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THE EFFECT OF METFORMIN ON SALIVARY GLANDS OF RATS WITH STREPTOZOCIN-INDUCED DIABETES

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ABSTRACT

Background: Diabetes mellitus, a chronic metabolic condition with rising incidence rates, burdens public health. Xerostomia, induced by salivary gland dysfunction, is a common diabetes consequence that causes oral infections as well as speech and mastication problems. Metformin is an efficient antidiabetic drug, however, its effects on salivary gland function are unclear. This study aimed to investigate the effect of diabetes on the salivary gland and to assess the potential ability of metformin to counter the diabetes effect on the salivary gland.

Methodology: Eighteen adult male albino rats were divided into three groups: control, streptozotocin, and metformin. In streptozotocin, and metformin groups diabetes was induced and only the metformin group received oral administration of metformin 100 mg/kg. After 28 days, all rats were euthanized, and the parotid glands were dissected. Hematoxylin & Eosin stain and quantitative real-time PCR analysis were performed to evaluate histological changes and gene expression, respectively.

Results: The streptozotocin group showed significant histological and morphometric changes in the salivary gland compared to the control group. However, the metformin group showed significant improvement in the histological picture compared to the streptozotocin group. The gene expression of TNF-α and IL-6 was significantly elevated in the streptozotocin group, while metformin administration significantly reduced their expression.

Conclusion: The study suggests that metformin has a potential protective effect on salivary gland function in diabetic rats. These results provide valuable insights into the potential therapeutic use of metformin in treating xerostomia in individuals with diabetes.

KEYWORDS Diabetes, xerostomia, salivary gland, metformin.

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INTRODUCTION

Diabetes mellitus is a globally prevalent chronic metabolic disorder with escalating incidence rates, significantly burdening public health [1]. The prevalence of diabetes was 425 million in 2017, according to the International Diabetes Federation. This number is anticipated to rise by 2045 to 629 million, with a 48% increase. In addition, the incidence of diabetes in the Middle East and North Africa was 39 million in 2017, which is expected to rise to 82 million by 2045, with a 110% increase in disease prevalence [2]. Egypt ranks as the ninth country regarding the highest incidence of diabetes worldwide. The incidence of diabetes in Egypt was 7.5 million in 2013 and is expected to increase to 13.1 million by 2035 [3].

Among diabetes numerous complications is xerostomia, or dry mouth. Xerostomia is a debilitating condition contributing to oral infections, dental caries, impaired mastication, and speech. Xerostomia arises from a reduction in salivary flow rate, primarily attributed to salivary gland dysfunction caused by hyperglycemia, inflammation, and oxidative stress [4-6]. Moreover, various inflammatory mediators arise from activating stress-signaling pathways interlinked with insulin resistance (IR) by diverse molecular mechanisms. Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) are two proinflammatory indicators whose prolonged production causes IR by inhibiting insulin signaling protein activation [7, 8].

Saliva, produced by the salivary glands, plays a vital role in maintaining oral health by lubricating and cleansing the oral cavity, neutralizing acids, and preventing bacterial proliferation. Consequently, compromised salivary gland function in individuals with diabetes has far-reaching implications for their oral health and overall quality of life [4].

Metformin (MET), an oral hypoglycemic agent widely prescribed as a first-line treatment for type 2 diabetes, exerts its glucose-lowering effects by suppressing hepatic glucose output, enhancing peripheral glucose uptake, and improving insulin sensitivity [9]. While MET has been established as an effective antidiabetic medication, its impact on salivary gland function remains inadequately clarified.

Several studies have attempted to explore the influence of diabetes and MET on salivary gland function, yet the findings have been inconclusive. Some investigations have proposed that diabetes negatively affects salivary gland function by diminishing salivary flow rate, altering saliva composition, and heightening susceptibility to oral infections [10, 11]. Conversely, MET's potential protective effect on salivary glands has been reported, as evidenced by increased salivary flow rate, reduced oxidative stress, and improved saliva quality [12]. However, these studies have been limited by small sample sizes, cross-sectional designs, and a lack of control groups. Therefore, this study aimed to investigate the effect of diabetes on salivary gland degeneration and to assess the potential ability of MET to counter the diabetes effect on salivary gland histology. By shedding light on the intricate mechanisms underlying the effects of diabetes and MET on salivary gland function, this study holds a promise in informing the development of innovative therapeutic strategies targeting xerostomia in individuals with diabetes.

