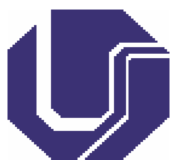

UNIVERSIDADE FEDERAL DE UBERLÂNDIA

FACULDADE DE ODONTOLOGIA

JÉSSICA RIBEIRO DAMASCENO

DETERMINAÇÃO DE BIOMARCADORES SALIVARES DE ESTRESSE OXIDATIVO, NÍVEIS DE ÓXIDO NÍTRICO E ATIVIDADE DA ALFA-AMILASE EM INDIVÍDUOS COM E SEM CÁRIE

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Trabalho de conclusão de curso apresentado à Faculdade de Odontologia da UFU, como requisito parcial para obtenção do título de Graduado em Odontologia

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RESUMO

DETERMINAÇÃO DE BIOMARCADORES SALIVARES DE ESTRESSE OXIDATIVO, NÍVEIS DE ÓXIDO NÍTRICO E ATIVIDADE DA ALFA-AMILASE EM INDIVÍDUOS COM E SEM CÁRIE

A cárie é uma doença resultante da desmineralização das estruturas dentais, em que a saliva desempenha função fundamental por sua característica tamponante. Além disso, a saliva possui em sua composição substâncias antimicrobianas e antioxidantes, fazendo parte dos mecanismos de defesa contra o estresse oxidativo e nitrosativo decorrente da geração de espécies reativas de oxigênio (ROS) e nitrogênio (RNS) nas doenças sistêmicas e bucais. Assim, este estudo teve como objetivo quantificar os marcadores de estresse oxidativo, níveis óxido nítrico (NO) e atividade da alfa-amilase (AA) na saliva de indivíduos com cárie e, com isso, estabelecer as características da cavidade oral relacionadas à esta doença. Foram coletadas amostras de saliva de pacientes com (n=30) e sem cárie (n=30), determinado o fluxo salivar e mensurado o pH. Foram realizadas as análises de: peroxidação lipídica (TBARS), capacidade antioxidante total (FRAP), níveis de glutationa reduzida (GSH), atividade de superóxido dismutase (SOD), atividade de catalase (CAT), atividade de AA e níveis de NO. Os resultados foram expressos em média ± EPM e comparados pelo teste t de student ($p<0,05$). Não foram observadas diferenças entre os grupos nas análises de fluxo, pH, TBARS, FRAP, GSH, AA e NO. No entanto, houve aumento da atividade de SOD e CAT no grupo cárie. A elevação da atividade destas enzimas pode estar relacionada à maior produção de radicais livres nesse grupo e possivelmente consiste em um mecanismo compensatório inicial responsável pela diminuição dos danos oxidativos. Tais resultados nos permite a melhor compreensão dos mecanismos de estresse oxidativo envolvidos nesta doença e fornece características bioquímicas orais relacionadas ao estabelecimento da cárie.

Palavras-chave: Saliva, radicais livres, status antioxidante

DETERMINATION OF SALIVARY BIOMARKERS OF OXIDATIVE STRESS, NITRIC OXIDE LEVELS AND ALPHA-AMYLASE ACTIVITY IN INDIVIDUALS WITH AND WITHOUT CARIES

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Short title: Salivary biomarkers for caries

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Abstract

Dental caries is a disease resulting from the demineralization of dental structures, in which saliva plays a fundamental role due to its buffering properties. In addition, saliva has antimicrobial and antioxidant substances in its composition, being part of defense mechanisms against the oxidative and nitrosative stress caused by the increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in systemic and oral pathologies. Thus, this study aimed to quantify the markers of oxidative stress, nitric oxide levels (NO) and alpha-amylase activity (AA) in saliva of individuals with and without caries, and therefore establish the characteristics of the oral cavity related to this disease. Saliva samples were collected from patients with (n=30) and without caries (n=30), the pH and salivary flow were measured and the following analyses were performed: lipid peroxidation (TBARS), total antioxidant capacity (FRAP), reduced glutathione levels (GSH), superoxide dismutase activity (SOD), Catalase activity (CAT), AA activity and NO levels. Results were expressed as mean ± SEM and compared by the student's t test ($p<0.05$). No differences were observed between the groups in the analyses of pH, salivary flow, TBARS, FRAP, GSH, AA and NO. It was observed an increase in SOD and CAT activities in the caries group. The increased activity of these enzymes may be related to the higher production of free radicals in this group and possibly consists of an initial compensatory mechanism responsible for the reduction of oxidative damage. These results allow us to better understand the mechanisms of oxidative stress involved in this disease and provide oral biochemical characteristics related to the establishment of caries.

