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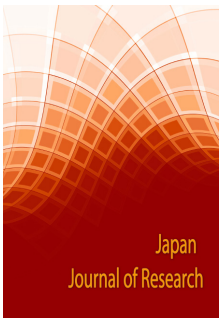

Evaluation of the Tissue Immune Microenvironment in the Diabetic Patients with Dental Implant

Slăvescu Dan[1], Moca Abel[1], Iurcov Raluca[1], Pop Alex Caleb[2], Cămărășan Andreea[3], Vaida Ligia[1], Vigdorovits Alon[3]

[1]Dental Science, Faculty of Dentistry, University of Oradea, Romania
[2]Bihor County Emergency Clinical Hospital, Romania
[3]Department of Morphological Disciplines, Medicine and Pharmacy Faculty, Univeristy of Oradea, Bihor County, Romania

Correspondence
Pop Alex Caleb
Bihor County Emergency Clinical Hospital, Romania
Email: popalex.caleb@yahoo.ro
Tel: +40 747672993

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Abstract

Long-term hyperglycemia in people with diabetes very frequently leads to damage and/or dysfunction of many tissues and organs of the human body, causing increased clinical morbidity. There is, however, an increased correlation between glycemic control and the onset of these consequences and in particular microvascular and macrovascular complications. The results of some studies have suggested that diabetes exerts an influence on the failure rates of implants compared to non-diabetic patients. One study demonstrates that HbA1c level (higher or lower than the threshold of 8.1) also influences the survival rate of dental implants. Biopsy fragments from 8 consecutive patients who had a titanium dental implant as well as diabetes were included in our study. The control group was represented by 4 patients with dental implants and who did not have diabetes. After the histological processing of the tissues, cell counts of CD4, CD8, CD20 and CD3 lymphocytes were performed by using QuPath. The findings characterize the composition of lymphocyte populations in the oral mucosa surrounding dental implants. In diabetic patients, poor glycemic control has been associated with a decreased CD4/CD8, contrary to our findings in tissue. The decreased number of CD20 positive lymphocytes could be associated with the dysfunction of humoral immunity generally observed in patients with diabetes. In our study, a 2.5 x higher value of TCD4+ lymphocytes was noted compared to the average of TCD8+ type lymphocytes. These results support the involvement of these types of inflammatory cells in the pot-implant bone healing processes. This process starts on the 10th day post-implant. Different studies raise, in these cases, the question of innate immune response vs adaptive immune response. Suppression of processes induced by CD8+ lymphocytes is associated with stimulation of bone formation. Our results support this hypothesis.

Introduction

Diabetes mellitus includes a group of chronic metabolic diseases characterized by hyperglycemia, resulting from deficiencies in the production of insulin by the pancreas, in the efficient use of insulin by the body, or both [1-3]. The most common type of diabetes is type 2, which accounts for 90-95% of people with diabetes [1]. It has been estimated that type 2 diabetes will affect more than 640 million adults by 2030 [4].

Long-term hyperglycemia in people with diabetes very frequently leads to damage and/or dysfunction of many tissues and organs of the human body, causing increased clinical morbidity [1,5]. Adverse effects of the disease include: poor response to infections [6], difficulty and delay in wound healing [7], macro- and microvascular complications [8], impaired bone metabolism and bone strength [9].

There is, however, an increased correlation between glycemic control and the onset of these consequences and in particular microvascular and macrovascular complications [10]. Good glycemic control in diabetic patients may delay the onset and progression of many vascular complications associated with this condition [3,11]. A patient with controlled diabetes is defined as a patient who maintains his/ her blood glucose level as close to normal as possible for as long as possible. This is determined by a test, which measures what percentage of the hemoglobin is glycated (HbA1c). People with diabetes who maintain a level of up to 6.5% HbA1c are considered to have controlled diabetes [12].

On the other hand, increased salivary glucose leads to changes in the oral cavity such as xerostomia, which, in turn, predisposes to increased bacterial plaque accumulation. This bacterial plaque is a determining factor in the

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onset of dental caries and periodontal disease, the complications of which can ultimately result in the loss of dental units [13,14].

