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ORIGINAL ARTICLE

Smoking increases salivary arginase activity in patients with dental implants

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Abstract It is believed that an increased arginase activity may lead to less nitric oxide production, which consequently increases the susceptibility to bacterial infection. Considering the hypothesis that smoking may alter the arginase activity and that smoking is considered a risk factor to dental implant survival, the present study aimed at evaluating the effect of smoking on the salivary arginase activity of patients with dental implants. Salivary samples of 41 subjects were collected: ten non-smoking and with no dental implants (group A), ten non-smoking subjects with dental implants (group B), ten smoking subjects with implants (group C), and 11 smoking subjects with no dental implants (group D). The levels of salivary arginase activity were determined by the measurement of L-ornithine and expressed as mIU/mg of protein. A significant increase in the salivary arginase activity was verified in groups C (64.26±16.95) and D (49.55±10.01) compared to groups A (10.04±1.95, p=0.00001 and p=0.0110, groups C and D, respectively) and B (11.77±1.45, p=0.00001 and p=0.0147, groups C and D, respectively). No significant difference was found between groups C and D (p=0.32). Within the limits of the present study, it can be concluded that salivary arginase activity is increased in smoking subjects with dental implants in contrast to non-smoking subjects with dental implants, therefore suggesting a possible mechanism by which cigarette smoking may lead to implant failure. The analysis of salivary arginase activity may represent an important tool to prevent implant failure in the near future.

Keywords Arginase · Saliva · Pathogenesis · Dental implant · Smoking

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Introduction

Implant failure is the result of a multifactorial process. Significant factors influencing the prognosis of dental implants include the length of the implant, bone quality, patient's gender, time of implant placement, location of the implant, and indication for implant treatment [12].

The vast majority of the literature implicates smoking as one of the prominent risk factors affecting the success rate of dental implants [1, 12, 17, 22, 29]. The failure rate of implants in smokers is reported to be more than twice that in non-smokers [1]. The exact mechanisms by which tobacco exerts its influence on the peri-implant tissues are still unknown. It is likely that smoking primarily has a systemic influence by altering the host response and/or by directly damaging the cells [21]. Some effects of nicotine have also been suggested to play a role in this process, such as vasoconstriction, increased levels of fibrinogen, hemoglobin and blood viscosity, excessive levels of carboxyhe-

moglobin in blood, compromised polymorphonuclear neutrophil function, as well as increased platelet adhesiveness [24].

L-Arginine, a semi-essential amino acid synthesized in the human liver and kidneys and found in large quantities in fish, chicken, and beans, is the common substrate for nitric oxide synthase (NOS) and arginase [10]. NOS converts L-arginine into nitric oxide (NO) and arginase converts L-arginine into ornithine and urea [18]. Due to its antimicrobial activity, it is considered an important molecule against infections [19]. Furthermore, considering that L-arginine is used as a common substrate by both arginase and NOS, it is believed that an increase in arginase activity would lead to a reduction in the NO production, consequently increasing the susceptibility to bacterial infection [28]. Accordingly, the arginase activity has been shown to be increased in periodontitis patients compared to healthy controls [9, 26, 30].

Recent studies have shown that cigarette smoking is associated with an increased expression of arginase in asthmatic subjects [2] and a reduced endothelial NO availability in the atherosclerotic vascular disease [32]. Therefore, it seems that smoking favors the arginase pathway, which might be attributable to the different components of the cigarette smoke, especially nicotine.

In the present study, we hypothesized that cigarette smoke influences the arginase activity in subjects with dental implants. In order to test this hypothesis, we compared the salivary arginase activity levels in smoking patients with dental implants with those in non-smoking individuals.

Materials and methods

Study population

Forty-one patients participated in the present study. Inclusion criteria were as follows: patients in good health who had not taken antibiotics, anti-inflammatory agents, immunosuppressants, or systemic contraceptives in the previous 6 months and who had no history of any chronic medical disease or conditions.

After that, we defined three groups:

- group A: control group consisting of non-smoking individuals with no dental implants (n=10);
- group B: non-smoking subjects with at least four dental implants (n=10).
- group C (n=10): subjects with at least 4 dental implants who have been smoking more than ten cigarettes per day for more than 10 years (information accessed by a questionnaire).
- group D (n=11): subjects with no dental implants who have been smoking more than ten cigarettes per day for more than 10 years (information accessed by a questionnaire).

Clinical examination

The periodontal and peri-implant probing depth was carried out with a periodontal probe (Hu-Friedy, Chicagol, IL, USA). Subjects showing absence of suppuration, bleeding on probing, and/or periodontal or peri-implant probing depth greater than 4 mm were included in the present study.

Data and personal information related to the medical and dental history of the subjects were obtained by questionnaire. All subjects signed an informed consent which was previously reviewed and approved by the institutional review board at the University of Taubaté, São Paulo, Brazil (protocol no. 0289/07).

Saliva collection

Stimulated whole saliva was collected at the same time of the day to avoid the circadian effect on composition of the saliva [7]. The subjects were instructed to rinse their mouth with water and to chew rubber bands to stimulate the saliva, 2.0 ml of which was collected. Saliva samples were centrifuged at 10,000×g for 10 min and at 4°C, and the supernatants were immediately stored at −20°C to be used latter to determine the arginase activity and the concentration of proteins.

