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ORIGINAL ARTICLE

Salivary VEGF and post-extraction wound healing in type 2 diabetic immediate denture wearers

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ABSTRACT

Objective: Oral wound healing in healthy could be promoted by VEGF in saliva, and immediate denture wearing, but data in type 2 diabetes are lacking. Aims were to investigate the timeline of extraction wound healing in diabetic participants wearing immediate dentures and its correlation to salivary VEGF, as well as to examine the impact of the palatal plate on tissue VEGF during palatal wound healing in rat diabetic model.

Material and methods: Healthy (42) and type 2 diabetic (36) denture wearers, candidates for teeth extractions were included. Extraction wound healing was followed *via* measurements of socket closure, gingival hyperaemia, pain and presence of necrosis on 3rd, 7th, 14th and on 21st-day post-extraction. Salivary VEGF was measured before and on the 3rd and 21st day after the extraction. In streptozotocin-induced diabetic (30) as well as non-diabetic rats (30), tissue VEGF was measured in palatal wounds healing under or without a palatal plate.

Results: Type 2 diabetic prosthetic patients exhibit delayed socket closure, with pronounced hyperaemia, pain and necrosis. Salivary VEGF is increased in diabetes and positively correlates to socket closure while negatively with pain on 21st day after the extraction. Palatal incision induced VEGF increase in non-diabetic and diabetic, but less pronounced in diabetic rats. Wound healing under the palatal plate exhibit higher tissue VEGF.

Conclusion: Type 2 diabetes-induced increase in salivary VEGF may mitigate diabetes-induced detrimental effects on extraction wound healing. Lack of adequate tissue VEGF response to injury may underly dysregulation of diabetic oral wound healing.

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Immediate denture; wound healing; diabetes mellitus type 2; VEGF

Introduction

Post-extraction wound healing is an integrative complex of cellular and molecular processes consisted of the following stages: hemostasis, inflammation, proliferative phase and bone tissue remodelling. This is achieved *via* the time-dependent interplay of cytokines and growth factors, activation of signalling pathways and modulation of genes creating a healing milieu [1]. Vascular endothelial factor (VEGF) is produced by many cell types that participate in wound healing: endothelial cells, fibroblasts, smooth muscle cells, platelets, neutrophils, and macrophages [2–4]. Local hypoxia caused by interruption of circulation due to injury is the primary stimulus for VEGF formation. The most important role of VEGF in the regulation of angiogenesis and growth of granulation tissue in the wound, thus providing oxygenation and nutrition of damaged tissues with the deposition of the fibrin network necessary for wound healing [5]. Wounds in the oral cavity heal faster, with fewer scars than wounds in other parts of the body. The main factor that contributed to this phenomenon is saliva. Saliva promotes the healing of oral wounds by creating a humid environment, which improves the functioning of inflammatory cells crucial for wound healing, and contains a variety of growth factors involved in the various stages of intraoral wound healing. For instance, the proliferation of epithelial cells is promoted by growth factors in saliva, such as VEGF, which exhibit up to three times higher concentrations in saliva than in plasma [6].

Diabetes mellitus type 2 (T2DM) is a complex metabolic disorder associated with microvascular and macrovascular disease [7,8]. One of the most debilitating complications in diabetes is delayed wound healing attributed to both microvascular and macrovascular changes. Microvascular abnormalities lead to altered permeability of capillaries and reduced response to tissue injury [9]. Macrovascular changes, by affecting large vessels cause maldistributed blood flow and angiogenesis impairment [10]. It has been shown that at the molecular level, expression of VEGF and VEGF receptors are significantly reduced during diabetes in wounds of animals and humans [11,12]. At the same time, our previous

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study revealed an increase in VEGF salivary levels in type 2 diabetic (T2D) subjects, and its association with oral mucosal disorders in T2DM, such as denture stomatitis [13]. Still, there are no reports of salivary VEGF relation to oral mucosal healing in T2D subjects.