Dental implants are considered to be the most functionally and esthetically effective solution to rehabilitate the edentulous areas of the dental arches and represent a modern and durable alternative [15,16]. Despite their obvious benefits, dental implants can pose some challenges for patients with diabetes, as this condition can affect the healing process and increase the risk of post-operative complications [17].

There are numerous studies showing that the negative effects of diabetes on the body also affect the stability of dental implants, raising some concerns about the long-term survival of dental implants in diabetic patients due to the influence of blood glucose levels on the general condition of oral tissues [18- 20].

The results of some studies have suggested that diabetes exerts an influence on the failure rates of implants compared to non-diabetic patients [14,21].One study demonstrates that HbA1c level (higher or lower than the threshold of 8.1) also influences the survival rate of dental implants [22]. However, there are also authors who claim that there is no significant difference in the survival rate of dental implants in diabetic versus non-diabetic patients [23].

Patients with type 2 diabetes mellitus appear to have a significantly better prognosis in terms of dental implant survival rates compared to those with type 1 diabetes mellitus [22,24]. The failure rate of dental implants has been shown to be higher in the first year after implantation in patients with microvascular complications associated with diabetes [25].

Materials and methods

Biopsy fragments from 8 consecutive patients who had a titanium dental implant as well as diabetes were included in our study. The control group was represented by 4 patients with dental implants and who did not have diabetes. The fragments obtained were represented by the peri-implant gingival mucosa. The type of implant used in the patients included in the study was identical.

All oral mucosa fragments were fixed in 10% buffered formalin (pH 7.4) for up to 48 h and processed automatically (Excelsior Epredia) in the paraffin blocks.

The paraffin-embedded tissue was sectioned using the Leica RM2125 microtome (Leica Biosystem, Buffalo Grove, IL, US) at 4 microns stained with hematoxylin & eosin (HE) with the Gemini automatic Stainer (Epredia-Portsmouth, New Hampshire-USA) and examined with the Leica DM 3000 LED microscope.

Using an Autostainer Link 48 (Agilent Technologies, Santa Clara, CA, USA) the sections were incubated with anti-CD8 (clone C8/144B), mouse monoclonal antibody, anti-CD4 (clone 4B12),mouse monoclonal antibody, anti-CD3 rabbit polyclonal antibody, anti-CD20 (clone L26),mouse monoclonal antibody (Agilent Technologies, Santa Clara, CA, USA). Positive control slides made from appendix tissue were utilized for every case. The negative control was made through the same processing steps but by omitting the antibody used and replacing it with an IgD.

After performing cell counts using QuPath, the average number of CD4, CD8, CD20 and CD3 lymphocytes across all cases were calculated, as well as the CD4/CD8 and CD20/CD3 ratios. The average values of these parameters were compared between patients that suffered from diabetes and those that were non-diabetic. For statistical evaluation, the Mann-Whitney U test was used to compare means between groups. A formal statistical significance level of 0.05 was chosen. Statistical analysis as well as figure generation were performed using Microsoft Excel.

Results

Examination of the morphological and immunohistochemical aspects of the peri-implant gingival mucosa revealed an abundant inflammatory infiltrate composed mainly of B cells (Figure 1A and 1B). The composition of the T cell population in patients with diabetes revealed an increased CD4/CD8 ratio (Figure 1C and 1D).

