Original Article
Insulin on changes in expressions of aquaporin-1, aquaporin-5, and aquaporin-8 in submandibular salivary glands of rats with Streptozotocin-induced diabetes

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Abstract: Objective: This study aimed to explore the relationship between diabetic xerostomia and changes in aquaporin-1 (AQP1), aquaporin-5 (AQP5), and aquaporin-8 (AQP8) expression in the submandibular glands (SMGs), to further study the pathogenesis of diabetic xerostomia and to observe the therapeutic effect of insulin (INS). Methods: Thirty SD rats were randomized equally into 3 groups: control group, diabetic model (DM) group and insulin (INS) group (n=10, respectively). The control group received no treatment. DM group and INS group were induced by a high-fat diet and streptozotocin intraperitoneal injection. After establishment of a diabetic rat model, the rats in INS group were treated with insulin. Then all rats were fed continuously with ordinary diet for 2 months. H&E staining was used to describe morphologic changes in the SMGs of rats. Immunohistochemistry was used to analyze the expressions and localization of AQP1, AQP5, and AQP8 in the SMGs. Computer image analysis was used to detect the mean optical density (MOD) values of AQP1, AQP5, and AQP8 expression, and changes in the diameters of acini and ducts. Results: The acini were mildly atrophied and the acinar cells were rearranged in an irregular way. The morphology of insulin-administered diabetic SMGs was similar to that of the control group. The acinar average circumference and GCT average diameter in DM group were significantly reduced (P<0.05). The acinar average circumference and GCT average diameter of INS group were significantly increased (P<0.05). The expressions of AQP1, AQP5, and AQP8 were significantly reduced in DM group (P<0.05). The expressions of AQP1, AQP5, and AQP8 in INS group were significantly increased (P<0.05). Conclusion: The decreased expressions of AQP1, AQP5, and AQP8 led to decreased salivary secretion of SMGs in diabetic rats, which may be involved in the pathogenesis of diabetic xerostomia. Insulin could up-regulate the expressions of AQP1, AQP5 and AQP8, and play a protective role in the secretory function of diabetic SMGs.

Keywords: Aquaporin-1, aquaporin-5, aquaporin-8, diabetes mellitus, submandibular gland, rat

Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin resistance, or both. Symptoms of hyperglycemia include polyuria, polydipsia, polyphagia, and weight loss. Xerostomia is one of the most typical symptoms of type 2 diabetes mellitus [1]. The administration of Streptozotocin (STZ) to mice causes an insulin insufficiency by destroying B cells of the pancreas, leading to an increase in plasma glucose level and osmotic pressure of urine. A common complaint associated with DM is dry mouth or xerostomia [2]. High blood glucose can cause thirst, and it has been reported that urine sugar increases urine osmotic pressure, leading to polyuria and circulating blood volume reduction in patients. This causes the central reflex of thirst in patients with diabetes to be excited, the thirst is enhanced, and the amount of drinking water is increased significantly [3], Current research has shown diabetic xerostomia is also related to reduced secretory function of submandibular glands (SMGs) [4]. The abnormal morphology of

SMGs is one of the important reasons for the reduced salivary secretion in diabetes mellitus. However, the underlying mechanisms of diabetic xerostomia remain unexplored [5].

The discovery of aquaporin membrane water channels by Agre and coworkers [6] answered a long-standing biophysical question of how water specifically crosses biologic membranes, and provided insight, at the molecular level, into the fundamental physiology of water balance and the pathophysiology of water balance disorders. Aquaporins (AQPs) are critically involved in the maintenance of ionic and osmotic balance in response to osmotic gradients and differences in hydrostatic pressure [7]. Of 13 AQPs isoforms in humans, at least AQP1, AQP3, AQP4, AQP5, and AQP8 are known to be present in the salivary glands of humans and rodents, participating in water transfer and electrolyte balance in saliva secretion [8, 9]. AQPs are membrane-associated proteins mainly involved in the transmembrane transport of water driven by osmotic gradients [10]. It was reported that AQP5 is largely involved in water transport during saliva secretion of SMGs. Previous studies with regard to AQPs have shown significantly down-regulated expressions of AQP1 [11] and AQP5 [12] in the ductal epithelial cells in SMGs of the Sjogren's syndrome (SS) mouse. The AQP5 protein levels were decreased in the parotid glands of the diabetic rats compared with control parotid glands. In addition, expressions of AQP0 and AQP1 were reduced in late diabetic cataract [13], and the AQP3 expression was significantly down-regulated in diabetic kidney disease [14]. The relation between reduced saliva secretion in diabetic rats and altered expressions of AQPs has not still been clarified. The aim of this study was to explore the changes of AQP1, AQP5, and AQP8 expression in SMGs of diabetic rats, which may help to elucidate molecular mechanism of reduced saliva secretion in SMGs of diabetic rats.