Diabetic patients have a higher prevalence of periodontal disease, followed by more missing teeth and indication for extraction than controls [14,15]. In order to obtain adequate mastication necessary for a regular daily dietary regime during the post-extraction period, an immediate denture is a necessary appliance for diabetics, candidates for teeth extractions. The presence of immediate dentures improves hemostasis, prevents trauma, and promotes wound healing in healthy yet [16], its impact on diabetic wound healing is not known.

Therefore, the aims of the present study were to investigate: 1) clinical parameters and time-line of extraction wound healing process in T2D participants wearing immediate complete dentures as well as its correlation to salivary VEGF levels, and 2) in the rat diabetic model, examine the impact of the palatal plate on tissue VEGF level during palatal wound healing.

Material and methods

Study population

The selected population consisted of 78 participants aged 45 to 64 years. Participants with diagnosed T2DM ($N = 36$) (referred by Clinic for Endocrinology, Zvezdara University Medical Centre, Belgrade) and participants without diabetes ($N = 42$) were recruited from the Department of Prosthodontics, School of Dental Medicine, University of Belgrade. The inclusion criteria were: wearing of maxillary partial removable dental prostheses and mandibular complete dentures, the presence of 3 teeth with poor or hopeless prognosis [17], defined by tooth mobility class II-III, class II inaccessible or class III furcation involvement and 50–75% attachment loss, in the anterior and premolar maxillary regions. For diabetic participants – a disease history of at least 2 years, use of oral hypoglycaemics or insulin to control the disease, and with glycosylated haemoglobin levels less than 8 (HbA1c <8). The exclusion criteria were smoking, a non-regular wearing of new dentures and treatment with corticosteroids.

Pre-prosthetic procedures included: attendance of a program of professional dental hygiene to nullify differences in preoperative hygiene conditions and atraumatic extractions of remaining 3 maxillary teeth without elevation of the full-thickness flap to preserve the bone ridges and soft tissue. The alveolar nerve block was obtained by using 2% mepivacaine. Both groups of participants, with and without diabetes, were indicated for immediate complete denture in order to provide T2DM participants with adequate mastication and to standardize the study conditions. The existing mandibular complete dentures were replaced on the day of receiving maxillary immediate complete dentures. The surgical protocol and clinical evaluation were conducted at the Clinic of Oral Surgery, School of Dental Medicine in Belgrade. The fabrication of new dentures (maxillary immediate complete dentures and mandibular complete dentures) and post-insertion denture adjustments necessary for removing difficulties that included pain and discomfort were performed at the Department of Prosthodontics, School of Dental Medicine in Belgrade. The study received approval from the Ethics Committee of the School of Dental Medicine, University of Belgrade (No. 32/36 in 2012 year) and was conducted in accordance with the Helsinki Declaration.

Clinical examinations

Dental records for all participants were provided at the Department of Prosthodontics and Clinic of Oral Surgery, School of Dental Medicine, University of Belgrade. Regular follow-up of the post-extraction wound healing in healthy and T2D immediate denture wearers was scheduled on the 3rd, 7th, 14th and 21st-day post-extraction, in order to obtain the records of post-extraction wound healing and discomfort under the immediate denture. The investigator, who was unaware of diabetic status, evaluated post-extraction socket width, obtained by measuring buccolingual diameter (using dental calliper). The reduction in the socket width (socket closure) is expressed as a percentage of the width measured immediately after the extraction (day 0). At the same observational time-points, wound healing outcomes such as gingival hyperaemia (ranged as slight middle or severe and presented by 3-point scale), gingival ulceration and gingival superficial necrosis (both recorded as yes or no and presented as frequency) as well as post-extraction pain (measured by a 100-mm visual analogue scale –VAS, from value 0-no pain to 100-max pain) were recorded. The presence of any side effects or complications (symptoms of dry socket and infection) were recorded. No pharmacologic therapy was prescribed and recourse to antibiotics was considered a negative postoperative parameter, a sign of postoperative complications from infections. The use of anti-inflammatory drugs was avoided and paracetamol was recommended for pain control.