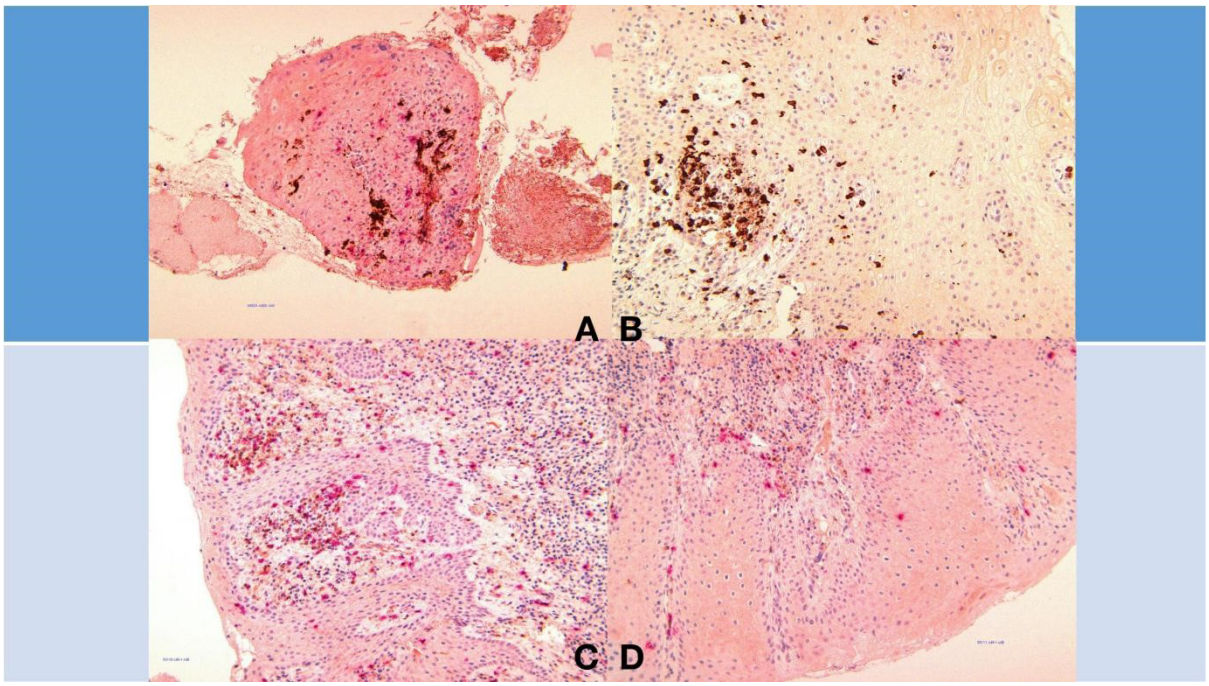

Figure 1 *– A and B: CD20 (brown) and CD3 (magenta); C and D: CD4 (brown) and CD8 (magenta)*

The evaluation of CD8 positive lymphocytes in the oral cavity revealed a higher average count in patients with diabetes than in non-diabetic patients but the difference did not reach statistical significance (95.66 vs.73.6, respectively; Mann-Whitney U p-value = 0.234) (Table 1).

The analysis showed the presence of a larger average number of CD4 positive lymphocytes in diabetic patients when compared with the non-diabetic group (136.5 vs. 47.6, respectively; Mann-Whitney U p-value = 0.022) (Table 1).

***Table 1**. Comparison of means between CD8, CD4, CD3, and CD20 positive lymphocytes in diabetic vs. non-diabetic patients (Mann-Whitney U test p-value);*

Positive lymphocytes (means)	Diabetic patients	Non-diabetic patients	Mann-Whitney U test p-value
CD8	95.66	73.6	0.234
CD4	136.5	47.6	0.022
CD3	127.8	153.5	0.417
CD20	24.8	144	0.004

