https://doi.org/10.46389/rjd-2021-1037
www.rjdnmd.org

Rom J Diabetes Nutr Metab Dis
2021; volume 28, issue 3, pages 242-254

Original Research

Combine treatment with N-acetylcysteine, anti-CD4/CD8 antibodies and physical exercise reduces histopathological damage in salivary glands of spontaneously diabetic mice

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Received: 23 May 2021 / Accepted: 5 July 2021

Abstract

Background and aims: The reactive oxygen species (ROS) shows intensification of tissue damage caused by type I diabetes mellitus, and N-acetylcysteine (NAC) can modulate this process by reducing its action. However, in diabetics, NAC seems to potentiate these damages because it stimulates a high infiltration of CD4 and CD8 T lymphocytes, which leads to an important therapeutic association with anti-CD4 / CD8 antibodies. Besides, physical exercise can complement this treatment by stimulating the body's natural antioxidant defense. The purpose of this study was to analyze the effects of a combined treatment with NAC, anti-CD4 / CD8, and physical exercise on the reduction of damage in the salivary tissues on NOD mice. **Material and methods:** Female mice were divided into 5 groups with 6 animals each, composed by: Balb/c; NOD; NOD + Ex; NOD + NAC + CDs NOD + NAC + CDs + Ex. The NAC was applied intraperitoneally for 21 days at a dosage of 50mg/kg. The anti-CD4/CD8 were applied intravenously at a dosage of 25 µg/ml on days 0, 7, 14, and 21. During the experimental period, the animals of the exercise groups were subjected daily to 30 minutes of swimming exercise. After 21 days, the tissues were assessed by light microscopy analysis, stereological and immunofluorescence techniques for verification of INS-R insulin receptors. **Results:** For animals in the NOD + Ex groups; NOD + NAC + CDs; NOD + NAC + CDs + Ex, it was noticed a decrease in lymphocytes and inflammatory processes, as well as a significant increase in the relative neutrophils numbers. There was also a statistically significant difference in the increase of nuclear, and cytoplasmic volume, and an intense INR-S marking when compared to the NOD group without treatment. **Conclusion:** The therapy used, when associated with NAC to anti-CD4/CD8 promoted the recovery of glandular architecture and INS-R, along with benefit in the reduction of inflammatory processes. It was also found that physical exercise potentiated the therapeutic action of the drug in the tissue restructuring of the salivary glands.

Keywords: diabetes mellitus type I; oxidative stress; N-acetylcysteine; physical exercise.

Background and aims

Hyperglycemia, characteristic of type I diabetes mellitus, can lead to an increase in the production of reactive oxygen species (ROS). These oxidants, the final products of a small part of O2, among them the superoxide anion radical, hydrogen peroxide, and hydroxyl radicals are aggressive and can further potentiate cell damage [1].

Thereby, along with the hyperglycemia, the ROS promote the activation of PK-C isoforms (C kinase protein) and the increase in the formation of glycosylated derivatives that stimulate a rise in the flow of glucose at the aldose-reductase pathway, which results in a sorbitol accumulation, also toxic to tissues [2, 3]. For this reason, studies have tried to evaluate the effects of antioxidants in addition to other therapies for the prevention and treatment of these complications [1, 3–6]. In this regard, physical exercise in patients with diabetes can promote several benefits to the whole organism, such as heart rate regulation, improved lipid profile, increased insulin sensitivity, decreased insulin replacement, and obesity, in addition to vascular dysfunction decrease. It could also be observed that physical exercise can influence cell homeostasis, balancing the oxidative attack, and improving the antioxidant defense mechanism [7–9].

Besides physical exercise, there are antioxidant agents that could promote recovery from damage caused by diabetes. Among these are NAC (N-acetylcysteine), which is considered a potent antioxidant agent. Its action refers to the capacity of stimulating the synthesis of reduced glutathione, one of the enzymes responsible for the antioxidant defense system [10, 11]. However, the studies observed that despite NAC presenting beneficial effects, their protective effects on beta-pancreatic cells it is still not exemplified.

The immune system is very sensitive to cysteine action, which is a compound of NAC. With this, the high intracellular amount of cysteine benefits higher infiltration of T cells in the tissues, leading to the progression of the disease [12]. However, studies that have been exploring the specifics antibodies, anti-CD4 / CD8, have been obtained success in blocking the infiltration of CD4 and CD8 T lymphocytes in beta-pancreatic cells of non-obese diabetic animals [13, 14]. Accordingly, would be possible to associate NAC and these two therapeutic agents, in an attempt to block the infiltration of T lymphocytes, promoted by NAC, allowing only their antioxidant activity. Therefore, diabetes is related to the oxidative stress process and, low-intensity physical exercise can assist in the adaptation of antioxidant enzymes [10, 15–17]. Also, NAC can be an antioxidant and a potential therapeutic agent when associated with CD4 and CD8 antibodies, that can improve insulin sensitivity and modulate the production of free radicals [10, 18, 19].

Thus, in salivary glands, which have morphologically structure similar to the pancreas and possibly affected in the same way by the hyperglycemic condition, the use of the antioxidants could decrease oxidative stress and inflammation, likewise recovering the morphology of salivary tissues. Thereby, the aim of this study was to verify the effects of a combined treatment with NAC, anti-CD4/CD8, and physical exercise jointly on the reduction of damage in the salivary tissues of non-obese diabetic mice (NOD).

Material and method

Animals and experimental conditions

In the present study, 30 female mice were initially used, divided equally and randomly into 5 groups: healthy controls (*Balb/c*); diabetic controls (*NOD*); Diabetic NOD + Physical Exercise (NOD + Ex); Diabetic NOD treated with Nac + Anti-CD4/CD8 (NOD + NAC + CDs); Diabetic NOD treated with Nac + Anti-CD4/CD8 + Physical Exercise (NOD + NAC + CDs + Ex).

All animals were 15 weeks aged and weighing 24 g on average. Maintained in a standardized manner regarding the environment, food, and treatment according to the norms about Animal Experimentation of the Laboratory. The work was approved by the ethics committee of the Institute (CEUA / FMJ process N °: 342/11).

The weekly monitoring of all animals was done to assess blood glucose levels (mg/dl). The animals that presented glycemic values above

300 mg/dl were considered diabetic [20]. The blood of these animals was collected and analyzed in the Accu-Chek Performa device (Roche, NY, USA). Blood samples were obtained by cardiac puncture for metabolic analysis. After confirmation of diabetes using the method described above, animals receive 50 mg/kg of antioxidant NAC daily intraperitoneally for 21 days [21]. The NOD + Ex; NOD + NAC + CDs and NOD + NAC + CDs + Ex animals were submitted to an exercise program (swimming) during 30 minutes, performed 5 times a week for a period of 21 days in total [22]. Associated with the treatment, the group of NOD + NAC + CDs and NOD + NAC + CDs + Ex received a dose of 25 µg of anti-CD4 / CD8 every 7 days by intravenous route (respectively on days: 0, 7, 14 and 21), as settled in previous studies [23].

