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Effects of Diabetes and Hypertension on Oral Mucosa and TGFβ1 Salivary Levels

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The aim of this study was to investigate salivary levels of TGFβ1 and proliferation/maturation of epithelial mucosa cells in diabetic and hypertensive patients. Design: in this cross-sectional study, whole stimulated saliva and oral mucosa exfoliative cytology specimens were collected from 39 patients that were healthy (control, n=10) or presented history of arterial hypertension (HAS, n=9), diabetes mellitus (DM, n=10) or both (DM+HAS, n=10). Salivary flow rate (SFR), TGFβ1 level in saliva, AgNORs and the epithelial maturation were evaluated. Non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparison post-test and the Spearman test correlation analysis were used. SFR showed a significant decreased in DM and DM+HAS (0.47±0.11 and 0.64±0.43 mL/min) when compared to control (1.4±0.38 mL/min). DM+HAS presented the highest value of TGFβ1 concentration (24.72±5.89 pg/mL). It was observed a positive correlation between TGFβ1 and glycaemia (R=0.6371; p<0.001) and a negative correlation between TGFβ1 and saliva (R=-0.6162; p<0.001) and glycaemia and SFR (R=-0.5654; P=0.001). AgNORs number and status of maturation of mucosa cells were similar for all conditions. DM and DM+HAS presented the lowest SFR, which correlated with increased TGFβ1 levels. Despite the higher TGFβ1 secretion it was not observed changes in the morphology or proliferation of epithelial cells when diabetes or hypertension was present.

Key Words: salivary flow, AgNORs, exfoliative cytology, hyperglycemia, blood pressure.

Introduction

Diabetes mellitus (DM) is an important independent risk factor to development of cardiovascular diseases and therefore the association between DM and hypertension has been investigated (1). These chronic diseases may promote dysfunction in different tissues and cell types (2), including oral health components as the salivary glands. Patients with glycaemia poorly controlled may present reduction of salivary flow rate, and, as consequence, an increased risk to develop oral injuries and impairment on velocity and quality of wound healings (3). Moreover, the presence of hypertension increases the probability of xerostomia (associated or not to salivary flow deficiency) as the number of cardiovascular drug administration increases (4).

Transforming growth factor beta1 (TGFβ1) has been implicated on hypertension physiopathogenesis and also has been reported dysregulated in diabetic patients (5,6). During embryogenesis of salivary gland, TGFβ1 expression is responsible for interrupted glandular branch and has an important role in regulating epithelial cell proliferation and differentiation, and, for this reason may influence the oral mucosa homeostasis (7). Also, it was already demonstrated in animal models that diabetes influences the production of TGFβ isoforms in the salivary glands (8).

It is possible that the association of diabetes and hypertension enhance the decreased in amount and quality of saliva and that this impairment be impairment of saliva may reflect in alteration on oral tissues. Thus, here we report the concentration of TGFβ1 in saliva collected from healthy, hypertensive and diabetic patients as well as oral exfoliated mucosa cells proliferation and maturation analysis.

Material and Methods

Study Population

Subjects for this cross-sectional study comprised patients under treatment at a public health service (Morro da Cruz, South of Brazil). Informed consent was obtained from 10 healthy volunteers (control group) and 29 patients with a history of arterial hypertension (HAS group, n=9), diabetes mellitus (DM group, n=10) or both (DM+HAS group, n=10), confirmed by previous exams obtained from the medical records. Fasting plasma glucose concentration of 126 mg/dL and above was the diagnostic criteria for diabetes mellitus (9). Patients with medical records of Systolic Arterial Pressure higher than 140 mmHg were considered as presenting systolic arterial hypertension condition (10). All subjects should be ≥40 and ≤60 years-old and not use tobacco or chronic psychotropic or any other medicaments which can alter the mucosa and the flow salivary rate.

The study was carried out in accordance with the guidelines of Declaration of Helsinki for experiments involving humans and was approved by the Ethical Committee of Federal University of Rio Grande do Sul, Brazil (CAAE: 04588512.6.0000.5347; 2012).