Salivary measurement of VEGF

Saliva specimens were collected from all participants before teeth extraction and prosthetic treatment and on 3rd and 21stday of denture insertion and wearing. The mouth was rinsed with tap water before the saliva specimen was collected. All participants were asked to retain mixed saliva in their mouth for 5 min without swallowing and then to expectorate into a clean plastic container. The specimens were frozen in liquid nitrogen and stored at -70°C until analysis. Before VEGF measurements, salivary samples were thawed, centrifugated (10000 g, 5 min at 4 °C) to remove cellular debris and assayed for VEGF (Human VEGFA ELISA Kit, RayBiotech, USA). The minimum detectable dose of Human VEGF-A was determined to be 10 pg/ml.

Table 1. Demographic characteristics of study participants.

PARTICIPANTS		
	Non-diabetic	Type 2 diabetic
N	42	36
Age (mean ± SD)	56.5 ± 4.3	57.3 ± 3.4
Sex (M/F)	30/12	22/14
Duration of wearing dentures (years)	5.7 ± 0.3	6.2 ± 0.2
Type 2 diabetes duration (years)	/	4.2 ± 2.1
HbA1c (%)	/	7.8 ± 0.8

Experimentally induced DM

Sixty male Wistar rats weighing approximately 250 g each were used in this study. The rats were divided equally into non-diabetic (control) and diabetic groups. Diabetes was induced using a single intraperitoneal injection of the pancreatic β-cell toxin monohydrate alloxan (140 mg/kg; Sigma, St. Louis, MO, USA) and rats were allowed to drink 5% glucose water for 24 h to prevent acute hypoglycaemia. Serum glucose levels were measured after 7 d (Accu-Chek; Roche Diagnostic, Indianapolis, IN, USA) and the diabetic state was confirmed by a decrease of body weight and increase of blood glucose levels (more than 10 mmol/L). Both groups were divided into 3 subgroups, with ten rats in each: a group without incision wound and without palatal base, a group with incision wound and without palatal base and a group with incision wound under the palatal base. All animal procedures were approved by the Ethics Committee of the School of Dental medicine, University of Belgrade (No. 32/36 in 2012 year).

Preparation of experimental wound and palatal plates and tissue VEGF measurement

The animals were anaesthetized by an intraperitoneal injection of 200 mL/kg thiopental sodium and a full-thickness incision wound was created in palatal mucosa in the posterior region, close to molars. For the animals in the group with palatal plate, the precision impression of the upper jaw was taken using vinyl silicone impression material (Elite HD + putty soft normal, Zhermack, Italy). Methil-metacrylate plate (Biokril, Galenika, Serbia) extending from the first to the third molar teeth on both sides of the palate was made on the plaster cast (New Fujirock, GC) and cured. The palatal base was set on the palatine mucosal surface over the wounds and was fixed to the molar teeth on both sides using the same resin. In order to obtain animal tissue samples, all rats were sacrificed 3 d after palatal plate wearing and wound healing, using an overdose of thiopental sodium. Maxilla was removed with palatal plate, and after removal of the palatal base, palatal tissues were excised and were homogenized with a lysis buffer provided in the ELISA kit. Quantification of VEGF was performed by using Enzyme-Linked Immunosorbent Assay (Rat VEGFA ELISA Kit, RayBiotech, USA) according to the manufacturer's instructions. Optical densities were measured at 450 nm with a microplate reader and the minimum detectable level of the test was 5.0 pg/mL.

Statistics

Results were presented as frequencies or as mean ± SD. Two-way ANOVA with repeated measures was used for estimation of overall effect (time-points, groups). One-way ANOVA with repeated measures and *posthoc* Bonferroni test was applied within groups. Comparisons between appropriate group-time point was done by using Student's *t*-test for the independent samples. Further, the results were analysed using Pearson's correlation coefficient. Significant differences were considered for $p<.05$. Data were analyzed with SPSS 18.0, IBM Statistics, USA.

Results

Characteristics of the subjects

The mean age of participants was not different between non-diabetic and T2D participants however, males represent 71% of non-diabetic and 61% of the diabetic group. There were no differences between groups regarding the duration of wearing dentures. The median HbA1c value among T2D participants was less than 8% (Table 1).