Total protein concentration

The protein concentration of the salivary samples was determined by a colorimetric protein assay (Bio-Rad Laboratories, CA, USA) based on the method of Bradford [3]. Briefly, the concentration of solubilized protein was measured at 595 nm with a spectrophotometer. Comparison to a standard curve calculated using bovine serum albumin provided a relative measurement of the protein concentration (mg/dL).

Arginase activity determination

The arginase activity assay was carried out by means of a colorimetric assay measuring the L-ornithine formed by L-arginine hydrolysis according to the protocol established by Chinard [8]. A 5 μl of each sample was incubated at 37°C for 30 min with 500 μL of 60 mM carbonate buffer (pH 9.8) containing 20 mM L-arginine and 1 mM $MnCl_2$; the reaction was stopped with the addition of 1.5 ml of glacial acetic acid. Afterwards, 0.5 ml of ninhydrin solution

(0.2 ml H_3PO_4 6M; 0.3 ml glacial acetic acid; 12.5 mg ninhydrin) was added to each sample, vortexed, sealed, and boiled at 100°C for 60 min then cooled in a water bath at room temperature and the absorbance measured at 505 nm. The blanks were treated in the same way, except for the addition of glacial acetic acid before the sample. Each sample was analyzed in duplicate. Fresh solutions containing 10–200 nmol of ornithine were used to construct a standard curve. Arginase activity was expressed as nanomoles of ornithine formed per minute per milligram of protein (mIU/mg protein).

Statistical analysis

One-way analysis of variance was used to compare means among groups. In case of significant differences among the groups, post hoc two-group comparisons were assessed with Student's *t* test. A *P* value <0.05 was considered statistically significant. Data were expressed as mean± SEM.

Results

Table 1 describes the demographics of the patients. The control group (A) consisted of non-smokers individuals with no dental implants: six women and four men, with a mean age of 23.7±2.62 years. The patients with at least four dental implants were separated into two subgroups according to their smoking habit: implant group (B) which included eight women and two men, with a mean age of 59.8±5.75 years and who had never smoked, and smoking-implant group (C) which included subjects who have been smoking an average of 19±2.2 cigarettes per day for more than 10 years (18.1±8.2 years). This group comprised six women and four men, with a mean age of 51±14.1 years. Group D consisted of 11 smoking individuals (47.18± 10.59 years of age) who have been smoking an average of 18.45±8.55 cigarettes per day for 24.54±7.97 years.

No significant difference (p=0.1361) with regards to the salivary protein concentration was verified among groups.

Figure 1 shows that a significant increase in the salivary arginase activity was verified in groups C (64.26±16.95) and D (49.55±10.01) compared to groups A (10.04±1.95, p=0.00001 and p=0.0110, groups C and D, respectively) and B (11.77±1.45, p=0.00001 and p=0.0147, groups C and D, respectively). No significant difference was found between groups C and D (p=0.32).

Discussion

Arginase is an enzyme of the urea cycle which catalyzes the hydrolysis of L-arginine to urea and ornithine [16]. There are two different isoforms of arginase: arginase I which is localized in liver and arginase II localized in extrahepatic tissues [6]. Increased systemic arginase activity has recently been found to be involved in the pathophysiology of sickle cell disease and several pulmonary diseases such as allergic asthma, chronic obstructive pulmonary disease, and cystic fibrosis [23]. In addition, a previous study conducted by our group [9] showed that the salivary arginase activity levels were increased in patients with chronic periodontitis compared to healthy controls and that periodontal therapy decreased the activity of such enzyme.

Ornithine is the precursor of polyamines which are nutritionally important to many bacteria [11]. Therefore, high arginase activity may contribute to bacterial growth further contributing to infectious disease process. Moreover, an enhanced arginase activity by the host is

Table 1 Distribution of total population according to group, gender, age, time of masticatory functional loading (MFL) of the implant, number of cigarettes per day, and time of smoking habit

	n	Age (years)		MFL (years)		Cigarettes/day		Smoking (years)	Habit
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Group A	10	23.7	2.62						
Male	4	25.5	3.41						
Female	6	22.5	1.04						
Group B	10	59.8	5.75	2.5	1.58				
Male	2	63	0	2	0				
Female	8	59	6.23	2.62	1.76				
Group C	10	51	14.1	4.8	2.14	19	2.2	18.1	8.2
Male	4	49.25	16	4	1.63	18.75	1.5	17.25	11
Female	6	52.16	14.1	5.33	2.42	19.16	2.6	18.66	7
Group D	11	47.18	10.59			18.45	8.55	24.54	7.97
Male	4	49.75	14.05			23.25	12.47	27.25	8.77
Female	7	45.71	9.01			15.71	4.49	23	7.74