MATERIALS AND METHODS

Sample size calculation

As per previous study [13], a total sample of 18 rats (6 per group) was found sufficient to detect an effect size of 0.9 a power of 0.8, a two-sided hypothesis test, and a significance level of 0.05. Sample size was calculated using the G*power program, Germany (Kiel, 2007).

Animals

This experiment was performed in the animal house of the Faculty of Medicine, Cairo University,

Egypt, under the guidance and approval of the Institutional Animal Care & Use Committee of Cairo University (CU-IACUC) approval number (CU-III-F-C-69-23) and in accordance with the ARRIVE guidelines for in vivo animal research. In the animal laboratory of the Faculty of Medicine at Cairo University, 18 adult male albino rats weighing between 150 and 200 g (Rattus norvegicus albinus, Wistar strain) were obtained and bred. Each animal was sustained in separate cage with unhindered access to food and water.

Induction of Diabetes and experimental design

Following the protocol outlined by Wilson and Islam [14], type 2 diabetes was induced in rats after a one-week adaptation period by replacing their water intake with a 10% fructose solution for two weeks to induce insulin resistance, while the control group received only water. Rats received an intraperitoneal injection of Streptozotocin (STZ) (40 mg/kg body weight) in citrate buffer (pH 4.5) to induce partial pancreatic cell dysfunction. Rats in the control group were given the same amount of citrate buffer. One week after diabetes was induced, the non-fasting blood glucose level was measured in the tail vein blood of all rats using an Accu-chek glucometer (Roche Diagnostics GmbH, Mannheim, Germany). Rats with blood glucose levels above 300 mg/dL for seven consecutive days were categorized as hyperglycemic and chosen for the study. Rats were divided into three groups (n=6 per group) as follows:

Control group: Six healthy rats received citrate buffer (2 mL/mg body weight) orally for 28 days.

STZ group: Six diabetic rats received citrate buffer (2 mL/mg body weight) orally for 28 days.

MET group: Six diabetic rats received oral administration of MET 100 mg/kg [15] body weight for 28 days.

Animal sacrifice and tissue preparation

After 28 days, all rats were euthanized by an intra-cardiac overdose of sodium thiopental (80 mg/kg). Parotid glands were dissected into two halves. Specimens from one half of the gland were prepared for Hematoxylin & Eosin (H&E) stain for histological evaluation. While specimens from the other half were used to measure TNF-α and IL-6 gene expression.

Investigation methods

1. Light Microscopic Examination

Samples were fixed for 48 hours in 10% buffered formalin at the Faculty of Dentistry, Cairo University, then dehydrated in ethyl alcohol, cleaned in xylol, and embedded in paraffin wax. Sections of approximately 4-6 um thickness were cut, mounted on glass slides, stained with H&E stain, and then examined using a light microscope (Leica, Switzerland) at magnifications of x40 and x100.

2. Histomorphometric Analysis

Density of inflammatory cells

The number of inflammatory cells was estimated in the connective tissue capsules and septa through analysis of light microscopic images of H&E stained sections acquired under magnification x40 via image analysis software (Image J 1.53d) [16]. For each field, the total number of inflammatory cells was acquired, and thereafter, density of inflammatory was computed by dividing the total number of inflammatory cells by the total area of the studied field as previously described [17-19].

Area percent of vacuolation within acinar cells

Area percent of vacuolation within acinar cells was measured through analysis of light microscopic images of H&E-stained sections acquired under magnification x40 using image analysis software

(Image J 1.53d) [16]. vacuolation within acinar cells was perceived as white, rounded, defined spacing within the acinar cells [20]. The total area percentage of vacuolation within acinar cells was calculated by dividing the area of acinar vacuolation by the total area of the studied field [20].

