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The Effects of Diabetes on Salivary Gland Protein Expression of Tetrahydrobiopterin and Nitric Oxide Synthesis and Function

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Abstract

Background—Xerostomia is defined as dry mouth resulting from a change in the amount and/or composition of saliva and often a major oral health complication associated with diabetes. Studies have shown that xerostomia is more common in females in the onset of diabetes. Evidence suggests that nitric oxide (NO) plays a critical role in healthy salivary gland function. However, the specific mechanisms by which NO regulates salivary gland function at the onset of diabetes have yet to be determined. This study had two aims: 1. To determine whether protein expression and/or dimerization of NO synthase enzymes (nNOS, eNOS) are altered in the onset of diabetic xerostomia and 2. To determine whether the changes in nNOS/eNOS protein expression/dimerization are correlated with changes in NO cofactor, tetrahydrobiopterin (BH₄) biosynthetic enzymes (GTP Cyclohydrolase-1, Dihydrofolate reductase).

Methods—Functional and western blot studies were performed in streptozotocin-induced diabetic (type 1 diabetes) and control Sprague Dawley female rats using standardized protocols. Confirmation of xerostomia was determined by increased water intake and decreased salivary flow rate.

Results—In diabetic female rats, salivary hypofunction is correlated with decreased submandibular and parotid gland sizes. Furthermore, our results show a decrease in NOS and BH₄ biosynthetic enzyme in submandibular glands.

Conclusion—Our results indicate that a decrease in submandibular NO-BH₄ protein expression may provide insight pertaining to mechanisms for the development of hyposalivation in diabetes-induced xerostomia. Furthermore, understanding the role of NO-BH₄ pathway may give insight to possible treatment options for the diabetic patient experiencing xerostomia.

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Keywords

diabetes mellitus; xerostomia; nitric oxide; nitric oxide synthesis; saliva; 5; 6; 7; 8-tetrahydrobiopterin

Diabetes mellitus (DM) is a metabolic disease in which patients' long term prognosis is dependent upon the consistency of their fasting plasma glucose levels remaining above 126mg/dl.¹ Approximately 29.1 million people are affected by diabetes in the United States.² Diabetes mellitus-induced oral health problems are highly prevalent in minority populations particularly African Americans, Hispanics and American Indians.² DM is thought to promote xerostomia, a qualitative and/or quantitative absence of saliva in the oral cavity.^{3,4} Decreased salivary flow can cause complications in the oral cavity by allowing excessive accumulation of bacteria leading to numerous oral infections, extreme thirst (especially at night), an alteration in the taste of food, rampant tooth decay, and halitosis.³ Decreased salivary production can be caused by a number of other conditions, i.e. medications⁵, radiotherapy involving the salivary glands⁶, as well as autoimmune diseases. A reported 43% of individuals experience xerostomia, also known as dry mouth syndrome, as a result of the onset of DM.⁴ Notably, 82% of DM patients with xerostomia are females.⁴ The reason this gender difference occurs is not discussed in the literature.

It has been suggested that nitric oxide (NO) does play a critical role in normal salivary gland function and saliva secretion.⁷ NO is a free radical and the first known gas to act as a biological messenger. NO was initially recognized as a potent vasodilator, however it was quickly found to impact angiogenesis, act as a neurotransmitter, as well as play a vital role in host defense mechanisms and pathogenesis of numerous inflammatory and autoimmune diseases.⁷ It is synthesized by three isoforms of the enzyme, termed nitric oxide synthases. These isoforms include neuronal (nNOS, NOS I), inducible (iNOS, NOS II), and endothelial (eNOS, NOS III), all of which produce NO which functions in different capacities within the central nervous system, immune system, and circulatory system, respectively.⁸