After the treatment period, the animals of all groups were submitted to the anesthetic Ketamine (130 mg/kg) and Xylazine (6.8 mg/kg) (1:1) and the salivary gland samples were collected. Then the animals were sacrificed with an additional anesthetic procedure (according to the principles of experimentation on animals-COBEA/Concea). The samples obtained were submitted to stereological analysis.

Light microscopy and stereology

The samples of the parotid and submandibular salivary glands were fixed in Bouin's Solution (Picric Acid Solution), with subsequent inclusion in plastic resin (Paraplast Plus, Oxford Lab, USA) and stained with hematoxylin and eosin (HE) [24, 25]. The acinar cells volume, such as nuclear and cytoplasmic of the parotid and submandibular glands, were measured using slides for light microscopy. These volumes were obtained after analysis of 50 cells of each animal totaling 300 acini per experimental group by the method of counting points similar to what Weibel describes (1979) [26]. For this current study, were considered intact and circular or ellipsoid acini nuclei with defined limits. These procedures were performed on 100x plan-achromatic objective at NIKON Eclipse microscope (Nikon, Japan) coupled to the SD-3.3 CCD image acquisition system of the University's Department of Morphology and Basic Pathology.

Immunofluorescence

For immunofluorescence, the samples from the salivary glands and frozen sections were post-fixed using alcohol and acetone (1: 1) at 40°C for 3 minutes and followed by 4% paraformaldehyde for 10 minutes, then the sections were washed in buffered saline (PBS). In due course, all sections were subjected to a 3% hydrogen peroxide blocking solution in water, and later they were placed in a second blocker for the nonspecific protein-protein binding sites with bovine serum albumin in PBS buffer, at room temperature for 1 hour. For the labeling of insulin receptors, the samples were incubated in a specific primary antibody (alpha INS-R, Santa Cruz, CA, USA), diluted in blocking solution (1:250), and applied over the samples for 12 hours at 4°C. Following this, the sections were washed in PBS buffer and incubated in secondary fluoresce-conjugated antibody (IgG-FITC, Santa Cruz, CA, USA) diluted in a blocking solution (1:100), due avoiding unspecific markings. The cuts were once again washed with PBS and mounted in 1.4-diazabicyclo [2.2.2] octane (DABCO, for fluorescent microscopy) (Sigma, St. Louis, USA), observed and photographed under the Fluorescence Microscope. To obtain all images, 20× and 40× objectives were used. For the control of negative immunostaining, parts of even samples were not incubated in the primary antibody.

Statistical analysis

The data normality was verified using the Shapiro-Wilk test. The analysis of variations in glucose concentrations, volume of the glandular acini nucleus, volume of the glandular acini cytoplasm, and the number of intergroup inflammatory cells was performed using the one-way ANOVA, and when relevant, the Tukey test was used as a post-test. To the accuracy of the significance between the comparisons of the main effects, as well as those of the post-test, a value

https://doi.org/10.46389/rjd-2021-1037

of p≤0.05 was adopted. All cited tests were performed using the Prism 8.0.1 software.

Results

When compared to the Balb/c group, the ANOVA test showed that all other groups presented high glucose levels [F (4, 11) = 147.0; p=0.0001]. However, in both groups, NOD + Ex and NOD + NAC + CDs was found that the treatment demonstrated efficiency in reducing the values, although it does not return to the Balb/c group values. Besides, the association between treatment and physical exercise at the NOD + NAC + CDs group showed superior efficiency in reducing blood glucose when compared with the treatment alone (Table 1).

In mice of the Balb/c group, no inflammatory infiltrates were found. In opposition, there was an inflammatory infiltrate found at the salivary glands of the NOD group, in both the submandibular and parotid glands, presenting a relative number of lymphocytes and macrophages significantly higher than in the control and treated groups (Figure 1A–E, respectively). In the NOD + Ex, NOD + NAC + CDs, and NOD + NAC + CDs + Ex groups it is notice a decrease in lymphocytes (Figure 1A, D) [F (3, 8) = 12.36; p=0.0023] and) [F (3, 8) = 27.10; p=0.0002], respectively, and inflammatory processes in submandibular glands (macrophages, Figure 1B) [F (3, 10) = 4.596; p=0.0286], as well as a significant increase in the relative number of neutrophils (Figure 1C, F) [F (3, 8) = 8.869; p=0.0063] and [F (3, 10) = 13.01; p=0.0009], respectively.

The seromucous acini with mucous columnar cells and basal nuclei were noted in Balb/c group when observed by light microscopy and stereological analysis of the submandibular glands. A slight intercellular space was observed in the acini, salivary ducts (Figure 2A). In NOD mice involuted cells and a space increase between the acini were identified, also the nuclei were located in the basal region (Figure 2C). About the NOD + Ex, NOD + NAC + CDs and NOD + NAC + CDs + Ex group, involuted acini were noticed compared with the Balb/c group, however, related to the NOD mice the acini were significantly recovered (Figure 2E, G, I).

Regarding the parotids gland, in the Balb/c group, serous acini with columnar cells with pyramidal shape were observed. The basophilic cytoplasm and the basal nucleus were detected. Through the acini, a slight stromal space and salivary ducts were seen (Figure 2B). In NOD mice, cells presented an increase in the interacinar space and involution. The nuclei were located in the basal region (Figure 2D). In the NOD + Ex, NOD + NAC + CDs and NOD + NAC + CDs + Ex group, even though there were involuted serous acini, it recovered in relation to NOD animals (Figure 2F, H, J).

Nuclear and cytoplasmic volumes in both submandibular and parotid glands of the Balb / c group have presented in normal standards meanwhile, in NOD animals, these volumes were unevolved comparing to the Balb / c group [F (4, 10) = 508.7; p=<0.0001] and [F (4, 10) = 14.01; p=<0.0004] to nuclear volume of submandibular and parotid glands, and [F (4, 10) = 26.14; p=<0.0001] and [F (4, 10) = 16.36; p=<0.0002] to cytoplasmic volume of submandibular and parotid glands. However, animals of the NOD + Ex, NOD + NAC + CDs, and NOD + NAC + CDs + Ex group have demonstrated significant recovery at nuclear and cytoplasmic volumes when compared to the control group without NOD treatment (Figure 3).

Table 1: Fasting glycemic values.