3. Quantitative Real Time-PCR (qRT-PCR) Analysis

Total RNA was extracted from the acquired samples using a total RNA isolation kit (Qiagen, USA), as directed by the kit's manufacturer. The RNA obtained from the specimens was subjected to reverse transcription using a cDNA Reverse Transcriptase reagent (Fermentas, USA) following the instructions provided in the kit's protocol. The Biosystem with software version 3.1 (Step OneTM, USA) was employed to amplify and analyze cDNA. The comparative CT method was employed to normalize the relative mRNA gene expression by comparing it to the mean critical threshold values of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences (Thermo-Fisher Scientific, USA) for the TNF-α, IL-6, and GAPDH genes are provided in Table 1.

4. Statistical Analysis

Data were described in terms of mean and standard deviation (SD). Since data were normally distributed, ANOVA test followed by Tukey post hoc test when ANOVA results were statistically significant, was used to analyze the data. Data analysis was carried out through statistical package SPSS version 22.

RESULTS

Histological examination:

Histological examination of the control group revealed the presence of serous acini with normal architecture and histology. Cells of the serous acini displayed the presence of normal basal nuclei. Several normal intralobular ducts in between the serous acini, and normal interlobular ducts within the connective tissue separating the gland lobes and lobules were detectable. All ducts revealed normal epithelial lining and no stagnant secretions were detectable within their lumen. Few inflammatory cells scattered within the connective tissue were obvious (figure 1).

Examination of the STZ group specimens, on the other hand, revealed marked distortion in the histology and morphology of the serous acini. Several degenerated acini were detectable, intracellular vacuolations were obvious within acinar cells upon higher magnification. Some nuclei displayed pleomorphism and hyperchromatism, with a tendency toward a more central displacement. Some intralobular ducts revealed signs of degeneration and intracellular vacuolation. interlobular ducts, detected within connective tissues, showed stagnant, retained secretions within their lumen, their lining showed areas of intracellular vacuolation, while some areas of their lining showed a marked increase in epithelial thickness. Dilated, congested blood vessels in addition to marked inflammatory cell infiltrate were detectable within the connective tissue. Several areas of hyalinization could be detected between the acini and within the connective tissue (figure 2).

TABLE (1) Primer's sequence of all studied genes.

Gene Symbol	Primer sequence (From 5' to 3')	Gene bank
TNF-α	F: 5′-AAATGGGCTCCCTCTCATCAGTTC	NM001135009.1
	R:5′- TCTGCTTGGTGGTTTGCTACGAC	
IL-6	F: 5′-TTCCATCCAGTTGCCTTCTT-3′	NM001314054.1
	R:5′-ATTTCCACGCGATTTCCCAGAG-3′	
GAPDH	F: 5′- CCATTCTTCCACCTTTGATGCT-3′	NM017008.4
	R:5′-TGTTGCTGTAGCCATATTCATTGT-3′	

