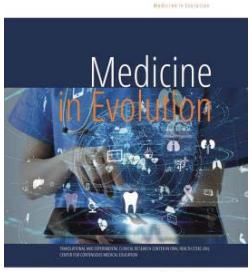
Prospective study on Glutathione expression and immune response in the oral cavity of diabetic patients



Slăvescu D. A.[1], Frăţilă O.[2], Moca A. E.[1*], Vaida L. L.[1], Iurcov R.[1], Lixandru N.[3], Rusu M.[3], Vasca E. M.[4]

[1]Department of Dentistry, Faculty of Medicine and Pharmacy, University of Oradea, 410073 Oradea, Romania
[2]Department of Medical Disciplines, Faculty of Medicine and Pharmacy, University of Oradea, 410073 Oradea, Romania
[3]PhD Student, Physician of the Diabetes, Nutrition and Metabolic Diseases Department, Faculty of Medicine, Victor Babes University of Medicine and Pharmacy, Timisoara, Romania
[4]Department of Dentistry, Faculty of Dental Medicine, "Vasile Goldis" Western University of Arad, Romania

Correspondence to:
Name: Moca Abel Emanuel
Address: 10 Piaţa 1 Decembrie Street, 410073 Oradea, Romani
Phone: +40 746662967
E-mail address: abelmoca@yahoo.com

Received: 4 March 2024; Accepted: 3 May 2024; Published: 30 June 2024

Abstract

Aim and objectives: This study aimed to investigate the association between antioxidant protection factors (glutathione and MTH1/MUTH) and immune response (CD3-lymphocyte T and CD20-lymphocyte B) in the oral cavity mucosa of diabetic patients. Material and methods: Glutathione levels and immune cell expressions (CD3 and CD20) were measured in the oral mucosa of diabetic patients and compared with a control group using immunohistochemical analysis. Results: Diabetic patients had significantly lower glutathione levels and altered immune responses, with higher oxidative stress in B lymphocytes (CD20) and T lymphocytes (CD3). The T/B lymphocyte ratio was significantly higher in diabetic patients, indicating immune imbalance. Conclusions: Diabetic patients showed reduced glutathione levels and a higher T/B lymphocyte ratio in the oral cavity, suggesting that oxidative stress negatively impacts immune function. This highlights the need for antioxidant therapies in managing oral health in diabetic patients.

Keywords: glutathione, diabetic patients, oral cavity

INTRODUCTION

Diabetes is a complex metabolic disease characterized by hyperglycemia, which results from anomalies in insulin secretion or insulin action [1]. The pathological effects of hyperglycemia adversely impact the function of various organs [2]. Diabetes is one of the most prevalent and challenging contemporary chronic diseases, significantly affecting patients' quality of life and life expectancy [3]. The global prevalence of diabetes reached 9% in 2019, and the number of newly diagnosed cases continues to rise [4].

Obesity, high carbohydrate consumption, processed red meat, alcohol, and refined grains are among the most commonly implicated risk factors for diabetes [5]. Additionally, a sedentary lifestyle and reduced physical activity are associated with a higher risk of prediabetes and type 2 diabetes [6].

Regarding oral health implications, periodontal disease and dental caries are diagnosed more frequently in patients with diabetes [7]. Periodontal disease and hyperglycemia share common risk factors, and individuals with diabetes are more susceptible to developing periodontal disease [8]. Moreover, in cases of uncontrolled diabetes, the severity of periodontal disease is greater than in other clinical contexts [9]. Dental caries have a higher prevalence among individuals with diabetes, likely due to alterations in the quality and quantity of saliva [10]. Other common complications in diabetic patients include salivary dysfunctions, tooth loss, oral lesions, candidiasis, and changes in taste [11].

Glutathione is a crucial non-enzymatic antioxidant present in mammalian cells, which protects cells against free radicals and pro-oxidants [12]. In patients with type 2 diabetes, glutathione concentration is reduced, though the mechanisms underlying this reduction are not fully elucidated [13]. Several clinical studies have indicated that glutathione and its precursors can reduce oxidative stress biomarkers and decrease insulin resistance [14]. The diminished synthesis of glutathione in patients with type 2 diabetes can be ameliorated through dietary supplementation, which also reduces systemic oxidative stress [14,15].

