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“JÚLIO DE MESQUITA FILHO”
Campus de Araçatuba

LUKAS MENDES DE ABREU

**Oxidative changes in exfoliated buccal cells in patients with major
depressive disorder**

Araçatuba – SP
2023

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Oxidative changes in exfoliated buccal cells in patients with major depressive disorder

Dissertação apresentada à Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista “Júlio de Mesquita Filho” - UNESP, como parte dos requisitos para obtenção do título de Mestre em Odontologia - Área de concentração em Estomatologia e Psiconeuroimunologia.

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Coorientador: Prof. Dr. Daniel Galera Bernabé

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***“We must not allow other people’s limited
perceptions to define us.”***

(Virginia Satir)

Abreu LM. Alterações oxidativas em células bucais esfoliadas em pacientes com transtorno depressivo maior [dissertação]. Araçatuba: Faculdade de Odontologia da Universidade Estadual Paulista; 2023.

RESUMO

Objetivos: Avaliar a ocorrência de estresse oxidativo nas células esfoliadas bucais de pacientes com transtorno depressivo maior (TDM) em comparação à voluntários sem a doença. **Métodos:** Neste estudo de caso-controle foram avaliados vinte pacientes com TDM diagnosticado de acordo com a quinta edição do Manual Diagnóstico e Estatístico de Transtornos Mentais (DSM-5) e vinte participantes controles sem TDM. Células esfoliadas bucais colhidas dos participantes de ambos os grupos foram analisadas semi-quantitativamente quanto aos níveis intracelulares de espécies reativas de oxigênio (ROS), potencial de membrana mitocondrial ($\Delta\Psi_m$), atividade de caspase-3 e -7 e glutathiona reduzida (GSH), visando investigar o estado oxidativo entre os grupos. **Resultados:** Os níveis intracelulares de ROS ($p= 0,0012$) e atividade de caspase-3 e -7 ($p= 0,0171$) foram significativamente maiores nas células esfoliadas bucais do grupo TDM em comparação ao grupo controle. Pacientes com TDM também apresentam células orais com menor expressão de $\Delta\Psi_m$ (0,0265). Não foram encontradas diferenças nos níveis intracelulares de GSH (0,8908) entre os grupos TDM e controle. **Conclusões:** Células bucais esfoliadas de pacientes com TDM expressam níveis intracelulares aumentados de indicadores de estresse oxidativo. Os resultados do presente estudo sugerem o uso de células bucais como método de avaliação do estresse oxidativo em pacientes com transtornos depressivos.

Palavras-chave: Transtorno depressivo maior. Depressão. Estresse oxidativo. Mucosa bucal. Doença bucal.

Abreu LM. Oxidative changes in exfoliated buccal cells in patients with major depressive disorder [dissertation]. Araçatuba: UNESP - São Paulo State University; 2023.

ABSTRACT

Objectives: To evaluate the occurrence of oxidative stress in the cells of the oral mucosa of patients with major depressive disorder (MDD). **Methods:** This case-control study included twenty patients diagnosed with MDD according to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) and twenty participants without MDD (control). Buccal exfoliated cells were collected from the participants in both groups and assessed for the intracellular levels of reactive oxygen species (ROS), mitochondrial membrane potential ($\Delta\Psi_m$), caspase-3 and -7 activity, and reduced glutathione (GSH). **Results:** The intracellular levels of ROS ($p= 0.0012$) and caspase-3 and -7 activity ($p= 0.0171$) were significantly higher in the buccal exfoliated cells from the MDD group compared to control group. MDD patients also display oral cells with lower expression of $\Delta\Psi_m$ (0.0265). No differences were found in GSH (0.8908) intracellular levels between MDD and control groups. **Conclusions:** Buccal exfoliated cells from patients with MDD presented increased intracellular levels of oxidative stress. The current study suggests the use of buccal cells as a method for assessing oxidative stress in patients with depressive disorders.

Keywords: Major depressive disorder. Depression. Oxidative stress. Oral mucosa. Oral disease.

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LISTA DE ABBREVIATURAS, SÍMBOLOS E SIGLAS

% percentage

* values are considered statistically significant at $p < 0.05$

** values are considered statistically significant at $p < 0.001$

> superior

°C degree Celsius

^a t-test for independent groups

AFU arbitrary fluorescence units

ANOVA analyses of variance

^b Chi-square test

CAPES Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CEAPS Specialized Psychosocial Care Center

DAPI 4'6-diamino-2-phenylindole dihydrochloride

DMSO dimethylsulfoxide

DSM-5 Diagnostic and Statistical Manual of Mental Disorders

FAPESP São Paulo State Research

GSH reduced glutathione

H₂DCFDA 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate

H₂O₂ hydrogen peroxide

min minute

ml milliliter

mL milliliter (unit of measurement equivalent to 10⁻³ l)

n number

p p-value

PBS phosphate buffered saline

PVA polyvinyl alcohol

REDOX oxidation-reduction

ROS reactive oxygen species

SD standard deviation

SE standard error

SP São Paulo

SPSS Statistical Package for the Social Science

UNESP São Paulo State University

USA United States of America

$\Delta\Psi_m$ mitochondrial membrane potential

μL microliter (unit of measurement equivalent to 10^{-6} l)

μM micromolar (unit of measurement equivalent to 10^{-6} one-millionth)

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1 INTRODUCTION*

Major Depressive Disorder (MDD) is a chronic and recurrent mood disorder commonly found throughout the world (1,2). This disorder affects at least 3.8% of the population, being twice as common in females as in males (3–5). Although MDD is mainly characterized by persistent emotional symptoms such as guilt and anhedonia (1,3,4), somatic symptoms and other symptoms that affect cognitive functions and compromise physical and mental health are commonly manifest (1,4). MDD pathogenesis involves genetic, environmental, psychological, biological factors (3) and even molecular mechanisms that lead to disorders in the metabolism of neurotransmitters such as serotonin and dopamine (6,7). Its occurrence and progression is associated with elevated systemic levels of pro-inflammatory cytokines, and mitochondrial dysfunction (6–8), which are consistent with the generation of oxidative stress observed in (8–10).

Oxidative stress refers to an imbalance between the oxidant and antioxidant systems in favor of the oxidants (11). This imbalance can result in the degradation of essential biological structures for the organic cell functioning, such as oxidative modifications of lipids, proteins, DNA, enzymes, and the activity of certain genes (11). Patients diagnosed with MDD have elevated systemic levels of enzymes involved in the production of reactive oxygen (ROS)/nitrogen (RNS) compared to healthy controls, indicating that the disease may be associated with changes in the activity of enzymes related to oxidative stress (9,10,12). Increased levels of xanthine oxidase and ROS were observed in the blood and brain of MDD patients. Moreover, studies have demonstrated high levels of oxidative damage products in peripheral blood, red blood cells, mononuclear cells, urine, cerebrospinal fluid, and postmortem brains of MDD patients (8). Oxidative damage products to malondialdehyde (MDA), 8-F 2 - isoprostane (8 - iso -PGF 2 α), carbonyl protein, 8-hydroxy-2-deoxyguanosine (8-OHdG) and 8-oxo-7,8 -dihydroguanosine (8-oxoGuo) were found in patients with MDD (9,10).