	Balb/C (n=6)	NOD (n= 6)	NOD+Ex (n=6)	NOD+NAC+ CDs (n=6)	NOD+NAC+ CDs+Ex (n=6)
Glucose (mg/dl)	143.5±21.8	605.25±31.2[a]	450.3±16.7[ab]	515.6±39.3[ab]	417.7±14.4[abd]

Values expressed as the mean ± standard deviation. The letters show the differences between the groups with a minimum significance of p<0.05. [a]Different from the Balb/c group. [b]Different from the NOD group. [d]Different from the NOD + NAC + CDs group.

Related to immunofluorescence analysis, as in the submandibular and parotid glands, the expression of the insulin receptors (INS-R) from the Balb/c group was intensely and uniformly observed close to salivary ducts (Figure 4A, B). In NOD mice the INS-R expression was mild (Figure 4C, D and Table 2), and there was also an increase of INS-R expression at the NOD + Ex group (Figure 4E, F and Table 2), whereas, in animals from NOD + Ex+ NOD + NAC + CDs and NOD + NAC + CDs +

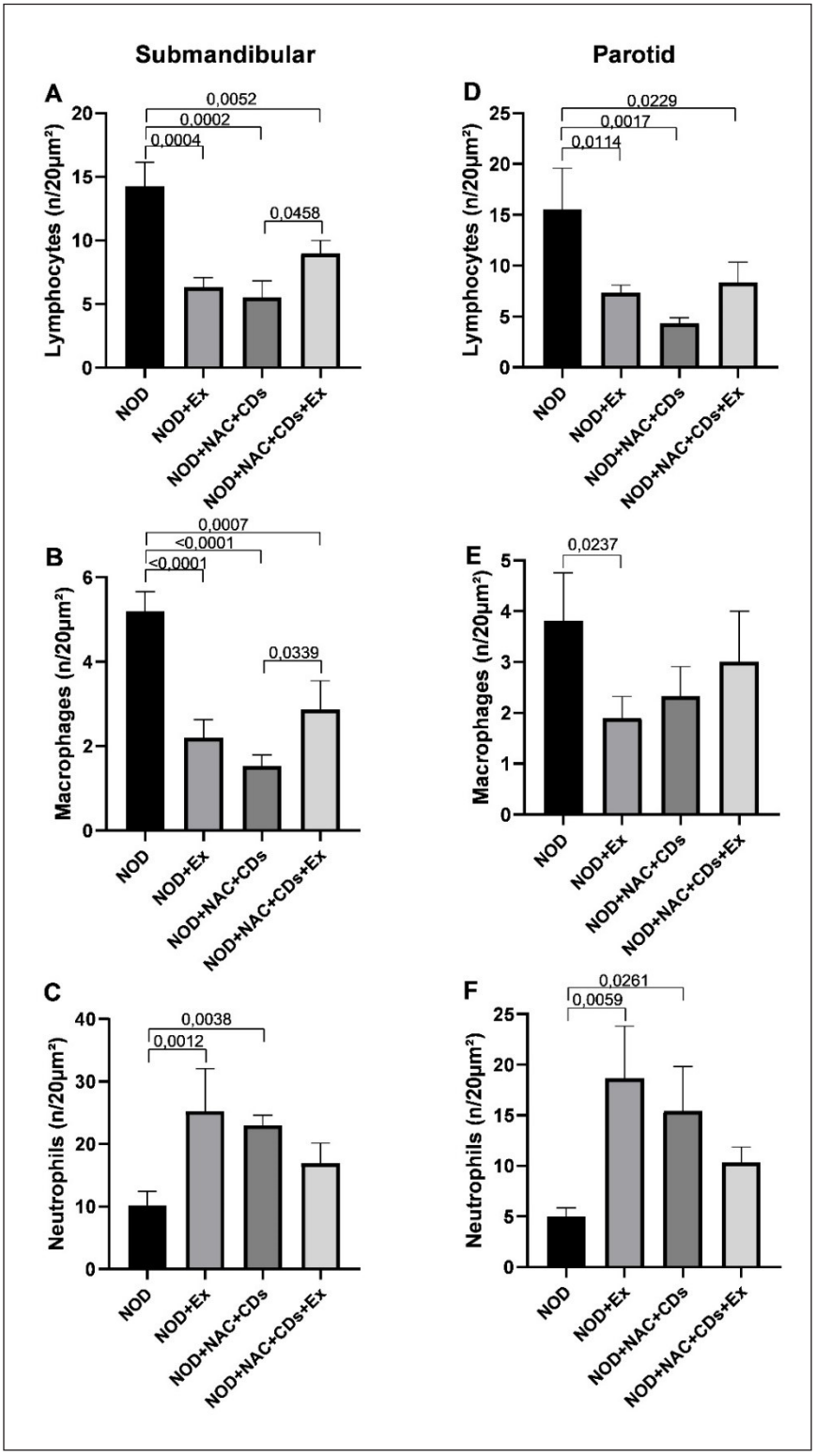

Figure 1: Expressed values as the mean ± standard deviation. The control group omitted because there are no inflammatory infiltrates. The p value of the pairwise comparisons are above brackets.