Traditionally, glutathione has been investigated in the blood, plasma, or serum of patients [16]. However, saliva is a valuable diagnostic tool and can also be used to measure glutathione levels [17]. Additionally, crevicular fluid [18] and oral tissue biopsies [19] can be utilized to evaluate oral glutathione levels. Once measured, glutathione levels can be compared between diabetic and non-diabetic individuals to assess any differences. Furthermore, glutathione levels can be correlated with other oral health parameters such as periodontal status, dental caries, and oral mucosal health to elucidate the relationship between glutathione and oral health in diabetic patients.

Aim and objectives

Based on the current state of knowledge, this study aimed to evaluate the possible association between antioxidant protection factors (glutathione and MTH1/MUTH) and cellular immune response (CD3-lymphocyte T), as well as the humoral immune response (CD20-lymphocyte B). Specifically, we aimed to assess the increase in glutathione as an oxidative protection factor in the oral cavity mucosa of patients with diabetes and to evaluate the involvement of immune mechanisms by quantifying the expression of CD20 and CD3.

MATERIAL AND METHODS

This study was designed as a prospective analysis involving the evaluation of 10 consecutive oral mucosa biopsies from patients with diabetes, compared to a control group of 10 non-diabetic patients, all sourced from the Resident Laboratory in Oradea, Romania. The

histological and immunohistochemical examination of the tissues necessitated their preservation in a condition resembling that in vivo. The research process comprised several successive stages:

- Tissue fixation: Tissue fragments were immersed in formalin, an aqueous solution, to prevent autolysis and necrobiosis. It was crucial to fix the tissue immediately after sampling, ensuring cold and warm ischemia times were under one hour.
- Dehydration: Water was extracted from the tissues through successive alcohol baths of increasing concentrations (70°, 90°, and 100°). This step aimed to replace tissue water with paraffin for solidification and sectioning.
- Clarification: The dehydrating agent was replaced with a paraffin solvent, such as toluene or xylene.
- Impregnation: Tissues were impregnated with paraffin.
- Embedding: The paraffin-impregnated tissue was embedded in a paraffin block, preparing it for the next step.
- Tissue sectioning: Tissue sections were cut at 3, 4, and 5 microns using a microtome, resulting in ribbons of tissue embedded in paraffin.
- Spreading: The sections were stretched on glass slides using warm water or heated platinum.
- Staining: The sections were deparaffinized, rehydrated, and stained using an automated Gemini-Epredia robot, following the manufacturer's Hematoxylin-eosin staining protocol.
- Mounting: The stained sections were protected with a glass slide.

Immunohistochemical analyses were conducted on an automated staining platform, the Ventana Benchmark GX, according to the manufacturer's instructions. Slides were deparaffinized with EZprep solution (Ventana Medical Systems, Inc.) at 90°C. All reagents and incubation times followed the instructions on the antibody inserts. Slides were prepared using the OmniMap DAB (3,3'-diaminobenzidine) detection kit (Ventana Medical Systems, Inc.) and counterstained with Hematoxylin.

Sections were incubated with polyclonal anti-glutathione primary antibody (ab9443, rabbit, IgG, cytoplasmic, Abcam, Cambridge, CB2 0AX, UK) following the manufacturer's protocol. Similarly, sections were incubated with rabbit polyclonal antibodies CD3 (clone 2GV6-ready-to-use, Ventana) and CD20 (clone L26-ready-to-use, Ventana) according to the respective protocols. Negative controls were established by omitting the primary antibody on identical sections.

Images of the tissues were captured using a Leica 300DM microscope equipped with an HD video camera, and analyzed using software provided by Leica. Specimens were assessed using the H-score, which is calculated by multiplying the percentage of cells at each intensity (scored from 0 to 3) and summing the results, with a maximum score of 300. In this system, <1% positive cells are considered a negative result.

Values were presented as mean values ± standard deviation (SD) using GraphPad Prism 8.0 software. Continuous variables were compared using paired and independent Student's t-tests, with p-values < 0.05 considered statistically significant.

