Streptozotocin- induced changes of aquaporin 1 and 4, oxidative stress and autophagy in submandibular and parotid salivary glands and the possible ameliorative effect of intermittent fasting on these changes: immunohistochemical and molecular study in rats.

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Abstract

Salivary glands are greatly responsible for maintaining oral tissue homeostasis by the secretion of saliva. This study was designed to investigate the changes of aquaporin 1 and 4, oxidative stress and autophagy in submandibular and parotid salivary glands of diabetic rats and the possible ameliorative effect of intermittent fasting on these changes. Fifty adult male rats were divided into control and experimental groups. Experimental diabetes was induced by a single intraperitoneal injection of streptozotocin. After diabetic induction, the experimental group was divided into two groups (diabetic without intermittent fasting and diabetic with intermittent fasting). Animals were sacrificed two and four weeks after induction of diabetes. Intermittent fasting significantly decreased malondialdehyde and significantly elevated reduced glutathione (GSH) in the submandibular and parotid glands compared to those in diabetic rats. The salivary secretions were also significantly spared histologically in diabetics with intermittent fasting groups. As well, intermittent fasting up-regulated aquaporin 1 in both glands, while aquaporin 4 was only elevated in submandibular gland. Immunolocalization and gene expression of Lc3-II was higher in diabetic salivary glands than in fasting glands. In conclusion, these findings highlight the pathological role of autophagy in diabetic sub-mandibular and parotid glands and provide potential target for therapeutic role of intermittent fasting to ameliorate submandibular and parotid glands dysfunction in type I diabetes mellitus.

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Abha 61421, Saudi Arabia. Email: m.alghamdi@kku.edu.sa.[4]Department of endocrinology and diabetes treatment center King Khalid University, College of medicine, Internal medicine department, Abha 61421, Saudi Arabia. Email: hanan.al_shehri@hotmail.com[5]Internal medicine department King Khalid University.Saudi Arabia.Email: aaamerai@kku.edu.com. ORCID ID:0000-0002-1517-9221[6]Department of Anatomy, College of Medicine, Imam Abdulrahman Bin Faisal University, P.O. Box 2114, Dammam 31451, Saudi Arabia. Email: kalkhater@iau.edu.sa. Tel # 00966504903833 raaldahhan@iau.edu.sa https://orcid.org/0000-0001-9933-9837[7]Department of Histology and Cell Biology college of Medicine, Benha University, Egypt. Email: eman.alnashshar@fmed.bu.edu.eg ; enashar@kku.edu.sa*https://orcid.org/0000-0002-2883-6761****Corresponding author**E-mail address: anamed1076@yahoo.com (R.I. Taha). Full postal address: Mansoura University Post Office, Mansoura, Egypt. Postal code: 35516.**Abstract:** Salivary glands are greatly responsible for maintaining oral tissue homeostasis by the secretion of saliva. This study was designed to investigate the changes of aquaporin 1 and 4, oxidative stress and autophagy in submandibular and parotid salivary glands of diabetic rats and the possible ameliorative effect of intermittent fasting on these changes. Fifty adult male rats were divided into control and experimental groups. Experimental diabetes was induced by a single intraperitoneal injection of streptozotocin. After diabetic induction, the experimental group was divided into two groups (diabetic without intermittent fasting and diabetic with intermittent fasting). Animals were sacrificed two and four weeks after induction of diabetes. Intermittent fasting significantly decreased malondialdehyde and significantly elevated reduced glutathione (GSH) in the submandibular and parotid glands compared to those in diabetic rats. The salivary secretions were also significantly spared histologically in diabetics with intermittent fasting groups. As well, intermittent fasting up-regulated aquaporin 1 in both glands, while aquaporin 4 was only elevated in submandibular gland. Immunolocalization and gene expression of Lc3-II was higher in diabetic salivary glands than in fasting glands. In conclusion, these findings highlight the pathological role of autophagy in diabetic sub-mandibular and parotid glands and provide potential target for therapeutic role of intermittent fasting to ameliorate submandibular and parotid glands dysfunction in type I diabetes mellitus.**Keywords:** diabetes type I, fasting, autophagy, aquaporin 1, aquaporin 4**Highlights:**

- Intermittent fasting up-regulated aquaporin 1 in both submandibular and parotid glands.
- Intermittent fasting up-regulated aquaporin 4 in submandibular gland.
- Intermittent fasting ameliorated the oxidative stress induced by diabetes.
- Immunolocalization and gene expression of Lc3-II was higher in diabetic salivary glands than in fasting glands.

1. Introduction

Diabetes mellitus (DM) is a disease characterized by elevation of fasting blood sugar and impaired carbohydrate, lipid, and protein metabolism [1]. The worldwide prevalence of DM is estimated to be 4.4% in 2030, involving about 366 million people [2]. DM affects multiple organs including the eyes, kidneys, nervous systems, and salivary glands [3-6].

Salivary glands are greatly responsible for maintaining oral tissue homeostasis by the secretion of saliva. Saliva contains many components that play an important role in defense against microorganisms, protection of teeth (buffering, lubrication, remineralization, protection against demineralization), and food handling (bolus formation, taste, digestion) [7].

Saliva secretion is adversely impacted in DM which can lead to many complications such as xerostomia, caries, periodontal diseases, and infections [8,9]. In humans, about 60% of resting saliva and 40% of stimulated saliva are secreted by the submandibular gland [10]. Saliva secretion is mainly accomplished through transcellular and paracellular pathways [11].

Aquaporins (AQPs) are a family of transmembrane protein water channels, which selectively promote the transport of water, small solutes (including cations and glycer-ines), and gases through cell membrane [12,13]. These AQPs are regulated by osmotic gradient and play various roles in physiological processes [14] (e.g., salivary secretion) [15]. Among the family of AQPs, AQP1, AQP4, AQP5, and AQP8 are expressed

in salivary glands. AQP5 has an essential function in secretion of saliva13. Interestingly, Steinfeld et al. [16] observed abnormal distribution of AQP5 in minor salivary glands of patients with Sjögren's syndrome. However, only a few reports addressed the role of AQP1 and AQP4 in salivary gland secretions.

Fasting is a non-pharmacological approach characterized by complete food deprivation with intervals of normal food intake. According to duration of fasting, it is classified into periodic fasting (successive fasting period for more than 24 hours), alternate day fasting, and intermittent fasting [17]. Intermittent fasting includes alternating cycles of feeding and fasting (typically 16 hours fasting with complete deprivation of food, but not water, and 8 hours eating with normal food intake) [18]. The popularity of intermittent fasting has been gained due to its beneficial role in regulating inflammation and in promoting health in many systems [19,20].

Autophagy is known to be a catabolic process that maintains homeostasis of cells by degrading cellular components, and its dysfunction is involved in the development of metabolic disorders, such as DM and its complications [21-23]. Macro autophagy is the major type of autophagy in which organelles and cytosol are sequestered within double-membrane vesicles (auto phagosome) that deliver their contents to the lysosome/vacuole for enzymatic degradation and product recycling [24]. Auto phagosome formation is controlled by autophagy related genes (Atg)25, Unc-51-like kinase, phosphatidylinositol 3-kinase (PI3K), ATG12–ATG5–ATG16L1 complexes, and microtubule-associated protein light chain 3 (MAPLC3) family members [26,27]. In mammalian cells, the conversion of LC3BI to LC3BII is a marker of autophagy [28]. However, the exact role of autophagy and whether it is involved in the dysfunction of submandibular and parotid glands in DM is still unknown.

Antunes et al. [29] had described the role of autophagy in tumorigenesis and the potentiality of using fasting as a complementary therapy for cancer treatment. Recently, Yuan et al. [30] investigated the therapeutic value of intermittent fasting on neuronal survival after acute spinal cord injury in rats, and its underlying mechanism related to autophagy regulation. They found remarkable improvement in both behavioral performance and neuronal survival at the injured segment of the spinal cord of animals previously subjected to intermittent fasting.

The aim of this study was to investigate the changes of aquaporin 1 and 4, oxidative stress and autophagy in submandibular and parotid salivary glands and the possible ameliorative effect of intermittent fasting on these changes in diabetic rats. This was carried out by studying changes in oxidative stress markers and examining histopathological, histochemical, and immunohistochemical changes and gene expression.

2. Results

2.1.Effects of diabetes and intermittent fasting on oxidative stress markers (MDA and GSH) in the submandibular and parotid glands

To assess the extent of oxidative stress in case of diabetes with or without intermittent fasting, the levels of lipid peroxidation biomarker MDA (Table 1) and the antioxidant enzyme reduced GSH (Table 2) were measured in both parotid and submandibular gland tissues of each group and compared to control group. Diabetes significantly increased MDA in submandibular glands compared to the control group. However, intermittent fasting significantly decreased MDA compared to diabetic values, at both time points of experiment (two and four weeks after induction of diabetes). Regarding the parotid gland, diabetes increased MDA level compared to control values at both time points, but this difference was only significant four weeks after induction of diabetes. As to the level of the reduced GSH, diabetes significantly decreased its level in the submandibular glands compared to the control group. However, intermittent fasting significantly increased its level compared to diabetic values, at both time points of the experiment. Regarding the parotid gland, diabetes decreased GSH level compared to control values, at both time points, but this reduction was significant compared to control value only four weeks after induction of diabetes.