https://doi.org/10.46389/rjd-2021-1037

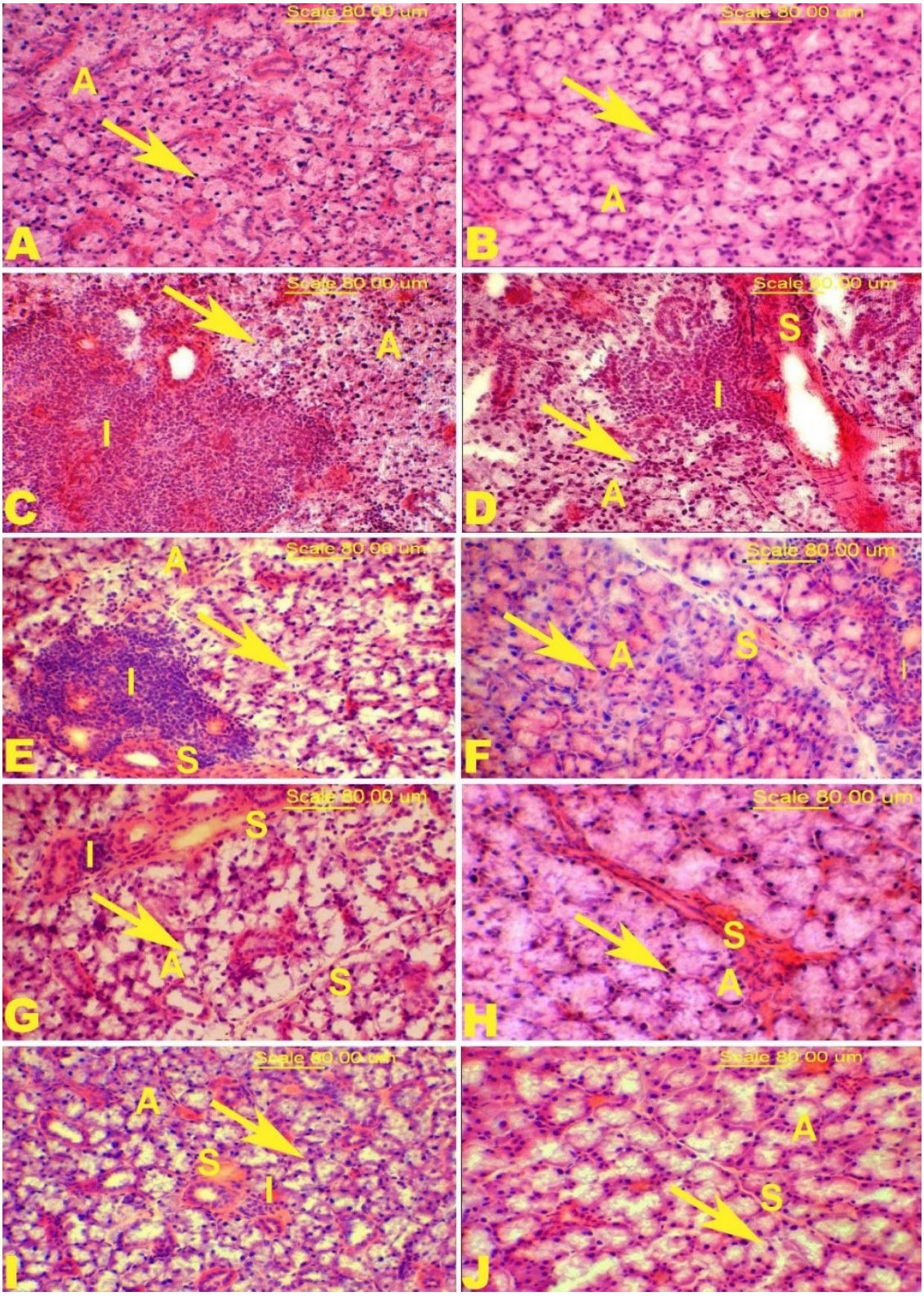

Figure 2: Photomicrograph of the submandibular salivary gland (left column) and parotid (right column). A and B: In the Balb / c mice, a slight stromal space was observed between the acini (arrow). Normal serous acoustics (A) and normal nuclei (arrow) in these animals. C and D: In the NOD mice, the presence of major stromal space (S) was observed. Inflammatory infiltrates (I), acini (A), and involuted nuclei (arrow). E, F, G, H, I and J: In mice from NOD + PE groups; NOD + NAC + CDs and NOD + NAC + CDs + Ex, there was a reduction of the inflammatory infiltrate (I), the stromal space between the acini (S) and significant recovery of the acini (A) and nuclei (arrow).

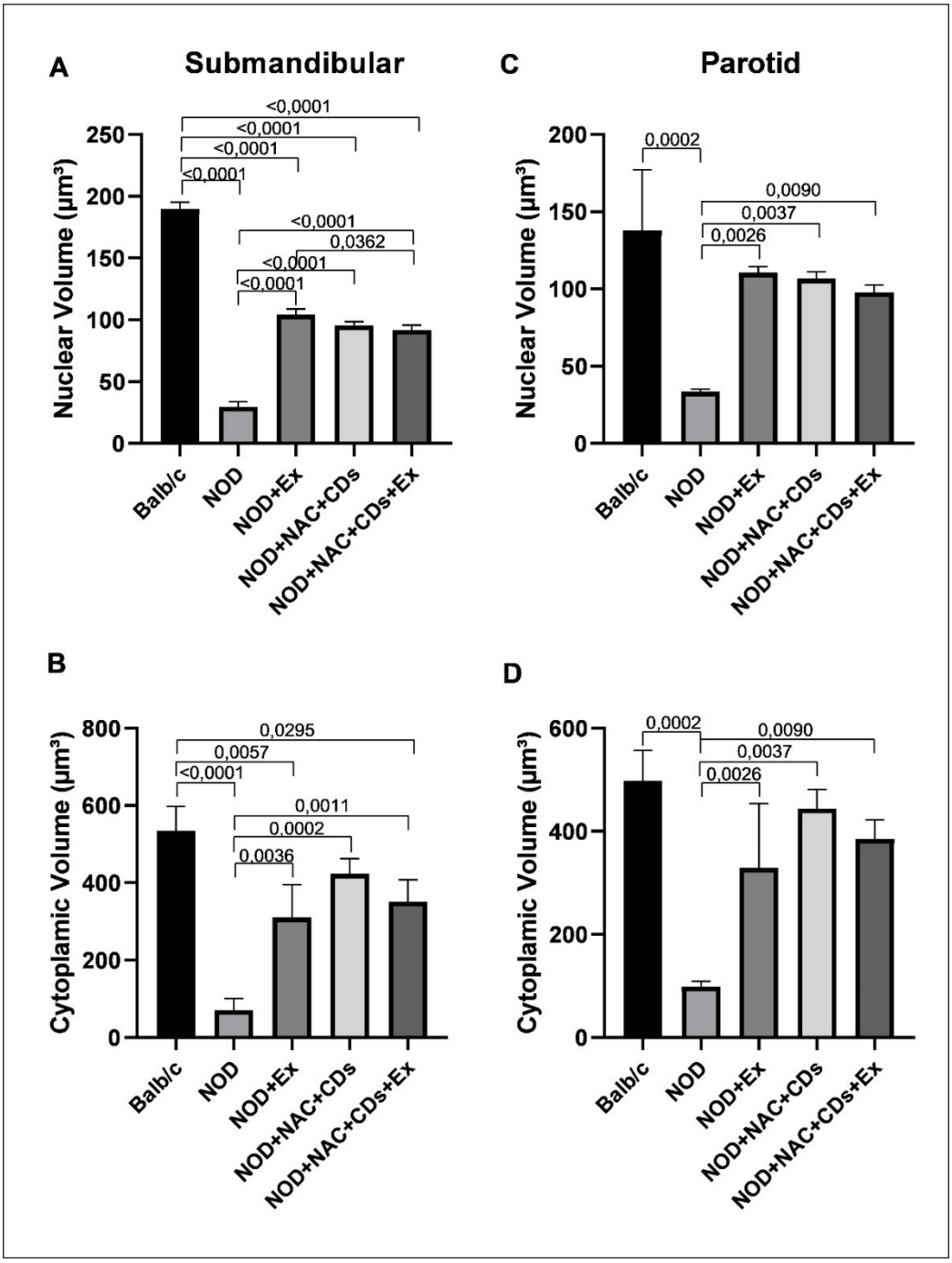

Figure 3: Expressed values as mean ± standard deviation. The p value of the pairwise comparisons are above brackets.