RESULTS

The analysis of mean H-score values for GLUT revealed a significant difference between biopsies from patients with diabetes and those from patients with strictly oral pathology. The mean value and standard deviation (SD) of GLUT in the control group was 278.5 ± 22.73, compared to the mean value in patients with diabetes, which was 15 ± 8.49 (Table I).

Table I. Values for GLUT in the study group and the control group

No.	0	1+	2+	3+	SG-H-score	CG-H-Score
1	90	10	0	0	10	270
2	70	30	0	0	30	280
3	90	10	0	0	10	300
4	80	20	0	0	20	250
5	90	10	0	0	10	230
6	80	20	0	0	20	290
7	80	20	0	0	20	285
8	80	20	0	0	20	300
9	0	0	0	0	0	295
10	90	10	0	0	10	285
Mean value and SD					**15 ± 8,49**	**278,5±22,73**

SG – Study Group; CG – Control Group

The results of the T-test indicated that the variation in mean values and standard deviations of glutathione levels between the study group and the control group possesses high statistical significance ($p < 0.05$).

Microscopic analysis revealed a high expression of the H-score in the majority of control cases. The maximum H-score value was 300. Immunostaining for the presence of glutathione (GLUT) was visualized as a brown color. High chromogenic intensity was observed in the oral mucosa of control group patients, corroborated by the reaction presence in most of the examined cells (Figure 1A). In certain cases within the control group, a decrease in immunohistochemical expression for GLUT was noted, particularly in the superficial layers of the non-keratinized stratified squamous mucosa (Figure 1C). Among patients with diabetes, two distinct patterns were observed: the first pattern exhibited a total absence of GLUT expression (Figure 1B), while the second showed minimal expression, primarily in the basal layers (Figure 1D).

The variability in the balance between the cellular immune response (T lymphocytes) and the humoral immune response (B lymphocytes) is presented in Table II. The comparative evaluation of CD3 and CD20 expression in patients with diabetes revealed a significant difference between these two types of immune response. The mean value and standard deviation (SD) of CD3 in the control group was 265.5 ± 141.63, whereas the mean value and SD of CD20 in patients with diabetes was 60.5 ± 82.85 (Table II).

The results of the T-test revealed a high statistical significance in the analysis of the two parameters, CD3 (cellular immune response) and CD20 (humoral immune response) ($p < 0.05$).

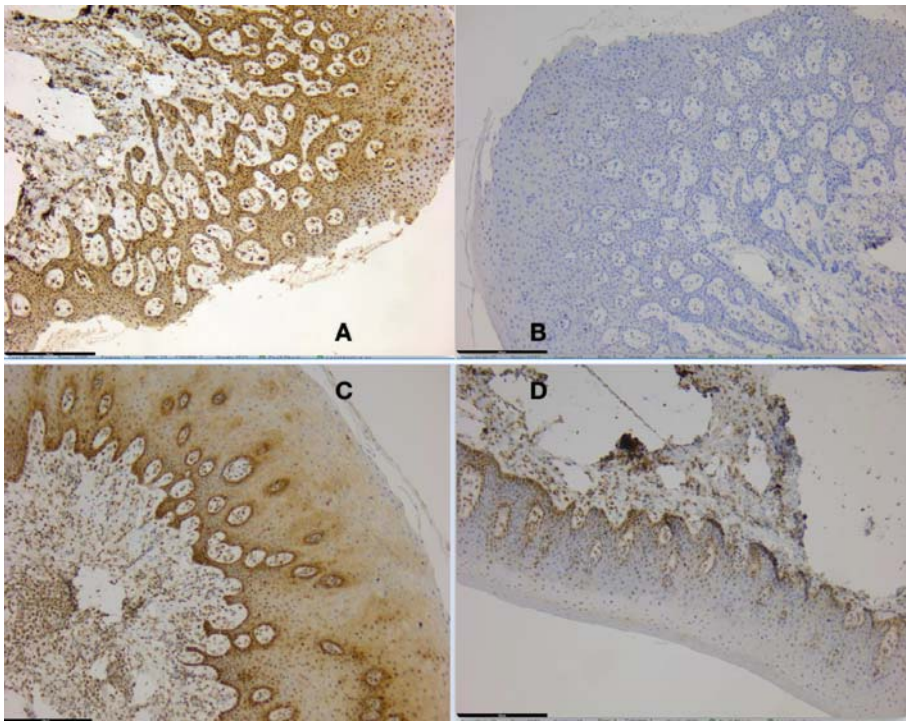


Figure 1. Immunohistochemical expression of glutathione in patients with diabetes and in the control group