Saliva Collection, Clinical Examination and Questionnaire

Patients were oriented to avoid brushing teeth, eating, drinking or practicing exaggerated exercises at least one hour before the collection time. The salivary samples were collected in the morning, respecting the circadian rhythm of salivary flow. Whole saliva was collected during stimulation by parafilm chewing during 6 min. After the first minute, the saliva was discarded and the following produced fluid was collect every minute in a 15-mL falcon tube. The salivary samples were kept on -20°C to mucin precipitation, unfrozen and centrifuged (2,000 rpm; 12 min). The supernatant was kept on -80 °C to analyze TGFβ1 by ELISA. All patients underwent a clinical smooth oral tissues examination to identify oral mucosa alterations. The patients with oral lesions were referred to clinical treatment. Data were obtained by only one operator (B.C.S) under the same field conditions, using plane mouth mirrors. At the time appointment, questions concerned to oral condition self-perception were recorded.

Glycemic Control

Glycemic levels of fasting plasma glucose in mg/dl were used to investigate the glycemic status of patients at the same day of the saliva collection. The glycemic levels were registered on patient medical records.

Enzyme-linked Immunosorbent Assay (ELISA) for the Quantitation of TGFβ1 in Whole Saliva Samples

Salivary levels of TGFβ1 were measured by ELISA using the commercial kit Human TGFβ1 (BD Biosciences) according to the manufacturer's instructions. The optical density of the color in each well was measured at 450 nm. TGFβ1 levels are reported as mean±SD, expressed in pg/mL. Clarified saliva was used undiluted.

Oral Exfoliative Cytology

Before smear collection, patients were instructed to remove prostheses and to rinse the mouth with water for 1 minute. Exfoliative cytology specimens were collected from the buccal mucosa using a cytobrush and the material was spread onto a labelled glass slide for microscopy. Smears were fixed in absolute alcohol and stored in plastic vials. The smears were processed for analysis of argyrophilic nucleolar organizer regions (AgNORs) and Papanicolaou technique. One blinded observer (N.C.S) to the patient history reports performed the AgNORs counting and examined the maturation pattern in all slides. For Papanicolaou evaluation, one hundred isolated cells were counted on each slide horizontally, from left to right, at 400-x magnification. Cells were classified as anucleated, superficial with nuclei, intermediate and parabasal. In AgNORs counting, fifty isolated cells were counted on each slide, according to the criteria established by Crocker et al (11). The mean number of AgNORs per nucleus (mAgNORs) and mean percentage of nuclei with 1, 2, 3, 4 or more AgNORs per nucleus (pAgNORs) were registered.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism (GraphPad Software, Inc., CA, USA). According to data distribution, differences between groups were assessed by the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparison post-test. Statistical significance was set at $p<0.05$. The correlation analysis between variables was obtained with Spearman test correlation.

Results

Oral lesions were more detectable in diabetic patients. To characterize the subjects of the study, information of age, gender, medicine treatment distribution, and oral health self-reported from all patients were recorded and are showed in Table 1. During the clinical examination of soft tissues was registered that two subjects from DM group presented white plaque lesions located one in labial mucosa (20 mm of extension), that was biopsied and diagnosed as actinic cheilitis, and other in gingival tissue (two sites with 6 and 8 mm of extension), that were kept in follow up and at end classified as a frictional hyperkeratosis. Some patients from DM (n=2) and DM+HAS (n=2) groups also present candidiasis, an oral manifestation frequently associated to salivary flow impairment. As candidiasis could be involved in the differential expression of TGFβ1, we have checked the TGFβ1 values for patients with candidiasis and we have noticed that the values were not outliers in relation to whole group. To verify a possible alteration in results, we performed TGFβ1 analysis without these patients, however the statistical difference was similar than performed considering all patients.