Materials and methods

Animal models and experimental groups design

Entire procedures were approved by Institutional Animal Care and Use Committee in Anhui, China. 30 Male Sprague-Dawley (SD) rats (provided by Anhui Medical University Animal Center, Hefei, China) were randomized equally into control group (control group), diabetic model (DM group) and insulin (INS) treatment group (INS group) (n=10, respectively). All rats were fasted for 12 hours, and the fasting blood glucose (FBG) level was measured by taking blood from the tail vein. The rats in the control group were fed with ordinary diet without any treatment. The rats of the DM group and INS group were fed a high-fat diet (with 2% cholesterol, 10% lard, 88% basal feed) for 2 months to make the rats produce impaired glucose tolerance and insulin resistance, then a single intraperitoneal injection of 2% streptozotocin (STZ, 35 mg/kg body weight) [15-18], selectively destroyed pancreatic β-cells [17], and rats were fed with an ordinary diet. One week after the diabetes model was established, the rats of DM group and INS group were fasted for 12 hours, and blood was drawn from the tail vein to detect the FBG level. When the FBG was greater than 7.0 mmol/L (>7.0 mmol/L) [18], it indicated that the model of type 2 diabetes mellitus was successfully established. After one week, the rats in INS group were treated with insulin (3 u/d). The rats in each group were fed continuously with ordinary diet for 2 months. During this period all rats were not restricted for diet and water.

Tissue extraction

After 2 months, all rats were anesthetized and the thoraces were opened to draw 5 milliliter blood from peritoneal venous for assaying FBG. The tissue of SMGs were removed and subsequently fixed in 10% formalin for 24 h and then rinsed with 0.01 M phosphate-buffered saline (PBS) (pH 7.4) to remove the formaldehyde. The tissues were dehydrated in graded ethanol and xylene and paraffin-embedded. For light microscopic observation, transverse 5 mm sections were cut and serially mounted on slides.

Haematoxylin-eosin (HE) staining

The dewaxed sections were stained with hematoxylin and eosin, then they were subsequently sealed with a neutral resin and examined under light microscopy.

Immunohistochemical staining

Unless indicated otherwise, immunohistochemistry was performed as described earlier.
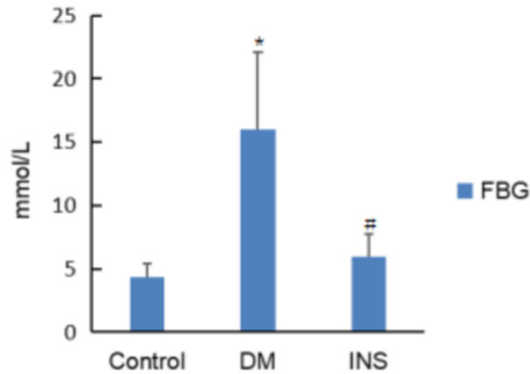
Figure 1. Change of fasting blood glucose (FBG) in each group. The FBG of DM group was significantly increased in comparison with the control group. The FBG of the INS group was significantly reduced in comparison with the DM group. Compared with control group, *$P<0.05$; compared with DM group, #$P<0.05$.

Tissue sections were dewaxed in xylene, washed in alcohol and rehydrated in PBS. Antigen retrieval was performed in a 700-W microwave oven in 0.02 M citrate buffer solution (pH 6.0) for 7 min. The sections were cooled at room temperature and washed again in PBS. Endogenous peroxidase was blocked through incubation in 3% hydrogen peroxide (H_2O_2) at 37°C for 15 min. After washing in PBS three times, the goat serum (dilution 1:50) was used for 30 min to avoid any non-specific reactions. Overnight incubation at 4°C was performed with anti-AQP1 antibody (rabbit, 1:100) or anti-AQP5 (rabbit, 1:100) antibody or anti-AQP8 (rabbit, 200) antibody. Goat anti-rabbit biotin conjugate (1:200) diluted in PBS was used as secondary antibody. For IgG, the primary antibody was coupled to polyclonal rabbit anti-mouse peroxidase conjugate (1:200) secondary antibody. Sections were incubated for 30 min at 37°C. Biotin labeling was followed by incubation at 37°C for 30 min, and the reaction was stopped with water. Sections were counterstained with Mayer's haematoxylin, rinsed with running tap water, dehydrated and cleared in xylene and covered with neutral balsam. Substituting the primary antibody with 0.01 M, PBS created negative controls.