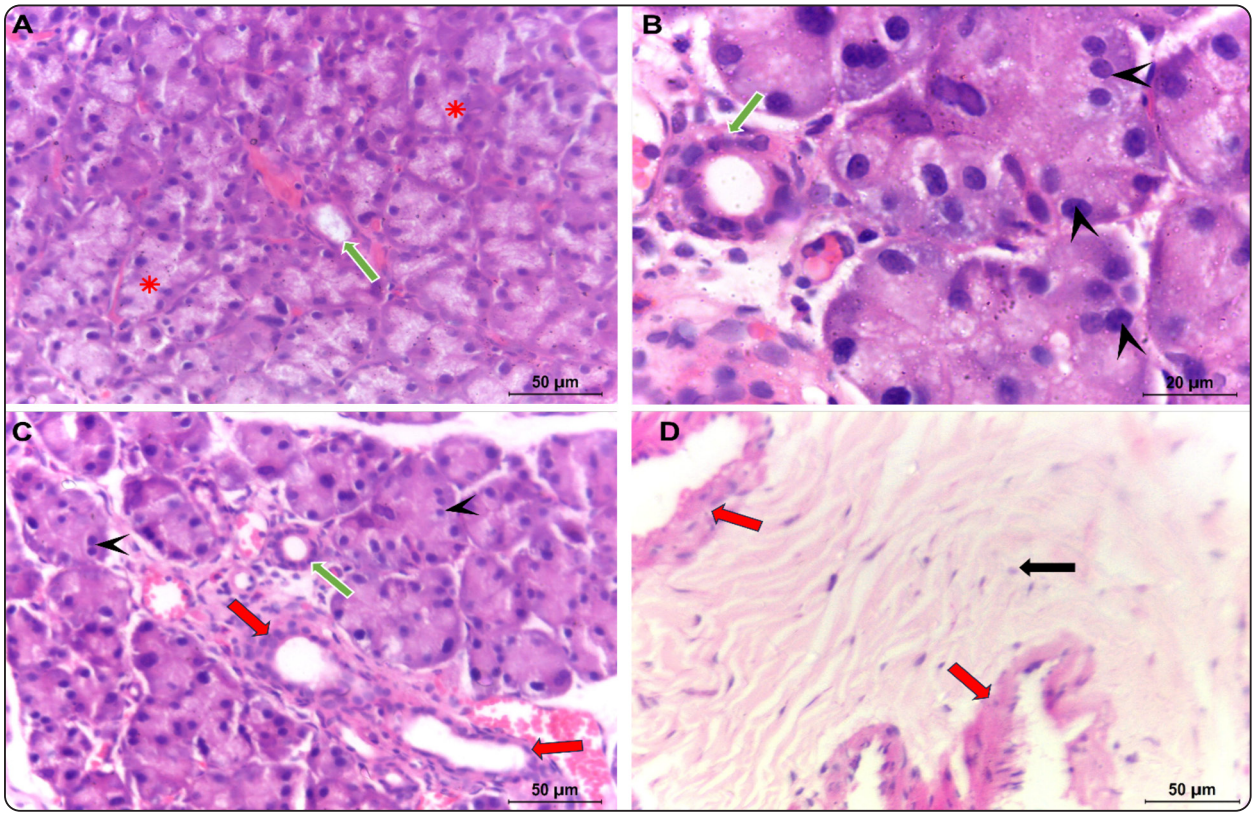

Fig. (1): Light microscopic picture of control group showing: normal acinar histology (red asterisk), with normal basally aligned nucleus (black arrow heads), intralobular duct (green arrows) and interlobular ducts (red arrows) showing normal morphology and normal lining, and few scattered inflammatory cells in connective tissue (black arrows). (Figures A, C & D original magnification X 40, figure B original magnification X 100).

Histological examination of the MET group showed improvement in the histological picture as compared to STZ group. However, some areas of acinar degeneration, in addition to some acinar cells with intracellular vacuolation were detected. Some nuclei displayed hyperchromatism and central displacement. Similar to STZ group, some intralobular ducts showed areas of degeneration and intracellular vacuolation. interlobular ducts showed stagnant secretion within their lumen, and areas of intracellular vacuolation were detected within their lining. Dilated blood vessels in addition to inflammatory cell infiltrate were detected within the connective tissue (figure 3).

Morphometric results:

One way ANOVA followed by Tukey's post hoc test demonstrated that a significantly higher mean density of inflammatory cells was recorded in the STZ group as compared to both MET and control groups. A significantly higher mean was also recorded in MET group as compared to the control groups ($p<0.05$) (Table 2).

STZ showed the highest mean value for area percentage of vacuolation within acinar cells as compared to both MET and control groups. A statistically significant higher mean value was detected in MET group as compared to the control group ($p<0.05$) (Table 2).

PCR results:

A significant increase in gene expression of TNF-α and IL-6 was detected in the STZ group as compared to control. A statistically significant decrease in gene expression of TNF-α and IL-6 was recorded in the MET group as compared to STZ group. While the difference in gene expression between MET and control groups was statistically insignificant (Table 3).