Lower levels of salivary rate and increased TGFβ1 concentration were more evident in the presence of DM. To identify if the systemic healthy condition as diabetes, hypertension or both could be interfering in saliva parameters, the whole stimulated saliva was collected from each patient and the levels of fasting plasma glucose from blood were reviewed. The glycaemia was 1.92 and 1.87 times higher in DM and 1.71 and 1.66 in DM+HAS patients in relation to control and HAS groups respectively, confirming the misbalance in blood glucose concentration, despite of the use of therapeutic treatment ($p<0.0001$) (Fig. 1A). Saliva flow rate showed a substantial decreased when compared DM and DM+HAS groups (0.47±0.11 and 0.64±0.43 mL/min) and healthy patients (1.4±0.38 mL/min). All subjects from DM group present a hyposalivation condition (Fig.

1B). Detectable salivary levels of TGFβ1 were observed in all saliva samples and DM only or associated to HAS presented the highest values for this growth factor concentration (21.44±4.47 and 24.72±5.89 pg/mL) (Fig. 1C), which were 29.16 and 48.92% higher than control group (16.60±0.81). The levels of TGFβ1, glycaemia and the rate of salivary flow were significantly correlated in subjects studied (Fig. 2). Positive correlation was showed between TGFβ1 and glycaemia (R=0.6371; p<0.001 and a negative correlation between TGFβ1 and saliva (R=-0.6162; p<0.001) and glycaemia and saliva flow (R=-0.5654; p=0.001). Control and HAS group showed no difference for all parameters. This set of data suggests that diabetes was more responsible by the lower levels of salivary rate and increased TGFβ1

Table 1. Study subjects demographic, self-reported and clinical data

Characteristics	Groups			
	Control (n=10)	HAS (n=9)	DM (n=10)	DM + HAS (n=10)
Sex (n)				
Male	-	2	1	3
Female	10	7	9	7
Age (years± SD)	47.6± 7.07	52.6± 8.45	52.7± 10.17	60.5± 7.88
Oral Manifestations (n)				
Candidiasis	-	-	2	2
Frictional hyperkeratosis	-	-	1	-
Tongue fissure	-	-	1	-
Cheilitis	-	1	1	-
Oral Health self-reported (n) [a]				
Xerostomia	1	3	4	5
Halitosis	1	5	3	-
Gingival bleeding [b]	-	1	-	2
Cardiovascular drug class (n)				
ACE inhibitors	-	5	-	5
Angiotensin II inhibitors	-	1	-	2
Alpha-adrenergic blockers	-	-	-	1
Beta-adrenergic blockers	-	5	-	4
Calcium-channel blockers	-	2	-	1
Diuretics	-	6	-	5
Statins	-	2	-	6
Nitrates	-	-	-	1
Platelet aggregation inhibitors	-	3	1	5
Others	-	-	-	2
Diabetes Drugs (n)				
Insulin	-	-	5	7
Metformin	-	-	6	7
Glibenclamide	-	-	7	4
Others Drugs (n)	-	1	-	2

HAS, arterial systolic hypertension; DM, diabetes mellitus; [a]'n' represents the number of patients that self-declared to feel xerostomia, halitosis or gingival bleeding frequently in the last year; [b]one patient from HAS group was edentulous.

concentration than the hypertension presence.

Mucosa epithelial cells proliferation and morphology were not dependent of TGFβ1, glycaemia or salivary flow levels. Using AgNORs technique was possible to show the velocity of the cell cycle among the groups. The values of mAgNORs in control (2.89±0.26), HAS (2.75±0.44), DM (2.99±0.28) and DM+HAS (2.71±0.63) group were not different statistically, what may indicate that the unhealthy conditions were not able to interfere in the velocity of the cell proliferation compared to healthy subjects. Additionally, the values of pAgNORs and correlations between AgNORs and cell types versus TGFβ1, glycaemia and the rate of salivary flow (data no showed) were also not significant. Thus the alterations in saliva seem not induce alteration in the mucosa epithelial cells proliferation. In order to complement the proliferative information, we used the cell type's quantification of exfoliated oral mucosa by Papanicolaou staining to assess the epithelial maturation process. The presence of diabetes and/or hypertension did not interfere in cell morphology since that the most frequent cell type in oral mucosa were intermediate (mean of 47.22 to 54.25) and superficial (mean of 45.50 to 52.67%) independently the group evaluated. All information about the velocity of cell proliferation and the status of maturation of mucosa cells were described in Table 2.