Thus, as in the previously tested fluid and tissue samples, oxidant and antioxidant systems play an important role in the physiological functions of buccal mucosal cells (14). Oral mucosal cells mirror health status, reflecting changes in systemic conditions such as the oxidative stress (15,16). Oxidative stress has been implicated in the pathogenesis or aggravation of several oral diseases, including periodontal disease, recurrent aphthous ulcers, salivary gland diseases, autoimmune disorders, oral leukoplakia, and squamous cell carcinoma (17). This involvement is related to constant exposure of the oral mucosa to endogenous and exogenous

* Normalização segundo a revista Oral Diseases - <https://onlinelibrary.wiley.com/journal/16010825>

oxidative stress sources (17–20). Furthermore, saliva spreads dissolved antioxidants and oxidants from different sources, reaching oral cells (18,19). However, there is a lack of knowledge regarding the occurrence of oxidative stress-related changes in the oral mucosa of MDD patients.

In this way, knowing that there is an increased oxidative stress condition in individuals with MDD (9,10) and that the imbalance of the oxidant-antioxidant state causes irreversible damage to cells of the oral mucosa that are implicated in the pathogenesis of oral diseases (17), it becomes essential to investigate the occurrence of redox disorders in the oral mucosa. To date, no studies have investigated specific molecular changes related to oxidative stress in the oral mucosa of patients with MDD. Thus, to test the hypothesis that patients with MDD present increased oxidative stress in buccal mucosa cells, this study was designed to evaluate the expression of molecules associated with oxidative stress in buccal exfoliated cells from patients with MDD compared to those from healthy individuals.

2 MATERIALS AND METHODS

2.1 Ethics considerations

This study was approved by the Committee of Human Studies of the Sao Paulo State University (UNESP), School of Dentistry, Araçatuba, São Paulo, Brazil (nº. 5.719.154), and informed consent was obtained from all participants.

2.2 Study design

A case-control observational study was carried out to assess oxidative stress in exfoliated oral cells of patients diagnosed and undergoing treatment for MDD in 2022 at the Specialized Psychosocial Care Center (CEAPS), Araçatuba, São Paulo, Brazil. A sample calculation was carried out using specific software G*Power considering the difference between means for independent groups (T-test) with an alpha error of 5% and test force of 80% (21). The calculation result for the study sample was at least 17 participants per group.

2.3 Participant

A review was carried out in the medical records of patients with MDD at CEAPS and, when eligible for the study, a semi-structured interview and an intraoral examination were performed. Seventy-two patients were initially considered for inclusion in this study. Patients younger than 18 years old ($n = 2$), those with severe comorbidities (eg. cancer, human immunodeficiency virus and pneumonia) ($n = 5$), with oral diseases ($n = 2$), smokers ($n = 11$) were excluded. All patients had the diagnostic of MDD made by a single psychiatrist using a semi-structured interview according to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), considering 2 or more weeks of depressed mood and/or loss of interest and pleasure, along with other symptoms including sleep, weight, and energy changes (22). Patients with an Axis I disorder other than MDD or with an Axis II disorder, as well as patients with MDD with psychotic, bipolar, cyclothymic, dysthymic symptoms, and absent features of MDD were excluded ($n = 8$). Twenty-four patients declined to participate in the study. All patients were on drug treatment with antidepressants and group behavioral therapy. After the selection, the total number of recruited participants for the MDD group was 20 (16 women, 4 men; ages ranging from 27 to 62 years old).

In order to carry out a case-control study, volunteers without MDD were recruited among the general population to compose the control group. An interview and an intraoral examination were used to evaluate these volunteers. They were matched with the MDD group according to age and gender. Similarly to MDD patients, participants with severe illnesses, alcoholism, smoking, psychotropic drug use, oral diseases, and unsatisfactory oral hygiene. Controls were excluded if they had a diagnosis or family history of a psychiatric disorder. Therefore, 20 volunteers were selected to compose the control group (16 females, and 4 males; ages ranging from 28 to 62 years old).

2.4 Evaluation of oxidative stress in buccal exfoliated cells

2.4.1 Collecting exfoliated buccal cells

The participants from the MMD and control groups were subjected to the collection of exfoliated buccal cells. Cell collections were performed in the morning, between 09:00 am and 11:00 am, in one participant from each group on the same day. The participants were instructed not to eat and not to brush their teeth for at least two hours before collection. Immediately before the collection, they were instructed to rinse their mouths three times with distilled water. The cells were collected with the aid of a cytobrush (Adlin, Santa Catarina, BR) from the lateral border of the tongue and floor of the mouth, which are regions most affected by oral cancer (17). The cytobrush was applied to the surface of the oral mucosa with a circular expansion movement starting at the lateral border of the tongue and gradually increasing the circumference until reaching the floor of the mouth. To maximize cell sampling and eliminate any bias that could be caused by sampling from only one side, the cells were exfoliated in the regions of interest on the right and left sides using different brushes. Analyzes were carried out immediately after cell collection.

2.4.2 Cell preparation

After exfoliation, the brushes were placed in a single 15 mL conical (Sigma-Aldrich, St. Louis, MO, USA) containing 5 mL of fresh phosphate buffered saline (PBS) with 1 mg/mL of polyvinyl alcohol (PBS/PVA; Sigma-Aldrich, St. Louis, MO, USA). The

cytobrushs were then vigorously rotated to dislodge and release the cell to the bottom of the tube. The tube containing cells from both sides of the oral mucosa was centrifuged at 1160 *g* for 5 min at 37 °C. The supernatant was discarded and replaced with 5 mL fresh PBS/PVA. The washing procedure was repeated twice. Next, cells were counted using a hemocytometer and diluted in PBS/PVA to a final concentration of 50,000 cells/mL. After setting the cell concentration, 2 mL of cell suspension was retrieved and the volume was divided equally into two microtubes (1 mL of cell suspension in each microtube). Microtubes were centrifuged at 1160 *g* for 5 min at 37 °C. At the end of the centrifugation, the supernatant was removed and the pellet containing the cells was subjected to staining protocols.

