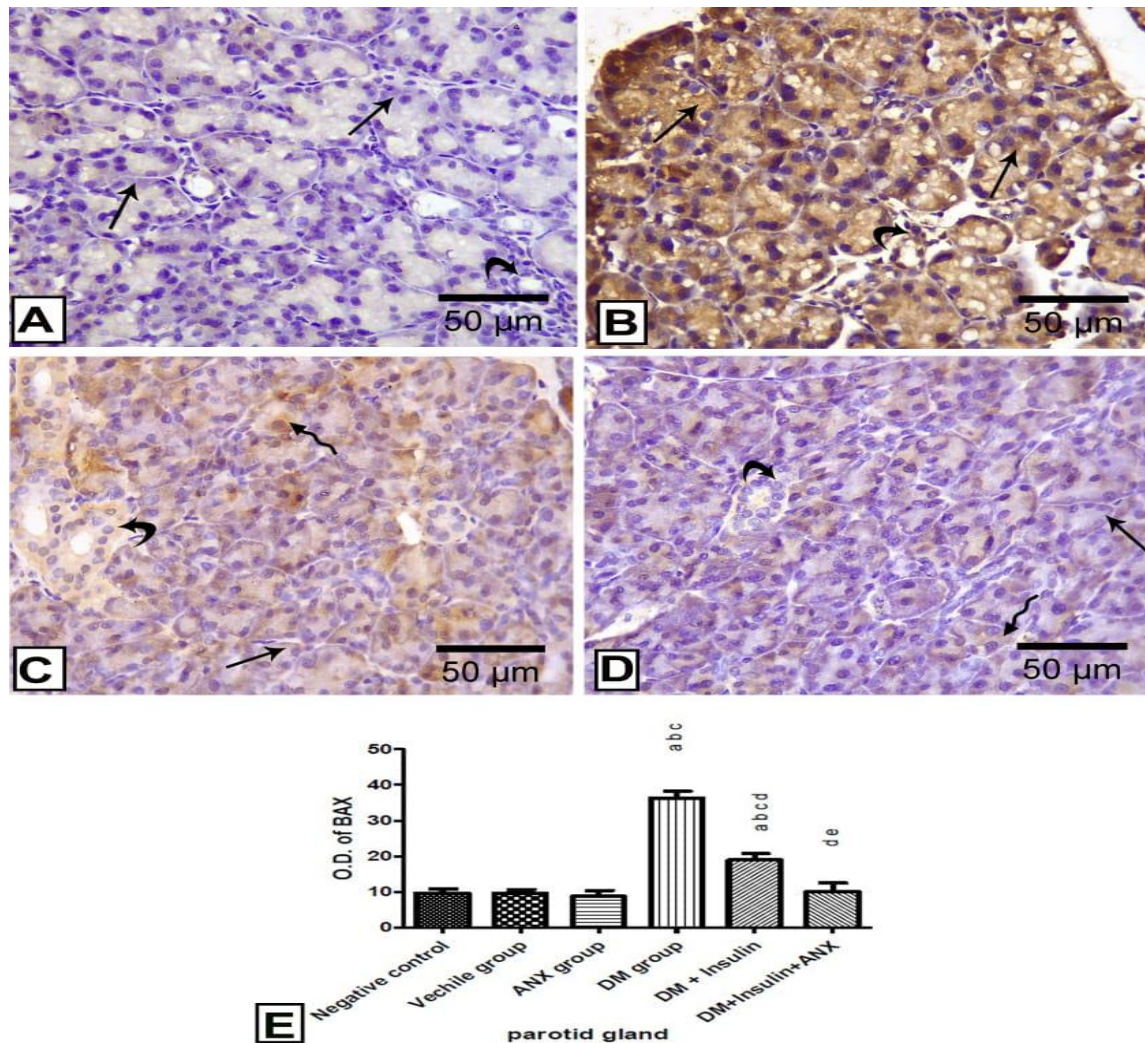
Fig. (5): Photomicrographs *of parotid gland sections from different studied groups; (A): control group shows negative cytoplasmic immunoreactivity for BAX in the acini (arrow) and ducts (curved arrow). (B): Diabetic group exhibits abundant strong positive immunoreaction for BAX in cytoplasm acini (arrow) and ducts (curved arrow). (C):Diabetic + insulin group some acini (arrow) and ducts (curved arrow) shows weak immunoreactivity for BAX (arrow) while other acini show moderate cytoplasmic reaction (wavey arrow) (D): Diabetic +insulin + ANX group show negative immunoreactivity to BAX in the acini (arrow) and ducts (curved arrow) but some acini appear with faint immunoreactivity to BAX (wavey arrow) (BAX immunostaining , X 400) (E): Effect of ANX plus insulin on the OD of BAX in parotid gland streptozotocin-induced diabetic rats is shown in a Bar graph. When P-value <0.05, the difference is significant. [a] versus negative control group; [b] versus vehicle group; [c] versus ANX group; [d] versus DM group and [e] versus insulin treated group.*