Keywords: Saliva**,** free radicals, antioxidant status,

49 **Introduction**

50 Dental caries is a multifactorial and dynamic disease arising from the interaction of
51 biofilm present on the tooth surface, diet sugars and microorganisms that result in the
52 demineralization of the enamel (Pitts *et al.*, 2017). This occurs due to the fermentation of
53 carbohydrates ingested in the diet, promoted by *Streptococcus mutans*, *Streptococcus sobrinus*
54 and *Lactobacillus spp.* These microorganisms produce acids, increasing the concentration of
55 hydrogen ions that will promote the dissolution of minerals present in the dental structure,
56 weakening the enamel and facilitating its deterioration, thus favoring the development of caries
57 (Leites, Pinto and Sousa, 2006). The presence of microorganisms is essential for the appearance
58 of caries, however, their development is the result of a complex interaction between diet, host
59 susceptibility, the presence of a specific microbiota and time (Twetman, 2015).

60 Saliva is a primary secretion for the maintenance of oral health and has several functions,
61 such as facilitating chewing and swallowing, phonation and also protecting the oral
62 environment from possible infections, due to its antibacterial characteristics. In addition, this
63 fluid has a direct relationship with the process of demineralization of dental structure and
64 consequently with the establishment of diseases such as caries, mainly due to its buffering
65 capacity that promotes resistance to variations in pH of the oral environment (Pinheiro, 1982).

66 The collection of salivary samples is simple and non-invasive, and this fluid contains
67 specific biomarkers that allow laboratory investigations and possible diagnoses (Malamud,
68 2011). Saliva composition can be affected by local oral status, enabling the use of this fluid for
69 diagnosis and monitoring oral diseases, such as periodontitis, caries, oral precancerous, and
70 other local oral pathologies that are associated with oxidative stress (Tothova *et al.*, 2015).

71 Oxidative stress is characterized by an imbalance between oxidants and antioxidants in
72 favor of oxidants, causing a disturbance of signaling and redox control, what can leads to the
73 installation and development of inflammatory oral diseases (Gutteridge and Halliwell, 2018).

74 The salivary fluid presents enzymatic and non-enzymatic components (antioxidants) that avoid
75 possible complications of oxidation reactions, being the first line of defense against oxidative
76 stress (Battino *et al.*, 2002).

77 The antioxidant system has mechanisms that are capable of neutralizing ROS and
78 attenuate oxidative damage (Michiels *et al.*, 1994; Sies, 1997). The enzyme superoxide
79 dismutase (SOD) is responsible for catalyzing the dismutation of the superoxide anion (O^{2-})
80 into oxygen (O^2) and hydrogen peroxide (H_2O_2) (Gilgun-Sherki, Melamed and Offen, 2001) .
81 This compound is then neutralized mainly by the glutathione system and the catalase (CAT)
82 activity (Dringen, 2000). In the glutathione system, glutathione peroxidase (GPx) converts
83 reduced glutathione (GSH) into oxidized glutathione (GSSG), removing H_2O_2 and forming H_2O
84 (Schneider and Oliveira, 2004). The CAT enzyme catalyzes the decomposition of hydrogen
85 peroxide (H_2O_2), transforming it into H_2O and O_2, thus acting as an antioxidant protection
86 system (Schanaider, 2010).

87 Another enzyme related to caries is salivary alpha-amylase. This protein plays role in
88 the degradation of carbohydrates in the oral cavity as an energy source for several
89 microorganisms, including *S. mutans*. There are studies that affirm that alpha-amylase binds
90 in this bacterium and favors its growth and nutrition, being also found in the bacterial plaque in
91 the tooth enamel, which favors the demineralization of the tooth (Scannapieco, Torres and
92 Levine, 1993). However, there are controversies regarding the influence of alpha amylase on
93 the development of dental caries (Borghi *et al.*, 2017).

94 In saliva it's possible to also find nitrate and nitrite, which are nitric oxide (NO)
95 metabolites. NO is involved in many biological and physiological processes, such as
96 homeostasis and vascular regulation, but its relationship with oral physiological and
97 pathological conditions is less studied. NO is a short-lived gas that participates in nonspecific
98 defense mechanisms. This compound can easily penetrate the bacteria membrane and cause

99 severe damage through different pathways, such as inhibition of cellular respiration in
100 mitochondria (Mobarak and Abdallah, 2011), besides increasing cytotoxicity mediated by
101 saliva macrophages (Eagappan *et al.*, 2016). There are studies that affirm that increased
102 concentrations of NO can be bacteriostatic and even bactericidal for bacteria that cause caries
103 (Doel *et al.*, 2004) .

104 Considering all this, the analysis of salivary biomarkers in individuals with caries could
105 promote a better understanding about the biochemical mechanisms involved in this disease and
106 could be useful to establish the characteristics of the oral cavity that promote the development
107 of caries. Thus, the aim of this work was to evaluate different biomarkers of oxidative stress,
108 nitric oxide and amylase activity in the saliva of individuals with and without caries.

109

110 Materials and methods

111 *Subjects*

112 This study was performed with 60 adults with (n=30) and without caries (n=30) that
113 attended at the dental department of the Clinical Hospital of the Federal University of
114 Uberlândia. Clinical examination was performed by dentists to verify the number of caries
115 lesions.