Ex group, the INS-R expression was intense, similar to the one detected at the animals of the Balb/c group (Figure 4G, H, I, J and Table 2).

Discussion

This study intended to analyze the effects of the combined treatment with NAC, anti-CD4/CD8 plus physical exercise for reducing the damage of salivary tissues in female mice. The main findings of this study were: I. The glucose concentrations of the animals in the treated groups (NOD + Ex, NOD + NAC + CD and NOD + NAC + CDs + Ex had a significant reduction when compared to the group without NOD treatment, but with no returning to the normal values of the Balb/c group; II. Significant reduction of lymphocytes and for the inflammatory process, as well as an important elevation in the relative number

https://doi.org/10.46389/rjd-2021-1037

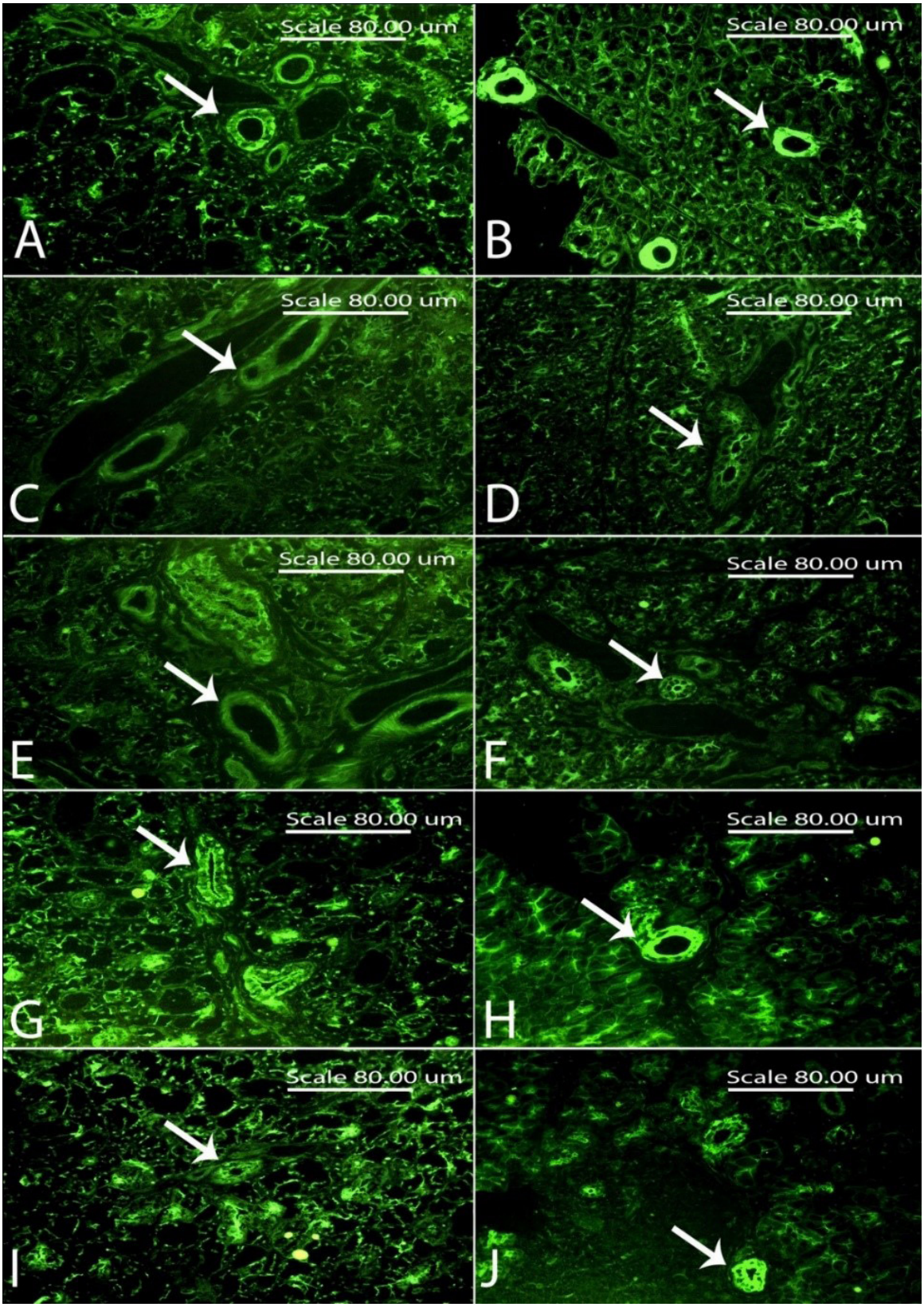

Figure 4: Photomicrograph of INS-R immunostaining in the submandibular (left spine) and parotid (right spine) glands. A, B: In the Submandibular and parotid glands of Balb / c mice, intense INS-R marking was noted (Arrow). C, D: In the NOD mice, a slight marking of these receptors was observed (Arrow) E, F: In the NOD + Ex mice, there was also a slight recovery of the INS-R marking (Arrow). G, H, I, J: In the NOD + NAC + CDs and NOD + NAC + CDs + Ex mice, intense marking of these receptors was noted. H.E. (40×).

Table 2: Expression of insulin receptors (INS-R) in the salivary glands from different studied groups.

	Submandibular	Parotid
Balb/C	+++	+++
NOD	+	+
NOD+ Ex	++	++
NOD+ NAC+ CDs	+++	+++
NOD NAC+ CDs + Ex	+++	+++

Immunostaining: + Light; ++ Moderate; +++ Intense.

of neutrophils at the treated groups, when compared to the NOD group; and III. The tissue restructuring of the submandibular and parotid glands has presented a significant statistical difference in the increase of nuclear and cytoplasmic volume and an intense INR-S marking when compared to the NOD group without treatment.

The high concentration of glucose in the blood due to the decrease in insulin in diabetic animals alters the metabolism, causing an increase in food consumption, consequently growing the feeling of thirst and excretion in the urine. Regarding the insensitive animals, glucose levels are 180 mg/dl when fasting. In diabetic animals, on the other hand, these levels reach or exceed 300 mg/dl, thus showing or effectively the diabetic state, according to related studies in the literature [20, 27]. This condition is established due to the destruction of the pancreatic beta cells, which are responsible for insulin production, a hormone that allows glucose absorption and glycemic control [20, 27]. Current studies have been demonstrated that physical exercise, as well as the use of NAC and treatment with monoclonal anti-CD4/CD8 in isolation, can preserve the function of pancreatic beta cells, also improving insulin secretion and consequently promoting glycemic control of type I diabetes [7, 8, 14, 21].