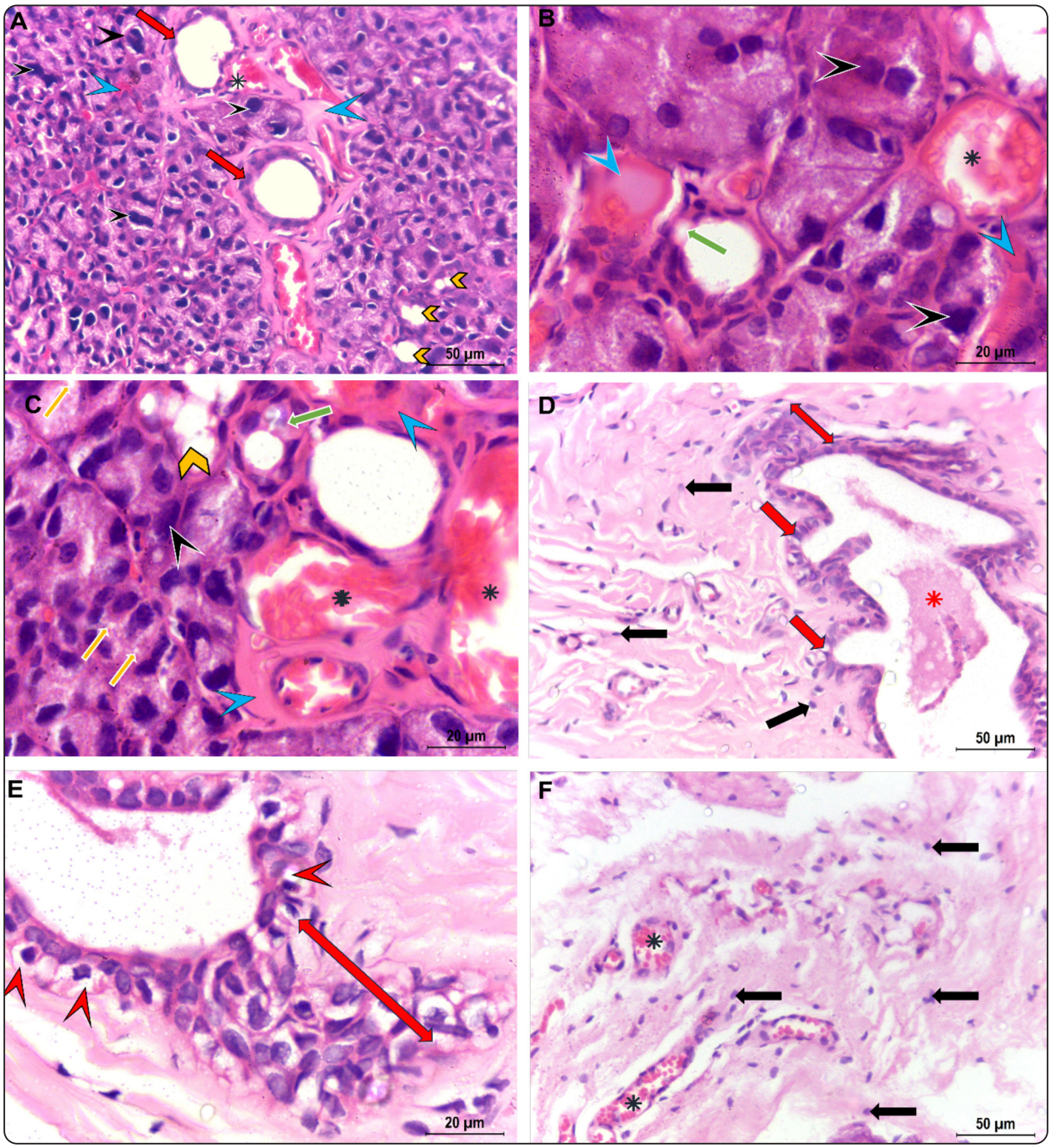

Fig. (2): Light microscopic picture of STZ group showing: areas of acinar degeneration (yellow arrowheads), interlobular ducts (red arrows), nuclear aberration and nuclear hyperchromatism with some nuclei showing central displacement (black arrow heads), dilated and congested blood vessels (black asterisks), intracellular vacuolation (yellow arrows), areas of hyalinization (blue arrow heads), intralobular duct with evidence of degeneration and intracellular vacuolation (green arrows), scattered inflammatory cells in connective tissue (black arrows), interlobular ducts (red arrows) showing stagnant secretions (red asterisk), and lining of interlobular ducts showing areas of vacuolation (red arrow heads) and areas of increased thickness (double arrow head). (Figures A, D & F original magnification X 40, figures B, C & E original magnification X 100).