Table (1): Effects of diabetes and intermittent fasting (IF) on MDA in submandibular and parotid glands in comparison to control group.

			Control group	Diabetic group	Diabetic Fasting group	P
MDA	**parotid**	**2wks**	**83.71±3.23**	**115.10±47.30**	**86.42±27.84**	**0.19**
		4wks	**83.71±3.23**	**325.23±30.74#**	**128.83±17.46#**	**<0.001***
				P1=<0.001*	**P2=0.005***	
					P3=<0.001*	
	submandibular	**2wks**	**34.42±3.52**	**126.53±42.65**	**47.56±14.30**	**<0.001***
				P1=<0.001*	**P2=0.66**	
					P3=<0.001*	
		4wks	**34.42±3.52**	**153.59±16.44**	**34.52±4.18**	**<0.001***
				P1=<0.001*	**P2=1.00**	
					P3=<0.001*	

Data expressed as mean ± SD, *: significance < 0.05

Test used: One way ANOVA followed by post-hoc Tukey for comparison between groups

P1: significance between Control & diabetic.

P2: significance between Control & diabetic fasting.

P3: significance between diabetic fasting & diabetic.

#: indicate significance between Week4 vs Week2 within each group

wks = weeks

Table (2): Effects of diabetes and intermittent fasting on GSH in submandibular and parotid glands in comparison to control group.

			Control group	Diabetic group	Diabetic Fasting group	P
GSH (reduced)	**Parotid**	**2wks**	**71.32±6.06**	**64.79±7.53**	**69.62±2.76**	**0.16**
		4wks	**71.32±6.06**	**56.55±9.36**	**65.30±5.13**	**0.009***
				P1=0.007*	**P2=0.33**	
					P3=0.11	
	Submandibular	**2wks**	**34.09±5.11**	**26.62±3.68**	**31.11±3.31**	**0.02***
				P1=0.017*	**P2=0.44**	
					P3=0.17	
		4wks	**34.09±5.11**	**20.65±5.79**	**28.05±4.66**	**0.002***
				P1=0.001*	**P2=0.14**	
					P3=0.065	

Data expressed as mean ± SD, *: significance < 0.05

Test used: One way ANOVA followed by post-hoc tukey for comparison between groups

P1: significance between Control & diabetic.

P2: significance between Control & diabetic fasting.

P3: significance between fasting & diabetic.

#: indicate significance between Week4 vs Week2 within each group

wks = weeks

2.2. Histopathological changes in the submandibular and parotid glands in response to diabetes and intermittent fasting

Examination of haematoxylin and eosin-stained sections of submandibular and parotid glands was performed by light microscopy. The control submandibular gland showed normal acini and duct parts [intercalated duct (ID), granular convoluted tubule (GCT), and striated duct (SD)]. On the other hand, the seromucous acini of diabetic submandibular gland were atypical and showed cytoplasmic vacuolization. These degenerative changes (atrophic acini and GCT) were more marked in submandibular gland of four weeks post diabetes induction group. In diabetic fasting group, there was hypertrophy of acini and regeneration of GCT two weeks post diabetes induction. However, the submandibular gland appeared like control four weeks post diabetes with fasting **(Figure 1).**

Control parotid gland showed normal serous acini and ducts with no histopathological findings. There was cytoplasmic vacuolization seen in the acinar cells and some SD cells in two weeks diabetic group. Moreover, pyknotic nuclei and a few polymorphonuclear leucocytic infiltration in connective tissue were observed. More atrophy and degenerative changes of acini and ducts with increase in the space between the acini were observed in four weeks diabetic group. Two weeks diabetic fasting parotid gland showed nearly normal appearance of acini, but some vacuolization was found. Four weeks diabetic fasting parotid gland appeared almost normal (**Figure 2**).

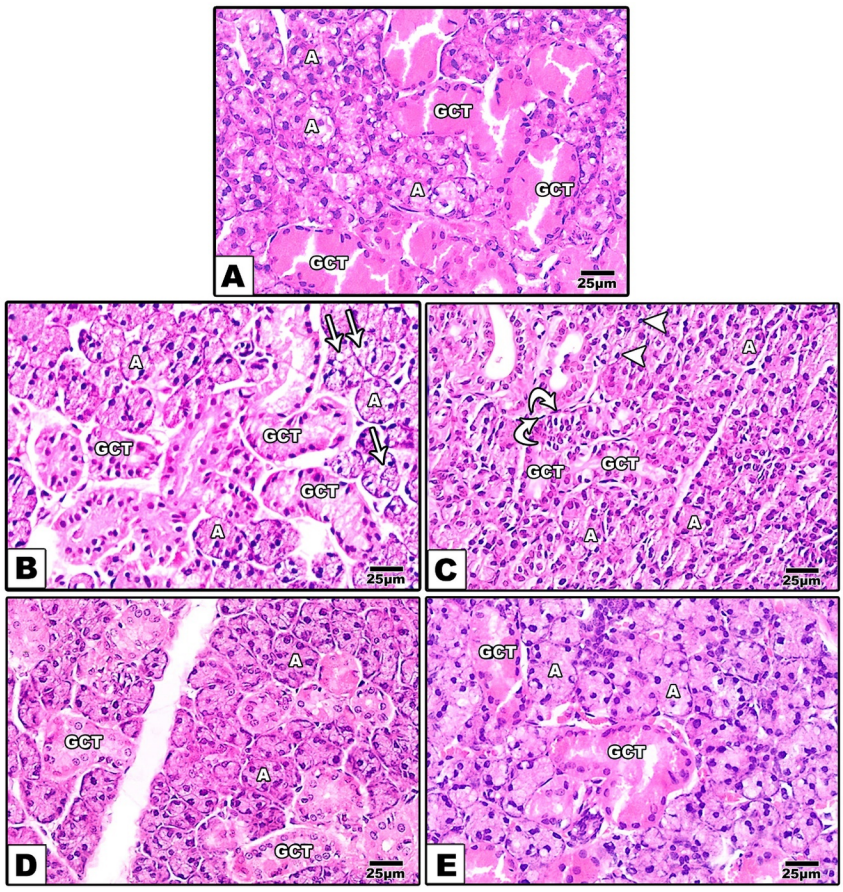


Figure 1. photomicrographs represent the haematoxylin and eosin-stained submandibular glands of rat in response to diabetes and intermittent fasting. A: a photomicrograph of control ratsubmandibular gland showing normal-appearing acini (A) and granular convoluted tubules (GCT) with no histological abnormality. B and C: photomicrographs of diabetic rat submandibular gland two (B) and four (C) weeks post diabetes induction showing atrophic acini (A) with cytoplasmic vacuolization (arrows) and pyknotic nuclei (arrowhead) and atrophicgranular convoluted tubules (GCT) and polymorphonuclear leucocytic infiltration (curved arrows).D: a photomicrograph of rat submandibular gland of two weeks post diabetes (fasting group) showing hypertrophy of acini (A) and regenerating granular convoluted tubules (GCT). E: a photomicrograph of rat submandibular gland of four weeks post diabetes (fasting group) showing apparently normal acini (A) and granular convoluted tubules (GCT). Magnification x400. Scale bar: 25 microns.