The activity of NOS depends on the dimerization of two polypeptides. Dimerization results in the creation of high affinity binding sites for tetrahydrobiopterin (BH₄; an NOS cofactor) and arginine (in the oxygenase domain) which enables electron transfer between the flavin and heme groups.^{9,10} Enzymatic uncoupling of NOS due to lack of BH₄ may account for a decrease in NO production and increased oxidative stress.⁹ In the specific case of DM, it has been documented that dimerization of NOS is interrupted during the onset of diabetes leading to low tissue levels of NO⁹. Rosignoli and Leiros (2001) carried out immunoblotting experiments in a disease model for sialadenitis in non-obese diabetic (NOD) mice and showed that nNOS was decreased in both the parotid and submandibular glands.⁷ No changes were documented in the expression of eNOS.⁷ It is of note that the female NOD mice used in the Rosignoli and Leiros experiment were considered pre-diabetic, with their weekly glucose sera levels showing no significant difference from the control mice. Although, a role of NO has been demonstrated in salivary gland function and saliva secretion⁷, the role and regulation of NO in the onset of diabetes-induced hyposalivary function and xerostomia has not yet been demonstrated.

The purpose of this study is therefore, to examine whether diabetic rats display xerostomia; if so, investigate whether protein expression of NOS [neuronal (n) /endothelial (e) NOS] as a result of diabetes induction. In addition to examine whether NO cofactor, BH₄ biosynthetic enzymes [GTP cyclohydrolase-1 (GCH-1) and dihydrofolate reductase (DHFR)] are altered and correlated with NOS changes in female diabetic rats.

2. Materials and Methods

2.1 Induction of Diabetes Mellitus With Streptozotocin (STZ)

All procedures were approved by the Institutional Animal Care and Use Committee at Meharry Medical College in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Sprague Dawley female rats (9-10 weeks old)^{||} arrived at the animal care facility and were held in quarantine for seven days. Animals were allowed free access to water and rodent food. Diabetes was induced in rats (n = 7) with a single intraperitoneal (55 mg/kg body weight; i.p.) injection of streptozotocin (STZ)^{||} in 5 mM citrate buffer, pH 4.0. Control group (n = 7) received only citrate buffer. Animals were sacrificed 6 weeks after the STZ injection. To confirm diabetes was induced in the diabetic (DB) animals, blood glucose levels (overnight fasting animals) were obtained 48 hours after STZ injection. Blood (~ 0.4 µL) was taken from the tail vein, and glucose levels were analyzed using a glucometer.[#] This method for measuring glucose levels were repeated weekly throughout the duration of study. Fasting glucose levels were also obtained from controls. Diabetes was confirmed if blood glucose levels ranged from 300-500 mg/dL. Age-matched control rats demonstrated normal glucose levels ranging from 80-100 mg/dL.

2.2 Evidence of Xerostomia in Diabetic Female Rats

Control and DB rats were given free access to 250 mL of water for an overnight period of 16 hours. The water intake (mL/hour) was measured at the conclusion of this 16 hour period. Saliva samples were collected after the water intake measurement by applying three drops of 4% pilocarpine hydrochloride into each rat's mouth. Saliva samples were collected with the animals placed in ventral decubitus in the operator's hands. The amount of stimulated saliva collected was measured at the end of an experimental period of 2 minutes.¹² The salivary flow rate was expressed in mL/min.

2.3 Gland Size Measurements and Western Blotting

After rats were sacrificed using CO₂, all three pairs of salivary glands were excised from both DB and control rats. Parotid (P), submandibular (SM), and sublingual glands (SL) were placed in pre-weighed vials, weighed, and stored at -80 °C until used for western blotting analysis. In this study, we used SM glands from both the DB and control groups because a significant portion of saliva comes from submandibular glands, producing over 70% of total saliva production.¹³ Submandibular glands were homogenized at 4°C in RIPA lysis buffer. All samples (n= 14) were centrifuged at 12,000 × g for 20 minutes at 4°C, then supernatants were separated for protein determinations. Cellular extracts (40 µg protein/lane), positive

^{||}Harlan Laboratories, Indianapolis, IN, USA.