2.4.3 Simultaneous evaluation of intracellular levels of reactive oxygen species and mitochondrial membrane potential

The intracellular ROS content (H_2O_2 , $\text{HO}\cdot$ and $\text{ROO}\cdot$) and $\Delta\Psi\text{m}$ were simultaneously evaluated using H_2DCFDA (6-carboxy-2',7'-dichlorodihydrofluorescein diacetate; Molecular Probes, OR, USA) and MitoTracker Red (CMXRos, Molecular Probes, Invitrogen, OR, US) probes, respectively. The tests were performed simultaneously according to the manufacturer's information. The buccal cells were incubated with 150 μL of a solution of 0.5 μM H_2DCFDA and 0.5 μM MitoTracker Red for 30 min at 37 °C, in the dark. Both probes were freshly diluted in PBS/PVA from stock solutions dissolved in dimethylsulfoxide (DMSO; Sigma), as recommended by the manufacturer. Then, the cells were washed twice with PBS/PVA and centrifuged for 5 min at 37 °C at 1160 *g*. The cells were then transferred to glass slides with a mounting medium [Vectashield with 4'6-diamino-2-phenylindole dihydrochloride (DAPI); Vector Laboratory, Inc, USA] and evaluated immediately under a microscope equipped with epifluorescence (IX51; Olympus, Tóquio, Japão).

2.3.4 Simultaneous evaluation of intracellular levels of reduced glutathione and caspase-3 and -7

The intracellular content of reduced glutathione (GSH) and caspase-3 and -7 were evaluated simultaneously using the fluorescent probes ThiolTracker™ Violet (Glutathione Detection Reagent; Molecular probes, Invitrogen, OR, USA) and Image-iT LIVE Red Caspase-3 and -7 (Molecular Probes, Invitrogen, OR, USA), respectively. The procedures were performed according to the manufacturer's information. Briefly, a 20 μM solution of

ThiolTracker™ Violet and a 30x solution of fluorescent caspase-3 and -7 inhibitors diluted in 150 µL of fresh PBS/PVA were added to the second microtube containing unstained buccal cells. Both probes were freshly diluted in PBS/PVA from stock solutions dissolved in DMSO. The cells were incubated at 37 °C for 45 min in the dark. The cells were washed twice with fresh PBS/PVA and placed on glass slides with mounting medium (Vectashield without DAPI) and immediately evaluated under microscopy.

2.3.5 Fluorescence intensity assessment

The fluorescence intensity of individual cells stained with each probe was measured for semi-quantitative assessment of stress biomarkers evaluated in the present study. Images of the cells were taken under a microscope equipped with epifluorescence (IX51; Olympus) and running Q-Capture Pro Image software. The excitation and emission wavelengths were, respectively: 495 nm and 520 nm for the H₂DCFDA probe; 615 nm and 587 nm for MitoTracker Red and Image-iT LIVE Red Caspase-3 and -7; and 404 nm and 526 nm for ThiolTracker Violet and DAPI probes. Images of 100 non-overlapping oral cells from each patient were taken under light and fluorescence at 20× magnification. The fluorescent pixel intensity value of the total area of each buccal cell was measured using a freehand tool to delineate the cytoplasm of each cell by ImageJ software (National Institute of Health, Bethesda, MD, USA). The background fluorescence was subtracted from each image before measuring and quantifying fluorescence. The mean values of the 100 buccal cells for each marker were expressed as arbitrary fluorescence units (AFU).

2.5 Statistical analysis

Data were analyzed by paired t-test using GraphPad Prism 8.21 (GraphPad Software Inc., San Diego, CA, USA). The chi-square test was used to explore associations between demographic and medical variables between groups. The AFUs were evaluated regarding normality using the D'Agostino-Pearson test. The paired t-test was used to assess the differences between the AFU of each biomarker between the groups. Differences were considered statistically significant when $p < 0.05$.

3 RESULTS

A total of 40 participants were included in this study. In the MDD group, there were 16 women (80%) and 4 men (20%) with a mean age of 51 years. Most participants with MDD were Caucasian (65%) and had high school education level (40%). The average body mass index of patients with MDD was 30.44 and 50% of these patients had some comorbidity, (eg. hypertension, diabetes, hypercholesterolemia and gastritis). As it would be expected from the paired design we used, the control group were similar the MDD group, not statistically different in terms of age, gender, race, education, comorbidities, and body mass index (Table 1).

Table 1. Characteristics of the sample

Variables	Controls (n=20)	MDD (n=20)	p-Value
Age (years $\pm$ SD)	49.95 $\pm$ 9.03	51.25 $\pm$ 9.10	0.653 ¹
Sex, n (%)			1.000 ²
Female	16 (80)	16 (80)	
Male	4 (20)	4 (20)	
Race, n (%)			0.144 ¹
White	17 (85)	13 (65)	
Black	3 (15)	7 (35)	
Education, n (%)			0.093 ²
less than high school	1 (5)	5 (25)	
high school	6 (30)	8 (40)	
postsecondary	13 (65)	7 (35)	
Comorbidities, n (%)			0.525 ²
Yes	8 (40)	10 (50)	
No	16 (60)	10 (50)	
Body mass index (mean $\pm$ SD)	27.08 $\pm$ 5.37	30.44 $\pm$ 7.76	0.220 ¹

Data are expressed as mean $\pm$ SD. A p value <0.05 was considered significant.

¹ paired t-test for independent groups.

²Chi-square test.

3.1 Intracellular expression of reactive oxygen

The intracellular expression of ROS was quantified in exfoliated buccal cells (Figure 1A) and results showed higher expression of intracellular ROS (expressed in AFU) in exfoliated buccal cells from patients with MDD compared to the control group (MDD: 599.3 ± 75.8 vs control: 288.0 ± 21.1 ; $p < 0.0012$). Representative photomicrographs of stained exfoliated buccal cells for evaluation of ROS are shown in Figure 1B.