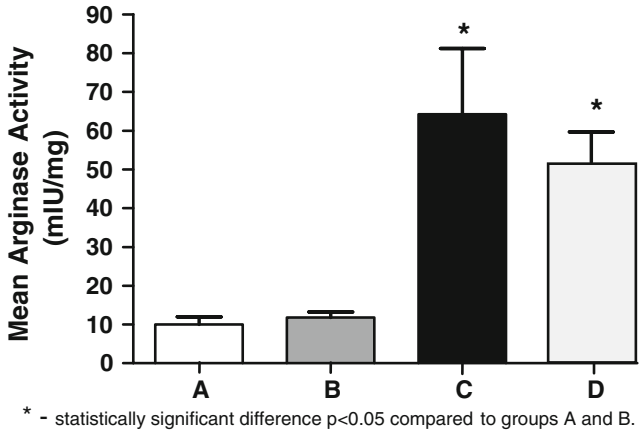

Fig. 1 Mean salivary arginase activity of groups A, B, C and D. *Statistically significant difference $p<0.05$ compared to groups A and B

accompanied by an increased consumption of L-arginine, common substrate for NOS thus resulting in less NO production. Besides its roles in wound healing, like angiogenesis and collagen formation, NO is suggested to play an anti-inflammatory role through the moderate inhibition of the nuclear factor-κB (NF-κB), which in turn leads to a down-regulation of several factors related to tissue destruction, such as cytokines, i.e., TNF-alfa, IL-1beta, and IL-6, and matrix metalloproteinases (MMP), MMP-2 and MMP-9 [14]. In addition, NO may act as a cytotoxic molecule against cells infected by fungi, protozoa, and bacteria as well as tumor cells [19]. Interestingly, the therapeutic potential of supplementation with L-arginine, which has been attributed to an enhanced synthesis of NO, has been documented to be beneficial in several conditions, including hypercholesterolaemia, hypertension, coronary artery disease, and diabetes [13]. Thus, arginine enzymes are highly associated with the host immune response and tissue breakdown.

Cigarette smoking results in a large amount of toxic elements that have the ability to disturb cell function and proliferation and to activate inflammatory cells [2, 5, 31, 32]. Recently, it has been found to be an important enhancer of the arginase activity. Accordingly, an increased expression of arginase mRNA has been reported in the airways of smoking compared with non-smoking asthmatic subjects [2]. In addition, a study by Butler et al. [4] assessed vascular responsiveness in smoking and non-smoking men using a venous occlusion plethysmography, concluding that cigarette smoking is associated with a significant reduced basal and stimulated NO bioactivity. Furthermore, cigarette smoke has been found to affect the endothelial L-arginine NOS pathway [32], resulting in reduced NO production, therefore providing a new biological insight into the basis of vascular diseases. Consistent with these findings, our present work provides the first demonstration that the salivary arginase activity is substantially higher in smoking compared with non-smoking subjects. In fact, the mean salivary arginase activity was almost sixfold higher in smoking (64.26±16.95) compared to non-smoking individuals (11.77±1.45, $p=0.00001$) with dental implants and almost fivefold higher in smoking (49.55±10.01) compared to non-smoking subjects with no dental implants (10.04±1.95). The influence of the time of exposure and intensity of smoking may have influenced our results once the smoking individuals of the present study were long-term heavy smokers who used to smoke approximately more than 20 cigarettes per day for more than 18 years.

An increased salivary flow rate has been described in smoking individuals compared to healthy controls [25]. However, no differences regarding the total salivary protein concentration were verified among groups, which therefore excludes the possibility that the salivary flow rate may have influenced our results.

Smoking has been found to have unfavorable effects in the oral cavity, such as increased plaque accumulation, higher incidence of gingivitis and periodontitis, higher rate of tooth loss, and increased alveolar bone resorption [27]. Smoking is also linked to the complications related to long-term survival and success rates of implant therapy. In a 5-year longitudinal study of post-loading implant failure, Hultin et al. [15] found that seven of nine patients who lost fixtures after loading were smokers. Smoking also increases the incidence of peri-implantitis and adversely affects the success of bone grafts [12, 20].

The exact mechanisms by which smoking leads to peri-implant tissue breakdown are not completely known. Several authors have suggested that smokers are more prone to develop peri-implantitis because of their impaired immune response, especially with regards to their compromised polymorphonuclear leukocyte functions such as delayed margination and diapedesis as well as compromised aggregation and adhesion to the endothelium in venules and arterioles [1, 24]. Since increased arginase activity and resulting decreased NO bioavailability may also be associated with host immune abnormalities, we believe that our findings on the salivary arginase activity in smoking patients may contribute to a better understanding of the pathophysiological link between smoking and adverse dental implant effects. Interestingly, in our present study, no adverse effect was clinically or radiographically detected in the implants of the smoking patients, therefore highlighting the importance of plaque control in oral health maintenance.

The present study demonstrated that salivary arginase activity is increased in smoking subjects with dental implants when compared to non-smoking individuals. The results observed in the present study justify the special

attention that clinicians should provide to smoking subjects with dental implants, especially with regards to the importance of plaque control. Future studies are needed to better clarify the specific role of increased arginase activity on dental implant therapy outcome in smokers.

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Conflict of interest statement The authors declare that they have no conflict of interest.

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