

CD4/CD8 ANTIBODIES REDUCE HISTOPATHOLOGICAL DAMAGE IN SALIVARY GLANDS OF SPONTANEOUSLY DIABETIC MICE

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received: April 16, 2019 accepted: June 10, 2019

available online: June 30, 2019

Abstract

Background and aims: Diabetes affects the metabolism promoting damage in different tissues, including salivary glands. Current treatments, such as insulin, are ineffective to recovery of these tissues. In this aspect, the immunotherapy has been tested, but it can be inefficient as an agent for the control of damage caused by diabetes. The aim of this study to evaluate the association in anti-CD4 and anti-CD8 monoclonal antibody in the recovery of salivary glands of diabetic NOD mice. **Material and methods:** Fifteen spontaneously diabetic mice (NOD) were divided into three groups with 5 animals each: group I (Balb/C control mice), group II (untreated NOD mice), group III (NOD mice treated with CD4 and CD8 antibodies). The CD4 and CD8 antibodies (IMUNY, Rheabiotech Ltda, Brazil) were administered by intravenously injections (25 ug/days: 0, 7, 14, and 21). After treatment salivary glands samples were analyzed by immunofluorescence, microscopy, light microscopy and stereology. (ethical approval process: 304/11), Analysis of variance (ANOVA) and Kruskal-Wallis nonparametric test were used. **Results:** Elevated levels of glucose (mg/dl) were observed in untreated animals (group II) (605.25 ± 31.23 , $p \leq 0.05$), whereas in treated animals (group III), were noted a decrease in these levels (464.77 ± 39.66 , $p \leq 0.05$). Tissue restructure, characterized by cell volume recovery, also was observed in group III (nuclear volume of parotid glands: (109.91 ± 02.03 , $p \leq 0.05$) and submandibular glands: (107.52 ± 02 , $p \leq 0.05$) (cytoplasmic volume of parotid glands: (356.14 ± 26.34 , $p \leq 0.05$) and submandibular glands: (331.22 ± 32.11 , $p \leq 0.05$). Intense signaling (+++) of insulin receptors was observed in animals of group I. On the other hand, in group II was noted a reduction of these receptors (+). In treated animals (group III) were observed a recovery of the insulin receptors (+++). **Conclusions:** This treatment was effective in the recovery of salivary acinar cells, contributed also to homeostasis of body metabolism. Thus, this immunomodulation promoted a beneficial effect on the recovery of these tissues.

key words: anti CD4 and CD8, immunotherapy, salivary glands, diabetes mellitus

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Background and aims

Type 1 diabetes is characterized by the infiltration of cells of the immune system into the pancreatic beta cells. Clinical diabetes is diagnosed when 80-90% of the Beta cell mass is already destroyed or has lost its function. However, this disease state can be reversed if the autoimmune response is rapidly suppressed. Studies in NOD mice and in diabetic patients indicate that CD4 + T CD8 + T lymphocytes are the primitive mediators of pancreatic beta cell destruction [1-5].

The hyperglycemic condition of type I diabetes presents several complications such as microangiopathies and tissue disruptions. It is also possible to observe inflammatory processes mediated by macrophages, which recognize autoantigens and cytotoxic T cells, which will act on tissue destruction, especially in pancreatic cells. Thus, tissue injuries occur throughout the organism due to the insulin alteration initiated by these processes, affecting in the same way the salivary glands. Thus, NOD mice, non-obese diabetics, are considered one of the best models for the study of the effects of type I diabetes mellitus. In these animal models, a lymphocytic infiltrate first appears in the pancreas, specifically in the "Islets of Langerhans", where there is destruction of beta cells. From there, the inflammatory process leads to the occurrence of the diabetic state and causes damages in different organs, including salivary glands [6-14].