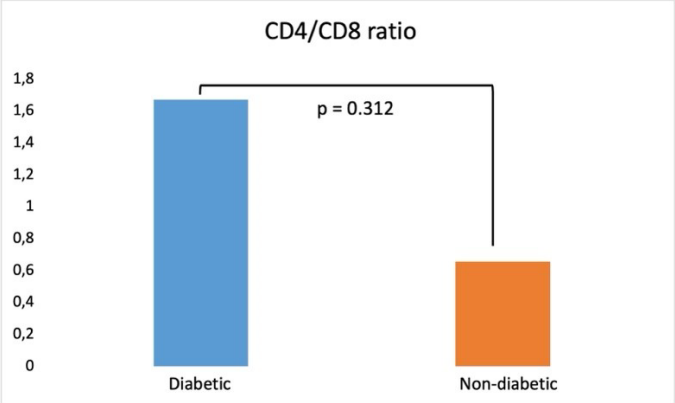

***Figure 2**. CD4/CD8 ratio*

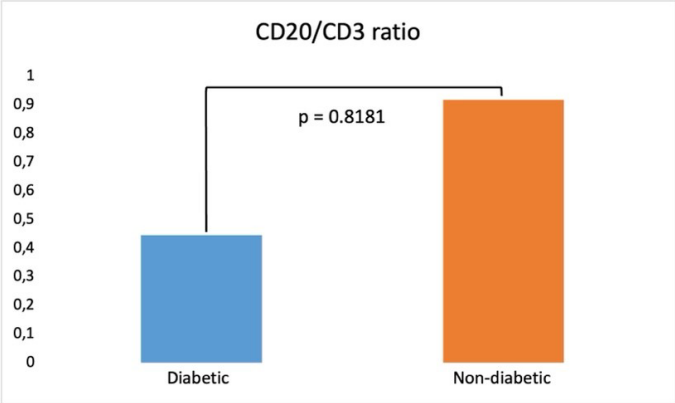

***Figure 3**. CD20/CD3 ratio*

We identified similar average numbers of CD3 positive lymphocytes in both groups (127.8 vs. 153.5, Mann-Whitney U p-value = 0.417) (Table 1).

Non-diabetic patients with dental implants showed a much higher average number of CD20 positive lymphocytes in the biopsies (144 vs. 24.8, Mann-Whitney U p-value = 0.004) (Table 1).

The average CD4/CD8 ratio was higher on average in patients diagnosed with diabetes without reaching formal statistical significance (1.67 vs. 0.65; Mann-Whitney U p-value = 0.312) (Figure 2), while the mean CD20/CD3 ratio appears to be higher in patients without diabetes, without reaching statistical significance (0.44 vs. 0.91; Mann-Whitney U p-value = 0.818) (Figure 3).

The average CD4/CD3 ratio was increased in patients with diabetes compared to non-diabetic patients without reaching formal statistical significance (1.81 vs. 0.39; Mann-Whitney U p-value = 0.0509) (Figure 4). Similarly, the CD8/CD3 ratio was larger in patients diagnosed with diabetes, without statistical significance (1.898 vs. 0.536; Mann-Whitney U p-value = 0.7113) (Figure 5).

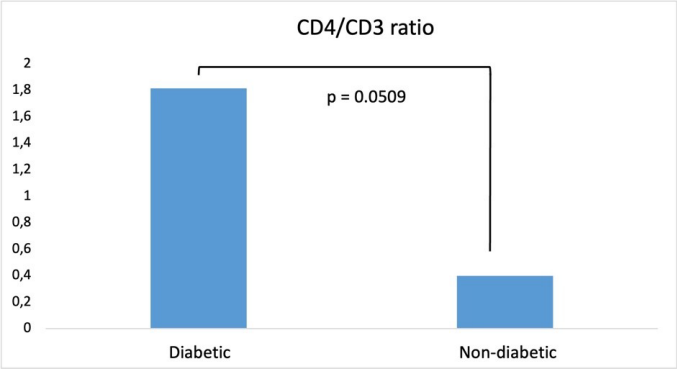

***Figure 4**. CD4/CD3 ratio*

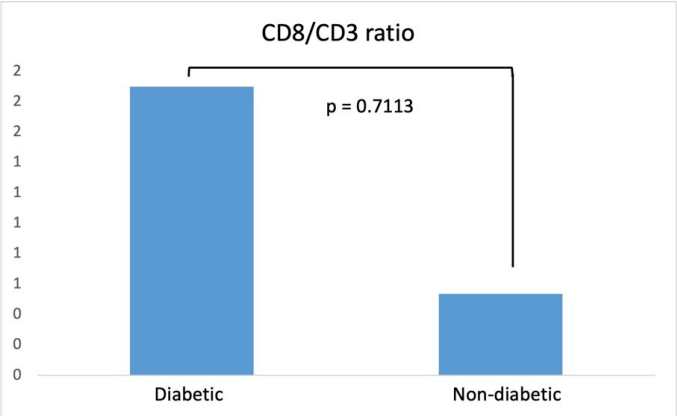

***Figure 5**. CD8/CD3 ratio*

Discussion

IHC staining of oral mucosa biopsies for CD4, CD8, CD20 and CD3 revealed several aspects regarding the immune response surrounding dental implants in diabetic and non-diabetic patients. In patients diagnosed with diabetes, CD4 positive lymphocytes seem to be increased on average (statistically significant), with CD8 being just slightly elevated, without reaching statistical significance.