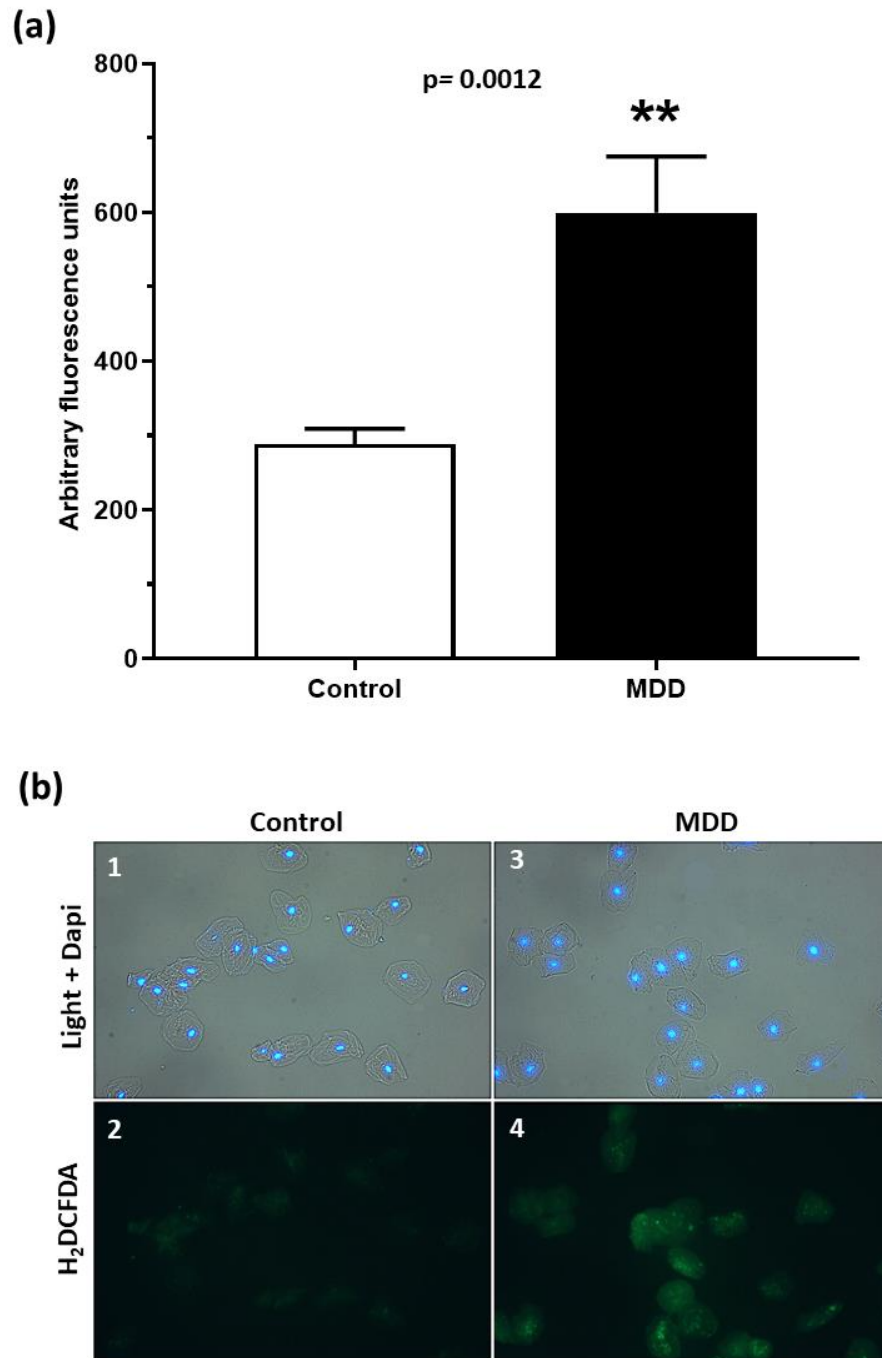


Figure 1. Intracellular expression of ROS in buccal exfoliated cells from patients diagnosed with major depressive disorder (MDD) and without the disorder (control). (a) The graph represents the mean intensities of fluorescent signals (pixels) measured in cells stained with the H₂DCFDA probe, expressed in arbitrary fluorescence units (AFU). **Values are statistically significant at $p < 0.001$. (b) Representative photomicrographs show the H₂DCFDA staining pattern (green) in exfoliated buccal cells in control (b-2) and MDD (b-4) groups. Figures b1 and b3 demonstrate the merge of images of exfoliated buccal cells under light microscopy and under fluorescence (DAPI: blue stained nuclei) in different groups.

3.2 Intracellular expression of mitochondrial membrane potential

The trial revealed that exfoliated buccal cells from patients with MDD exhibited lower expression of $\Delta\Psi_m$ (expressed as AFU) than those from the control group (MDD: 7.703 ± 527.3 vs control: 9.299 ± 443.6 ; $p < 0.0265$). Data are summarized in Figure 2A, while representative photomicrographs of stained exfoliated buccal cells for evaluation of $\Delta\Psi_m$ are shown in Figure 2B.