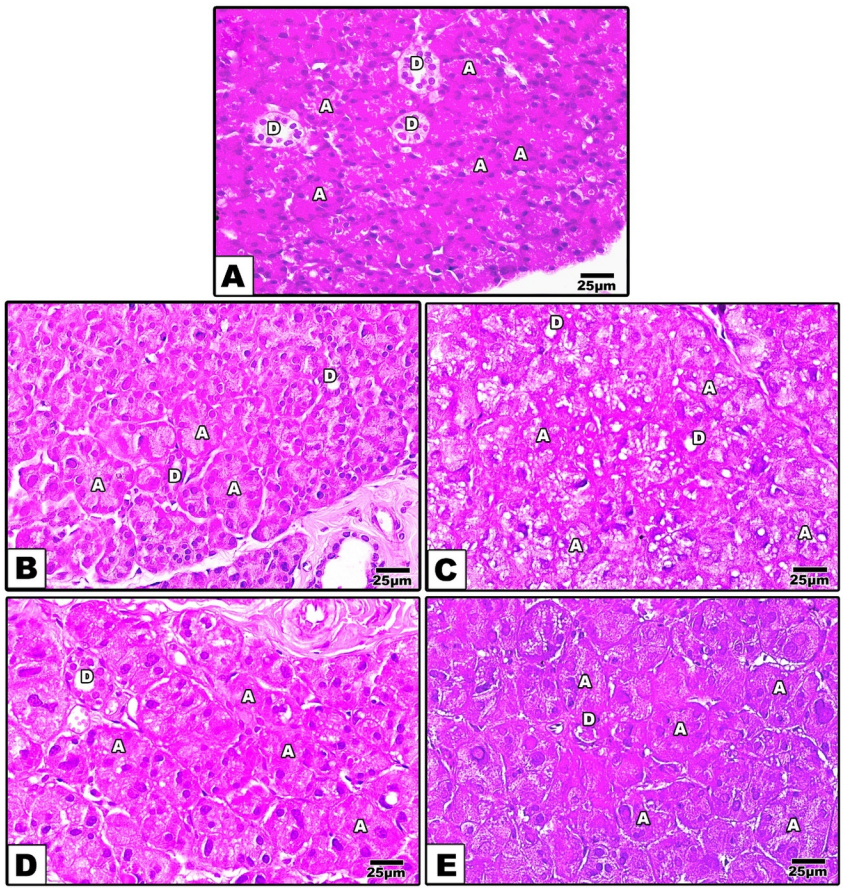
Figure 2. photomicrographs represent the haematoxylin and eosin-stained parotid glands of rat in response to diabetes and intermittent fasting. A: a photomicrograph of control rat parotid gland representing normal-appearing acini (A) and ducts (D) with no histological abnormality. B:a photomicrograph of two weeks diabetic rat parotid gland showing cytoplasmic vacuolization in the acinar cells and some striated duct cells (arrow), pyknotic nuclei (arrowhead), and a few polymorphonuclear leucocytic infiltration in connective tissue (curved arrow). C: a photomicrograph of four weeks diabetic rat parotid gland showing more atrophy and degenerative changes of acini (A) and ducts (D) and increase in the space between the acini (star). D and E: photomicrographs of two (D) and four (E) weeks diabetic fasting rat parotid gland showing nearly normal

appearance of acini (A) and ducts (D) but there was some vacuolization in some acini (arrow). Magnification x400. Scale bar: 25 microns.

Histochemical changes in the submandibular and parotid glands in response to diabetes and intermittent fasting

Histochemical examination of submandibular gland in the control group showed that acinar cell cytoplasm had strong positive reaction with PAS and AB stains, but the GCT showed strong positive reaction with PAS and negative reaction with AB. In submandibular gland of two weeks post diabetes group, acinar cell cytoplasm showed moderate reaction with PAS and darker reaction with AB, while GCT showed positive reaction with PAS only (weaker than acini). Four weeks post diabetes submandibular gland showed weak reaction in the atrophic acini with PAS and dark reaction with AB, and very mild reaction with PAS in the GCT. Two weeks post diabetes fasting sub-mandibular gland showed moderate PAS and AB reactions in acinar cell cytoplasm and moderate PAS reaction only in the GCT. Four weeks post diabetes fasting sub-mandibular gland showed strong positive PAS and AB reaction in the acinar cyto-plasm, whereas the GCT showed moderate reaction with PAS only (Figure 3). Regarding the parotid gland in control rats, acinar cell cytoplasm stained strongly positive with PAS and with AB. While in two weeks post diabetes parotid gland, acinar cell cytoplasm was stained weakly positive with PAS, and stained darker with AB than the control group. Four weeks post diabetes parotid gland, the PAS stain was observed in the lumen of atrophic ducts with no AB stain in the atrophic acini. Two weeks post diabetes (with fasting) parotid gland, the intensity of PAS stain decreased while that of AB stain increased, and in four weeks post diabetes (with fasting) parotid gland, the tissue looked like control group in both stains (Figure 4).

Figure 3 (a and b):photomicrographs represent the histochemical changes in the submandibular glands of rat in response to diabetes and intermittent fasting. (a) PAS and (b) AB. A: control, B: two weeks post diabetes, C: four weeks post diabetes, D: two weeks post diabetes (with fasting), E: four weeks post diabetes (with fasting). PAS: periodic acid Schiff, AB: alcian blue. Magnification x400. Scale bar: 25 microns. A: photomicrographs of control rat submandibular gland showing strong positive reaction of PAS (panel a) and AB (panel b) in acinar cell cytoplasm (A) and strong positive reaction of PAS (panel a) in granular convoluted tubules (GCT). B: photomicrographs of two weeks post diabetes submandibular gland showing positive reaction with PAS (panel a) which is stronger in acinar cell cytoplasm (A) than in granular convoluted tubules (GCT) and positive AB reaction (panel b) (darker than control) in acinar cell cytoplasm (A). C: photomicrographs of four weeks post diabetes submandibular gland showing weak PAS (panel a) and strong AB reactions (panel b) in acinar cell cytoplasm (A) and very mild PAS (panel a) reaction in granular convoluted tubules (GCT). D: photomicrographs of two weeks post diabetes (with fasting) submandibular gland showing moderate PAS (panel a) and AB reactions (panel b) in acinar cell cytoplasm (A) and moderate PAS (panel a) reaction only in granular convoluted tubules (GCT). E: photomicrographs of four weeks post diabetes (with fasting) submandibular gland showing strong positive PAS (panel a) and AB reactions (panel b) in acinar cell cytoplasm (A) and moderate PAS (panel a) reaction only in granular convoluted tubules (GCT).



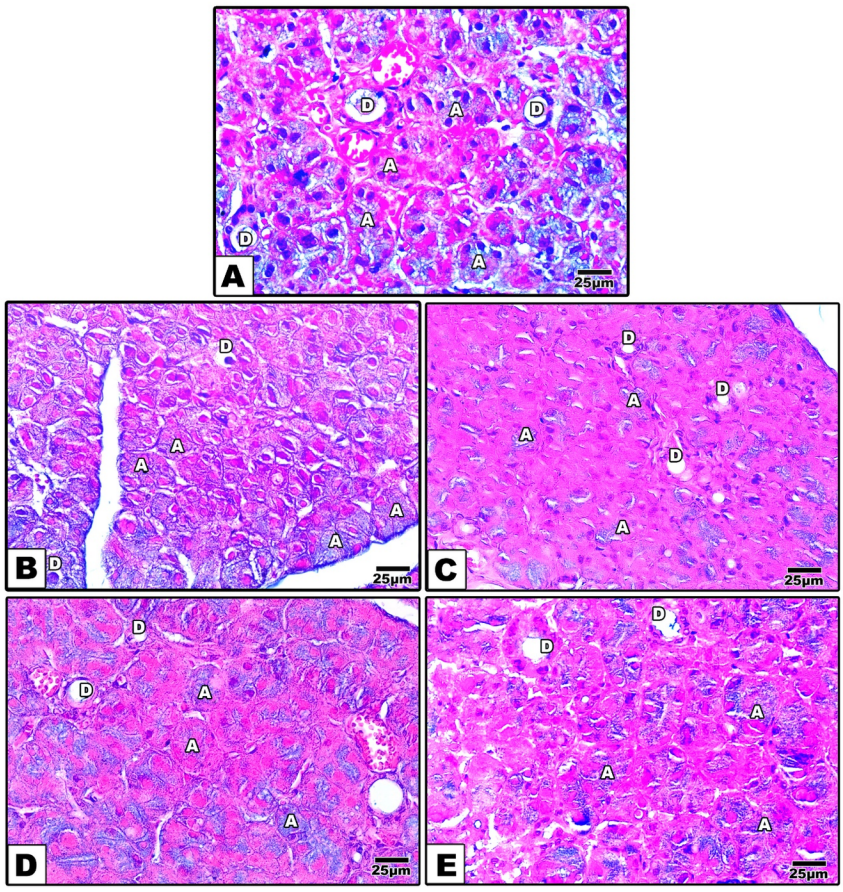


Figure 4 (a and b):*photomicrographs represent the histochemical changes in the parotid glands of rat in response to diabetes and intermittent fasting. (a) PAS and (b) AB. A: control, B: two weeks post diabetes, C: four weeks post diabetes, D: two weeks post diabetes (with fasting), E: four weeks post diabetes (with fasting). PAS: periodic acid Schiff, AB: alcian blue. Magnification x400. Scale bar: 25 microns. A: photomicrographs of control parotid gland showing strong positive PAS (panel a) and AB reaction (panel b) in acinar cell cytoplasm (A). B: photomicrographs of two weeks post diabetes parotid gland showing positive PAS reaction (panel a) (weaker than control) and weak positive AB stain (panel b) in acinar cell cytoplasm (A). C: photomicrographs of four weeks post diabetes parotid gland showing minimal PAS (panel a) and AB (panel b) reaction in atrophic acini (A). D: photomicrographs of two weeks post diabetes (with fasting) parotid gland showing weak positive PAS (panel a) and AB (panel b) reaction in acinar cell cytoplasm (A). E: photomicrographs of four weeks post diabetes (with fasting) parotid gland showing strong positive PAS (panel a) and strong positive AB (panel b) in acinar cell cytoplasm (A). D: duct.*