The CD4/CD8 ratio seems to be increased in patients with diabetes (not statistically significant). This ratio is due to the large increase in CD4 average counts that is observed in samples from diabetic patients. Levels of CD3 positive lymphocytes were similar between the two groups, while CD20 positive lymphocytes are much lower in biopsies of patients with diabetes (statistically significant). The CD20/CD3 ratio is lower in patients with diabetes, without reaching statistical significance.

These findings characterize the composition of lymphocyte populations in the oral mucosa surrounding dental implants. In diabetic patients, poor glycemic control has been associated with a decreased CD4/CD8, contrary to our findings in tissue. The decreased number of CD20 positive lymphocytes could be associated with the dysfunction of humoral immunity generally observed in patients with diabetes.

One previous study showed that in rabbits, titanium implants activate the immune system and suppress bone resorption during the first 4 weeks after femoral implantation [26]. Due to potential pH changes in the oral cavity, dissolution of titanium particles can occur. These particles lead to activation of the immune system that could lead to peri-implant disease [27]. In studies performed on healthy rats, titanium dental implants were shown to upregulate CD4 positive cells while suppressing CD8 positive cells, which could suggest a local reduction of the immune inflammatory response in order to promote tissue repair [26].

Macrophages appear to be yet another important cell type in the immune response to dental implants. There is evidence that titanium leads to the activation of macrophages either directly or by phagocytosis, which leads to the production of both pro and anti-inflammatory cytokines. Macrophage polarization between M1 and M2 phenotypes is a well-known determining factor in immune reactions to foreign materials [27]. Titanium has a reparative/anti-inflammatory M2 phenotype, while copper as well as PEEK have a mixed pro-inflammatory M1 and anti-inflammatory M2 reaction [27].

Literature reviews on this topic highlight the lack of consensus and the needed for large-scale studies. A systematic review by Wanger et al. revealed that patients with diabetes have shorter implant survival times.

An important mechanism of implant rejection is blocking the transformation of M1-type macrophages into M2-type macrophages. This transformation is controlled by inflammatory cells, especially T lymphocytes (CD3+), as well as various chemical mediators. The decrease or blockage of this transformation is the result of a failure of dental implant survival. According to the pilot study carried out by us, it shows that in the case of patients with diabetes, the number of T lymphocytes tends to decrease [28].

In our study, a 2.5 x higher value of TCD4+ lymphocytes was noted compared to the average of TCD8+ type lymphocytes. These results support the involvement of these types of inflammatory cells in the pot-implant bone healing processes. This process starts on the 10th day post-implant. Different studies raise, in these cases, the question of innate immune response vs adaptive immune response [5]. Suppression of processes induced by CD8+ lymphocytes is associated with stimulation of bone formation [27]. Our results support this hypothesis.

The CD20/CD3 ratio clearly indicates the intensity of a cellular immune response carried out by cytokines in the case of diabetic patients compared to the antigen-antibody type response

The present study did not aim to evaluate another subpopulation of T lymphocytes called reg T cells. This lymphocyte population is known to achieve immunological tolerance. The process of immunological tolerance is mandatory for the activation of regeneration and reparative processes [17].

More research on this topic as well as more highly powered studies are necessary to characterize the interactions between immune cells in detail. Robust characterization of local immune response could pave the way for various methods and tools to predict the success of dental implants. The potential of understanding the immune environment related to dental implants could lead to better outcomes for these procedures, particularly in patients with type 2 diabetes, which suffer from higher rates of dental implantation failure.

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