Table II. Values for CD3 and CD20

No.	CD20	CD3
1	10	106
2	5	287
3	20	177
4	120	490
5	195	267
6	15	50
7	5	259
8	15	193
9	200	389
10	20	437
Mean value and SD	**60,5± 82,85**	**265,5± 141,63**

Microscopic analysis of the immune response showed that the cellular immune response, as evidenced by CD3 expression, is higher (Figure 2A) compared to the humoral immune response, as evidenced by CD20 immunolabeling (Figure 2B). In the case of B lymphocytes (CD20), there is greater variability (ranging from a minimum of 5 lymphocytes/HPF to a maximum of 200 lymphocytes/HPF) compared to T lymphocytes (CD3), which exhibit much less dispersion (ranging from a minimum of 50 lymphocytes/HPF to a maximum of 437 lymphocytes/HPF).

Evaluating the ratio of the medians of the two lymphocyte populations, it is evident that T lymphocytes are much more numerous compared to B lymphocytes, with a T/B ratio of 4.38. T lymphocytes (CD3) are primarily located in a band at the dermo-epidermal interface (Figure 2C), whereas B lymphocytes (CD20) tend to localize deeper within the dermis, often forming nodular aggregates (Figure 2D).

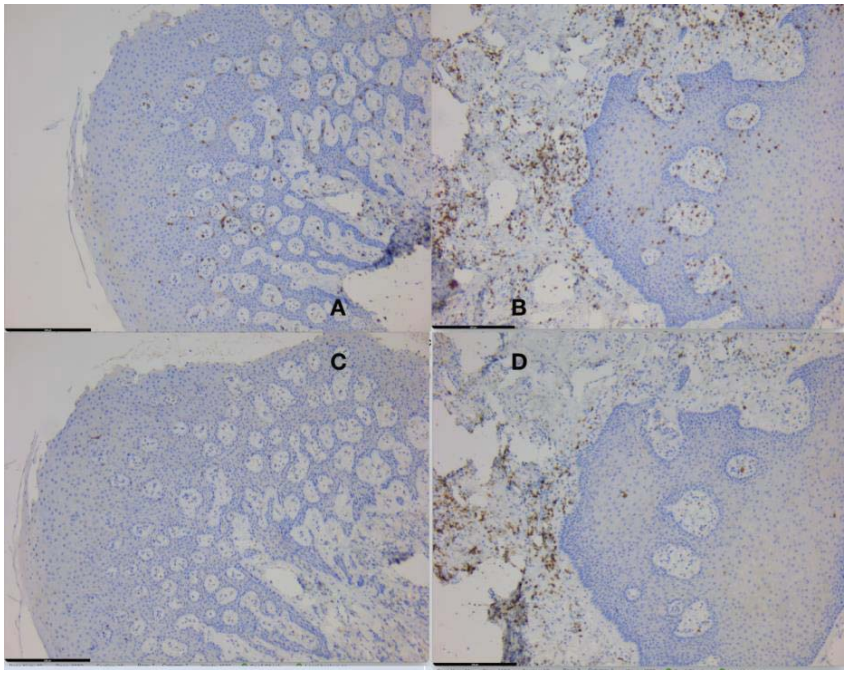


Figure 2. Immunohistochemical expression of CD3 and CD20 in patients with diabetes

DISCUSSIONS

The elevated blood sugar levels in patients with diabetes are associated with an increased release of reactive oxygen species (ROS), resulting in heightened oxidative stress. Elevated oxidative stress can induce the development of metabolic diseases [20]. High oxidative stress is linked to both increased cellular insulin resistance and the destruction of β-cells, as well as exacerbating diabetes-related complications such as angiopathies and neuropathies [20].