Discussion

The prevalence of diabetes mellitus and hypertension conditions usually increases with age as well their association to higher oral diseases manifestations (12). It is still unknown if the appearance of these pathologies is due to systemic profile of the DM and hypertension or caused by local manifestations as lower rate of salivary flow, buccal mucosa epithelium alterations or even saliva composition changes. So, this study investigated if the salivary levels of TGFβ1 and the proliferation and maturation of epithelial mucosa cells are modified in those patients.

We have used a convenience sampling composed by patients under treatment at a public health service (Morro da Cruz, South of Brazil). During the interval of sample selection, only healthy women had appointments in the service. This may be explained since women used health services more often than men. However, it is important highlight that some papers have related that women tend to have better dietary practices when compared to men, while others have shown that women with diabetes may be at higher risk for developing cardiovascular disease than men (13,14). Additionally, besides already have been related

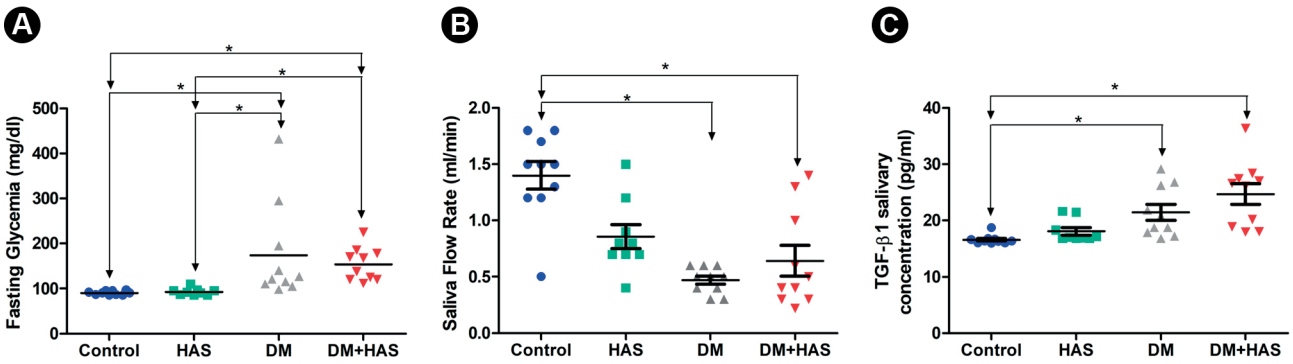

Figure 1. Fasting Glycaemia (mg/dL), Saliva Flow Rate (mL/min) and TGFβ1 concentration (pg/mL) distribution (A-C) in control, HAS, DM and HAS+DM patients. Statistical Analysis: Kruskal-Wallis test, followed by Dunn's multiple comparison post-test (p<0.05).