The literature findings corroborate with this study, for it was possible to observe a reduction in the glycemic indexes of the treated groups when compared to those of the NOD group without treatment, despite not maintaining similar levels to the control group Balb/c. It is important to highlight that NOD + NAC + CDs + Ex group had demonstrated lower glycemic levels when compared to NOD. This fact may be directly linked with the effects of NAC and CD4/CD8 antibodies in preserving the pancreatic tissues, along with positive physiological adaptations that physical exercise generally promotes in the metabolism at the type I diabetes organism [28–32].

Related to salivary glands, the NOD + Ex, NOD + NAC + CDs, NOD + NAC + CDs + Ex groups have shown a significant reduction in lymphocytes and inflammatory process. Also, a significant increase in the relative number of neutrophils, with an important nuclear recovery, cytoplasmic volume, and INR-S receptors when compared to the NOD group without treatment. In the literature, there are several effects of diabetes on the salivary glands. Studies have demonstrated the accumulation of lipid droplets that are characteristic of tissue damage processes and saliva. These morphological changes are more significant in the epithelium of the submandibular gland than in the parotid gland in mice with hyperglycemia [20, 28–30, 32–37].

In this context, the tissue recovery of the submandibular and parotid glands, presented in this study may be related to some aspects of the chosen therapy. Some research has shown that NAC could be a potent antioxidant and anti-inflammatory agent acting in hyperglycemic conditions, rebalancing the antioxidant enzymes, reducing inflammation, and recovering specific tissues [11, 21]. One study investigated the effect of NAC on the nicotinamide adenine dinucleotide phosphate oxidase expression (NADPH), antioxidant enzymes, and inflammatory markers for diabetic rats. The authors have found a satisfactory response for NAC which shows a protective and

 https://doi.org/10.46389/rjd-2021-1037

antioxidant effect in cardiac tissue [18]. Another study evaluated its action in reducing oxidative tissue damage from the liver and kidneys of diabetic rats, furthermore, they have noted a significant decrease in lipid peroxidation, especially in renal tissue [10]. However, it is important to indicate that these studies were made, in its majority, using type II diabetic animals or chemically induced.

On another hand, for type I diabetes, NAC seems to stimulate the disease, potentiating damage, as it contains a cysteine compound, which leads the immune system to major production of T lymphocytes [38]. In a research, a model for transferring explenocytes from NOD mice to NOD Scid mice was used to induce diabetes, using a NAC as a treatment. After two weeks of monitoring the treatment, an increase in the infiltration of T/CD4 and CD8 lymphocytes was observed, forward accelerating the autoimmune process in this animal model [38].

Consequently, in order to obtain antioxidant activity of NAC, an association with T/CD4 and CD8 lymphocyte blockers was necessary, as some studies have shown that the administration of specific anti-CD4 / CD8 antibodies was efficient in reducing the infiltration of T lymphocytes into pancreatic beta cells of non-obese diabetic animals [13, 14, 39, 40]. This demonstrates that an association between these two therapeutic agents is possible since the inhibitory action of anti-CD4 / CD8 in specific lymphocytes allowed the antioxidant action of NAC causing a decrease in inflammation and re-establishing the cell tissue structure.

Also, the animals in the NOD+Ex and NOD+NAC+CDs+Ex groups have shown significant recovery when compared to the animals in the NOD group. Several chemical reactions occur during the physical exercise causing EROs modulation. However, to protect tissues from possible damage caused by oxidizing elements during physical exercise, antioxidant enzymes such as SOD (superoxide dismutases), CAT (catalase) and GPX / GR (glutathione peroxidase) seem to respond in an adaptive manner, increasing their activity in tissues and organs of trained animals and humans. This reaction mainly appears in aerobic exercises of long duration and low intensity [3, 5, 11, 15–19], which can promote the protection and better tissue adaptation against these aggressive agents.

In addition to these beneficial effects described, a positive modulation was found in the reduction of lipid peroxidation after the exercise. This lipid peroxidation, when not modulated, could occur in cell membranes and possibly sake of biological damage promoted by free radicals released after training, knowing that practically all biomolecules are susceptible to this oxidation [17]. However, this increase in lipid peroxidation seems to be tissue-specific, showing some organ immunity to this process even after physical activity. A reduction in lipid peroxidation in kidneys and salivary glands was observed in running-trained mice compared to sedentary mice [17, 41].

Positive immunological effects of physical exercise have also been detected. Physical exercise can modify the state of organic homeostasis, leading to the reorganization of several system's response, including the immune system. Neutrophils, and other types of leukocytes as well, could be quickly released by stimulation, such as adrenaline rush or physical exercise [16], which may explain the expressive increase of these cells in salivary tissues of the NOD+Ex group. In this study, it was possible to notice the physical exercise enhanced as a therapeutic action in the tissue restructuring of salivary glands.

Nevertheless, this study has some limitations. The number of animal quantity per group, however, the statistics have shown significance in the comparisons made, even with a low number of animals. That suggests an increase in the number of animals per group could lead us to similar results, contributing negatively to the 3Rs rule [42]. To evaluate the effects of different treatments was beyond the purposes of this study, which focused only at evaluate the effect of treatment with the mentioned compounds, associated with exercise. Therefore, it is recommended that future studies could consider this assessment to verify the impact of different lengths of time treatment, as well as the doses of the medications used.

Conclusion

In conclusion, these results suggest that the therapy used, especially when associated NAC with anti-CD4/CD8, could promote recovery of glandular architecture and INS-R, besides the assistance to reduce inflammatory processes. Furthermore, physical exercise, due to its ability to modulate the general homeostasis of the organism, could also be an important ally to drug therapy. Therefore, it is implied that this treatment could act, initially blocking the inflammatory infiltrate in type I diabetes through CD4/CD8 antibodies and then allowing the NAC and physical exercise antioxidant action for the recovery of glandular homeostasis.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

We would like to acknowledge to the Coordination for the Improvement of Higher Education Personnel (CAPES) of the Brazilian, Government and the São Paulo State Research Support Foundation (FAPESP) and Graduate Nucleus of the Jundiaí School of Medicine (NAPED/FMJ). To the team members from the Laboratory of Tissue Morphology, Department of Morphology and Basic Pathology, Faculty of Medicine of Jundiaí.