[#]AlphaTRAK Blood Glucose Monitoring Pack (Abbott Laboratories, Abbott Park, IL, USA).

controls for each enzyme (nNOS, eNOS, GCH-1, DHFR), and molecular weight standards were subjected to 6, 6, 12, and 15% SDS-polyacrylamide gels, respectively. Proteins were then transferred to nitrocellulose membranes and the results were revealed with specific monoclonal antibodies^{**}. Changes in specific proteins were normalized with the housekeeping protein, beta-actin.[¶] Western blotting and NOS dimerization experiments were performed as described in our previous publication.¹¹

2.4 Statistical Analysis

Nitric oxide synthesis enzymes (nNOS, eNOS) and cofactor BH₄ biosynthesis enzymes were western blotted and ratios of protein expressions of each enzyme were compared to housekeeping protein, beta-actin. In the case of dimerization, the ratio of monomer and dimer of nNOS and eNOS were analyzed. Trends of increase or decrease were analyzed between nNOS/eNOS and BH₄ enzymes to determine if a direct correlation existed between NOS enzymes and BH₄ enzymes.

Data were presented as mean $\pm$ standard error (SE). All data was analyzed for statistical comparisons between groups with Student's t-test or Tukey test. P values less than 0.05 were considered significant.

3. Results

3.1 Glucose and Body Weight Comparison Between Diabetic and Control Rats

Glucose levels (mg/dL) were significantly increased (DB: 458 ± 12.6 , Control: 104 ± 0.71 ; $p = 0.0004$) in DB rats, while body weights (grams) were significantly decreased (DB: 252 ± 3.87 , Control: 282 ± 7.99 ; $p = 0.744$) in DB rats compared to the control group (table 1).

3.2 Evidence of Xerostomia in the Onset of Diabetes in Female Rats

Diabetic rats displayed a significantly larger volume of water intake (mL/hr) (DB: 8.14 ± 0.523 , Control: 1.81 ± 0.194 ; $p = 1.06E-05$ $P < 0.05$) compared to the control group. Diabetic rats also showed a decreased volume (mL/min) of saliva (DB: 0.027 ± 0.005 , Control: 0.235 ± 0.050 , $p = 0.002$) after pilocarpine stimulation (table 1).

3.3 Effects of Diabetes in Salivary Gland Weight

The submandibular (DB: 348 ± 20.7 , Control: 445 ± 20.4 ; $p = 0.025$) and parotid glands (DB: 132 ± 13.8 , Control: 188 ± 17.2 ; $p = 0.048$) in diabetic rats weighed significantly less ($P < 0.05$) compared to the control glands. The sublingual glands in diabetic rats also weighed slightly less than control sublingual glands, however, the difference was not found statistically significant (table 1).

3.4 Effects of Diabetes on NOS Protein and NOS Dimerization in Submandibular Glands

SM gland protein expression for both nNOS and eNOS were significantly ($P < 0.05$) decreased in diabetic animals compared to control group. Next, we examined changes in

^{**}Zymed Laboratories, Grand Island, NY, USA.

[¶]Sigma-Aldrich Chemical, St. Louis, MO, USA.

NOS (total) enzyme activity as measured by dimerization of nNOS and eNOS by using low-temperature SDS-PAGE. Our results show that the ratio of nNOS and eNOS dimers to monomers was significantly decreased in diabetic rats ($P < 0.05$) compared to control rats (Figures 1, 2).

3.5. Effect of Diabetes on BH₄ Biosynthesis Enzyme Protein Expression in Submandibular Glands

There was a decrease in expression of DHFR (BH₄ enzyme) in the submandibular glands from DB rats ($P < 0.05$). GCH-1 showed a decrease as well, but the change was not statistically significant. Our data suggests a direct correlation between BH₄ biosynthesis via the salvage pathway and a decrease in NOS dimerization (Figure 3).