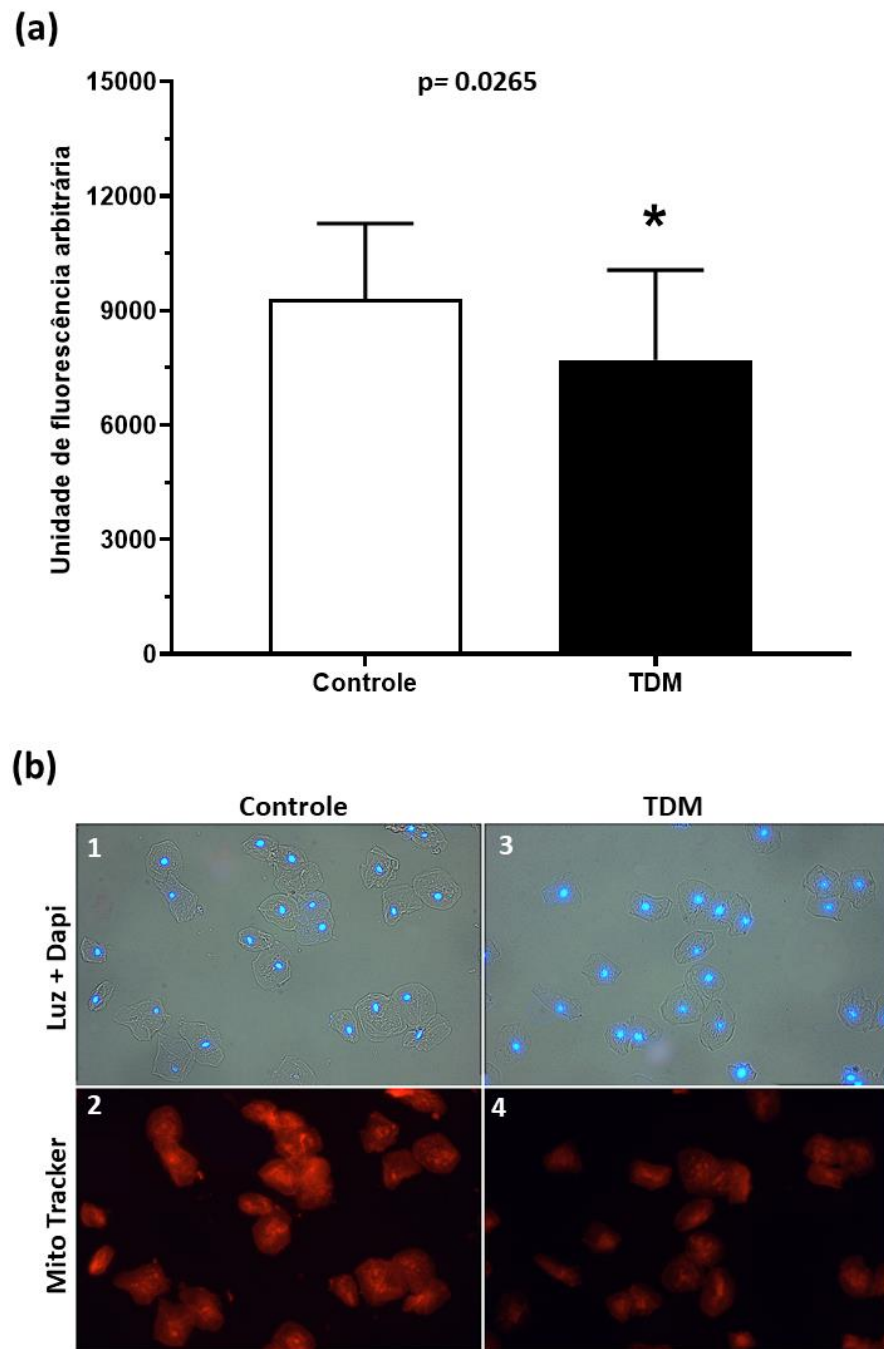


Figure 2. Intracellular expression of $\Delta\Psi_m$ in buccal exfoliated between patients diagnosed with major depressive disorder (MDD) and without the disorder (control). (a) The graph represents the mean intensities of fluorescent signals (pixels) measured in cells stained with the MitoTracker probe, expressed in arbitrary fluorescence units (AFU). *Values are statistically significant at $p < 0.05$. (b) Representative photomicrographs show the MitoTracker Red probe staining pattern (red) in exfoliated buccal cells in control (b-2) and MDD (b-4) groups. Figures b1 and b3 demonstrate the merge of images of exfoliated buccal cells under light microscopy and under fluorescence (DAPI: blue stained nuclei) in different groups.

3.3 Intracellular expression of caspase-3 and -7 activity

The intracellular expression of caspase-3 and -7 (expressed as AFU; Figure 3A) were higher in exfoliated buccal cells of patients with MDD than in control (MDD: 7.735 ± 452.8 vs control: 6.183 ± 398.1 ; $p < 0.0171$). Representative photomicrographs of stained exfoliated buccal cells for evaluation of intracellular levels of caspase-3 and -7 activity are shown in Figure 3B.

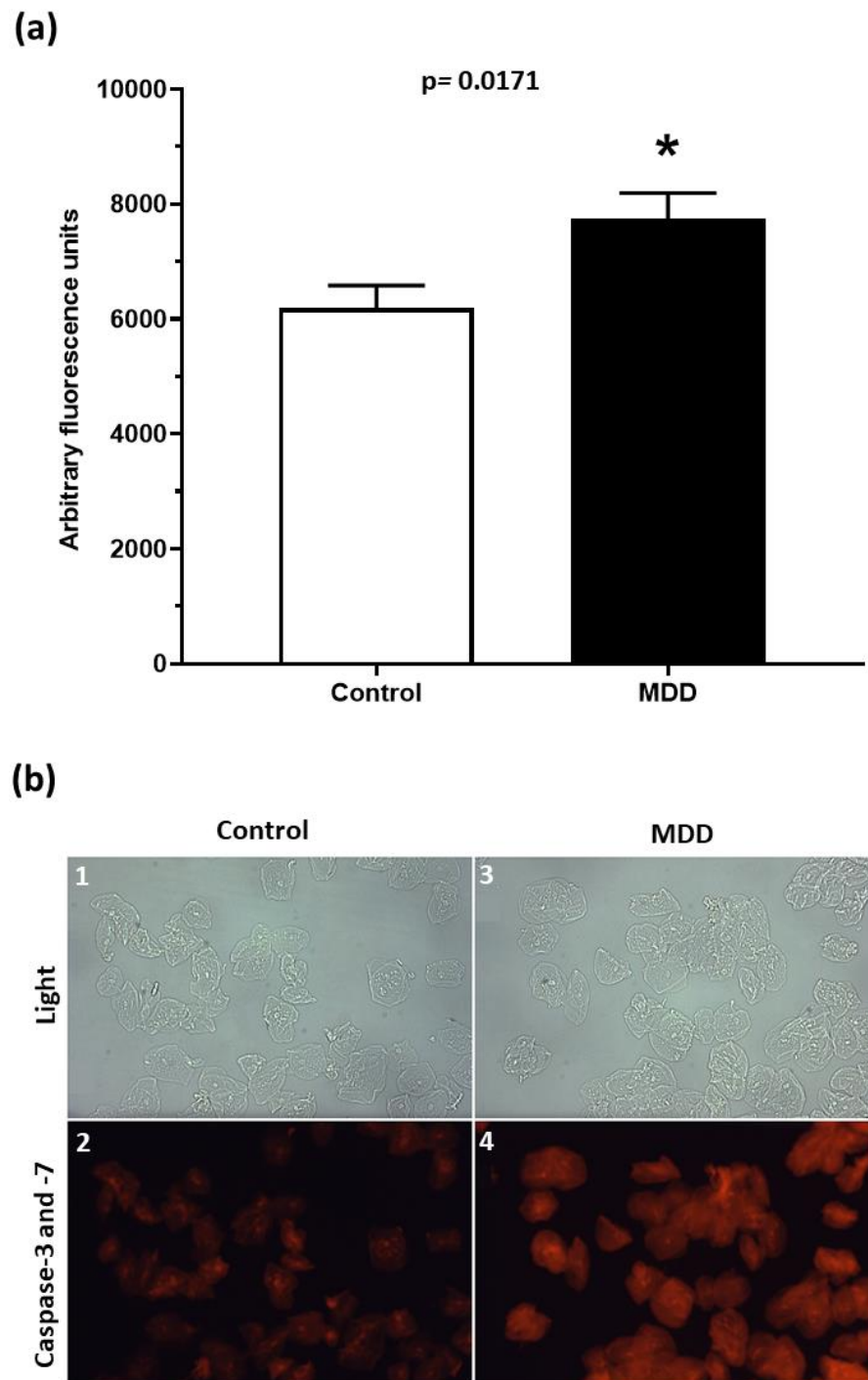


Figure 3. The intracellular expression of caspase-3 and -7 in buccal exfoliated between patients diagnosed with major depressive disorder (MDD) and without the disorder (control). (a) The graph represents the mean intensities of fluorescent signals (pixels) measured in cells stained with the Image-iT LIVE Red Caspase-3 and -7 probe, expressed in arbitrary fluorescence units (AFU). *Values are statistically significant at $p < 0.05$. (b) Representative photomicrographs show the Image-iT LIVE Red Caspase-3 and -7 staining pattern (red) in exfoliated buccal cells in control (b-2) and MDD (b-4) groups. Figures b1 and b3 demonstrate of images of exfoliated buccal cells under light in different groups.