Author contributions

Conception or design: R.O.R.F.N., E.J.C. Acquisition, analysis, or interpretation of data: R.O.R.F.N., E.G.M., M.J.A.B., L.O.C., R.D.M., R.E.S., V.A.R.F., R.F., R.S.B. Drafting the work or revising: R.F., D.C.C.B., R.O.R.F.N. Final approval of the manuscript: R.O.R.F.N., E.J.C.

References

1. Asmat, U., Abad, K., Ismail, K. (2016). Diabetes mellitus and oxidative stress—A concise review. *Saudi Pharm J* [Internet]. 24(5):547–553. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1319016415000766.
2. Oberley, L. W. (1988). Free radicals and diabetes. *Free Radic Biol Med* [Internet]. 5(2):113–124. Available from: https://linkinghub.elsevier.com/retrieve/pii/0891584988900366.
3. Phaniendra, A., Jestadi, D. B., Periyasamy, L. (2015). Free radicals: Properties, sources, targets, and their implication in various diseases. *Indian J Clin Biochem* [Internet]. 30(1):11–26. Available from: http://link.springer.com/10.1007/s12291-014-0446-0.
4. Jomova, K., Valko, M. (2011). Advances in metal-induced oxidative stress and human disease. *Toxicology* [Internet]. 283(2–3):65–87. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0300483X11000886.
5. Sailaja Rao, P., Kalva, S., Yerramilli, A., Mamidi, S. (2011). Free radicals and tissue damage: Role of antioxidants. *Free Rad Antioxid.* [Internet]. 1(4):2–7. Available from: http://www.phcogfirst.com/article/725.
6. Rani, V., Singh Yadav, U. C. (2015). Free radicals in human health and disease [Internet]. Rani, V., Yadav, U. C. S., editors. Free Radicals in Human Health and Disease. New Delhi: Springer India, pp. 1–430. Available from: http://link.springer.com/10.1007/978-81-322-2035-0.
7. Galassetti, P., Riddell, M. C. (2013). Exercise and Type 1 Diabetes (T1DM). In: Comprehensive Physiology [Internet]. Hoboken, NJ, USA: John Wiley & Sons, Inc.; pp. 1309–1336. Available from: http://doi.wiley.com/10.1002/cphy.c110040.
8. Fuchsjager-Mayrl, G., Pleiner, J., Wiesinger, G. F., et al. (2002). Exercise training improves vascular endothelial function in patients with type 1 diabetes. *Diabetes Care* [Internet]. 25(10):1795–1801. Available from: http://care.diabetesjournals.org/cgi/doi/10.2337/diacare.25.10.1795.
9. Zisser, H., Gong, P., Kelley, C. M., Seidman, J. S., Riddell, M. C. (2011). Exercise and diabetes. *Int J Clin Pract* [Internet]. 65(1):71–75. Available from: http://doi.wiley.com/10.1111/j.1742-1241.2010.02581.x.
10. Ribeiro, G., Roehrs, M., Bairros, A., et al. (2011). N -acetylcysteine on oxidative damage in diabetic rats. *Drug Chem Toxicol* [Internet]. 34(4):467–474. Available from: http://www.tandfonline.com/doi/full/10.3109/01480545.2011.564179.
11. Kamboj, S. S., Vasishta, R. K., Sandhir, R. (2010). N-acetylcysteine inhibits hyperglycemia-induced oxidative stress and apoptosis markers in diabetic neuropathy. *J Neurochem* [Internet]. 112(1):77–91. Available from: http://doi.wiley.com/10.1111/j.1471-4159.2009.06435.x.
12. Kinscherf, R., Fischbach, T., Mihm, S., et al. (1994). Effect of glutathione depletion and oral N-acetyl-cysteine treatment on CD4 + and CD8 + cells. *FASEB J* [Internet]. 8(6):448–451. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1096/fasebj.8.6.7909525.
13. Netto, R. O. R. F., Moura, E. G., Col, L. O., et al. (2019). CD4/CD8 antibodies reduce histopathological damage in salivary glands of spontaneously diabetic mice. *Rom J Diabetes Nutr Metab Dis.* 26(2).
14. Phillips, J. M., Parish, N. M., Bland, C., Sawyer, Y., De La Peña, H., Cooke, A. (2009). Type 1 diabetes development requires both CD4+ and CD8+ T cells and can be reversed by non-depleting antibodies targeting both T cell populations. *Rev Diabet Stud* [Internet]. 6(2):97–103. Available from: http://www.soc-bdr.org/e4/e887/volRdsVolumes7102/issRdsIssues7148/chpRdsChapters7149/strRdsArticles7742/index_en.html?preview=preview.