DISCUSSION

The high incidence of diabetes mellitus (DM) among persons was the primary impetus for doing this investigation. Streptozotocin (STZ) was used to initiate the insulin-dependent diabetic state and lower the plasma level of insulin in order to simulate this condition (*Lenzen, 2008*). According to a prior study that found that a dose of (50 mg/dL) of STZ is adequate to create a diabetogenic effect at 100% rat survival, this is the dose that was chosen for the current study (*Goyal et al., 2016*).

The malfunctioning of the parotid gland and alterations to its structure are frequent side effects for diabetic people. According to the current study, diabetic rats exhibit an increase in oxidative stress, as evidenced by decreased SOD and GPX levels, increased lipid peroxidation

caused by an increase in MDA level, as well as increased levels of IL1 and GPX. Prolonged hyperglycemia also causes a rise in the inflammatory mediators IL6 *(Abate et al., 2013; Ahmed, 2005; Brownlee, 2001).*

According to *Kang et al. (2021)*, these are the results of hyperglycemia, in which glucose converted to sorbitol consuming NADPH, which is required for the antioxidant function of glutathione and causing oxidative stress and cell damage *(Giacco and Brownlee 2010; Vincent et al., 2002).*

The parotid glands exhibited histological changes on account of DM, which was the study's goal. The findings demonstrated that DM can cause observable structural alterations, involving shrinkage and atrophy. Additionally, darkly stained acinar cells and compressed nuclei were found in the parotid gland tissues. These results concurred with existing literature on *Salem et al. (2021)*, which observed that DM caused acinar size to decrease along with gland atrophy and indicated that atrophy could be brought on by the infiltration of fibrous or fatty tissue.

Additionally, the acinar and ductal cells that make up the parotid glands displayed a variety of degenerative alterations, such as cytoplasmic vacuolation and swelling of acini. This result was explained by the accumulation of degenerative substances and fatty degeneration in the cytoplasm *(Mubarak, 2012)*. According to several researches, lipid droplets accumulate in the epithelial cells of the parotid glands *(Carda et al., 2006; Lilliu et al., 2015)* with increased free fatty acid production causing lipotoxicity *(Shikama et al., 2013)*. The pressure from the degenerative products, which were primarily of a fatty type, caused the cell edges to seem crushed and distorted *(Moubarak, 2008).*

According to earlier researches, these degenerative alterations may be a result of damage to parenchymal cells, which alters the integrity and permeability of the cell membrane and alters the cell volume's hemostasis *(Adjene et al., 2014).* Another study claimed that prolonged hyperglycemia in diabetic people caused the development of oxidative stress, which in turn had damaging and degenerative consequences on the structure of the parotid glands *(Büyük et al., 2015).*

In diabetic parotid glands, the acinar and ductal cells exhibited pyknotic dark stained nuclei. Similar to the present study, another one shown that these modifications are mostly the outcome of damage from lipid peroxidation and oxygen radicals that target the chromatin and induce DNA fragmentation, which eventually results in cell death *(Balaban et al., 2005).*

In this study, the epithelial lining of the excretory ducts was deteriorated, and the ducts were dilated with retained secretion. These results agreed with *(Elabasiry et al., 2018).* Moreover, a significant rise in the diabetes group compared to the control group supported the increase in acinar diameter. Similar information was presented in a prior study of *(Omar et al., 2018),* which further stated that such dilatation may be explained by glandular malfunction, ineffective secretion, and secretory granule buildup in the acinar cells. The current study also indicated that, when comparing the diabetes group to the control group, a highly significant difference developed in the epithelial height of the striated duct of the gland. These outcomes were in line with *(Anderson et al., 1994)* who reported that, in contrast to the control group, the diabetic rats' epithelial cell height in the granular and striated ducts continued to decrease, although it remained stable in the intercalated ducts.