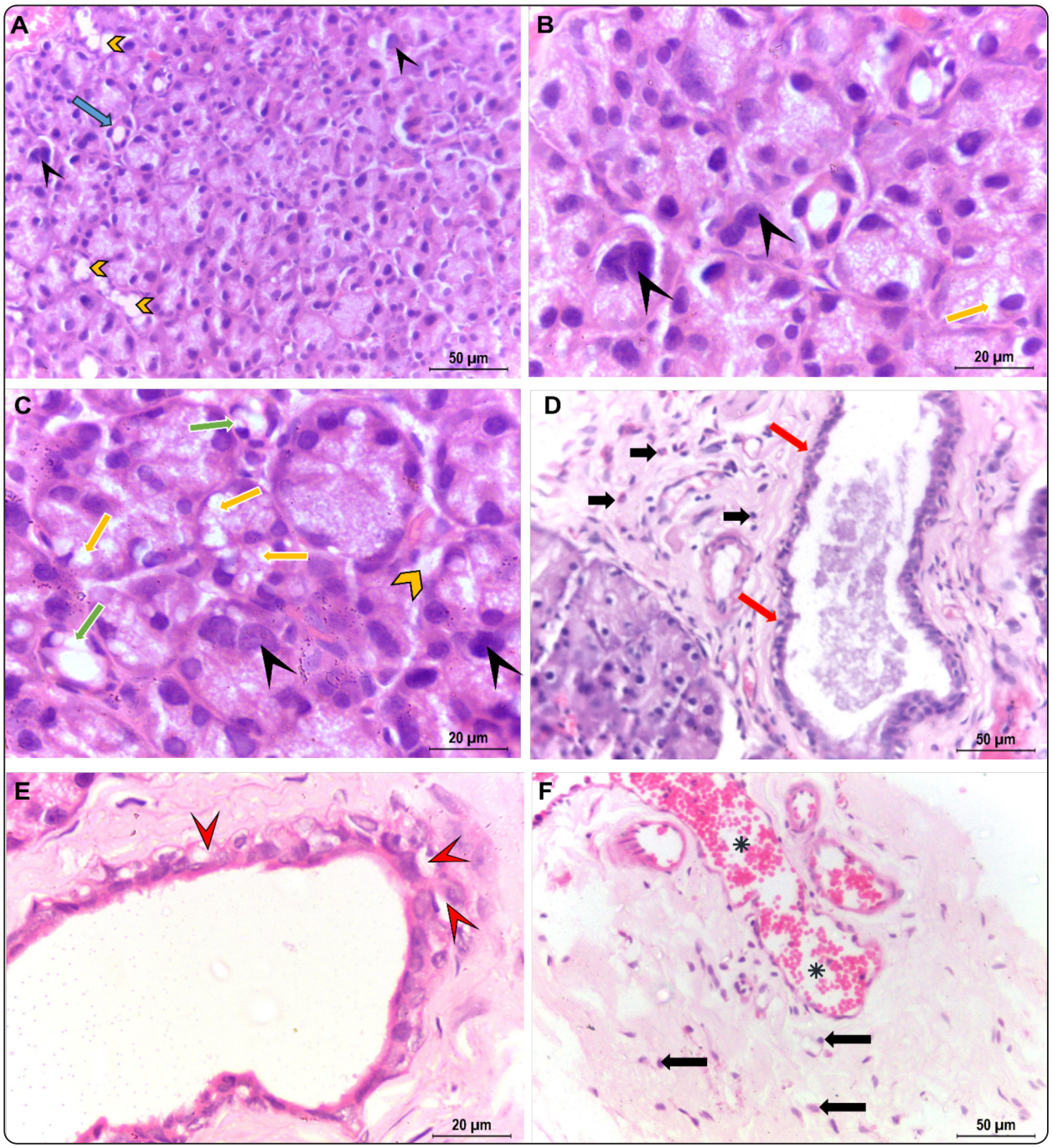

Fig. (3): Light microscopic pictures of MET group showing: areas of acinar degeneration (yellow arrowheads), intralobular duct (blue arrow), nuclear aberration and nuclear hyperchromatism, with some nuclei showing central displacement (black arrow heads) intracellular vacuolation (yellow arrows), intralobular duct with areas of vacuolization (green arrows), dilated and congested blood vessels (asterisk), scattered inflammatory cells in connective tissue (black arrows), interlobular ducts showing stagnant secretions (red arrows) and lining of interlobular ducts showing areas of vacuolation (red arrow heads). (Figures A, D & F original magnification X 40, figures B, C & E original magnification X 100).