3.4 Intracellular expression of reduced glutathione

The intracellular expression of GSH (expressed as AFU) in buccal exfoliated cells was similar between patients with MDD and controls (MDD: 7.336 ± 472.9 vs 7.258 ± 434.7 ; $p < 0.8908$), as demonstrated in Figure 4A. Representative photomicrographs of stained exfoliated buccal cells for evaluation of GSH levels are shown in Figure 4B.

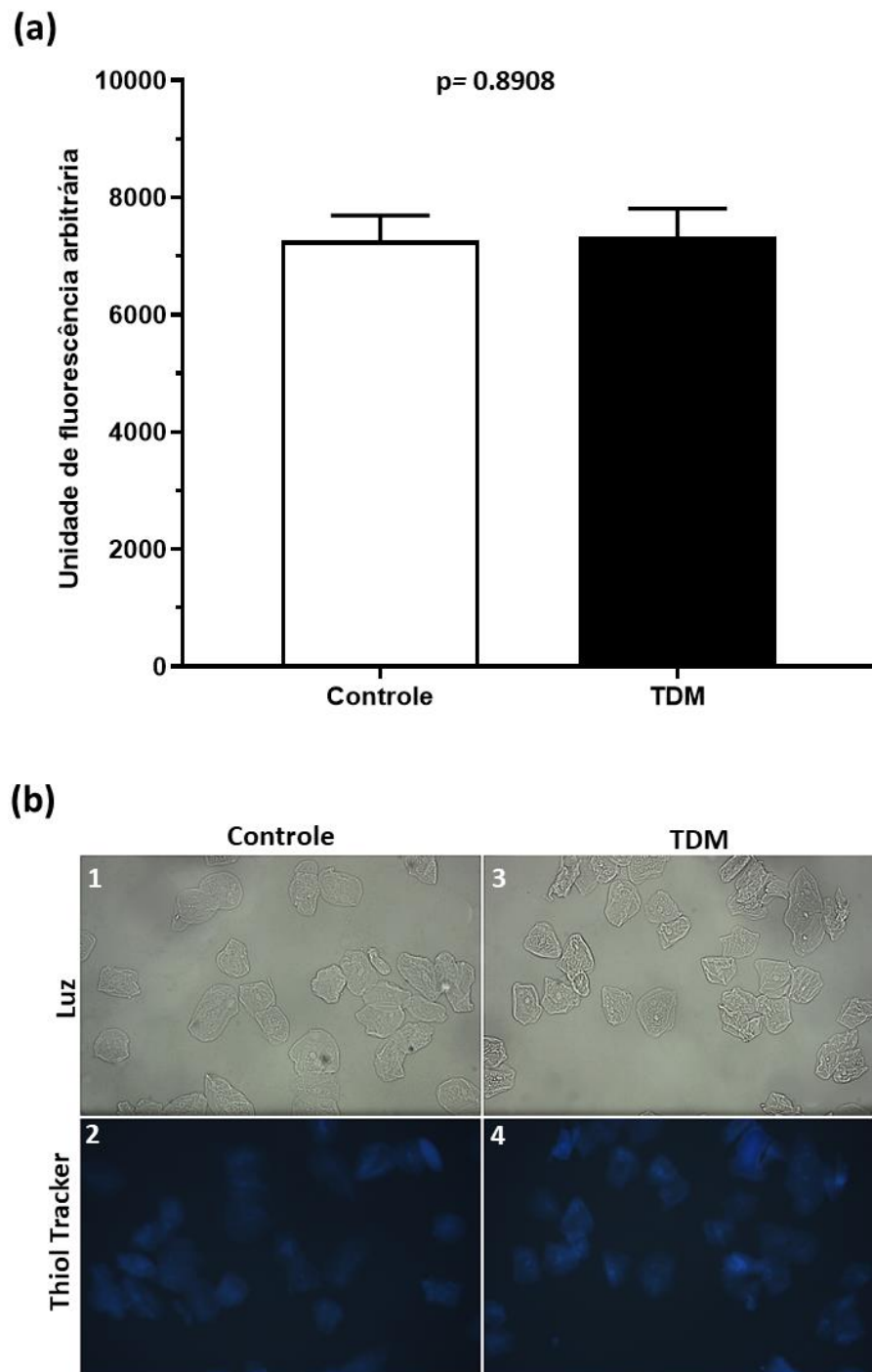


Figure 4. A comparison of intracellular expression of GSH in buccal exfoliated cells from patients diagnosed with major depressive disorder (MDD) and without the disorder (control). (a) The graph represents the mean intensities of fluorescent signals (pixels) measured in cells stained with the ThiolTracker™ Violet probe, expressed in arbitrary fluorescence units (AFU). (b) Representative photomicrographs show the ThiolTracker™ Violet staining pattern (blue) in exfoliated buccal cells in control (b-2) and MDD (b-4) groups. Figures b1 and b3 demonstrate of images of exfoliated buccal cells under light in different groups.

4 DISCUSSION

Studies have shown that patients with MDD have elevated levels of systemic oxidative stress (23). While increases in oxidative stress levels have been reported in serum/plasma and urine from MDD patients (10,24), these changes had not yet been demonstrated in oral mucosal epithelial cells. Although a minimal level of ROS is indispensable for cell survival, high levels of ROS in cellular compartments result in slow cell growth, cell cycle arrest and apoptosis (11). In this study, we demonstrated that exfoliated buccal cells from patients with MDD exhibit increased expression of this oxidative stress marker compared to volunteers without the psychiatric disorder. Elevated ROS levels were also reported in other tissues of patients with MDD, including serum (25). The molecular events that generate the oxidative stress in patients with MDD is still unknown, however, multiple studies have shown that such patients have increased levels of pro-inflammatory cytokines in the blood, which can lead to the formation and accumulation of ROS (26,27).

Excessive ROS production and accumulation can damage cellular components, including cell membranes and mitochondria, thus leading to mitochondrial dysfunction (28,29). A loss of $\Delta\psi_m$ is observed in damaged mitochondria, which leads to the opening of mitochondrial permeability transition pores and the leakage of cytochrome c into the cytoplasm (29,30). Cytochrome c is an important mediator of apoptosis, and its release into the cytosol can trigger programmed cell death (29,30). The reduction in $\Delta\psi_m$ has been reported as an early event and a possible cause of programmed cell death (31). Our results suggest that the intracellular increase in ROS levels in the buccal exfoliated cells of patients with MDD led to mitochondrial dysfunction by loss of $\Delta\psi_m$ in these patients cells. Although studies in MDD patients have not yet investigated the $\Delta\psi_m$, it is consistent in between studies that MDD is triggered, in part, by mitochondrial dysfunction (28–30,32–34). These and other studies suggest that increased ROS may contribute to mitochondrial dysfunction and neuronal death in MDD (28–30,32–34).