Computer image analysis

Under optical microscopy of 10×40 times, H&E slice images of the acini and ducts in SMGs were captured with Nikon digital color CCD camera in every group. Immunohistochemical staining slice images of AQP1, AQP5, and AQP8 were captured. 5 views were randomly selected in each slice. The mean optical density (MOD) value of AQP1, AQP5 and AQP8 immunoreactive cells was measured, using computer biological image analysis system.

Statistical analysis

SPSS13.0 was used for statistical analysis. Measurement data were shown as mean ± standard deviation (x ± s). Comparison between groups was conducted by univariate analysis of variance combined with q test, and $P<0.05$ was considered statistically significant.

Results

Change of fasting blood glucose

The FBG of control group and INS group was compared with that of DM group. The differences were significant ($P<0.05$). The FBG in INS group was compared with that of control group. The difference was not significant ($P>0.05$) (**Figure 1**).

Histopathologic changes of SMGs

H&E staining showed that the SMGs in control group were clear, containing the acinar portion and the duct portion. The SMGs consisted of mixed glands, including more serous glands and fewer mucous glands; the duct system included intercalated duct, granular convoluted tubule (GCT), striated duct, and excretory duct. The acinar portion in the control group was full and clear; GCT is a structure unique to rodents. The number of GCTs was more than that of the striated duct, but the diameter of GCTs was shorter than that of the striated duct. GCTs were surrounded by a single layer of columnar epithelial cells, which contained a large number of eosinophilic granules in the cytoplasm (**Figure 2A**). In comparison with the control group, the gland vacuoles were mildly atrophied in DM group, and the acinar cells were arranged irregularly. The components of connective tissue were not obviously increased (**Figure 2B**). The structure of SMGs in the INS group was similar to that of the control group (**Figure 2C**).

Changes in the expressions and localization of AQP1, AQP5, and AQP8 in each group

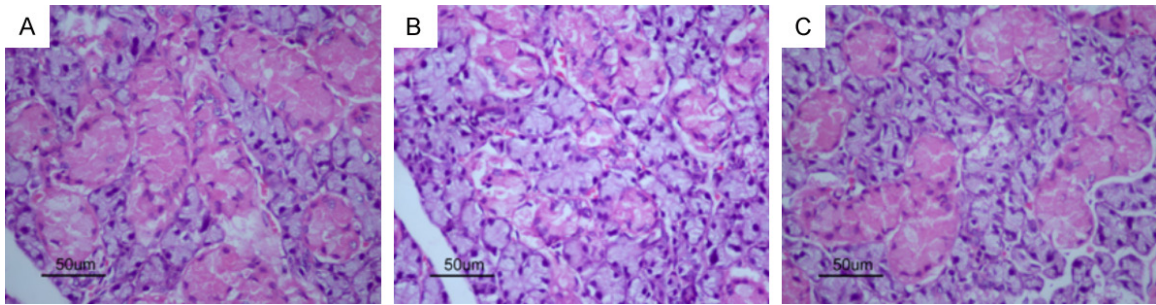Immunohistochemistry showed that AQP1 and AQP5 were mainly located in the granular