TABLE (2): Descriptive statistics and comparison between groups for density of inflammatory cells and area percent of vacuolation within acinar cells (ANOVA test and Tukey's post hoc test).

Parameter	Group	Mean±SD	Std. Error	Min	Max	95% Confidence Interval for Mean	One way ANOVA	
							F	P value
Density of inflammatory cells	Control	0.144±0.028 ^{C}	0.01148	0.108	0.183	(0.0933, 0.1938)	110.81	0.000*
	MET	0.393±0.058 ^{B}	0.024	0.302	0.474	(0.3428, 0.4433)		
	STZ	0.631±0.076 ^{A}	0.031	0.538	0.743	(0.5897, 0.6902)		
Area percent of vacuolation within acinar cells.	Control	5.5±1.87 ^{C}	0.764	3.000	8.000	(2.533, 8.467)	122.40	0.000*
	MET	26.13±1.73 ^{B}	0.705	24.500	29.100	(23.166, 29.101)		
	STZ	35.6±5.33 ^{A}	2.18	30.25	43.80	(32.66, 38.59)		

Significance level P<0.05, *significant.

Means with different superscript letters are significantly different.

TABLE (3): Descriptive statistics and comparison between groups for IL-6 and TNF-α gene expression (ANOVA test and Tukey's post hoc test).

Parameter	Group	Mean±SD	Std. Error	Min	Max	95% Confidence Interval for Mean	One way ANOVA	
							F	P value
IL-6	Control	1.00 ^{B}	0.00	1.00	1.00	(-0.038, 2.038)	22.22	0.000*
	MET	1.158±0.33 ^{B}	0.14	0.60	1.42	(0.121, 2.196)		
	STZ	5.052±2.04 ^{A}	0.83	2.04	8.17	(4.014, 6.089)		
TNF-α	Control	1.00 ^{B}	0.00	1.00	1.00	(0.481, 1.519)	76.01	0.000*
	MET	1.270±0.638 ^{B}	0.261	0.610	2.480	(0.751, 1.789)		
	STZ	4.805±0.813 ^{A}	0.332	3.600	5.511	(4.286, 5.324)		

Significance level P<0.05, *significant.

Means with different superscript letters are significantly different.

DISCUSSION

Through the current study, we investigated the efficacy of MET in restoring parotid salivary gland architecture and normal histology in rats with STZ-induced diabetes. STZ has been successfully used for the induction of diabetes, STZ-induced diabetes in rats provides a convenient and accurate animal model for studying the deleterious effect of diabetes on multiple systems [21-25]. STZ single injection can partially destruct the pancreatic β-cells and induce diabetes [21, 24]. Through the current study, a single intraperitoneal injection of STZ was used to induce type 2 diabetes in rats.

Histological and histomorphometric analysis of STZ specimens of the current study revealed a diabetes associated destruction of rats' parotid salivary glands normal histology and architecture. Several areas of degenerated acini and ducts in addition to several areas of intracellular vacuolation in acinar cells and duct cells as well as ducts with stagnant secretion were detected in the STZ group. Multiple inflammatory cells and dilated congested blood vessels were also obvious

within the connective tissue. Morphometric analysis of STZ group demonstrated a significant increase in the density of inflammatory cells and area percent of intracellular vacuolation within acinar cells. PCR results confirmed histological and histomorphometric analysis with significant upregulation of proinflammatory cytokines IL-6 and TNF-α in the STZ group.

The deleterious effect of diabetes on salivary glands was previously documented. Diabetes was associated with atrophic submandibular salivary gland changes, increased parenchymal cell vacuolation, congested blood vessels, and increased connective tissue septa thickness. Atrophic salivary gland changes were mediated through TGFβ / Smad2 and Smad3 pathway [26]. Diabetes was also associated with increased acinar cell vacuolization and increased laminin content of the parotid salivary gland [27]. Increased acinar vacuolization was also observed in acinar cells of the submandibular salivary gland of rats with STZ-induced diabetes [20].

Diabetes associated damage to multiple organs, including salivary glands, with subsequent consequences on reduced salivary production and hypo-salivation in patients, is directly attributed to diabetic hyperglycemia. Diabetes associated hypercalcemia induces a state of systemic chronic inflammation accompanied with increased levels of systemic inflammatory cytokines [28, 29]. Chronic inflammatory state results in accumulation of advanced glycation end products in multiple organs eventually upregulating reactive oxygen species accumulation and inducing oxidative stresses in multiple body systems [28-30].