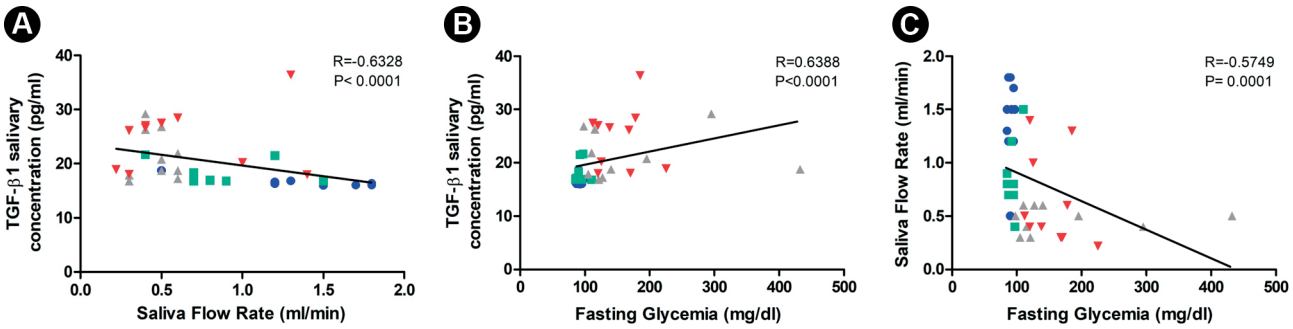

Figure 2. Fasting Glycaemia (mg/dl), Saliva Flow Rate (mL/min) and TGFβ1 concentration (pg/mL) Spearman's correlation (A-C) in control, HAS, DM and HAS+DM patients. Statistical Analysis: Kruskal-Wallis test, followed by Dunn's multiple comparison post-test (p<0.05).

in the literature differences in the pathophysiology of glucose homeostasis disorder between genders, in relation to TGFβ1 levels, we were not able to find in the literature studies considering gender as a variable (15).

Djukic et al (16) showed in a population of 387 hypertensive subjects that hypertension combined to diabetes promoted a significant salivary flow rate decrease that was dependent of the type of anti-hypertensive drug administrated during the treatment. In our data, salivary flow rate was also significantly different for diabetic patients (DM or DM+HAS group), which had salivary flow levels lower than 0.7 mL/min, while control and HAS individuals had normal flow condition (1-3 mL/min). Although the reduction on salivary flow, here only 50% of patients from DM+HAS group reported feel dry mouth sensation frequently, while has already been related that the most of patients presenting the association of these diseases declared to have xerostomia (27). Additionally, in our sample, patients with isolated HAS condition (not-associated to diabetes), even using several anti-hypertensive drugs, did not present difference in salivary flow rates when compared to control volunteers similar to Dodds et al (17) that reported only a trend of decrease of this parameter comparing these groups. Thus, it seems that diabetes but not hypertension was the main cause resulting in lower salivary flow.

Diabetic and hypertensive patients usually have wound healing impairment. This condition may be caused by the alteration of cytokine profile in saliva. It was showed by a proteomic evaluation that cell motility proteins-involved were up or down expressed in saliva from patients with DM versus controls (18). Higher levels of potassium and human α2-macroglobulin and lower expression of s-IgA and EGF secretion rates were also observed in diabetics when compared to healthy subjects by ELISA (19,20). It also has been demonstrated that animals diabetes-induced have important changes in the production of a several different extracellular matrix components and growth factors as TGFβ isoforms for example (8,21). Regarding this, we demonstrated that the levels of TGFβ1 in saliva were higher when diabetes was associated to hypertension as demonstrated by the positive correlation between the growth factor presence in saliva and fasting plasma glucose concentration in blood. It is possible that these changes in saliva composition may be involved in oral mucosa and gastrointestinal homeostasis imbalances, since the absorption of growth factors occurs in these sites.

TGF-beta family is present during the physiological gland development involved on the proliferation and differentiation of the epithelial cells and can be one of the molecules responsible by cell epithelial-mesenchyme transition in pathological process as cancer establishment (7, 22). However, no association of increase of TGFβ1 was observed with AgNOR and Papanicolaou quantification. These results suggest that oral effects caused by DM and presence of HAS are not related to changes in epithelial cell proliferation or maturation what may become possible the inclusion of diabetic and/or hypertensive patients in studies that involve the evaluation of such cells in oral carcinogenesis context.