4. Discussion

The purpose of this study was to determine the relationship between diabetes and the protein expression of NOS enzymes and BH₄ in salivary glands of rats experiencing salivary gland hypofunction and signs of xerostomia. It has been reported that diabetic patients often experience reduced saliva production due to salivary gland hypofunction (SGH) causing an increase in water intake.⁴ This may be a result of general dehydration experienced by many diabetic patients. Our lab suggests another proposed hypothesis for dry mouth seen in diabetic patients, including the idea of an alteration in the function or structure of salivary glands. Our current study examined specifically the alteration of protein expression of NO and its cofactor, BH₄ biosynthesis enzymes in the salivary glands of diabetic female rats. Human patients are able to dictate their symptoms associated with xerostomia such as dry mouth, trouble swallowing, and extreme thirst; this is not possible in an animal model. With the expression of clinical manifestations seen in the human model as opposed to the animal model, we were able to design experiments to confirm xerostomia in a DB animal model by demonstrating signs associated with salivary gland dysfunction such as an increase in water intake and decreased saliva production upon pilocarpine-stimulation of DB rats compared to the control counterparts.¹²

Saliva is an essential biological fluid in the body and is predominately produced by three pairs of major salivary glands [Parotid (P), Submandibular (SM), and Sublingual glands (SL)].¹³ Of these, a significant portion of saliva comes from the submandibular gland, which yields over 70% of total saliva production. In the current study, compared to healthy females, DB rats showed a significant decrease in saliva production. Previous studies reported that saliva contains several enzymes and serves as a non-invasive way to measure biological markers for various systemic as well as oral diseases.^{15,16,17} The identification of specific enzymes (either absent or in abundance) could reveal further information concerning the adverse clinical manifestations associated with xerostomia. One limitation in this study is the absence of histological evaluations of the salivary glands. It is possible that changes in cellular function, in this case, could lead to salivary gland hypofunction and hyposecretion.

Salivary gland functions are controlled by a number of neuronal and circulatory mechanisms.¹⁸ Salivary glands are innervated, either directly or indirectly, by the

parasympathetic and sympathetic branches of the autonomic nervous system that regulates salivary fluid secretion.¹⁸ Nitric oxide (NO) is a non-adrenergic and non-cholinergic neurotransmitter as well as potent vasodilator known to play a key role in salivary gland function and secretion of saliva.⁷ Neuronal nitric oxide synthase (nNOS) is expressed in nerve fibers surrounding the acini of the parotid and submandibular salivary glands in many mammalian species as well as surrounding acini in human labial salivary glands.^{19,20,21,22,23} Notably, the presence of NOS in human and rodent acinar cells has been demonstrated by functional studies measuring NO synthesis and by determining NOS expression.⁷ Very little information is available on the pathogenesis of NO in human oral diseases. The expression of nNOS was shown to be reduced in NOD animal models similar to Sjogren's syndrome (SS), a chronic autoimmune rheumatic disease characterized by a severe dryness of the mouth and other mucosal tissues. Similarly, our studies showed that n/e NOS protein expression and dimerization was altered in female diabetic rat SM glands. This suggests that the NOS mechanisms in salivary gland hypofunction, regardless of its etiology, can be generalized among various causes associated with xerostomia.

Further, the animal model established in this study can be used as a tool to investigate the underlying mechanisms that may be associated with salivary gland dysfunction and thus development of xerostomia in the onset of diabetes. Wang et al studies have shown abnormal subcellular localization of AQP5 as well as downregulation of AQP5 protein in parotid glands of streptozotocin-induced diabetic rats.²⁴ Previous research in humans have shown that salivary flow rate is reduced in the onset of diabetes mellitus.^{3,4} In the current study, we also found that the salivary volume and weights of all three salivary glands (P, SM, SL) were less in the diabetic rat compared to controls. However, only the P and SM glands displayed a significant difference. Thus further research is needed to examine other potential mechanisms that may impair NO pathways as well as impede function of the salivary glands.