On the other hand, diabetic parotid glands showed dilated congested blood vessels and infiltration with mononuclear cells that provide evidence of an inflammatory process associated with DM, which is consistent with past studies. An earlier study that showed DM is linked to increased expression of TNF, which causes inflammation and impaired parotid function, validated the findings of this study *(Li et al., 2019)*. In addition, a previous study *(Zahawi, 2015)* claimed that such inflammatory responses might be intended to increase blood flow to damaged tissue.

Insulin was able to ameliorate the histological appearance of the parotid gland in the insulin-controlled diabetic parotid glands, with some pyknotic nuclei remaining and some architectural changes being made. This histological architecture clearly improved after ANX and insulin co-administration, bringing them as near as possible to the control group. This was in line with a prior study that found daily supplementation with 1000 mg of Vitamin C can significantly lower blood sugar and lipid levels in Type 2 diabetic patients, lowering their risk of complications *(Prajapat et al., 2017b)*.

In the same line, another study confirmed that giving Type 2 diabetic patients an oral vitamin E supplement (900 mg per day) over a 4-month period helped reduce insulin resistance and hence enhance glucose absorption *(Pazdro and Burgess 2010)*. On the other hand, a link between vitamin A insufficiency and type 2diabetes has been discovered, suggesting that vitamin A supplementation plays a part in the biology of the disease *(Ziouzenkova et al., 2007)*.

The parotid glands in diabetic specimens showed substantial fibrous tissue replacing the glands' deteriorated parts in line Masson stain. According to this study, such changes are typically irreversible and the gland cannot regenerate *(Mata et al., 2004)*. Later research indicated that the primary mechanism stimulating -collagen expression with excessive collagen production was the lipid peroxidation process *(Novitskiy et al., 2006)*.

The morphometric examination of collagen fiber area percentage, which revealed extremely significant differences between the control and diabetes groups with no significant difference observed between the ANX and control group, provided statistical support for this. Conversely, simultaneous administration of Anx and insulin revealed a highly significant decrease in the area % of collagen fibers as compared to the diabetic group.

In comparison to the control group, the level of 8-OHdG immunoreactivity was considerably higher in diabetic parotid glands. These findings were consistent with other studies that claimed that ROS significantly contributes to the pathophysiology of the parotid glands in diabetes and modifies the antioxidant function of the parotid glands. Additionally, there was little to no or even negative cytoplasmic immunoreactivity to 8-OHdG in group VI (DM + Insulin + ANX).

Smooth muscle cells express a special marker termed -SMA early in development. The acini and intercalated ducts of the rat and human parotid glands contain myoepithelial cells that wrap each secretory component and are often visible at the borders of these structures. Squeezing the acini serves as a means of facilitating secretion *(Abelson et al., 1976)*.

Myoepithelial cells, intercalated ductal cells, and acinar cells have low baseline proliferation under normal circumstances, which supports the physiological regeneration of the acini and intercalated ducts. However, when glandular injury occurs, the proliferation index increases, especially in myoepithelial cells *(El Maadawi and Gabr 2011)*.

In the current investigation, diabetic parotid glands displayed a significantly higher expression of the α-SMA immunoreaction than the control group, indicating an excessive proliferation or hypertrophy of myoepithelial cells. The simultaneous treatment of Antox and insulin led to a mild immunoreaction expression of α-SMA like control, which appeared to improve.

This growth in the quantity and size of myoepithelial cells was intended to counteract the degeneration process that was brought about within the glands, and it was hypothesized that myoepithelial cells might function as stem or phagocytic cells and maintain the gland's secretory capabilities *(Cotroneo et al., 2008)*.

In agreement with this study findings, *Jung, (2021)* observed that when compared to the control group, the level of Bax (an apoptotic agonist) expression in the diabetic group increased noticeably. These results provide significant evidence for the hypothesis that DM causes apoptosis, but concurrent injection of insulin and ANX was able to cause a notable decrease in Bax expression.

The statistical analysis region percentage of 8-OHDG, α-smooth muscle actin, and Bax recording a high significant difference between control and diabetic groups served as proof for all prior findings. Contrarily, concurrent administration of ANX (group 5) did not show any appreciable variations from the control group. In agreement with the findings of this study, recent research found that the combination of vitamins A, C, and E (ANTOX) can significantly enhance the histological and biochemical structures of parotid glands in a rat model of STZ-induced diabetes *(Gozif et al., 2019)*.

CONCLUSION

In conclusion, this study showed that rat parotid structural changes caused by diabetes mellitus could be significant. However, simultaneous Antox therapy in rats with managed diabetes might mitigate such effects.

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