The body's mechanisms for counteracting the increased levels of reactive oxygen species include endogenous enzymatic systems such as catalase, superoxide dismutase, and glutathione peroxidase, as well as non-enzymatic mechanisms involving vitamins [21].

In our study, we observed a reduction in antioxidant factors within the oral mucosa. The antioxidant levels were assessed by quantifying the intensity of the immunohistochemical reaction of glutathione (GLUT) in the oral cavity mucosa. The role of glutathione as an intracellular antioxidant that balances oxidative stress is well established [22,23]. Glutathione deficiency, similar to other pathological states, has been investigated in the context of diabetes. Several studies have reported a decrease in erythrocyte glutathione concentration in diabetic patients [24-27]. Our analysis revealed a significantly lower glutathione level in diabetic patients, as evaluated by the H-score. The control group exhibited a mean H-score of 278.5 ± 22.73, compared to the diabetic group's mean of 15 ± 8.49. This low antioxidant level in the study group aligns with the observed high oxidative stress in the examined tissues.

Despite the extensive research on oxidative stress, the complexity of its mechanisms in type 2 diabetes patients remains incompletely understood. Considering the potential use of biomarkers to evaluate oxidative stress profiles, we propose the hypothesis of potential therapeutic options targeting these mechanisms. However, the literature provides conflicting data regarding the therapeutic benefits and limitations in the progression of diabetes [20]. Numerous studies have aimed to elucidate the impact of glucose levels on the immune system in diabetic patients.

The alteration of the immune response in patients with diabetes is a gradual process involving numerous cellular and humoral mechanisms. Patients with diabetes, especially those experiencing ketoacidosis, exhibit an increased incidence and severity of infections [28]. Many studies have posited that hyperglycemia serves as a metabolic substrate for various microorganisms [28]. More recent approaches focus on the impact of diabetes on the host's immune response to harmful factors.

A comparative analysis of the mean values of B and T lymphocytes reveals a lower number of B-type lymphocytes (CD20+), which directly results in decreased immunoglobulin levels [29]. Specifically, the number of B lymphocytes (CD20) varies widely (from a minimum of 5 lymphocytes/HPF to a maximum of 200 lymphocytes/HPF) compared to T lymphocytes (CD3), which range from 50 lymphocytes/HPF to 437 lymphocytes/HPF. The average ratio of T to B lymphocytes indicates that T lymphocytes are significantly more abundant (T/B=4.38).

Recent studies have shown that peripheral blood mononuclear cells stimulated with anti-CD3 and exposed to elevated blood glucose levels exhibit a significant decrease compared to non-diabetic patients [21]. Other studies have demonstrated the involvement of various interleukins (IL-2, IL-6, IL-10) in initiating the immune response, with extreme hyperglycemia associated with their reduced levels [30]. Despite significant advances in understanding the impairment of the immune response in diabetic patients, the underlying molecular mechanisms remain unclear. TGF-beta 1, which precedes the reduction of IL production, could serve as a useful biomarker in monitoring diabetic patients [30].

The analysis of lymphocyte subpopulations in the biopsies from this study revealed T lymphocytes predominantly at the dermo-epidermal interface, whereas B lymphocytes were primarily located in the deep dermis. Although not the primary focus of our study, it was noted that both B lymphocytes (CD20) and T lymphocytes (CD3) exhibited higher oxidative stress levels compared to the control group.

Numerous studies have highlighted the interrelationship between reactive oxygen species and immune response levels. A decrease in glutathione or other antioxidant factors is associated with inhibited synthesis and release of cytokines from T lymphocytes (CD3). Increased oxidative stress stimulates the production of IL-1, NF-kB, and TNF-α, altering the immune response [30].

Based on the existing literature, it is evident that not only the number of T lymphocytes is crucial for maintaining immune system homeostasis, but their functionality is also compromised by oxidative stress. In our study, the link between glutathione levels in the oral mucosa and immune system status could not be evaluated due to the limited number of cases. This small sample size diminishes the predictive value of the statistical data. Nonetheless, this research hypothesis remains significant for future studies.