Clinical characteristics of extraction wound healing process

Socket closure was progressive in both groups, being more prominent in non-diabetic compared to the diabetic group. The differences between investigated groups were statistically significant on 7th, 14th and 21st day after teeth extractions. T2D participants showed delayed and reduced socket closure three weeks after extraction (92% in diabetic vs 99% in non-diabetic) (Table 2), but all healed in four weeks with 19.8 ± 0.5 d needed in diabetic vs 16.9 ± 0.3 d needed in non-diabetic group. During 3 weeks of follow-up period, hyperaemia was observed in both groups, while score being significantly pronounced in diabetic patients on the 3rd, 7th and 14th days compared to controls. (Table 2). Pearson's tests revealed in the diabetic group a significant positive correlation between hyperaemia score and pain intensity on 3rd day ($r = 0.391$, $p = .02$) and on 7th day ($r = 0.441$, $p = .008$) post-extraction, while the negative correlation between socket closure and pain intensity ($r = -0.8$, $p < .01$) on 3rd-day post-procedure.

Clinical manifestations of disturbed post-extraction wound healing

Type 2 diabetic patients exhibit more frequently gingival necrosis, soft tissue ulcerations and pronounced pain compared to non-diabetics (Table 3), however, no infection or dry socket were identified. The frequency of necrosis and ulcerations as well as pain intensity peak on 3rd day post extraction and persist at high levels during two weeks after the extraction (Table 3).

Table 2. Clinical parameters of post-extraction wound healing in non-diabetic and T2D immediate denture wearers.

	Non-diabetic participants (42)		Type 2 diabetic participants (36)	
Observation period	Socket closure (%)	Hyperaemia (score)	Socket closure (%)	Hyperaemia (score)
Day of extraction	0	1	0	1
3rd day	$15.03 \pm 1.31^{\$\$\$}$	1.36 ± 0.53	$8.31 \pm 0.78^{\$\$\$}$	$1.91 \pm 0.78^{***}$
7th day	$46.59 \pm 0.72^{\$\$\$}$	1.43 ± 0.55	$32.33 \pm 1.23^{\$\$\$***}$	$2.0 \pm 0.54^{***}$
14th day	$82.7 \pm 0.70^{\$\$\$}$	1	$62.69 \pm 0{,}64^{\$\$\$***}$	$1.11 \pm 0.32^{*}$
21st day	$99.0 \pm 0.29^{\$\$\$}$	1	$92.3 \pm 0{,}50^{\$\$\$***}$	1

$^{***}p < .001$, $^{*}p < .05$ nondiabetic vs T2D.
$^{\$\$\$}p < .001$ time-point measurement vs. measurement on the day of extraction.

Table 3. Clinical manifestations of disturbed post-extraction wound healing in non-diabetic and T2D immediate denture wearers.

	Gingiva necrosis (%)		Soft tissue ulcerations (%)		Pain intensity-VAS (mm)	
			PARTICIPANTS			
Observation period	ND	T2D	ND	T2D	ND	T2D
Day of extraction	0	0	0	0	0	0
3rd day	0	31.4^{***}	21.4	42.9^{*}	35.1 ± 18.7	$51.42 \pm 26.14^{**}$
7th day	42.9	40.0	38.1	54.3^{*}	29.4 ± 15.4	$49.9 \pm 25.7^{**}$
14th day	0	20^{**}	0	11.4^{*}	12.8 ± 11.8	19.6 ± 26.5
21st day	0	20^{**}	0	0	0	$2.2 \pm 2.5^{**}$

ND-non-diabetic; T2D- type 2 diabetic.
$^{***}p < .001$, $^{**}p < .01$, $^{*}p < .05$ non-diabetic vs. T2D.