Figure 2. Histopathologic changes of the SMGs in each group. H&E staining, 400×. H&E staining showed the SMGs contained an acinar portion and a duct portion. The acinar portion in control group was full and clear.l, GCT was surrounded by a single layer of columnar epithelial cells, which contained a large number of eosinophilic granules in the cytoplasm (A). In comparison with the control group, the gland lumen was mildly atrophied and the acinar cells were arranged irregularly in the DM group. The components of connective tissue were not obviously increased (B). The structure of the SMGs in the INS group was similar to the control group (C).
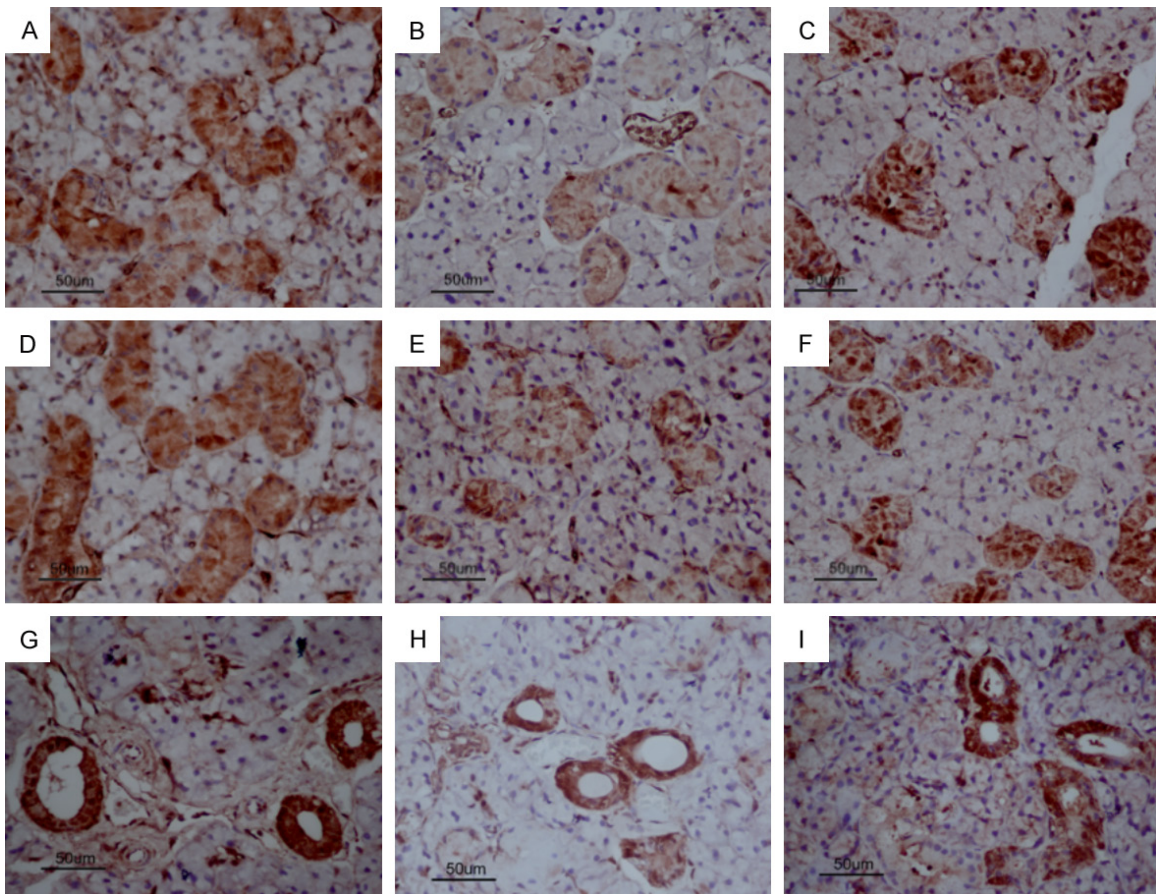

Figure 3. Expressions and localization of AQP1, AQP5, and AQP8 in the SMGs of rats in each group. SP, 400×. AQP1 and AQP5 were mainly located in the granular curved tube and striated tube of the SMGs in the control group (A, D). AQP8 was mainly located in the cytoplasm of striated duct epithelial cells (G). Compared with the control group, the expressions of AQP1, AQP5, and AQP8 in DM group were significantly reduced (B, E, H). Compared with the DM group, the expressions of AQP1, AQP5, and AQP8 in INS group were significantly increased (C, F, I).