Effects of diabetes and intermittent fasting on submandibular and parotid glands AQP1

The expression of aquaporin 1 was assessed in different experimental groups (**Table 3** and **Figures 5, 6**). In submandibular gland, the control group showed positive reaction in cell membranes of acini and cytoplasm of acini, myoepithelial cell, ID, GCT, and SD. Also, it was strong positive reaction in endothelial lining of blood vessels (OD = **0.425±.053**). Two weeks post diabetes submandibular gland, the reaction was very weak positive in acinar cell cytoplasm, weak positive in GCT and SD, but it was still strong in endothelial cells of blood vessels and myoepithelial cells (OD = **0.309±0.003**). Four weeks post diabetes submandibular

gland, there was weak positive reaction in GCT, no reaction in acinar cell cytoplasm, and strong reaction in endothelial cells of blood vessels and myoepithelial cells (OD =**0.259±0.012**). Two weeks post diabetes (with fasting) submandibular gland, the reaction was strong positive in all components except acinar cell cytoplasm where it was weak (OD =**0.306±0.011**). Four weeks post diabetes (with fasting) submandibular gland, it was strong positive in all components like control (OD = **0.415±0.033**). In parotid gland, the control group showed positive reaction in cytoplasm of acini and in endothelial lining of blood vessels (OD = **0.484±0.013**). In diabetic groups (two and four weeks) the reaction was weak positive in cytoplasm of acini but strong in endothelial lining of blood vessels (OD = **0.380±0.026**and **0.233±0.017,** respectively). Two weeks post diabetes (with fasting) parotid gland, the reaction was strong positive in cytoplasm of acini in comparison to control group and in endothelial lining of blood vessels (OD = **0.552±0.035**). Four weeks post diabetes (with fasting) parotid gland, the reaction was weaker in comparison to two weeks post diabetes (with fasting) parotid gland, but nearly equal to control group (OD = **0.510±0.045**).

Table (3): Effects of diabetes and intermittent fasting on optical density (OD) aquaporin 1 in submandibular and parotid glands in experimental rats in comparison to control group.

			Control group	Diabetic group	Diabetic Fasting group	P
OD of aquaporin 1	**Parotid**	**2wks**	**0.484±0.013**	**0.380±0.026**	**0.552±0.035**	**<0.001***
				P1=<0.001*	**P2=0.001***	
					P3=<0.001*	
		4wks	**0.484±0.013**	**0.233±0.017#**	**0.510±0.045**	**<0.001***
				P1=<0.001*	**P2=0.3**	
					P3=<0.001*	
	Submandibular	**2wks**	**0.425±.053**	**0.309±0.003**	**0.306±0.011**	**<0.001***
				P1=<0.001*	**P2=<0.001***	
					P3=0.98	
		4wks	**0.425±.053**	**0.259±0.012#**	**0.415±0.033#**	**<0.001***
				P1=<0.001*	**P2=0.88**	
					P3=<0.001*	

Data expressed as mean ± SD, *:significance < 0.05

Test used: One way ANOVA followed by post-hoc Tukey for comparison between groups

P1: significance between Control & diabetic.

P2: significance between Control & diabetic fasting.

P3: significance between fasting & diabetic.

#: indicate significance between Week4 vs Week2 within each group

wks = weeks

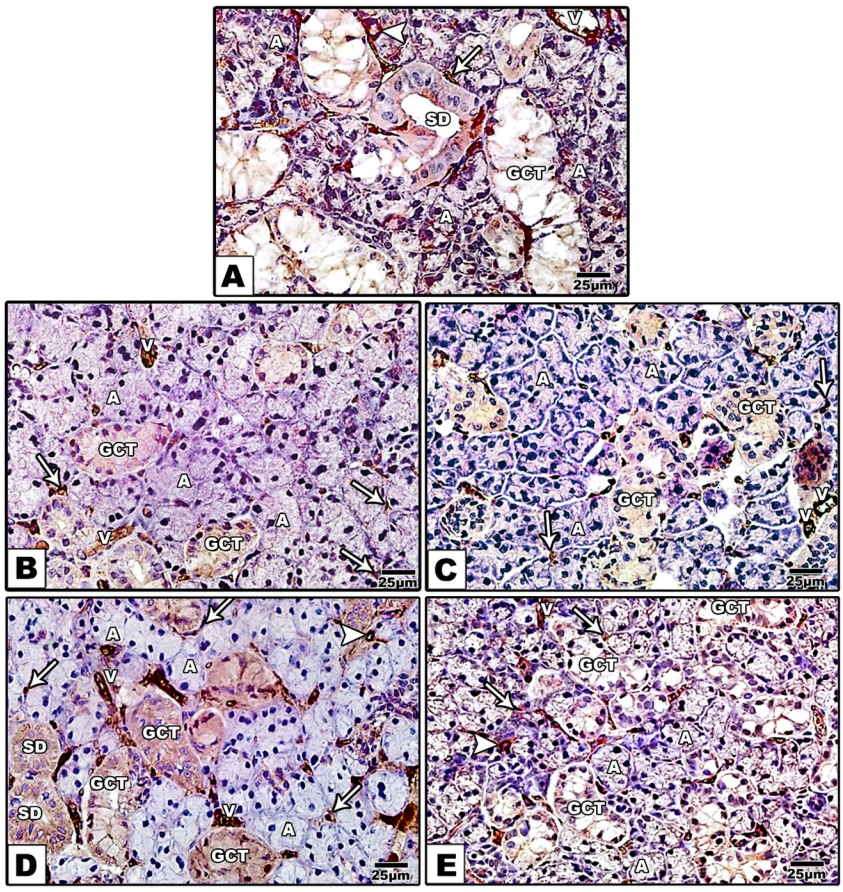


Figure 5. photomicrographs represent the effect of diabetes and intermittent fasting on the distribution of aquaporin 1 in the submandibular glands of rat. A: control, B: two weeks post diabetes, C: four weeks post diabetes, D: two weeks post diabetes (with fasting), E: four weeks post diabetes (with fasting). Magnification x400. Scale bar: 25 microns. A: a photomicrograph of control submandibular gland representing positive reaction in cell membranes and cytoplasm of acini (A), myoepithelial cell (arrow), intercalated ducts (ID) (arrowhead), granular convoluted tubules (GCT) and striated ducts (SD). Also, it shows strong positive reaction in endothelial lining of blood vessels (V). B: a photomicrograph of two weeks post diabetes submandibular gland showing very weak positive reaction in acinar cell cytoplasm (A), granular convoluted tubules (GCT), and striated ducts (SD), and strong positive reaction in endothelial cells of blood vessels (V) and myoepithelial cells (arrow). C: a photomicrograph of four weeks post diabetes submandibular gland showing weak positive reaction in granular convoluted tubules (GCT), no reaction in acinar cell cytoplasm (A), and strong reaction in endothelial cells of blood vessels (V) and myoepithelial cells (arrow). D: a photomicrograph of two weeks post diabetes (with fasting) submandibular gland showing strong positive reaction in all components except acinar cell cytoplasm where it was weak strong positive reaction in cytoplasm of acini (A). E: a photomicrograph of four weeks post diabetes (with fasting) submandibular gland showing positive reaction nearly equal to control group. (Aquaporin 1×400).