ROS levels were markedly increased in the salivary glands of diabetic rats [31]. Further, STZ-induced diabetes was associated with reduced glandular function owing to increased oxidative stresses and oxidative damage in parotid and submandibular salivary glands in rats [32]. Diabetes was also associated with a reduction in antioxidant enzymes superoxide dismutase and thiol in submandibular salivary glands of rats with alloxan-induced diabetes [33]. Additionally, diabetes was also associated with hypofunction and atrophy of submandibular and parotid salivary glands of diabetic rats in addition to reduced nitric oxide synthase enzymes, important for the function of the salivary gland [34].

Similar to PCR results, reported in the current study, STZ-induced diabetes in rats resulted in a significant increase in serum oxidative markers, and inflammatory markers including IL-6 and TNF-α [13]. Further, significant increase in expression of TNF-α, and IL-6, associated with significant destruction of acinar cells of parotid salivary glands, was observed in diabetic rats [35]. Similarly, histopathological analysis of the parotid salivary gland of diabetic rats revealed a significant infiltration of inflammatory cells, in addition to upregulated expression of IL-6 and TNF-α, and a significant increase in the markers of oxidative stresses [36]. Diabetes was also associated with an increase in both serum levels and mRNA levels of inflammatory cytokines including IL-6 and TNF-α in the salivary glands of diabetic rats [37].

The histological examination of the MET group revealed areas of salivary gland acini and duct degeneration. Some acinar cells and ductal cells showed intracellular vacuolation, interlobular ducts showed stagnant secretion within their lumen. Dilated blood vessels in addition to inflammatory cell infiltrate were detected within the connective tissue. Morphometric analysis of MET group demonstrated a significant decrease in density of inflammatory cells and area percent of intracellular vacuolation as compared to STZ while MET results were significantly higher as compared to the control group. PCR results revealed a significant reduction in IL-6 and TNF-α expression as compared to STZ, while the difference between MET and the control group was insignificant.

Similar to findings reported in the current study, MET successfully reduced expression levels of inflammatory markers including IL-6 and TNF-α and inflammatory cell infiltrate in salivary glands of diabetic rats and successfully restored salivary flow rate and salivary gland function as compared to untreated diabetic group [12]. MET was also associated with significant improvement in diabetes associated histopathological changes in the salivary gland of rats and restored its function [38]. Additionally, similar to findings reported in the current study, MET significantly reduced inflammatory markers in the salivary glands of diabetic rats including IL-6 and TNF-α [39].

On the other hand, MET at high doses was associated with signs of parotid salivary gland cellular necrosis and mild inflammatory infiltrate [40]. Further, it is noteworthy that transporters of MET include organic cation transporter (OCT) 1–3 [41]. OCT3 robust expression was detected in parenchymal elements of salivary glands, where it provides the way for MET accumulation in salivary gland [42], which may explain with metformin associated dysgeusia in diabetic patients [41].

From previous data, it can be deduced that even though MET group showed significant improvement as compared to STZ group, where MET group was associated with a significant decrease in inflammatory markers and density of inflammatory cells as compared to STZ group, yet, MET treatment did not effectively reverse the damaging effect of diabetes on the salivary gland and evidence of salivary gland degeneration was still detectable. The previous findings can be attributed to the accumulation of high concentrations of MET within the salivary gland causing some adverse effects.

CONCLUSIONS:

The present study provides strong evidence that the administration of STZ in rats induced significant histological and morphometric changes in the salivary gland. In contrast, the administration of MET resulted in significant improvement in parotid salivary gland histology and inflammatory markers as compared to STZ group, even though MET did not reverse all the damaging effects of diabetes on the salivary gland back to normal. These results highlight the potential beneficial effects of metformin in ameliorating diabetes-related salivary gland damage. Further studies with different timelines, comparing different dosages of MET and applying supplementations that can potentiate MET reparative effects are needed to better understand the underlying mechanisms and to determine the optimal dosing and duration of treatment with MET to improve salivary gland histology and function in diabetic patients.

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