The salivary glands in both humans and rodents have a morphofunctional unit characterized as tubulo-acinar and are divided into major and minor. The smaller ones are present on the lip, tongue, palate and cheek, have short ducts and produce mucoprotein-rich secretion varying according to their topographic position. In relation to the larger ones, we can

highlight the parotid glands, which mainly have serous acini with a single pyramidal-shaped cell layer distributed around a central lumen and the submandibular ones that present mixed secretory characteristics, that is, it presents mostly seromucous acini and the other secretory portions with mucosal cells. Besides these main ones, there are the sublingual ones, also classified as mixed, whose predominant cells are mucous and seromucous. The glandular parenchyma possesses acini with cells specialized in the production and secretion of saliva, whose function is to participate in the processes of defense and digestion of food. This fluid is slightly thick and presents ions, proteins, water, insulin, immunoglobulins and peptides, as some growth factors, including IGF [11-15].

These tissues include atypical epithelial cells, decreased cell volume, inflammatory processes, and alterations in the expression of Insulin-like Growth Factor (IGF) receptors, demonstrating the similarity with what occurs in the pancreas and the magnitude of the damage caused by this disease.

In this way, research is needed to test new therapies, mainly focusing on the modulation of this autoimmune action present in type I diabetes. There are many studies that present treatments, however they do it in isolation and without also focusing the tissue recovery after treatments. Some studies have used specific antibodies, anti-CD4 / CD8, demonstrating an effective result in blocking the infiltration of CD4/CD8 T lymphocytes into the beta-pancreatic cells of NOD animals [16-18].

Thus, the objective of this study was to evaluate the association of anti-CD4 and anti-CD8 monoclonal antibodies in the tissue recovery and insulin receptors of salivary glands of spontaneously diabetic mice

Material and methods

Fifteen spontaneously diabetic mice (NOD) were divided into three groups with 5 animals each: group I (Balb/C control mice), group II (untreated NOD mice), group III (NOD mice treated with CD4 and CD8 antibodies). All animals were aged 15 weeks weighing on average 24 grams from the Campinas State University Bioterrorism Center (CEMIB with ICLAS certification) and kept during the experiment under standard conditions in the Laboratory Animal Experimentation Sector (SEA- Anatomy - registered at the Brazilian College of Animal Experimentation/COBEA and at the Brazilian Society of Sciences in Laboratory Animals/SBCAL) of the Department of Morphology and Basic Pathology of the Medical School of Jundiaí.

To verify the glucose levels, the blood levels of these animals were checked and analyzed weekly with the Accu-Check Performa device according to the manufacturer's standards, thus proving the hyperglycemic condition. The animals in groups III received a total dose of 100 µg/kg of anti-CD4 / CD8 (IMUNY, Rheabiotech Ltda, Brazil) every 7 days (divided into 4 doses of 25 µg/kg respectively on days 0, 7, 14 and 21) by intravenous route, totaling 21 days of treatment [19].

After the experimental period, the female mice of all groups underwent general anesthesia with Ketamine (130 mg/kg) and Xylazine (6.8 mg/kg) (1:1). salivary glands. After this, the animals were euthanized with the deepening of the anesthetic procedure (according to the principles of animal experimentation-COBEA/Concea and CEUA/FMJ process number: 342/11).

Portions of the collected samples were post-fixed in aqueous solution of picric acid (Bouin Solution), prepared and included in plastic paraffin (Paraplast Plus, CA, USA), for

subsequent standard staining with Hematoxylin and Eosin (HE), for morphological study.

The sections were used for the three-dimensional quantification of the tissues, where the nuclear and cytoplasmic volume of the acinar cells of the salivary glands were evaluated using stereological methods. For this, the diameters of 50 nuclei of each animal were measured, being 250 nuclei per experimental group, totaling 750 nuclei studied, and this process was repeated for confirmation in four random regions of each histological slide. Nuclei with defined limits and flat cuts were chosen in all their extension. The measurements were performed with a 10x eyepiece with a ruler and attached to the light microscope, and the observations were fixed with the objective of 100x.