Tetrahydrobiopterin deficiency leads to n/e NOS uncoupling and reduced enzyme activity; n/e NOS activity represents a critical signaling node for regulating salivary gland function.²⁵ The catalytic activity of n/e NOS depends on a dimerization step, aided by BH₄.^{9,25} BH₄ is synthesized from GTP de novo by the rate-limiting enzyme GCH-1 or from a salvage pathway (via dihydrofolate reductase, DHFR) using arginine as a substrate.^{9,25} Numerous studies have identified suppression of n/e NOS activity/loss of NO generation as central to the development of endothelial dysfunction and gastroparesis in non-diabetics and diabetics, respectively.^{26,27,28,29,30,31,32,33} We have previously demonstrated that diabetic gastric tissue have reduced BH₄ availability and that supplementation with dietary BH₄ or its precursor, sepiapterin, improves nNOS dimerization, enzyme activity, nitroergic relaxation and gastric emptying in females but not in males.^{11,27,34,35} A significant amount of BH₄ has been quantified in salivary glands.³⁶ Our current study showed that DHFR protein expression (BH₄ biosynthetic enzyme via salvage pathway) is reduced in SM glands of 6 week diabetic female rats.

5. Conclusion

Our preliminary studies provided evidence that a reduction in submandibular gland (SM) DHFR protein expression correlated well with altered n/e NOS protein expression and

dimerization in female diabetic rats. The alterations in BH₄-NO synthesis are correlated with decreased salivary flow rate and increased water intake a classic symptom of xerostomia in diabetic and non-diabetic humans.^{7,8,36} However, it is not clear whether BH₄-NO signaling pathway is altered in all salivary glands (P, SM, SL) or when these changes actually will occur in the onset of xerostomia (early vs. late diabetes). In addition, it is not known whether supplementation of BH₄ will ameliorate NOS activity and NO production in P, SM, SL glands and improve salivary secretion and water intake. Examining more definitive time points in the onset of xerostomia along with BH₄-mediated NO signaling and salivary function will provide a more complete understanding of the role of BH₄-NO pathway in diabetes-induced xerostomia. Further, it may help to develop new chemotherapeutic compound(s) to treat signs and symptoms of xerostomia in diabetic patients.

Notably, dietary BH₄ has been shown to be useful and safe in improving endothelium-dependent relaxation in patients with diabetes gastroparesis²⁸, patients with type II diabetes³⁷, chronic smokers³⁸, hypercholesterolemia³⁹, as well as venous conduits used for coronary artery bypass graft surgery⁴⁰. Future studies will be highly significant because they will help delineate the role of NO in the pathogenesis of xerostomia in a diabetic rat model and determine if BH₄ supplementation ameliorates any observed exocrine defects.

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Figure 1.

Histogram and immunoblots of submandibular homogenates showing the decrease in protein expression of nNOS (155 kDa) and eNOS (140kDa). Also shown are changes in specific proteins normalized to beta-actin (housekeeping protein). Data are mean $\pm$ SEM, 4 DB animals and 4 controls. * p <0.05 compared to control group (t-test).

Figure 2.

Histogram and immunoblots of submandibular gland homogenates showing the ratio of nNOS dimers (A: 310 kDa) to monomers (155 kDa) and eNOS dimers (B: 280 kDa) to monomers (140 kDa). Data are mean $\pm$ SEM, 4 DB animals and 4 controls. * p <0.05 compared to control group (t-test).

Figure 3.

Representative immunoblots of submandibular homogenates showing the significant decrease in protein expression of DHFR (25 kDa) but not GCH-1 (26 kDa). Also shown are changes in specific proteins normalized to beta-actin levels (house-keeping protein). Data are mean $\pm$ SEM, 4 DB animals and 4 controls. * p <0.05 compared to control group (t-test).

Table 1
Effect of Diabetes on Glucose Levels, Salivary Gland Weights and Xerostomia Symptoms

	Control	Diabetes
Glucose (mg/Dl)	104 ± 0.71	458 ± 12.6 *
Body Weight (mg)	282 ± 7.99	252 ± 3.87 *
Water Intake (mL/hour)	1.81 ± 0.194	8.14 ± 0.523 *
Salivary Flow Rate (mL/min)	0.235 ± 0.050	0.027 ± 0.005 *
Parotid Gland Weight (mg)	188 ± 17.2	132 ± 13.8 *
Submandibular Gland Weight (mg)	445 ± 20.4	348 ± 20.7 *
Sublingual Gland Weight (mg)	93 ± 29.6	85.5 ± 22.9

P values less than 0.05 are considered significant and denoted with an

*
n=7 in each group.