Most of the studies have investigated the AgNOR counts in buccal cells exposed to carcinogens. When we

Table 2. Quantification of AgNORs and cells pattern stained by Papanicolaou. Values are expressed in mean± SD

	Control (n=9)	HAS (n=8)	DIA (n=9)	DIA+HAS (n=9)	p value*
mAgNORs	2.89 ± 0.26	2.75 ± 0.44	2.99 ± 0.28	2.71 ± 0.63	0.18
pAgNORs					
>1	89.78 ± 7.84	86.00 ± 11.81	90.44 ± 5.64	81.1 ± 11.92	0.38
>2	61.11 ± 9.55	54.25 ± 16.54	64.00 ± 9.43	51.56 ± 19.99	0.27
>3	29.56 ± 9.04	26.75 ± 13.35	33.78 ± 10.65	27.56 ± 20.44	0.32
>4	8.00 ± 3.74	7.50 ± 4.87	10.22 ± 5.95	9.11 ± 13.35	0.52
Papanicolaou					
Anucleated	-	-	-	-	-
Superficial	52.67 ± 6.12	45.50 ± 8.72	51.11 ± 7.47	47.67 ± 6.96	0.16
Intermediate	47.22 ± 6.18	54.25 ± 8.62	48.56 ± 7.46	52.22 ± 6.76	0.16
Parabasal	-	-	-	-	-
Binucleated	0.11 ± 0.33	0.25 ± 0.46	0.33 ± 0.5	0.11 ± 0.33	0.58

*Kruskal-Wallis test, followed by Dunn's multiple comparison post-test.

compare the AgNOR quantification of our control group with the control groups of other studies that assessed the buccal mucosa, usually the mAgNOR values were lower when compared with Jindal et al. (23) and Mondal et al. (24). This may due to different criteria for quantification and different mean age of patients. We have found only another study that investigated the expression of AgNORs in relation with diabetes mellitus which also did not show any effect of diabetes mellitus on the AgNOR quantification, although it was an animal model study (25).

Our results are in accordance to Burzlaff et al (26) that also observed that the most frequent cell type in health oral mucosa were intermediate and superficial. The percentages are different; however this may due to the fact that they assessed the border of the tongue while in this study the buccal mucosa was analyzed. Although we have not found significant differences on cell maturation pattern, other studies have investigated the effects of type 2 diabetes mellitus on oral cells. Studies performed cytometric analysis, showing that nuclear area is significantly higher in the diabetic group when compared to control non-diabetic group, and also observed increased karyorrhexis and binucleation in diabetic patients (27,28).

Diabetes but not hypertension was associated with lower salivary rate and increased TGFβ1 levels; however, the alterations in saliva were not correlated to changes on the maturation and proliferation profile of the mucosa epithelial cells.

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Resumo

O objetivo deste estudo foi investigar os níveis de TGFβ1 na saliva e a proliferação/maturação das células epiteliais da mucosa em paciente diabéticos e hipertensos. Neste estudo transversal, saliva estimulada e amostras de citologia exfoliativa de mucosa oral foram coletadas de um total de 39 pacientes que se apresentavam saudáveis (controle, n=10) ou com história de hipertensão arterial (HAS, n=9), diabetes mellitus (DM, n=10) ou ambos (DM+HAS, n=10). Taxa de fluxo salivar (SFR), níveis de TGFβ1 na saliva, AgNORs e maturação epitelial foram avaliados. Teste não-paramétrico de Kruskal-Wallis, seguido de comparação múltipla de Dunn e correlação de Spearman foram utilizados para as análises. SFR diminuiu significantemente em DM e DM+HAS (0,47±0,11 e 0,64±0,43 mL/min) quando comparado ao controle (1,4±0,38 mL/min). DM+HAS apresentou os maiores valores de concentração de TGFβ1 (24,72±5,89 pg/mL). Foi observada uma correlação positiva entre TGFβ1 e glicemia (R=0,6371; p<0,001) e uma correlação negativa entre TGFβ1 e saliva (R=-0,6162; p<0,001) e glicemia e SFR (R=-0,5654; p=0,001). Número de AgNORs e o padrão da maturação das células epiteliais foram similares entre os todos grupos. DM e DM+HAS apresentaram os menores valores de SFR, os quais foram correlacionados com o aumento nos níveis de TGFβ1. Apesar da maior secreção de TGFβ1, não foram observadas mudanças na morfologia ou proliferação das células epiteliais quando o paciente apresentava diabetes ou hipertensão.

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