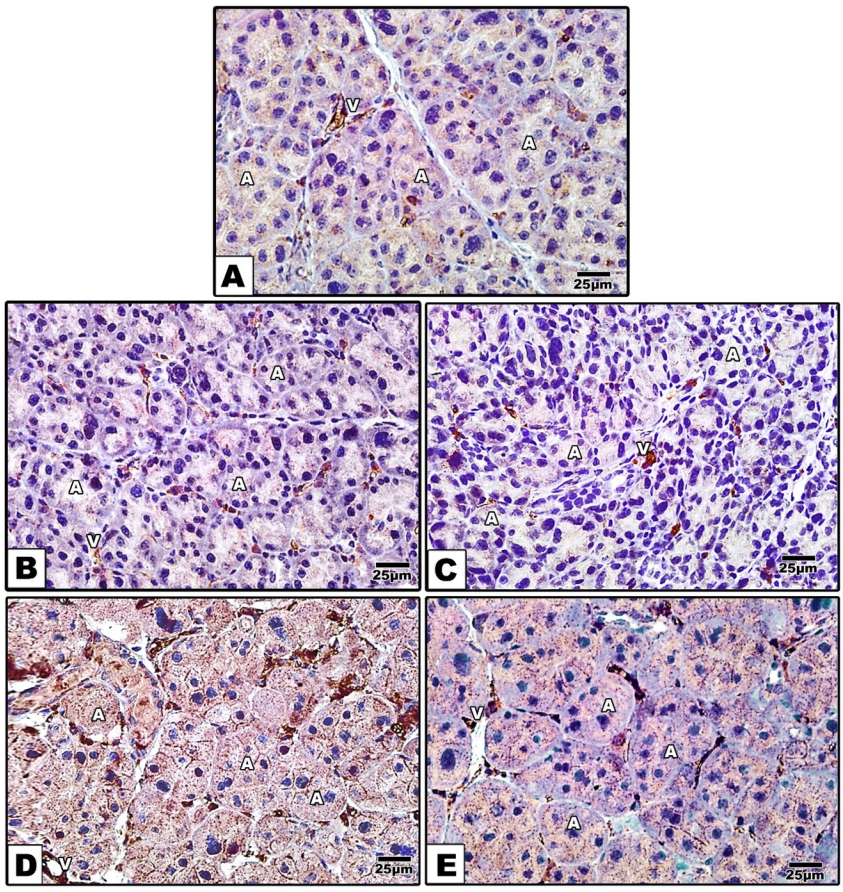


Figure 6. photomicrographs represent the effect of diabetes and intermittent fasting on the distribution of aquaporin 1 in the parotid glands of rat. A: control, B: two weeks post diabetes, C: four weeks post diabetes, D: two weeks post diabetes (with fasting), E: four weeks post diabetes (with fasting). Magnification x400. Scale bar: 25 microns. A: a photomicrograph of control parotid gland representing positive reaction in cytoplasm of acini (A) and in endothelial lining of blood vessels (V). B and C: photomicrographs of two (panel B) and four (panel C) weeks post diabetes parotid gland showing weak positive reaction in cytoplasm of acini (A) and strong reaction in endothelial lining of blood vessels (V). D: a photomicrograph of two weeks post diabetes (with fasting) parotid gland showing strong reaction in cytoplasm of acini (A) compared to control and in endothelial lining of blood vessels (V). E: photomicrograph of four weeks post diabetes (with fasting) parotid gland showing positive reaction nearly equal to that in control group.

Effects of diabetes and intermittent fasting on submandibular and parotid glands AQP4

The expression of aquaporin 4 was studied in different experimental groups (**Table 4** and **Figures 7, 8**). In submandibular gland, the control group showed strong positive reaction in cell membranes and cytoplasm of acini, weak positive in cytoplasm of ID, GCT, and SD (OD=**0.396±.036**). Two weeks post diabetes submandibular gland, the reaction was strong positive in acinar cell membrane and cytoplasm and very weak positive in GCT and SD (OD=**0.342±0.029**). Four weeks post diabetes submandibular gland, the reaction was very weak in the cytoplasm of acini and GCT. Some acini showed positive reaction in their cell membrane (OD=**0.279±0.012**). Two weeks post diabetes (with fasting) submandibular gland, the reaction was very strong in cell membrane and cytoplasm of acini and moderate in SD and GCT (OD=**0.377±0.010**

). Four weeks post diabetes (with fasting) submandibular gland, the reaction was strong in cell membrane and cytoplasm of acini, GCT, and SD (OD=**0.401±0.014**).

In parotid gland, the control group showed positive reaction in cytoplasm of acini (OD=**0.545±.017**). In diabetic groups, the reaction was strong positive in cytoplasm of acini in two weeks post diabetes gland, but it was moderate in four weeks gland (OD=**0.529±0.093** and **0.456±0.051,** respectively). Two weeks post diabetes (with fasting) parotid gland, the reaction was strong positive in cytoplasm of acini in comparison to control group (OD=**0.552±0.034**). Four weeks post diabetes (with fasting) parotid gland, the reaction was weaker in comparison to two weeks post diabetes (with fasting) parotid gland, but nearly equal to control group (OD=**0.423±0.038**).

Table (4): Effects of diabetes and intermittent fasting on optical density (OD) aquaporin 4 in submandibular and parotid glands in experimental rats in comparison to control group.

			Control group	Diabetic group	Diabetic Fasting group	P
OD of aquaporin 4	**parotid**	**2wks**	**0.545±.017**	**0.529±0.093**	**0.552±0.034**	**0.78**
		4wks	**0.545±.017**	**0.456±0.051** **P1=0.003***	**0.423±0.038#** **P2=<0.001*** **P3=0.33**	**<0.001***
	submandibular	**2wks**	**0.396±.036**	**0.342±0.029** **P1=0.01***	**0.377±0.010** **P2=0.49** **P3=0.095**	**0.01***
		4wks	**0.396±0.036**	**0.279±0.012#** **P1=<0.001***	**0.401±0.014#** **P2=0.9** **P3=<0.001***	**<0.001***

Data expressed as mean ± SD, *: significance < 0.05

Test used: One way ANOVA followed by post-hoc tukey for comparison between groups

P1: significance between Control & diabetic.

P2: significance between Control & diabetic fasting.

P3: significance between fasting & diabetic.

#: indicate significance between Week4 vs Week2 within each group

wks = weeks

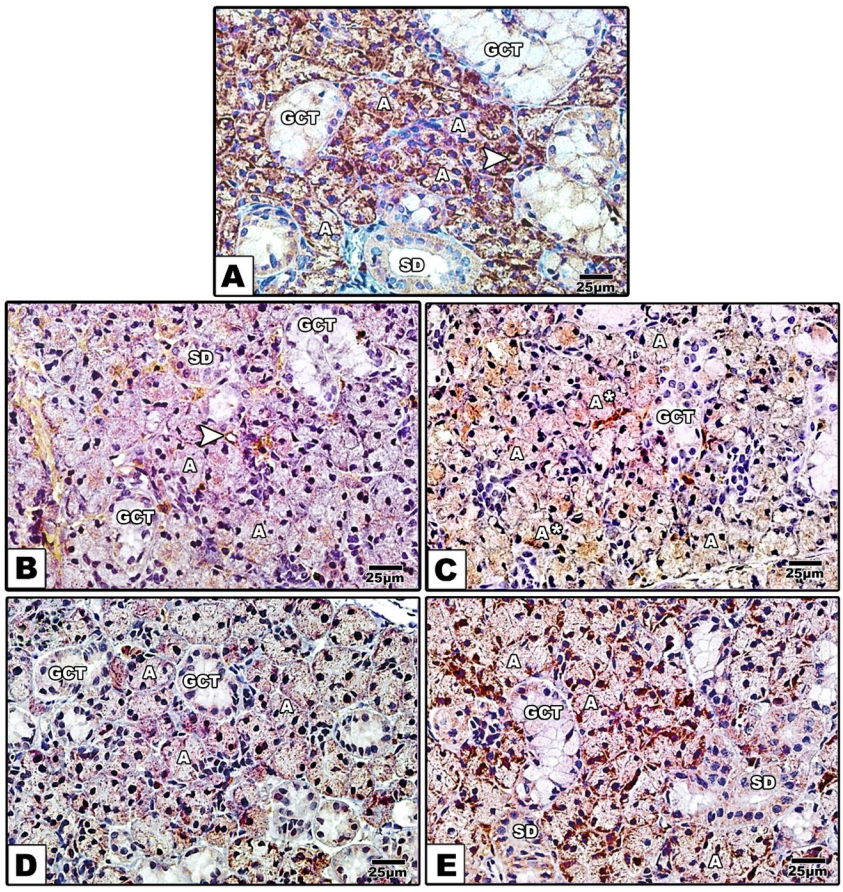


Figure 7. photomicrographs represent the effect of diabetes and intermittent fasting on the distribution of aquaporin 4 in the submandibular glands of rat. A: control, B: two weeks post diabetes, C: four weeks post diabetes, D: two weeks post diabetes (with fasting), E: four weeks post diabetes (with fasting). Magnification x400. Scale bar: 25 microns. A: a photomicrograph of control submandibular gland representing strong positive reaction in cell membranes and cytoplasm of acini (A) and in cytoplasm of intercalated ducts (ID) (arrowhead) and moderate in granular convoluted tubules (GCT) and striated duct (SD). B: a photomicrograph of two weeks post diabetes submandibular gland showing weak reaction in acinar cell cytoplasm (A) and very weak positive in granular convoluted tubules (GCT) and striated ducts (SD). C: a photomicrograph of four weeks post diabetes submandibular gland showing moderate reaction in the cytoplasm of acini (A) and no reaction in granular convoluted tubules (GCT). Some acini showed positive reaction in their cell membrane (A). D: a photomicrograph of two weeks post diabetes (with fasting) submandibular gland showing strong reaction in cytoplasm of acini (A) and moderate in granular convoluted tubules (GCT). E: a photomicrograph of four weeks post diabetes (with fasting) submandibular gland showing strong reaction in cell membrane and cytoplasm of acini (A), granular convoluted tubules (GCT), and striated ducts (SD). Figure 8. photomicrographs represent the effect of diabetes and intermittent fasting on the distribution of aquaporin 4 in the parotid glands of rat. A: control, B: two weeks post diabetes, C: four weeks post diabetes, D: two weeks post diabetes (with fasting), E: four weeks post diabetes (with fasting). Magnification x400. Scale bar: 25 microns. A: a photomicrograph of control parotid gland representing positive reaction in cytoplasm of acini (A).B: a photomicrograph of two weeks post diabetes parotid gland showing strong positive reaction in cytoplasm of acini (A). C: a photomicrograph of four weeks post diabetes parotid gland showing moderate*

positive reaction in cytoplasm of acini (A). D: a photomicrograph of two weeks post diabetes (with fasting) parotid gland showing strong reaction in cytoplasm of acini (A) compared to control. E: photomicrograph of four weeks post diabetes (with fasting) parotid gland showing positive reaction nearly equal to that in control group. D: duct.