For immunofluorescence the salivary glands and frozen sections were fasted using alcohol and acetone (1:1) at 40°C for 3 minutes and 4% paraformaldehyde for 10 minutes, then the sections were washed in brine buffered saline (PBS). Subsequently, all sections were subjected to the 3% hydrogen peroxide blocking solution in water and subsequently placed in a second blocker for non-specific protein-protein binding sites with bovine serum albumin in PBS buffer for 1 hour at room temperature. Next, for labeling of insulin receptors, the samples were incubated in specific primary antibody (INS-R alpha, Santa Cruz, CA, USA), which was diluted in blocking solution (1:250) and applied over the cuts during 12 hours at 40°C. Subsequently, the sections were washed in PBS buffer and incubated in fluorescein-conjugated secondary antibody (IgG-FITC, Santa Cruz, CA, USA) and diluted in blocking solution (1:100), thus avoiding nonspecific labeling. After this procedure, the sections were washed again with PBS, mounted on 1.4-diazabicyclo [2.2.2] octane (DABCO, for fluorescence microscopy) (Sigma, St. Louis, USA), observed and photographed in

the Fluorescence Microscope. To obtain all the images were used 20x and 40x objective. For negative control of the immunostaining, even parts of the samples were not incubated in the primary antibody.

For the statistical analysis of glucose levels (mg / dl), variance analysis (ANOVA) was used comparing each of the groups and complemented with the non-parametric analysis by the Kruskal-Wallis test for the three-dimensional study of the tissues. The entire study was performed with at least 5% significance.

Results

Table 1. Levels of glucose (mg/dL) between the studied groups.

	Levels of glucose (mg/dL)
Group I (Balb/c Healthy)	143.50 ± 21.79 ^{a,c}
Group II (Untrated NOD)	605.25 ± 31.23 ^{a,b}
Group III (CD4 and CD8 NOD)	464.77 ± 39.66 ^{b,c}

Values expressed by the mean ± standard deviation.
a, b, c Different in significance (P <0.05)

Elevated glucose levels (mg/dL) were observed in untreated animals (group II). Whereas in the treated animals (group III) a significant decrease of these levels was observed.

Table 2. Nuclear and cytoplasmic volume of Acinar Cells (μm^3) of the parotid glands present in the studied groups.

	Nuclear Volume (μm^3)	Cytoplasmic Volume (μm^3)
Group I (Balb/c Healthy)	137.82 ± 37.27 ^{a,c}	497.74 ± 58.81 ^{a,c}
Group II (Untrated NOD)	33.46 ± 01.68 ^{a,b}	98.37 ± 10.95 ^{a,b}
Group III (CD4 and CD8 NOD)	109.91 ± 02.03 ^{b,c}	356.14 ± 26.34 ^{b,c}

Values expressed by the mean ± standard deviation.
a, b, c Different in significance (P <0.05)

It was possible to verify the restructuring of the tissue, characterized by the recovery of the cellular volume, where a significant difference in the nuclear and cytoplasmic volume of the parotid glands and submandibular glands was observed in group III when compared to group II.

Light microscopy and stereological analysis

Submandibular Gland

In group I, seromucous acini were noted with mucosal columnar cells and basal nuclei. Among the acini we observed salivary ducts and discrete intercellular space ([Figure 1A](#) and [Table 2](#)). In untreated diabetic mice (group II), we observed involute cells and increased space between the acini. The nuclei were located in the basal region ([Figure 1C](#) and [Table 2](#)). In treated diabetic animals (group III), inverted acini were observed when compared to control animals, but significantly recovered when compared to diabetic animals without treatment ([Figure 1E](#) and [Table 2](#)).

Parotid Gland

In group I, serous acini were observed with pyramidal-shaped columnar cells. The basophilic cytoplasm and the basal nucleus were noted. Among the acini we observed discrete stromal space and salivary ducts ([Figure 1B](#) and [Table 2](#)). In the untreated diabetic mice (Group II), the cells were involuntary and with an increase in the interacting space. The nuclei were located in the basal region ([Figure 1D](#), and [Table 2](#)). After treatment (Group III), serous acini animals were still involved when compared to healthy animals but recovered when compared to untreated animals ([Figure 1F](#) and [Table 2](#)).