Salivary VEGF levels during post-extraction wound healing

Type 2 diabetic patients exhibit higher salivary VEGF expression comparing to non-diabetic at all time points of measurements: before teeth extraction and prosthetic treatment and on the 3rd and 21st day of denture insertion (Table 4). In both groups, salivary VEGF levels peak on 3rdday and persisted at similar levels on the 21st day after teeth extractions (Table 4). Pearson's tests revealed in the diabetic group a significant positive correlation between salivary VEGF expression and socket closure on the 21st day ($r = 0.557$, $p < .001$) as well as a negative correlation between salivary VEGF and pain intensity on the 21st day ($r = -0.432$, $p < .01$) post-extraction.

Oral mucosal VEGF expression and palatal wound healing in the diabetic rat

Expression of VEGF levels in palatal mucosa showed no significant differences between diabetic and non-diabetic rats. Surgical injury-induced VEGF expressions in both groups but being more prominent in the non-diabetic group (Table 5). Wounds healing under the palatal plate show significantly higher VEGF levels in both groups, while those in the diabetic group reach levels comparable to wounds in non-diabetics, which heal without the plates. (Table 5).

Discussion

The present findings show that T2D prosthetic patients exhibit delayed and more frequently disturbed post-extraction wound healing, with pronounced signs of inflammation (hyperaemia and pain) and a higher incidence of healing problems. Namely, present time-line analysis of post-extraction wound epithelialization under immediate complete denture showed significantly slower and reduced socket closure in T2D compared to non-diabetic participants, during three weeks of observation. Moreover, hyperaemia and pain were more pronounced in T2D patients, especially during first-week post-procedure. Having in mind that the inflammatory phase of oral wound healing occurs during the first week after the procedure, these results suggest a disturbance in the inflammatory phase of healing during T2DM, as we previously showed for diabetic dental pulp [18] and bone [19] healing, and pointed out recently by Patel et al. also [20]. Furthermore, presently observed a positive correlation between pain and hyperaemia and a negative correlation between pain and socket closure in T2D patients support the contribution of more intense inflammation in T2D to disturbance of healing progression. Our results show that T2DM is associated with the occurrence of healing complications also, reflected by more frequent ulceration and necrosis as well as prolonged and more intense pain observed in T2D compared to non-diabetic participants. It has been shown that the prevalence of oral mucosal lesions is significantly higher in patients with T2DM than in non-diabetics and that it could be due to slower healing rates and it induced prolonged pain in T2DM denture wearers [21,22]. Noteworthy, in a larger cohort study by Huang et al., patients with T2DM on oral hypoglycemics showed no significant socket healing disturbances comparing to non-diabetics [23]. This could be due to different inclusion criteria applied in this study compared to ours: not known duration of diabetes, not using HbA1c but blood glucose levels *ad hoc* as a parameter, and a cohort of patients on oral hypoglycemics, while our cohort comprised both oral hypoglycemics and insulin-treated T2D. Indeed, a recent study by Gadicherla et al. showed that based on the HbA1c values, the diabetic group shows a larger socket size on the 7th postoperative day and more frequent complications like swelling and infection than non-diabetic and prediabetic patients [24].

Healing after a tooth extraction is a multistage process depending on cytokines and growth factors such as VEGF.

Table 4. Salivary VEGF concentration during post-extraction wound healing in non-diabetic and T2D immediate denture wearers.

Participants (*N*)	VEGF (pg/ml)		
	Before extraction	3rd day after extraction	21st day after extraction
Non-diabetic (42)	113.75 ± 34.9	463.1 ± 125.9$^{\$\$}$	338.5 ± 82.7$^{\$}$
Type 2 diabetic (36)	564.9 ± 133.4***	1962.6 ± 366.4$^{\$\$}$***	1948.3 ± 364.9$^{\$}$***

***$p < .001$ non-diabetic vs. T2D.
$^{\$}p < .05$
$^{\$\$}p < .01$ after vs. before extraction.

Table 5. Tissue VEGF concentrations in palatal mucosa and in palatal wound healing under palatal plate in non-diabetic and diabetic rats.