curved tube and striated tube of the SMGs. The expression of AQP1 in control group was strong and dark brown (**Figure 3A**); the expression of AQP1 in DM group was significantly reduced
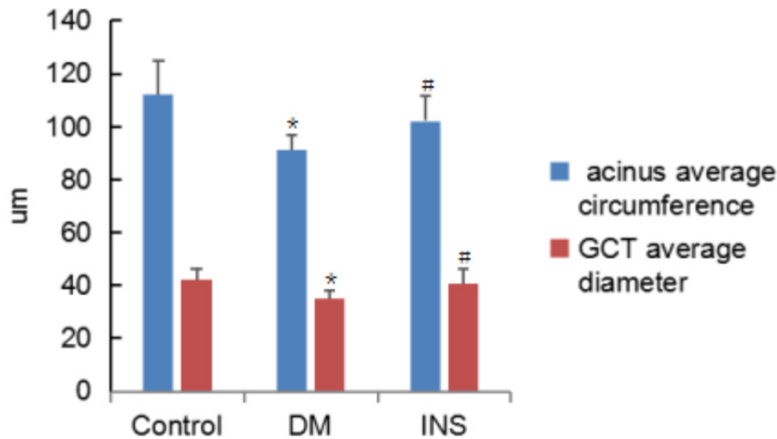
Figure 4. Changes in the acinar average circumference and GCT average diameter by histomorphometric analysis. The acinar average circumference and GCT average diameter of the SMGs in the DM group were significantly reduced. The acinar average circumference and GCT average diameter of the SMGs in the INS group were significantly increased. Compared with control group, *P<0.05; compared with DM group, #P<0.05.
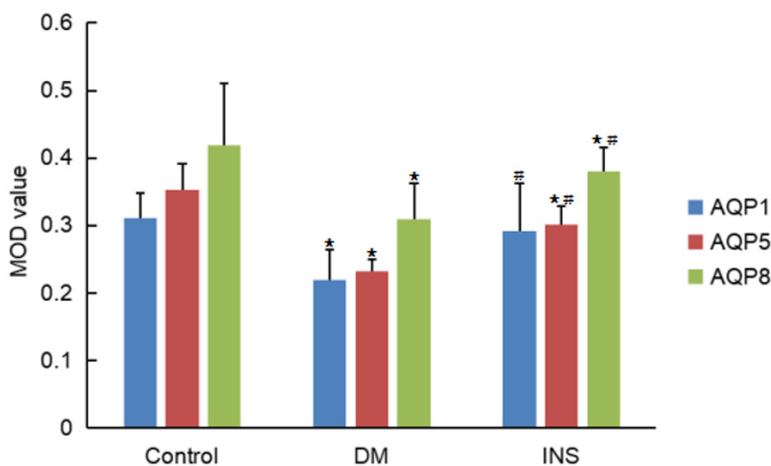

Figure 5. Changes of AQP1, AQP5, and AQP8 expression MOD value by computer biologic image analysis system in each group. Compared with the control group, the expressions of AQP1, AQP5, and AQP8 were significantly reduced in the SMGs of the DM group, and up-regulations of AQP1, AQP5, and AQP8 were observed in the INS group in comparison with the DM group. Compared with control group, *P<0.05; compared with DM group, #P<0.05.

plasm of striated duct epithelial cells. The expression of AQP8 in control group was strong dark brown (**Figure 3G**); the expression of AQP8 in DM group was brown and was significantly reduced (**Figure 3H**); the expression of AQP8 in the INS group was brown and was significantly increased compared with the DM group (**Figure 3I**).

Changes of the acinar average circumference and GCT average diameter by histomorphometric analysis

The acinar average circumference and GCT average diameter of the DM group were respectively compared with those of the control group, and the differences were significant (*P<0.05). The acinar average circumference and GCT average diameter of the INS group were respectively compared with those of DM group, and differences were significant (*P<0.05). The acinus average circumference and GCT average diameter of INS group were respectively compared with those of the control group, and the differences were not significant (*P>0.05) (**Figure 4**).

Changes in AQP1, AQP5, and AQP8 expression MOD value by computer biologic image analysis system

Using computer imaging analysis, the MOD values of AQP1, AQP5, and AQP8 in the DM group were respectively compared with those of the control group. The differences were respectively significant (*P<0.05). The MOD values of AQP1, AQP5, and AQP8 in the INS group were respectively compared with those of DM group. The differences were respectively significant (*P<0.05). Comparison between the MOD value of AQP1 in the control group and that of the INS

and was light brown (**Figure 3B**); the expression of AQP1 in INS group was similar to the control group and was brown (**Figure 3C**). The expression of AQP5 in the control group was strong and brown (**Figure 3D**); the expression of AQP5 in DM group was significantly reduced and was light brown (**Figure 3E**); the expression of AQP5 in the INS group was brown, and was significantly increased compared to the DM group (**Figure 3F**). AQP8 is mainly located in the cyto-

group was not significant ($P>0.05$). The MOD values of AQP5 and AQP8 in the INS group were respectively compared with those of the control group. The differences were significant ($P<0.05$) (**Figure 5**).