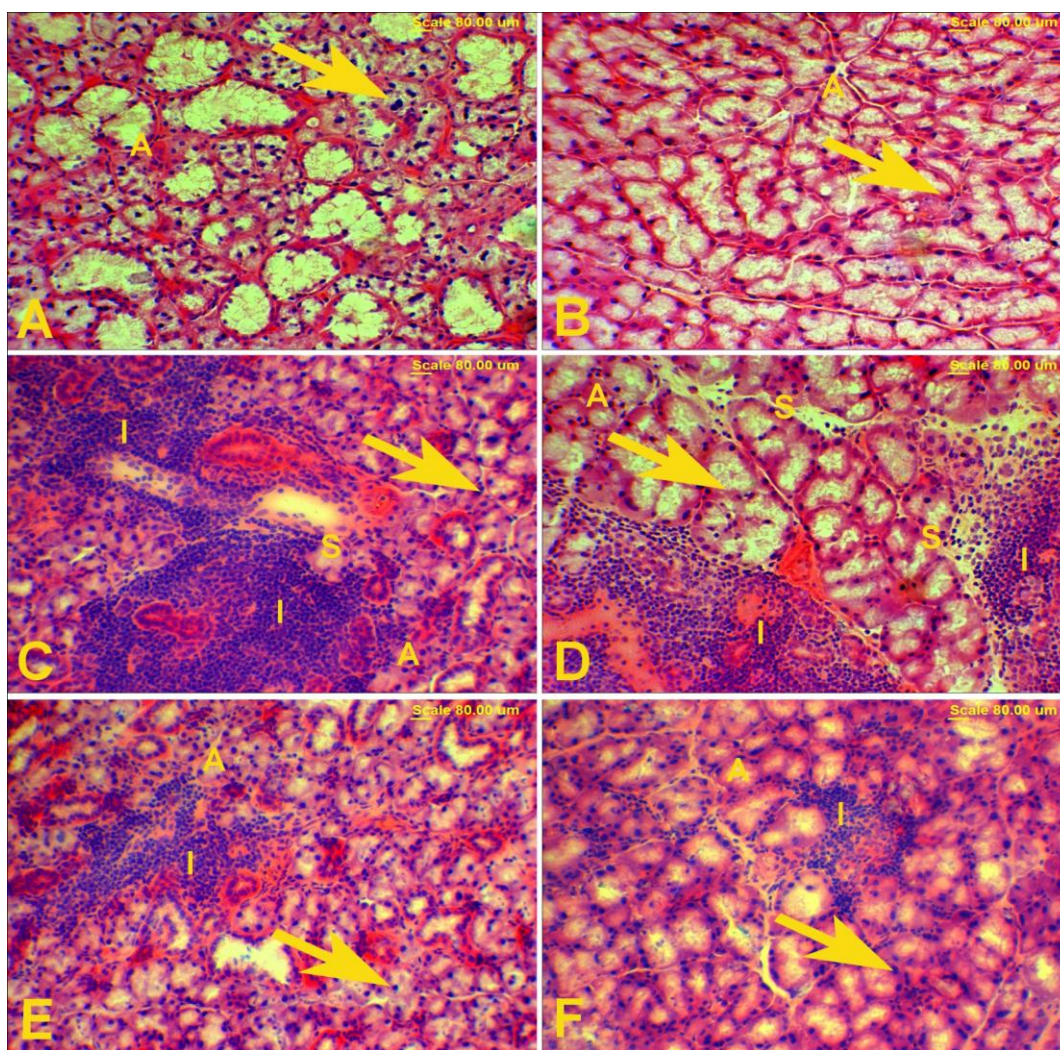


Figure 1. Photomicrography of the submandibular and parotid salivary glands. A, B: In the controls mice, discrete stromal space was observed between the acini (arrow) and seromucous and serous acini (A). C, D: In untreated diabetic mice, the stromal space (S) was affected (A) the presence of inflammatory infiltrate (I) and acinar nuclei (arrow). E, F: In the treated diabetic mice, seromucous and serous acini were observed (A), also acinar nuclei (arrow) and reduced inflammatory infiltrate (I).

Immunofluorescence

Submandibular Gland

The expression of the INS-R in the submandibular glands of the control animals (group I) presented in an intense and uniform way near the salivary ducts ([Figure 2A](#) and [Table 3](#)). In the untreated diabetic animals (group II) the expression of INS-R was mild ([Figure 2C](#) and [Table 3](#)), while in the animals of the treated groups (group III) the expression of INS-R was intense, similar to that observed in the animals of group I ([Figure 2E](#) and [Table 3](#)).