Effects of diabetes and intermittent fasting on parotid and submandibular glands LC3-II Immunohistochemical expression and area percentage

In submandibular gland, positive reaction was observed in both acini and GCT (more in GCT than in the acini) in control group. In diabetic rats, the intensity of reaction increased in both acini and GCT (at the two time points). In case of diabetes with fasting, the reaction was intense in GCT in the two weeks group. However, the reaction in four weeks post diabetes (with fasting) gland appeared like that in the control (**Figure 9**).

In parotid gland, positive reaction was observed in acini of control group. The reaction increased in diabetic groups (two and four weeks). However, in diabetes with fasting groups, the reaction decreased markedly in two weeks gland and increased in four weeks gland (**Figure 10**)

This was verified by calculating the area percentage of LC3-II reaction in different groups, and the results were presented (**Table 5**).

Figure 9. photomicrographs represent the effect of diabetes and intermittent fasting on the distribution of LC3-II in the submandibular glands of rat. A: control, B: two weeks post diabetes, C: four weeks post diabetes, D: two weeks post diabetes (with fasting), E: four weeks post diabetes (with fasting). Magnification x400. Scale bar: 25 microns. A: a photomicrograph of control submandibular gland representing positive reaction in the cytoplasm of cells of acini (A) and granular convoluted tubules (GCT) (more in GCT than in acini). B and C: photomicrographs of two (B) and four (C) weeks post diabetes submandibular gland showing an increase in the intensity of reaction in acinar cell cytoplasm (A) and in granular convoluted tubules (GCT). D: a photomicrograph of two weeks post diabetes (with fasting) submandibular gland showing an increase in the intensity of the reaction in granular convoluted tubules (GCT). E: a photomicrograph of four weeks post diabetes (with fasting) submandibular gland showing positive reaction in cell cytoplasm of acini (A) and in granular convoluted tubules (GCT); the tissue looks like control. Fig. 10. A: a photomicrograph of control parotid gland representing positive reaction in cytoplasm of acini (A). B and C: photomicrographs of two (B) and four (C) weeks post diabetes parotid gland showing an increase in the intensity of the reaction in cytoplasm of acini (A). D: a photomicrograph of two weeks post diabetes (with fasting) parotid gland showing marked decrease of the reaction in cytoplasm of acini (A) compared to control. E: a photomicrograph of four weeks post diabetes (with fasting) parotid gland showing an increase in the intensity of the reaction in the cytoplasm of acini (A) nearly equal to that in the control group. D: duct.

Table (5): Effects of diabetes and intermittent fasting on area percentage of LC3- II in submandibular and parotid glands in experimental rats in comparison to control group.

			Control group	Diabetic group	Diabetic fasting group	P
Area % of LC3-II	**Parotid**	**2wks**	**22.53±1.92**	**27.03±5.39**	**16.60±2.53**	**0.002***
				P1=0.16	**P2=0.06**	
					P3=0.002*	
		4wks	**22.53±1.92**	**45.15±11.60#**	**36.24±8.20#**	**0.003***
				P1=0.003*	**P2=0.054**	
					P3=0.24	
	Submandibular	**2wks**	**18.21±3.02**	**25.26±2.61**	**31.73±5.18**	**<0.001***

			Control group	Diabetic group	Diabetic fasting group	P
				P1=0.03*	**P2=<0.001*** **P3=0.047***	
		4wks	**18.21±3.02**	**45.90±2.42#** **P1=<0.001***	**28.84±1.58** **P2=<0.001*** **P3=<0.001***	**<0.001***

Data expressed as mean ± SD, *: significance < 0.05

Test used: One way ANOVA followed by post-hoc tukey for comparison between groups

P1: significance between Control & diabetic

P2: significance between Control & diabetic fasting

P3: significance between fasting & DM.

#: indicate significance between Week4 vs Week2 within each group

wks = weeks

Effects of diabetes and intermittent fasting on parotid and submandibular glands LC3-II gene expression

Relative gene expression of LC3-II (main autophagy marker) was performed to confirm autophagy activation in the samples. In parotid gland, the expression of LC3 in two weeks post diabetes (with fasting) group was downregulated by **0.23±0.02** compared to the control group, and was upregulated by **4.57±0.78** , **4.56±1.73** , and**20.81±9.02** in four weeks post diabetes (with fasting) group, two weeks post diabetes group, and four weeks post diabetes group, respectively, compared to the control group. While in the submandibular gland, compared to the control group, the expression of LC3 was upregulated in all experimental groups by **7.74±1.80** ,**9.25±1.70** , **110.39±22.16** , and **279.84±134.05** in the two weeks post diabetes (with fasting) group, four weeks post diabetes (with fasting) group, two weeks post diabetes group, and four weeks post diabetes group, respectively (**Table 6**).

Table (6): Effects of diabetes and intermittent fasting on LC3-II gene expression in submandibular and parotid glands in experimental rats in comparison to control group.

			Control group	Diabetic Fasting group	Diabetic group	P
R-LC3	**parotid**	**2wks**	**1.00±0.11**	**0.23±0.02** **P1=0.39**	**4.56±1.73** **P2=<0.001*** **P3=<0.001***	**<0.001***
		4wks	**1.00±0.11**	**4.57±0.78#** **P1=0.48**	**20.81±9.02#** **P2=<0.001*** **P3=<0.001***	**<0.001***
	submandibular	**2wks**	**1.01±0.14**	**7.74±1.80** **P1=0.64**	**110.39±22.16** **P2=<0.001*** **P3=<0.001***	**<0.001***
		4wks	**1.01±0.14**	**9.25±1.70** **P1=0.98**	**279.84±134.05#** **P2=<0.001*** **P3=<0.001***	**<0.001***

Data expressed as mean ± SD, *: significance < 0.05

Test used: One way ANOVA followed by post-hoc tukey for comparison between groups

P1: significance between Control & diabetic fasting

P2: significance between Control & diabetic

P3: significance between fasting & diabetic.

#: indicate significance between Week4 vs Week2 within each group

wks = weeks

3. Discussion

The present study demonstrated that autophagy plays a crucial role in diabetic changes in submandibular and parotid glands in the form of aquaporin 1 and aquaporin 4 degradation, which contributes to the dysfunction of these glands. Intermittent fasting is also involved in upregulation of aquaporin1 and aquaporin 4 (in submandibular), which helped in improving salivary glands secretions.

The relationship between the decrease of submandibular and parotid glands secretion, changes of secretion dynamics, and symptoms of xerostomia in diabetic patients attracted the attention of scholars [31, 32]. The clinical study of Lima et al. [33] showed that 92.5% of elderly patients with type II DM suffered from low salivary flow rate. Here, we intended to gather the changes that occur in both submandibular and parotid glands in STZ-induced type I diabetic rat model and discuss the role of intermittent fasting in improving these changes.

Type I diabetes in the present model was induced by STZ. The diabetic status is STZ dose dependent. The usual dose of STZ, to induce diabetes type I in animals, ranges from 45 to 100 mg/dL, STZ doses less than 40 mg/dL may not induce diabetes and doses more than 80 mg/dL may lead to high mortality in the rat [34]. Based on our own experience, we chose a dose of 45 mg/dl, because this dose is enough to induce diabetes with 100% rat survival over the period of the experiment. This agrees with previous reports [35, 36]. STZ induces diabetes as it causes selective toxicity to beta (?) cells of the pancreas [37]. According to Goyal et al. [34], this animal model is clinically relevant model, and it is commonly used to mimic human type I DM.

In this study, we evaluated the oxidative damage level and progression in the diabetic and fasting submandibular and parotid salivary glands using two different markers of oxidative stress (MDA and reduced GSH). We provided evidence that intermittent fasting significantly improved oxidative stress (MDA and GSH values) in both parotid and submandibular glands, but it did not return it to the control values, except for MDA value in the submandibular gland.