CONCLUSIONS

In this study, the average value of glutathione was lower in patients with diabetes, and the difference between the average value in the control group and that in the diabetic group was statistically significant. The reduced levels of antioxidant factors in the oral mucosa were frequently associated with ulcerations and microangiopathies. Additionally, the analysis of T (CD3) and B (CD20) lymphocyte subpopulations in the oral mucosa of diabetic patients revealed a significant difference in the immune response between these two lymphocyte types. Consistent with other data from the specialized literature, it can be concluded that oxidative stress interacts with and impairs T lymphocyte function.

REFERENCES

1. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2014; 37 (Suppl 1):S81-90.
2. Banday MZ, Sameer AS, Nissar S. Pathophysiology of diabetes: An overview. Avicenna J Med. 2020; 10(4):174-188.
3. Sun H, Saeedi P, Karuranga S, Pinkepank M, Ogurtsova K, Duncan BB, Stein C, Basit A, Chan JCN, Mbanya JC, Pavkov ME, Ramachandaran A, Wild SH, James S, Herman WH, Zhang P, Bommer C, Kuo S, Boyko EJ, Magliano DJ. IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. Diabetes Res Clin Pract. 2022; 183:109119.
4. Chan JCN, Lim L-L, Wareham NJ, Shaw JE, Orchard TJ, Zhang P, et al. The Lancet Commission on diabetes: using data to transform diabetes care and patient lives. Lancet. 2020; 396 (10267):2019–2082.
5. Kyrou I, Tsigos C, Mavrogianni C, Cardon G, Van Stappen V, Latomme J, Kivelä J, Wikström K, Tsochev K, Nanasi A, Semanova C, Mateo-Gallego R, Lamiquiz-Moneo I, Dafoulas G, Timpel P, Schwarz PEH, Iotova V, Tankova T, Makrilakis K, Manios Y; Feel4Diabetes-study Group. Sociodemographic and lifestyle-related risk factors for identifying vulnerable groups for type 2 diabetes: a narrative review with emphasis on data from Europe. BMC Endocr Disord. 2020; 20(Suppl 1):134.
6. Khetan AK, Rajagopalan S. Prediabetes. Can J Cardiol. 2018; 34(5):615–623.
7. Borgnakke WS, Poudel P. Diabetes and Oral Health: Summary of Current Scientific Evidence for Why Transdisciplinary Collaboration Is Needed. Front Dent Med. 2021; 2:709831.
8. Kocher T, König J, Borgnakke WS, Pink C, Meisel P. Periodontal complications of hyperglycemia/diabetes mellitus: epidemiologic complexity and clinical challenge. Periodontol 2000. 2018; 78 (1):59–97.
9. Miller A, Ouanounou A. Diagnosis, management, and dental considerations for the diabetic patient. J Can Dent Assoc. 2020; 86:k8.
10. Singh I, Singh P, Singh A, Singh T, Kour R. Diabetes an inducing factor for dental caries: A case control analysis in Jammu. J Int Soc Prev Community Dent. 2016; 6(2):125-129.
11. Chen H, Hill R, Baysan A. Systematic review on dental caries preventive and managing strategies among type 2 diabetic patients. Front Oral Health. 2022; 3:998171.
12. Averill-Bates DA. The antioxidant glutathione. Vitam Horm. 2023; 121:109-141.
13. Lutchmansingh FK, Hsu JW, Bennett FI, Badaloo AV, McFarlane-Anderson N, Gordon-Strachan GM, Wright-Pascoe RA, Jahoor F, Boyne MS. Glutathione metabolism in type 2 diabetes and its relationship with microvascular complications and glycemia. PLoS One. 2018; 13(6):e0198626.
14. Tuell D, Ford G, Los E, Stone W. The Role of Glutathione and Its Precursors in Type 2 Diabetes. Antioxidants (Basel). 2024; 13(2):184.
15. Sekhar RV, McKay SV, Patel SG, Guthikonda AP, Reddy VT, Balasubramanyam A, Jahoor F. Glutathione synthesis is diminished in patients with uncontrolled diabetes and restored by dietary supplementation with cysteine and glycine. Diabetes Care. 2011; 34(1):162-167.
16. Derindağ G, Akgül HM, Kızıltunç A, Özkan Hİ, Kızıltunç Özmen H, Akgül N. Evaluation of saliva glutathione, glutathione peroxidase, and malondialdehyde levels in head-neck radiotherapy patients. Turk J Med Sci. 2021; 51(2):644-649.
17. Koregol AC, Kalburgi NB, Pattanashetty P, Warad S, Shirigeri NS, Hunasikatti VC. Effect of smokeless tobacco use on salivary glutathione levels among chronic periodontitis patients before and after non-surgical periodontal therapy. Tob Prev Cessat. 2020; 6:15.
18. Savita AM, Sarun E, Arora S, Krishnan S. Evaluation of glutathione level in gingival crevicular fluid in periodontal health, in chronic periodontitis and after nonsurgical periodontal therapy: A clinicobiochemical study. Contemp Clin Dent. 2015; 6(2):206-210.
19. Deshpande KC, Kulkarni MM, Rajput DV. Evaluation of glutathione peroxidase in the blood and tumor tissue of oral squamous cell carcinoma patients. J Oral Maxillofac Pathol. 2018; 22(3):447.
20. Bhatti JS, Sehrawat A, Mishra J, Sidhu IS, Navik U, Khullar N, Kumar S, Bhatti GK, Reddy PH. Oxidative stress in the pathophysiology of type 2 diabetes and related complications: Current therapeutics strategies and future perspectives. Free Radic Biol Med. 2022; 184:114-134.

21. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. Curr Biol. 2014; 24(10):R453-62.
22. Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. Biomed Pharmacother. 2003; 57(3–4):145-155.
23. Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. J Nutr. 2004; 134(3):489–492.
24. Whiting PH, Kalansooriya A, Holbrook I, Haddad F, Jennings PE. The relationship between chronic glycaemic control and oxidative stress in type 2 diabetes mellitus. Br J Biomed Sci. 2008; 65(2):71–74.
25. Sundaram RK, Bhaskar A, Vijayalingam S, Viswanathan M, Mohan R, Shanmugasundaram KR. Antioxidant status and lipid peroxidation in type II diabetes mellitus with and without complications. Clin Sci (Lond). 1996 ;90(4):255–260.
26. Murakami K, Kondo T, Ohtsuka Y, Fujiwara Y, Shimada M, Kawakami Y. Impairment of glutathione metabolism in erythrocytes from patients with diabetes mellitus. Metabolism. 1989; 38(8):753–758.
27. Memisogullari R, Taysi S, Bakan E, Capoglu I. Antioxidant status and lipid peroxidation in type II diabetes mellitus. Cell Biochem Funct. 2003; 21(3):291–296.
28. Casqueiro J, Casqueiro J, Alves C. Infections in patients with diabetes mellitus: A review of pathogenesis. Indian J Endocrinol Metab. 2012; 16 (Suppl 1):S27-36.
29. Lu ZH, Yu WL, Sun Y. Multiple immune function impairments in diabetic patients and their effects on COVID-19. World J Clin Cases. 2021; 9(24):6969-6978.
30. Ferlita S, Yegiazaryan A, Noori N, Lal G, Nguyen T, To K, Venketaraman V. Type 2 Diabetes Mellitus and Altered Immune System Leading to Susceptibility to Pathogens, Especially Mycobacterium tuberculosis. J Clin Med. 2019; 8(12):2219
31. Ciora E.D., Miron M.I., (Bojoga) Mocuţa D.E., Dragoş B., Luca M.M., Evolution of periodontal disease in patients with type 2 diabetes in the context of initial therapy - systematic review, Medicine in Evolution Volume XXVIII, No. 3, 2022
32. Potra-Cicalău G.I., Todor L., Ciavoi G., Scrobotă I., Baciu O. A., Jurca M.C, Clinical study on the frequency of dental examinations and oral prophylaxis in patients diagnosed with type 2 diabetes mellitus and periodontal disease, Medicine in Evolution Volume XXVIII, No. 2, 2022