Parotid Gland

The expression of INS-R was shown to be intense and uniform in the parotid glands of control animals (group I), especially close to the salivary ducts ([Figure 2B](#) and [Table 3](#)). In untreated diabetic animals (group II) the expression of these receptors was mild ([Figure 2D](#), [Table 3](#)). Similar to that observed in the control animals, the expression of INS-R was intense in the parotid glands of treated diabetic animals (group III) ([Figure 2F](#) and [Table 3](#)).

Table 3. Expression of insulin receptors (INS-R) in the salivary glands in the different groups studied.

	Submandibular	Parotid
Group I (Balb/c Healthy)	+++	+++
Group II (Untrated NOD)	+	+
Group III (CD4 and CD8 NOD)	+++	+++

Imunmarcation: + Mild, ++ Moderate, +++ Intense.

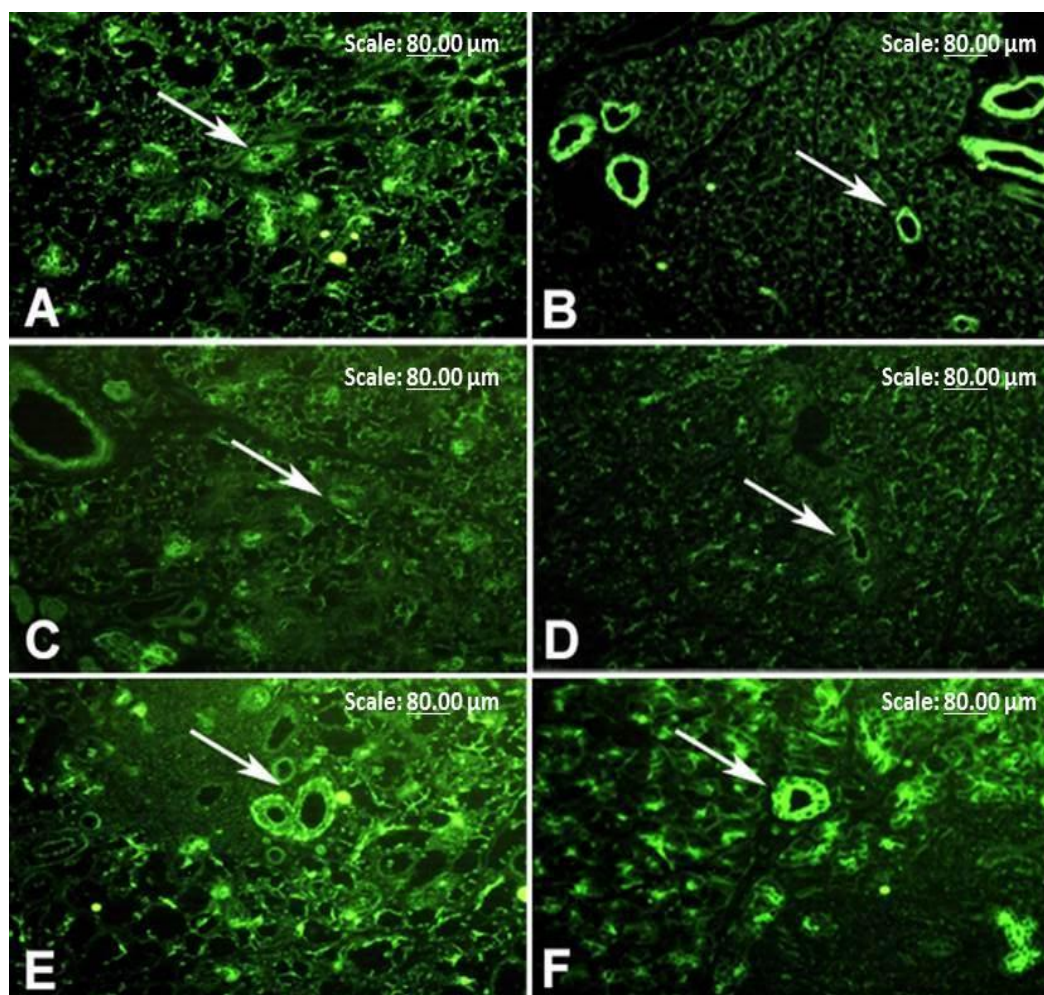


Figure 2. Photomicrography of INS-R immunostaining in the submandibular glands (A, C, E,) and parotid gland (B, D, F). A, B: In the submandibular and parotid glands of the healthy mice, intense labeling of INS-R (Seta) was noted. E, F: In the diabetic mice treated anti-CD4 and CD8, marked marking of these receptors was observed. H.E. (40x)

Discussion

After the trial period and results were obtained, the effects of diabetes and the action of anti-CD4 and anti-CD8 treatment on body metabolism, animal salivary tissues and insulin receptors could be observed. Regarding metabolic control, the animals were considered healthy when the glycemia was below 180 mg/dL. When glucose levels increased from 300 mg/dL, the animals were considered to be

diabetic, similar to those reported in the literature.

Diabetes mellitus occurs as a consequence of the reduction of beta cell mass in the pancreatic islets, which are responsible for the production of endogenous insulin, which in turn is responsible for the uptake of glucose by the cells and maintenance of glycemic homeostasis [20,21].

All of these processes described above also occur in NOD animals. Therefore, they are considered models for the study of type 1 diabetes mellitus. It is also important to note that in relation to the glandular organs, especially in relation to the salivary tissues, the females of these rodents present a greater amount of acini in their glandular epithelium. In addition, some authors point out that in females, the incidence of this disease is around 60 to 95% in this lineage, demonstrating the importance of this lineage when studying diabetes and glandular tissues [22-24].