Nogueira et al. [38] showed that MDA level increased in submandibular gland of rats in long term STZ-induced diabetes. Deconte et al. [39] demonstrated increased MDA content in rat parotid glands 30 days after STZ-induced diabetes. Zalewska et al. [40] supposed that changes in MDA depend on the type of the salivary glands and diabetic duration in rats. In agreement with Knas' et al. [41], the parotid glands seem more risky than submandibular glands to an oxidant attack generated during STZ-induced diabetes.

In the present study, we observed an atrophy of the submandibular gland acini and GCT as well as parotid gland acini, which was marked in four weeks post diabetes group. These findings were in accordance with a previous report [32]. Cytoplasmic vacuolization was seen in the acinar cells and some striated duct cells in the diabetic group. This vacuolization appeared to be of lipid nature since they were removed during fixation and processing of the samples. According to Anderson et al. [42], there was an accumulation of lipid in the acinar cells ultra-structurally. The destructive effect of sustained hyperglycaemia, and in turn, oxidative stresses induction, might be the cause of this finding [43, 44]. The present study reported that intermittent fasting significantly saved the salivary gland histopathology. We found that the atrophic changes in diabetic group were partially corrected in both glands by intermittent fasting.

Staining with PAS and AB revealed an obvious alteration in the content of neutral and acidic muco-polysaccharides secretions of acinus epithelium of both submandibular and parotid glands in diabetic group (at both time points of the experiment) in comparison to the control group. The diabetic fasting group was closer to the control group and neutral and acidic muco-polysaccharides contents were slightly changed. In the assessment of PAS and AB staining, the changes in the content of neutral and acidic muco-polysaccharides in diabetic group could be explained by drop in the rate of the acyl lipids [45], decrease in the synthesis of granules, the secretion change in the direction of the hyperglycaemia-induced oxidative stress, or due to impairment of aquaporin content and distribution [46, 47].

The present work recorded that intermittent fasting significantly saved the salivary secretions as evidenced by increased PAS staining intensity in acinar cell cytoplasm and GCT in submandibular gland and in acinar cell cytoplasm in parotid gland which was obvious in four weeks post diabetes (with fasting) glands.

As intermittent fasting could return the salivary secretions to be comparable to the control group, intermittent fasting may play a role in correcting the rate of acyl lipids, decreasing the oxidative stress (as evidenced in this work), or changing the aquaporin distribution.

It has been reported that xerostomia in Sjogren's syndrome, radiation, and inflammation was associated with down-regulation and abnormal distribution of AQP5 in salivary glands [48-51]. AQP1 plays an important role in the regulation of transmembrane water flow to maintain intracellular and intercellular fluid balance [52]. In this work, we focused on distribution of AQP1 and AQP4 in submandibular and parotid glands. The expressions of AQP1 and AQP4 in this study were reduced significantly in the submandibular and parotid glands of diabetic rats, but they were up-regulated markedly by intermittent fasting. This was evidenced by measuring the optical density of both immunohistochemical stains in both submandibular and parotid glands. The present results agree with those of Fangqin et al. [53] regarding AQP1 in submandibular gland in diabetic group.

This result showed that a reduction of AQP1 and AQP4 might contribute to reduced secretion of submandibular gland in diabetic rats. Reduced expressions of AQP1 and AQP5 in submandibular gland of the mouse resulted in decreased salivary secretion volume [54]. After the adenovirus vector encoding human aquaporin-1 (hAQP1) was introduced into the subjects with low function of radioactive saliva, the expression of hAQP1 was increased, which could significantly restore salivary secretion and relieve symptoms [55].

Other studies have shown that synthesis and secretions of AQPs may be decreased by impaired granular endoplasmic reticulum in diabetic rats [56]. In addition, the presence of anti-AQP1 [48] and anti-AQP5 [57] antibodies may also be related to a reduction of salivary volume in diabetic patients, which needs further study. At least, we can deduce that impairment of distribution and content of AQP1 and AQP4 in diabetic submandibular and parotid glands is associated with impairment of the nature of salivary secretions as proved with PAS and AB stains. Furthermore, intermittent fasting could partially help in resuming normal distribution and content of these AQPs helping in improving the nature of salivary secretions.

Morgan-Bathke et al. [58] discussed the role of autophagy in salivary glands homeostasis and stress responses in relation to radiation, ductal ligation, autoimmune diseases, and salivary cancer. Up to our knowledge, autophagy has not been discussed in salivary glands in case of diabetes alone and in case of diabetes combined with intermittent fasting. Therefore, to evaluate the autophagic process in different groups, the present work selected measurement of LC3-II protein, which is known as a hallmark of autophagy that results from conjugation of LC3-I with phosphatidylethanolamine [59,60].

First, we assessed immunohistochemical expression and area percentage of LC3-II. It was detected in acini and GCT in control submandibular gland and in acini of control parotid gland. The intensity and area percentage increased in diabetic submandibular and parotid glands at both time points of the experiment. However, in the diabetic fasting groups, the intensity and area percentage of LC3-II decreased in parotid of two weeks post diabetes (with fasting) group and increased in parotid of four weeks post diabetes (with fasting), in submandibular of two weeks post diabetes (with fasting), and in submandibular of four weeks post diabetes (with fasting) groups.

To further examine the degree of autophagy in both submandibular and parotid glands, RT-PCR LC3-II gene expression in both glands was measured. The values increased in both diabetic glands, but the values were very high in the submandibular gland. This may be due to difference in the response of each gland to the metabolic changes of diabetes. In the submandibular gland of the diabetic and fasting group, R-LC3-II gene expression increased at both time points of the experiment, but the values were markedly less than the diabetic values. In contrast, the mean value was reduced at first in parotid of two weeks post diabetic with fasting, then increased again in parotid of four weeks post diabetes with fasting group.

Interestingly, as mentioned above, the immunohistochemistry and gene expression of LC3-II were higher in salivary glands of diabetic rats compared to glands of diabetic and fasting rats. Autophagy is mainly activated by nutrient starvation [61], oxidative stress [62], endoplasmic reticulum stress [63], and energy deficiency [64]. Jia et al. [65] stated that expression of LC3 and Beclin-1 increased due to inflammatory cytokines such as TNFα, and this, is in turn, induced autophagy in plaque cells. Beclin-1 has an important role in the regulation of the interaction between autophagy and apoptosis [66,67]. In diabetes, due to hyperglycaemia, glycosylation of proteins occurs, and glycosylation products attach to the receptors of the cells, inducing inflammation that stimulates immune cells to release cytokines [68]. Moreover, nutrient deprivation, hypoxia, or oxidative stress destroy endoplasmic reticulum homeostasis [69-71]. However, excessive endoplasmic reticulum stress can induce autophagy in tumour cells leading to cell apoptosis [71-73]. Mowers et al. [74] stated that stimulated autophagy pathways by chemotherapeutic drugs induce apoptosis of tumour cells through activation of caspase 3. Furthermore, it has been reported that cell death in colorectal cancer can occur by inducing autophagy-dependent apoptosis pathways [72,75-78]. Previous studies on diabetic salivary glands indicated that diabetes causes damage to major salivary glands, in the form of DNA damage and cellular hypertrophy, atrophy, and hyperplasia [79,80].

Regarding intermittent fasting, an in vitro study showed that it improves cellular stress adaptation (mainly against ROS generation), decreases inflammation and DNA damage, decreases mTOR (mammalian target of rapamycin) expression [81], and promotes autophagy [82]. The signalling pathway mTOR plays a key role in cell survival, growth, proliferation, and cell metabolism by increasing various anabolic processes (e.g., lipids and proteins synthesis) and by inhibiting catabolic processes, such as autophagy [83,84].

As discussed above, cell death could result from insufficient, impaired, or excessive autophagy. So, finding a link between autophagy and apoptosis in salivary glands of diabetics should be searched.

In summary, this study demonstrated that intermittent fasting plays a crucial role in the pathological changes of salivary glands (submandibular and parotid) in diabetes in the form of AQP1 and AQP4 degradation, which contributes to the dysfunction of these glands. Furthermore, intermittent fasting is involved in upregulation of AQP1 and AQP4 (in submandibular gland), which helps in improving salivary glands secretions. These findings highlight the pathological role of autophagy in submandibular and parotid glands in response to diabetes, and provide a potential target for therapeutic role of intermittent fasting to ameliorate submandibular and parotid glands dysfunction in type I DM.