 https://doi.org/10.46389/rjd-2021-1037

15. Osorio, R. A. L., Christofani, J. S., D'Almeida, V., Russo, A. K., Piçarro, I. C. (2003). Reactive oxygen species in pregnant rats: effects of exercise and thermal stress. *Comp Biochem Physiol Part C Toxicol Pharmacol* [Internet]. 135(1):89–95. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1532045603000784.
16. Peake, J., Suzuki, K. (2004). Neutrophil activation, antioxidant supplements and exercise-induced oxidative stress. *Exerc Immunol Rev* [Internet]. 10:129–141. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15633591.
17. Reddy Avula, C. P., Fernandes, G. (1999). Modulation of antioxidant enzymes and lipid peroxidation in salivary gland and other tissues in mice by moderate treadmill exercise. *Aging Clin Exp Res* [Internet]. 11(4):246–252. Available from: http://link.springer.com/10.1007/BF03339665.
18. Guo, Z., Xia, Z., Jiang, J., McNeill, J. H. (2007). Downregulation of NADPH oxidase, antioxidant enzymes, and inflammatory markers in the heart of streptozotocin-induced diabetic rats by N -acetyl- l -cysteine. *Am J Physiol Circ Physiol* [Internet]. 292(4):H1728–1736. Available from: https://www.physiology.org/doi/10.1152/ajpheart.01328.2005.
19. Pham-Huy, L. A., He, H., Pham-Huy, C. (2008). Free radicals, antioxidants in disease and health. *Int J Biomed Sci* [Internet]. 4(2):89–96. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23675073.
20. Shirai, H., Sato, T., Hara, T., Minagi, S. (1998). The effect of diabetes mellitus on histopathological changes in the tissues under denture base and without mechanical pressure. *J Oral Rehab*. [Internet]. 25(9):715–720. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1046/j.1365-2842.1998.00301.x.
21. Liu, Y., Zhang, H., Zhang, L., et al. (2007). Antioxidant N-acetylcysteine attenuates the acute liver injury caused by X-ray in mice. *Eur J Pharmacol*. [Internet]. 575(1–3):142–148. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0014299907008035.
22. Evangelista, F. S., Brum, P. C., Krieger, J. E. (2003). Duration-controlled swimming exercise training induces cardiac hypertrophy in mice. *Brazilian J Med Biol Res* [Internet]. 36(12):1751–1759. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0100-879X2003001200018&lng=en&tlng=en.
23. Cui, H., Tucker-Burden, C., Cauffiel, S. M. D., et al. Long-term metabolic control of autoimmune diabetes in spontaneously diabetic nonobese diabetic mice by nonvascularized microencapsulated adult porcine islets. *Transplantation* [Internet]. 88(2):160–169. Available from: http://journals.lww.com/00007890-200907270-00004.
24. Cowden, R. R., Weibel, E. R. (1982). Stereological methods. *Trans Am Microsc Soc* [Internet]. 101(1):115. Available from: https://www.jstor.org/stable/3225581?origin=crossref.
25. Hung, G. W. C. (1984). Stereological methods. Vol. 1: Practical methods for biological morphometry. *Microchem J* [Internet]. 29(3):397–399. Available from: https://linkinghub.elsevier.com/retrieve/pii/0026265X84901243.
26. Weibel, E. R. (1974). Selection of the best method in stereology. *J Microsc* [Internet]. 100(3):261–269. Available from: http://doi.wiley.com/10.1111/j.1365-2818.1974.tb03938.x.
27. Oki, K., Sato, T., Hara, T., Minagi, S. (2002). Histopathological changes in the tissues under a denture base in experimental osteoporosis with a non-pressure covering or bearing continuous pressure. *J Oral Rehab* [Internet]. 29(6):594–603. Available from: http://doi.wiley.com/10.1046/j.1365-2842.2002.00863.x.
28. Cagnon, V. H. A., Camargo, A. M., Rosa, R. M., Fabiani, R., Padovani, C. R., Martinez, F. E. (2000). Ultrastructural study of the ventral lobe of the prostate of mice with streptozotocin induced diabetes (C57BL/6J). *Tissue Cell* [Internet]. 32(4):275–283. Available from: https://linkinghub.elsevier.com/retrieve/pii/S004081660090123X.
29. Maekawa, E. T., Maioral, É. E., Metidieri, H. T., Picardi, P. K., Caldeira, E. J. (2011). Recovery of INS-R and ER-alpha expression in the salivary glands of diabetic mice submitted to hormone replacement therapy. *Arch Oral Biol* [Internet]. 56(10):1129–1136. Available from: https://linkinghub.elsevier.com/retrieve/pii/S000399691100094X.
30. Fávaro, W. J., Padovani, C. R., Cagnon, V. H. A. (2009). Ultrastructural and proliferative features of the ventral lobe of the prostate in non-obese diabetic mice (NOD) following androgen and estrogen replacement associated to insulin therapy. *Tissue Cell* [Internet]. 41(2):119–132. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0040816608000827.
31. Caldeira, E., Cagnon, V. A. (2008). IGF-I and INS receptor expression in the salivary glands of diabetic Nod mice submitted to long-term insulin treatment. *Cell Biol Int* [Internet]. 32(1):16–21. Available from: http://doi.wiley.com/10.1016/j.cellbi.2007.08.005.
32. Da Silva, R. E., De Karla Cervigne, N., Pinto, C. A. L., et al. (2017). Role of GAD peptides p217 and p290 in the repair of INS receptor in salivary tissues of type 1 diabetic mice. *Pak J Pharm Sci*. 30(5):1545–1550.
33. Straka, M. (2011). Oral manifestations of diabetes mellitus and influences of periodontological treatment on diabetes mellitus. *Bratisl Lek Listy* [Internet]. 112(7):416–420. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21744740.
34. Hu, Y., Nakagawa, Y., Purushotham, K. R., Humphreys-Beher, M. G. (1992). Functional changes in salivary glands of autoimmune disease-prone NOD mice. *Am J Physiol Metab* [Internet]. 263(4):E607–E614. Available from: https://www.physiology.org/doi/10.1152/ajpendo.1992.263.4.E607.
35. Kamata, M., Shirakawa, M., Kikuchi, K., Matsuoka, T., Aiyama, S. (2007). Histological analysis of the sublingual gland in rats with Streptozotocin-induced diabetes. *Okajimas Folia Anat Jpn* [Internet]. 84(2):71–76. Available from: http://joi.jlc.jst.go.jp/JST.JSTAGE/ofaj/84.71?from=CrossRef.
36. Caldeira, E. J., Camilli, J. A., Cagnon, V. H. A. (2005). Stereology and ultrastructure of the salivary glands of diabetic Nod mice submitted to long-term insulin treatment. *Anat Rec Part A Discov Mol Cell Evol Biol* [Internet]. 286A(2):930–937. Available from: http://doi.wiley.com/10.1002/ar.a.20236.
37. Metidieri, H. T., Mancio, R. D., Mayoral, É. E., et al. (2012). Effects of anti-CD3 monoclonal antibody in salivary glands of spontaneously diabetic mice. *Microsc Res Tech*.
38. Irie, J., Shimada, A., Oikawa, Y., Shigihara, T., Saruta, T. N-acetyl-cysteine accelerates transfer of diabetes into non-obese diabetic scid mice. *Diabetologia* [Internet]. 47(10):1803–1809. Available from: http://link.springer.com/10.1007/s00125-004-1529-x.
39. Yi, Z., Diz, R., Martin, A. J., et al. (2012). Long-term remission of diabetes in NOD mice is induced by nondepleting anti-CD4 and anti-CD8 antibodies. *Diabetes* [Internet]. 61(11):2871–2880.

Available from: http://diabetes.diabetesjournals.org/cgi/doi/10.2337/db12-0098.

40. Ildstad. S. T., Chilton, P. M., Xu, H., Domenick, M. A., Ray, M. B. (2005). Preconditioning of NOD mice with anti-CD8 mAb and costimulatory blockade enhances chimerism and tolerance and prevents diabetes, while depletion of αβ-TCR+ and CD4+ cells negates the effect. *Blood* [Internet]. 105(6):2577–2584. Available from: https://ashpublications.org/blood/article/105/6/2577/20179/Preconditioning-of-NOD-mice-with-antiCD8-mAb-and.
41. Avula, C. P. R., Fernandes, G. (1999). Modulation of lipid peroxidation and antioxidant enzymes in murine salivary gland by dietary fatty acid ethyl esters. *Life Sci* [Internet]. 65(22):2373–2383. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0024320599005044.
42. Tannenbaum, J., Bennett, B. T. (2015). Russell and Burch's 3Rs then and now: the need for clarity in definition and purpose. *J Am Assoc Lab Anim Sci* [Internet]. 54(2):120–132. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25836957.

 https://doi.org/10.46389/rjd-2021-1037