Mitochondrial dysfunction is associated with the regulation of the apoptosis process (35,36). Caspases 3 and 7 are the main effector caspases involved in the intrinsic pathway of apoptosis, which is triggered by cellular stress, damage, or programmed cell death (37). Our results revealed increased intracellular expression of caspase-3 and -7 in oral epithelial cells from the patients with MDD compared to control volunteers. Augmented activity of Caspase-3 and -7 was also found in the hippocampus of rats subjected to the depression model (38–40). Although caspase activity has not been reported in MDD patients, a previous study showed an association between the upregulation of 20 genes related to the apoptosis pathway in patients with MDD. (41). The increase in caspase activity observed in oral cells from MDD patients in our study may be related to the loss of $\Delta\psi_m$, which could result in the release of intermembrane cytochrome c into the cytoplasm (42,43). Cytochrome c binds to apoptotic protease activating factor 1 and recruits caspase-9 resulting in the formation of apoptosomes and activation of executor caspases-3 and -7 (37). This leads to the cleavage of various proteins, DNA

fragmentation, and cell death (30,37).

The breaking of cellular redox balance is essential for the progression of apoptosis and can be prevented by antioxidant actions (44). GSH is one of the most important intracellular antioxidants in the cell (11). In the present study, the intracellular expression of GSH in exfoliated buccal cells of patients with MDD was similar to that of voluntary controls. The relationship between MDD and antioxidant levels in the body is complex and not yet fully understood (6,10,24,45). Some studies suggest that depression may be associated with lower antioxidant levels, while other studies found no significant association (6,10,24,45,46). Studies also show that antidepressant treatment can have a positive effect, restoring antioxidant levels, which could justify our result, since the patients in this study were on antidepressant treatments (46,47). Furthermore, another explanation for our result comes from the saliva, which is an important source of antioxidants in the oral cavity, and may contribute to the local maintenance of antioxidant levels (14,18). Therefore, based on our results, we can suggest that GSH expression was not high enough to decrease ROS expression, which led to a loss of $\Delta\Psi_m$ expression and an increase of caspase-3 and -7 in patients with MDD. Thus, we can conclude that patients with MDD, even with compensatory defense mechanisms, showed mild to moderate oxidative stress in exfoliated cells of the oral mucosa.

Our results should be interpreted with some limitations. Although the sample size is consistent with that calculated by the statistics program, we were not able to explore the possible effects of some clinical parameters (eg. MDD severity and use of antidepressants) on the outcome. Finally, it is not yet possible to predict the long-term clinical consequences of increased intracellular levels of oxidative stress in patients with MDD. Pre-clinical and subsequent clinical studies may be designed for this purpose. However, the current study demonstrates that the exfoliated cells of the buccal mucosa are practical and non-invasive additional resources to assess oxidative stress in patients with MDD. Our findings showed a significant increase in ROS levels and caspase-3 and -7 activity and a significant decrease in $\Delta\Psi_m$ levels in the oral epithelial from the patients with MDD. Together, the results demonstrate for the first time the occurrence of oxidative stress in exfoliated cells of the mucosa of patients with MDD. Our data suggest the use of buccal cells as a method for assessing redox status in patients with MDD.

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ANEXO A – Parecer do CEP

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ARAÇATUBA/ UNIVERSIDADE
ESTADUAL PAULISTA "JÚLIO
DE MESQUITA FILHO"



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Alterações do estresse oxidativo em pacientes com depressão

Pesquisador: LUKAS MENDES DE ABREU

Área Temática:

Versão: 4

CAAE: 57628922.0.0000.5420

Instituição Proponente: Universidade Estadual Paulista Júlio de Mesquita Filho

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 5.719.154

Apresentação do Projeto:

A depressão é uma doença multifatorial com alta prevalência na população mundial que tem sido associada a outras comorbidades, como o câncer, e alta taxa de morbidade e mortalidade. O estresse oxidativo desempenha um papel na fisiopatologia de muitas doenças, incluindo a depressão e a ocorrência de doenças bucais, como o câncer de boca. Até o momento, não há estudos que avaliam a associação da depressão com alterações vinculadas ao estresse oxidativo na mucosa bucal. O objetivo do presente estudo é avaliar a ocorrência de estresse oxidativo e alterações morfológicas nas células da mucosa bucal de indivíduos com diagnóstico de transtorno depressivo em comparação à indivíduos com câncer de boca e indivíduos sem câncer de boca e depressão. Para isso, será realizado um estudo de caráter transversal-observacional cuja casuística será composta por 20 indivíduos com depressão (grupo caso), 20 indivíduos com câncer de boca (grupo câncer de boca) e 20 voluntários sem câncer de boca e depressão (grupo controle). Variáveis demográficas e a ocorrência ou a gravidade dos sintomas depressivos serão avaliadas. Os níveis de estresse oxidativo serão avaliados nas células coletadas da mucosa de revestimento de duas regiões

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Continuação do Parecer: 5.719.154

distintas da boca. A expressão das moléculas resultantes do estresse oxidativo (peróxido de hidrogênio, hidroxila e peroxila), glutatona reduzida e caspases 3 e 7, bem como o potencial de membrana mitocondrial serão avaliadas por imunofluorescência e as alterações celulares morfológicas através da técnica do Papanicolau. Será realizado a coleta de salivas dos dois grupos. Os valores obtidos relativos à expressão das moléculas associadas ao estresse oxidativo, as alterações celulares morfológica e os níveis de cortisol salivar serão submetidos a testes estatísticos para avaliar diferenças entre os grupos caso e controle. Esperamos que os dados do presente estudo possam trazer novas informações sobre a associação entre a depressão e alterações oxidativas da mucosa bucal.

Objetivo da Pesquisa:

Objetivo Primário:

Comparar as células da mucosa bucal de indivíduos com depressão, com indivíduos com câncer de boca e indivíduos sem câncer de boca e depressão quanto ao: Conteúdo intracelular das espécies reativas de oxigênio: Peróxido de Hidrogênio (H₂O₂), Hidroxila (HO) e Peroxila (ROO); Conteúdo intracelular de glutatona reduzida; Potencial de membrana mitocondrial e Atividade das caspases 3 e 7.