Discussion

Xerostomia is one of the classic symptoms of diabetes mellitus (DM). It is well known that the secretion of submandibular gland constitutes about 70% of salivary secretion volume. The relationship between the decrease of submandibular gland secretion, abnormal changes of secretion dynamics, and symptoms of xerostomia in diabetic patients has gradually attracted the attention of scholars [3]. This study demonstrated that the acini were mildly atrophied and acinar cells were arranged irregularly in the DM group, compared with a control group. Some studies have shown that the ultrastructure of submandibular gland in diabetic rats shows obvious abnormal changes [19]. Compared with controls, the acinar cells of diabetic rats exhibited variability in the density and structure of secretory granules, increased numbers of lysosomes and autophagic vacuoles, lipid droplets in the basal cytoplasm and folding and redundancy of their basal laminae [20]. The conclusion of this experiment is consistent with that reported by previous scholars [5]. The xerostomia in diabetic patients is primarily due to decreased saliva flow which seems to be related to degenerative changes of the salivary glands. The damage to cell structure, electrolyte imbalance, abnormal changes of cell osmotic pressure, changes of aquaporins content and distribution [21] in SMGs may be the causes of diabetic xerostomia. However, the underlying mechanism is still unclear and needs further study.

AQPs are a family of transmembrane protein channels of water channels, which selectively promote the transport of water and small solutes (including cations and glycerines) and gases in cell membrane [22, 23], regulated by osmotic gradient, and play various roles in physiologic processes [23, 24], including salivary secretion [6, 25]. AQP1 plays an important role in the regulation of transmembrane water flow to maintain intracellular and intercellular fluid balance [26], in which AQP1, AQP5 [27], and AQP8 [28] are abundant and play an important role in salivary secretion [29]. AQP5 plays a

role in fluid secretion/movement in many tissues, including exocrine glands and lungs [30]. AQP8 is a water channel protein with high permeability to water, that is expressed in salivary gland, kidney, and other tissues [31].

The expressions of AQP1, AQP5, and AQP8 were reduced significantly in the diabetic rats' SMGs in this experiment, but AQP1, AQP5, and AQP8 were up-regulated markedly by insulin treatment. This result showed that a reduction of AQP1, AQP5, and AQP8 might contribute to reduced secretion of SMGs in diabetic rats. Reduced expressions of AQP1 and AQP5 in SMGs of the SS mouse resulted in decreased salivary secretion volume [10]. 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 [32]. AQP5 plays an important role in the regulation of salivary flow and has been unequivocally identified as being associated with the fluid-secreting of salivary glands [33]. Knockout mice lacking AQP-5 showed markedly depressed rates of salivary secretion, and their salivary volume was decreased to 60% of that of a normal mouse [34]. The decrease of AQP5 expression in salivary glands of SS patients is a main cause of oral dryness [32, 35]. The decreased AQP5 expression in the ductal cells of the mandible gland may hinder the excretion of water in the ductal cells [36-38]. Altered expressions of AQPs also have been observed in other complications of diabetes. For instance, investigation showed rat myocardial cell edema was accompanied by decreased expressions of AQP1 and AQP3 in early diabetic cardiomyopathy. The AQP1 gene was up-regulated in the glomeruli of diabetic nephropathy patients.

The mechanisms of down-regulated AQP1, AQP5, and AQP8 expression in SMGs of diabetic rats remain unclear. AQP1, AQP5, and AQP8 were stained significantly weaker by immunohistochemical techniques in diabetic rats than in controls. After the blood glucose of diabetic rats was corrected with insulin, the expressions of AQP1, AQP5, and AQP8 were also up-regulated. Research showed that reinforced oxidative stress [39], inflammatory response [40], increased apoptosis [41] and insulin lack, or abnormal hormone levels change [42] play important roles in the patho-

genesis of DM. Thus it was speculated that the gene expression of AQPs in diabetic rats may be affected by the above factors. Other studies have shown that synthesis and secretions of AQPs may be decreased by impaired granular endoplasmic reticulum in diabetic rats [43]. In addition, the presence of anti-AQP1 [44] and anti-AQP5 [45] antibodies may also be related to a reduction of salivary volume in diabetic patients, which needs further study.

In conclusion, the decreased expressions of AQP1, AQP5, and AQP8 are closely related to the occurrence of diabetic xerostomia. This study provided a morphologic basis for a relationship between the changes of AQP1, AQP5, and AQP8 expression and the pathogenesis of diabetic xerostomia, and proved that insulin could up-regulate the expressions of AQP1, AQP5, and AQP8.

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Disclosure of conflict of interest

None.

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