Rats	VEGF (pg/ml)		
	Palatal mucosa	Palatal wound without plate	Palatal wound under plate
	Before incision	On 3rd day post incision	On 3rd day post incision
Non-diabetic	2.6 ± 0.5	5.3 ± 0.7$^{\$\$}$	11.8 ± 22.7$^{\#}$
Type 2 diabetic	2.8 ± 0.3	3.9 ± 0.9$^{\$\$}$***	6.6 ± 1.1***$^{\#}$

***$p < .001$ non-diabetic vs. diabetic.
$^{\$\$}p < .01$ before vs. after incision.
$^{\#}p < .01$ with vs. without palatal plate.

The presence of VEGF in saliva, which originated mainly from salivary glands, could be contributing factor for faster healing of oral comparing to skin wounds [25]. Keswani et al. [26] suggested that lack of salivary VEGF significantly impaired mucosal tissue repair and neovascularization following palatal injury. Present findings showed that salivary VEGF levels were higher in diabetics, regardless of the time of saliva sampling (before teeth extractions or during wound healing), and correlate with clinical parameters of extraction wound healing. Chronic hyperglycaemia has been reported to stimulate the synthesis and secretion of VEGF and plasma VEGF is higher in diabetics [27]. Likewise, diabetes increased salivary gland VEGF expression which by altering vascular reactivity, may mitigate diabetes-induced gland microvascular injury [28]. The present study shows that salivary VEGF increases during extraction wound healing, being the most prominent on the 3rd-day post-procedure, suggesting a salivary response to injury-induced inflammation. However, correlation of salivary VEGF with wound healing parameters such as pain and socket closure was not observed in the inflammatory stage, but only at the later time-point (measurements on the 21st-day post-procedure). Having in mind that at that time-point, differences between T2D and non-diabetic participants in clinical parameters of healing are the least, and salivary VEGF is positively related to socket closure, the beneficial role of salivary VEGF on diabetic wound healing could be proposed.

In order to investigate the possible underlying mechanism of disturbed socket healing in type 2 diabetic patients observed presently, we needed to consider oral mucosal tissue VEGF as well. However, due to ethical issues and limited experimental feasibility, we could not measure VEGF in human oral mucosa under the immediate denture. Therefore, we used a rat model of oral wound healing under the plate to investigate possible diabetes-induced alterations in oral mucosal tissue VEGF and the impact of immediate denture wearing under these circumstances. In this regard, present results in the experimental model suggest that lack of adequate tissue VEGF response to injury, rather than an alteration in tissue VEGF levels in diabetics, may contribute to healing dysregulation in diabetes. Regarding the possible mechanism underlying healing disturbances in T2DM, our present animal study suggested that lack of adequate VEGF response to injury may contribute to healing dysregulation in diabetes. Namely, surgical incision induced VEGF increases in both healthy and diabetic rats, but this increase was less pronounced in diabetic rats. In line with this, a role for fibroblast/VEGF dysfunction in the delayed wound healing observed in humans with T2DM was proposed [4]. Namely, it has been shown that diabetic fibroblasts exhibited a significant impairment in VEGF production and no adequate response of VEGF up-regulation in response to hypoxia [4], the main stimulus for VEGF after injury.

A noteworthy, present animal study revealed that wounds under palatal plate exhibit significantly higher VEGF expression compared to those healing without plate, and VEGF in diabetic rats reach levels comparable to non-diabetics. This may be due to the negative pressure occurring during plate movements since negative pressure has been associated with accelerated VEGF expression [29]. In the present clinical investigations, all participants were provided with immediate dentures and did not include patients without them, which disables immediate denture effectiveness estimation, representing a limitation of the study. However, based on the studies showing that negative pressure, in the humid environment can accelerate wound healing [30] as well as present results of wounds under plates exhibiting higher tissue VEGF levels, beneficial effects of the immediate denture on wound healing in diabetic patients could be proposed.

Conclusions

Type 2 diabetic patients exhibit more frequent signs of delayed and disturbed extraction wound healing while increased salivary VEGF levels may mitigate diabetes-induced detrimental effects on wound healing. Based on the data from the experimental model of wound healing under the plate, lack of adequate mucosal tissue VEGF response to

injury-induced hypoxia may contribute to dysregulation of diabetic oral wound healing while under such context, immediate denture application could be useful.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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