4. Materials and Methods

4.1. Animals

Fifty adult male Sprague-Dawley albino rats weighing 200-250 g (4-6 months) were used in this study. Rats were housed in stainless-steel cages with soft wood chips for bedding and were permitted free access to standard rat chow diet and water ad libitum for two weeks before the experiment for acclimatization and to ensure normal growth and behaviour. The rats were obtained from Medical Experimental Research Centre (MERC). Experiments were performed in Human Anatomy & Embryology Department, Mansoura Faculty of Medicine, Mansoura University, Egypt. The experiment was done in accordance with National Institutes of Health (NIH) guidelines for the maintenance and use of science lab animals; NIH Publication (EU Directive 2010/63/EU) and in conformity with local laws and regulations laid by the Animals' Experimentation Committee at Mansoura University **(R.21.02.1190 - 2021/10/13).**

4.2. Experimental design

Rats were divided randomly into two groups: control group (n=10) and experimental (diabetic) group (n=40). The control rats were fed on a normal chow diet. Experimental diabetes was induced in overnight fasting rats by a single intraperitoneal injection of STZ (Sigma-Aldrich, Egypt) (45 mg/kg body weight). Two days later, measurement of blood glucose level was done. Rats with blood glucose level above 250 mg/dl were considered diabetic [85]. After diabetic induction, the experimental group was divided into two equal groups [diabetic without intermittent fasting (n=20) and diabetic with intermittent fasting (n=20)]. Intermittent fasting includes alternating cycles of feeding and fasting (typically 16 hours fasting with complete deprivation of food, but not water, and 8 hours eating with normal food intake) [18].

4.3. Sacrifice of rats and specimen collection

The rats were sacrificed two or four weeks after the induction of diabetes. Following overnight starvation, rats were anesthetized with Ketamine (60 mg/kg intraperitoneal), and then sacrificed. Parotid and submandibular salivary glands were dissected and removed immediately, and then weighed and processed for further techniques.

4.4. Measurement ofMalondialdehyde (MDA) andreduced glutathione (GSH) levels in parotid and submandibular glands homogenate as oxidative stress markers

Initially, tissue homogenization was done. Teflon glass homogenizer and a buffer containing KCl (1.15%) were used for this step to get (1:10 w/v) the entire homogenate. Measurement of MDA level in parotid and submandibular glands homogenate was done according to an adjusted technique of Ohkawa et al. [86]. The concentration of GSH in parotid and submandibular glands homogenate was measured by a kinetic assay described by Ellman [87] via a dithionitrobenzoic acid recycling technique.

4.5. Histopathological study

The histological processing of the submandibular and parotid glands involved fixation in formalin, dehydration, clearing in xylene, and then embedding in paraffin wax. Thereafter, cutting into sections (5 μm) and staining were performed. The used stains were haematoxylin and eosin (H&E) for routine examination, periodic acid Schiff (PAS), and alcian blue (AB) to differentiate between acinar cell secretion. Staining with PAS was used to stain neutral muco-polysaccharides, whereas AB was used to stain acidic muco-polysaccharides.

4.6. Immunohistochemical study and morphometric analysis

Immunohistochemical staining technique was done according to Li et al. [88]. Initially, deparaffinization of submandibular and parotid glands sections was done followed by rehydration using descending grades of alcohol. Endogenous peroxidases were added to the sections for 0.5 h at 37 °C, followed by 3 times rinsing in phosphate-buffered saline (PBS) (pH 7.4). Heating of the sections was done (for 20 min) in sodium citrate buffer (0.01 M, pH 6.0). When room temperature was reached, incubation of slides with 1% bovine serum albumin (BSA) for 1 h was done and then incubation with primary antibodies overnight at 4 °C was performed. Three primary antibodies were used in this study: 1- rabbit polyclonal primary antibodies raised against aquaporin-1 (AQP1) (GB11311-1, Servicebio, China at 1:1000 dilution); 2- rabbit polyclonal primary antibodies raised against aquaporin-4 (AQP4) (GB11529, Servicebio, China at 1:1200 dilution) for assessment of their role in diabetes and intermittent fasting [89]; and 3- rabbit polyclonal primary antibodies raised against microtubule-associated proteins 1A/1B light chain 3B (MAP1LC3-II) (YPA1652, Chongqing Biospes, China at 1:200 dilution) for assessment of autophagy [90]. The sections were then rinsed with PBS followed by these three steps: Step 1, incubation with horseradish peroxidase (HRP) conjugated secondary antibodies for 0.5 h at 37 °C; Step 2, incubation with labelled streptavidin-biotin for 0.5 h at 37 °C; and Step 3, detection of the reactions with 3,3'-diaminobenzidine (DAB). Lastly, the sections were counterstained with haematoxylin and eosin, dehydrated with ascending alcohol grades, and then cleared with xylene. Ten non-overlapping fields from each slide were examined and photographed using Olympus® light microscope (X400) with Olympus® digital camera installed on it. The morphometric study was done using NIH Image J program (National Institutes of Health, Bethesda, MD, USA), according to the program instructions. The

optical density (OD) of AQP1 and AQP4 was estimated according to El-Tarhouny et al. [91]. The positive expression of LC3-II protein was considered as the area percentage occupied by brown pixels. It was estimated according to the slightly modified protocol of Schipke et al. [92].

4.7. Analysis of MAP1LC3B (LC3) gene expression by real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from submandibular and parotid tissues by RNeasy Plus Mini Kit (Qiagen, Germany). The concentration of extracted total RNA was measured by Implen NanoPhotometer P-Class (P330, GmbH, Germany), and 1 μg of total RNA was converted into cDNA using RevertAid First Strand cDNA Synthesis Kit (ThemoFisher Scientific, United States). Gene expression was evaluated for rat MAP1LC3B (LC3) gene, while the expression of the control samples in the two groups was used as negative control and rat GAPDH as an internal control. Amplifications were performed in a 20 μL reaction volume in each well that contains 10 μL 2X HERA SYBR® Green (Willowfort, England), 1 μL of cDNA template, 2 μL primers and 7 μL of nuclease-free water. The plate array was inserted in Piko-Real Real-time PCR System (Thermo Fisher Scientific) and programmed according to manufacturer instructions. Rat LC3 and GAPDH primers were manually designed on NCBI's Primer-blast library (Table 7). A mathematical model introduced by Pfaffl [93] was used for the relative quantification of target genes. For each sample, the procedure was carried out in triplicate.

Table 7. The primer sequences used for RT-PCR test in rats.

Gene	Forward Primer	Reverse Primer	PCR Product
LC3	**TTTGTAAGGGCGGTTG**	**GTGGCTGTATGTTC**	**123**
GAPDH	**TGCCACTCAGAAGAC**	**CAGGGATGAT**	**86**

LC3 (MAP1LC3B) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

5.8. Statistical analysis

Data were analyzed using Statistical Package for Social Science software computer program version 26 (SPSS, Inc., Chicago, IL, USA). The normality of variables was determined using the Shapiro-Wilk test. Data were normally distributed, so they were presented in mean and standard deviation. Student's t-test (unpaired) was used to compare two different groups while one-way Analysis of variance (ANOVA) and post-hoc Tukey were used for comparing more than two different groups. P value less than 0.05 was considered statistically significant.

5. Conclusions

We can deduce that impairment of distribution and content of AQP1 and AQP4 in diabetic submandibular and parotid glands is associated with impairment of the nature of salivary secretions as proved with PAS and AB stains. Furthermore, intermittent fasting could partially help in return of normal distribution and content of these AQPs, and thus, helping in improving the nature of salivary secretions. As intermittent fasting ameliorates the deleterious effects of diabetes evidenced by reducing lipid peroxidation biomarker malondialdehyde (MDA) and elevating the antioxidant enzyme reduced glutathione (GSH). It also decreases or modulates the autophagy process that occur in diabetes, which appeared to be a defence mechanism to protect cells at first, and then, becomes hazardous mechanism and may progress to programmed cell death (apoptosis pathway). Alternatively, intermittent fasting stimulates autophagy by another pathway rather than that stimulated by diabetes. This point needs further investigation.

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Software, Revision of the content, Final approval of the version to be submitted. Eman Ali Al Qahtani: Investigation, Supervision, Revision of the content, Final approval of the version to be submitted. Khulood Mohammed Al-khater: Funding acquisition, Supervision, Revision of the content, Final approval of the version to be submitted. Rashid A. Aldahhan: Funding acquisition, Revision of the content, Final approval of the version to be submitted. Eman Mohamad El Nashar: Investigation, Supervision, Funding acquisition, Revision of the content, Final approval of the version to be submitted.**Funding:**The authors would like to extend their appreciation to the Deanship of Scientific Research at King Khalid University for funding this project through the large research group program under grant number (R.G.P. 2/143/43).**Institutional Review Board Statement:** "The animal study protocol was approved by the Institutional Review Board of Animals' Experimentation Committee at Mansoura University (protocol code R.21.02.1190 and date of approval 2021/10/13)."**Informed Consent Statement:** "Not applicable."

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