In continuity with these characteristics, the persistent hyperglycemic state, as it is found in these animals, is considered a disease and promotes structural and morphological changes in the glandular organs, including in these salivary acini, as observed in the present study, mainly in the animals of groups II. Among the tissue changes observed, decreased cytoplasmic and nuclear volume of acinar cells, and the presence of inflammatory infiltrates. In the inflammatory processes, a greater number of neutrophils were observed in the animals with the associated treatments, possibly demonstrating an attempt to recover this tissue, as already reported in other studies, including this group of researchers, which related the presence of these cells with a possible functional tissue recovery. These alterations may also be related to changes in the activities of these organs, among them, the decrease of saliva secretion and its components. These changes occur both in the submandibular salivary glands and in the parotid glands. Apparently, these alterations, although similar in both glands, have different intensities in relation to the tissue responses [9,20,25-27].

The use of immunotherapies in an attempt to reverse this disease has also been advocated in the scientific literature. In this regard, the use of anti-CD4 / CD8 monoclonal antibodies has been tested in experimental studies with satisfactory results, since they are able to block the infiltration of CD4 and CD8 T lymphocytes in different tissues, and especially in pancreatic beta cells. Waldmann studies demonstrated that anti-CD4 and anti-CD8 together with splenocytes derived from donors, induced tolerance in allograft models, showing efficacy of this therapy. Another work tested tolerance induced by CD4 and CD8 antibodies demonstrating the efficacy of the therapy in inducing beta cell tolerance in diabetic NOD mice [18,19,28-30]. Importantly, despite these positive results, most of these studies still address the isolated use of these treatments.

Conclusion

The hyperglycemic condition promoted alterations in the general metabolism as well as structural alterations in both the parotid glands and submandibular glands. The expression of INS-R was also altered by this hyperglycemic condition. However, treatment with anti-CD4 and CD8 promotes a decrease in glycemic indexes and mainly of the glandular structure in both the parotid glands and the submandibular glands. It is important to highlight the recovery in the expression of INS-R in these tissues and the decrease of the inflammatory processes. Thus, the immunotherapy used was effective in the recovery of these glandular organs and may be a therapeutic alternative to revert the tissue damages caused by this disease.

Acknowledgements. FAPESP, CAPES, CNPq and NAPED/FMJ.

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