Objetivo Secundário:

Comparar as células da mucosa bucal de indivíduos com depressão, com indivíduos com câncer de boca e indivíduos sem câncer de boca e depressão Aumento da relação núcleo/citoplasma; Presença de micronúcleos; 3. Presença de núcleos aumentados; Hiper cromatismo nuclear; Alterações no padrão de distribuição da cromatina nuclear; Espessamento acentuado e irregular da membrana nuclear íntegra; Nucléolos múltiplos e proeminentes; Pleomorfismo nuclear; Mitoses atípicas e Os níveis de concentração de cortisol salivar.

Avaliação dos Riscos e Benefícios:

Riscos:

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Continuação do Parecer: 5.719.154

A participação nesta pesquisa oferece riscos mínimos, pois sua metodologia é: coleta de células esfoliadas da mucosa bucal, cuja técnica é conhecida e aplicada em laboratórios de diagnóstico. Neste projeto, a coleta de células será realizada por profissionais capacitados. O teste psicológico que será utilizado é validado na população brasileira e será aplicado por profissional treinado. Nenhum dos procedimentos que serão realizados irá oferecer riscos à dignidade do paciente. Todas as informações coletadas neste estudo serão estritamente confidenciais.

Benefícios:

Será oferecida ao grupo Caso e Controle uma avaliação clínica intrabucal com o objetivo de realizar o diagnóstico precoce de lesões benignas e malignas. Caso for identificada alguma lesão bucal o voluntário será encaminhado para tratamento na FOA/UNESP. Esperamos que os resultados do presente estudo possam trazer maiores evidências sobre a possível participação de fatores psicológicos ainda não investigados na etiopatogenia do câncer de boca. Além disso, os resultados poderão, em longo prazo, estimular o desenvolvimento de novos mecanismos psiconeurobiológicos, relacionados às alterações moleculares da mucosa bucal e consequente predisposição para doenças bucais, podem ser propostos.

Comentários e Considerações sobre a Pesquisa:

Nada a declarar

Considerações sobre os Termos de apresentação obrigatória:

Nada a declarar

Conclusões ou Pendências e Lista de Inadequações:

Nada a declarar

Considerações Finais a critério do CEP:

Salientamos que, de acordo com a Resolução 466 CNS, de 12/12/2012 (título X, seção X.1., art. 3, item b, e, título XI, seção XI.2., item d), há necessidade de apresentação de relatórios semestrais, devendo o primeiro relatório ser enviado até 01/03/2023. O CEP reitera a necessidade da entrega de 01 via (Não podendo ser Cópia) rubricada e assinada do TCLE.

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Continuação do Parecer: 5.719.154

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Recurso do Parecer	recurso.pdf	29/08/2022 10:47:44		Aceito
Projeto Detalhado / Brochura Investigador	Projetodepesquisadetalhado...pdf	29/08/2022 10:47:37	LUKAS MENDES DE ABREU	Aceito
Cronograma	CRONOGRAMA.pdf	29/08/2022 10:47:22	LUKAS MENDES DE ABREU	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	29/08/2022 10:46:58	LUKAS MENDES DE ABREU	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	29/08/2022 10:43:19	LUKAS MENDES DE ABREU	Aceito
Recurso do Parecer	recurso.pdf	31/08/2022 11:31:27		Aceito
Recurso do Parecer	recurso.pdf	02/08/2022 10:18:58		Aceito
Recurso Anexado pelo Pesquisador	Recurso_Lukas.pdf	02/08/2022 10:18:44	LUKAS MENDES DE ABREU	Aceito
Informações Básicas do Projeto	PB_INFORMACOES_BASICAS_DO_P ROJETO_1853595.pdf	15/03/2022 10:46:08		Aceito
Outros	HADS.pdf	15/03/2022 10:41:12	LUKAS MENDES DE ABREU	Aceito
Folha de Rosto	Folhaderosto.pdf	09/03/2022 11:42:26	LUKAS MENDES DE ABREU	Aceito
Outros	COPARTICIPANTES.pdf	09/03/2022 11:40:45	LUKAS MENDES DE ABREU	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

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Continuação do Parecer: 5.719.154

ARACATUBA, 24 de Outubro de 2022

Assinado por:
André Pinheiro de Magalhães Bertoz
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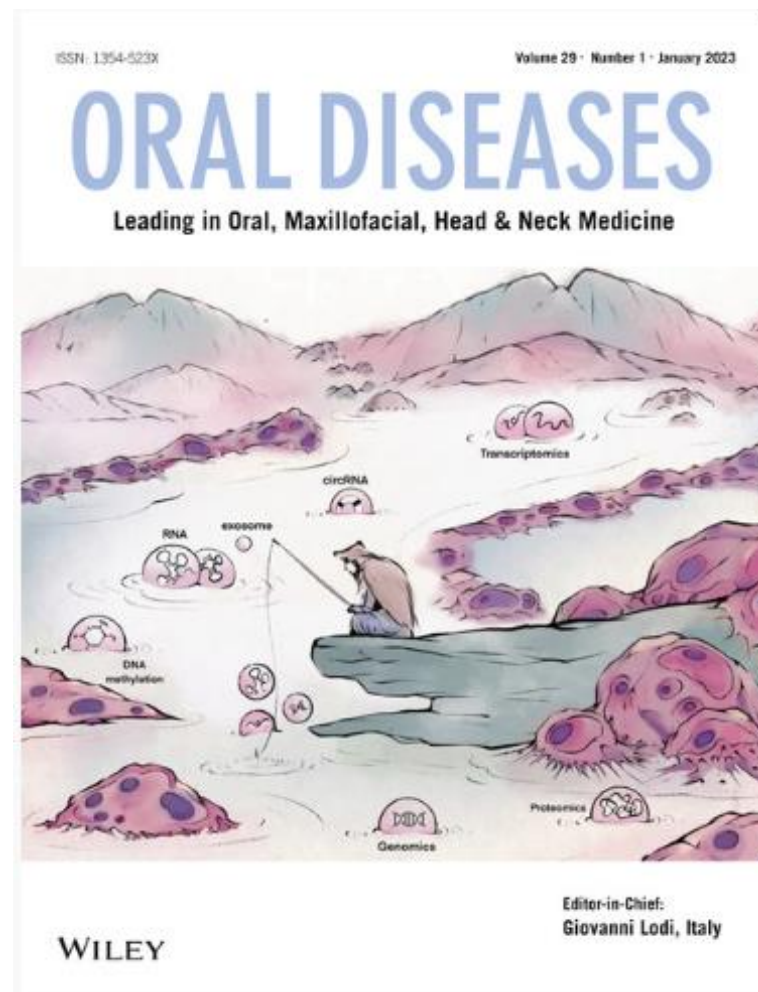
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ANEXO B – Normas de Publicação da Oral Diseases



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