116 All experimental procedures were carried out in accordance with The Code of Ethics
117 of the World Medical Association (Declaration of Helsinki) and were approved by the
118 Institutional Review Board of the Federal University of Uberlândia (nº090690). The subjects
119 were given informational briefings, and they provided voluntary, written informed consent for
120 participation.

121 *Saliva samples*

122 Unstimulated saliva samples were collected in plastic tubes by spitting method for 2
123 minutes. Volunteers rinsed their mouth with water and they were instructed swallow the
124 remaining water in the oral cavity and to wait a minute before saliva collection. All collection
125 procedures were performed in the morning.

126 Salivary pH was determined using a pH meter (GEHAKA/ PG1800). To determine the
127 salivary flow, the initial weight of the tubes was discounted from their weight after collection.
128 The resulting weight was divided by the total collection time. Saliva samples were centrifuged
129 at 3000 rpm at 4°C for 20 minutes, and the supernatant was aliquoted. All samples were kept
130 frozen at -80°C until analysis. All biochemical determinations were done in duplicates.

131 ***Salivary total protein***

132 Determination of total protein concentration was performed by the Bradford method,
133 using bovine serum albumin as standard (Bradford, 1976).

134 ***Salivary alpha-amylase activity***

135 The saliva samples were diluted in MES buffer (50 mM MES, 300 mM NaCl, 5 mM
136 $CaCl_2$ and 140 mM KSCN; pH 6.3), and pipetted into a microplate; then, pre-heated (37°C)
137 substrate solution (2-chloro-4-nitrophenyl-β-D-galactopyranosylmaltoside: GALG2-CNP) was
138 added. The optical density was read at 405 nm in one-min intervals for three minutes at 37°C
139 uing a microplate reader, then the enzyme activity was determined (Granger *et al.*, 2007).

140 ***Salivary nitric oxide (NO)***

141 Nitrite concentration, an indicator of the production of NO, was determined by a
142 colorimetric assay using the Griess reaction. Equal volumes of saliva and Griess reagent (1%
143 sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% phosphoric
144 acid) were mixed at room temperature. Absorbance was measured at 570 nm using a microplate

145 reader. The content of nitrite was calculated based on a standard curve constructed using sodium
146 nitrite ($NaNO_2$) (Bryan and Grisham, 2007).

147 ***Salivary biomarkers of oxidative stress***

148 ***Thiobarbituric acid reactive substances (TBARS)***

149 Lipid peroxidation was measured by the reaction between thiobarbituric acid (0.67%
150 TBA) and malondialdehyde (MDA). In the organic phase, fluorescence was measured at 515
151 nm (excitation) and 553 nm (emission). A standard curve of MDA allowed the quantification
152 of this compound in saliva samples by linear regression. The TBARS levels were calculated as
153 μmol TBARS/mg protein (Yagi, 1998).

154 ***Total antioxidant capacity by ferric reducing antioxidant power (FRAP) analysis***

155 The total antioxidant capacity was evaluated by the capacity of the samples to reduce
156 Fe^{+3} to Fe^{+2}, which is chelated by TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) leading to the
157 formation of an intense blue Fe+2-TPTZ complex. This complex was read on the
158 spectrophotometer at 593 nm (Teixeira *et al.*, 2017).

159 ***Reduced glutathione (GSH) concentration***

160 The protein content of the samples was initially precipitated by metaphosphoric acid
161 (MPA) in the ratio 1:1 (saliva/MPA). The samples were centrifuged at 7000 × g for 10 min.
162 The supernatant was collected and mixed with sodium phosphate buffer (100 mM, pH 8.0),
163 containing 5 mM EDTA and ortho-phthaldialdehyde (1 mg/mL in methanol). The mixture was
164 incubated in the dark at room temperature for 15 min and fluorescence was measured at 350
165 nm (excitation) and 420 nm (emission). A standard curve of GSH (0.001–0.1 mM) was used
166 for the linear regression (Teixeira *et al.*, 2017).

167 ***Superoxide Dismutase activity (SOD)***

168 The activity of SOD was evaluated by inhibiting the autoxidative capacity of pyrogallol
169 by the SOD present in the samples. The samples were mixed with Tris-HCl buffer 50 mmol.L-
170 1 (pH 8.2) containing 1 mmol.L-1 EDTA to deactivate metal-dependent enzymes such as
171 metaloproteases, 80 U.mL-1 CAT and 24 mmol.L-1 pyrogallol, and the activity of SOD was
172 evaluated by a spectrophotometer at 420 nm for 10 minutes, using an analytical curve built with
173 SOD as standard (Justino *et al.*, 2017).

174 ***Catalase activity (CAT)***

175 CAT activity evaluation was based upon hydrogen peroxide decomposition by CAT
176 present in the samples. Samples were mixed with 10 mmol.L^{-1} potassium phosphate buffer pH
177 7.0 containing 0.2% hydrogen peroxide. The hydrogen peroxide decomposition was monitored
178 at 240 nm during 10 min (Justino *et al.*, 2017).

179 **Results**

180 ***Clinical examination and sample characterization***

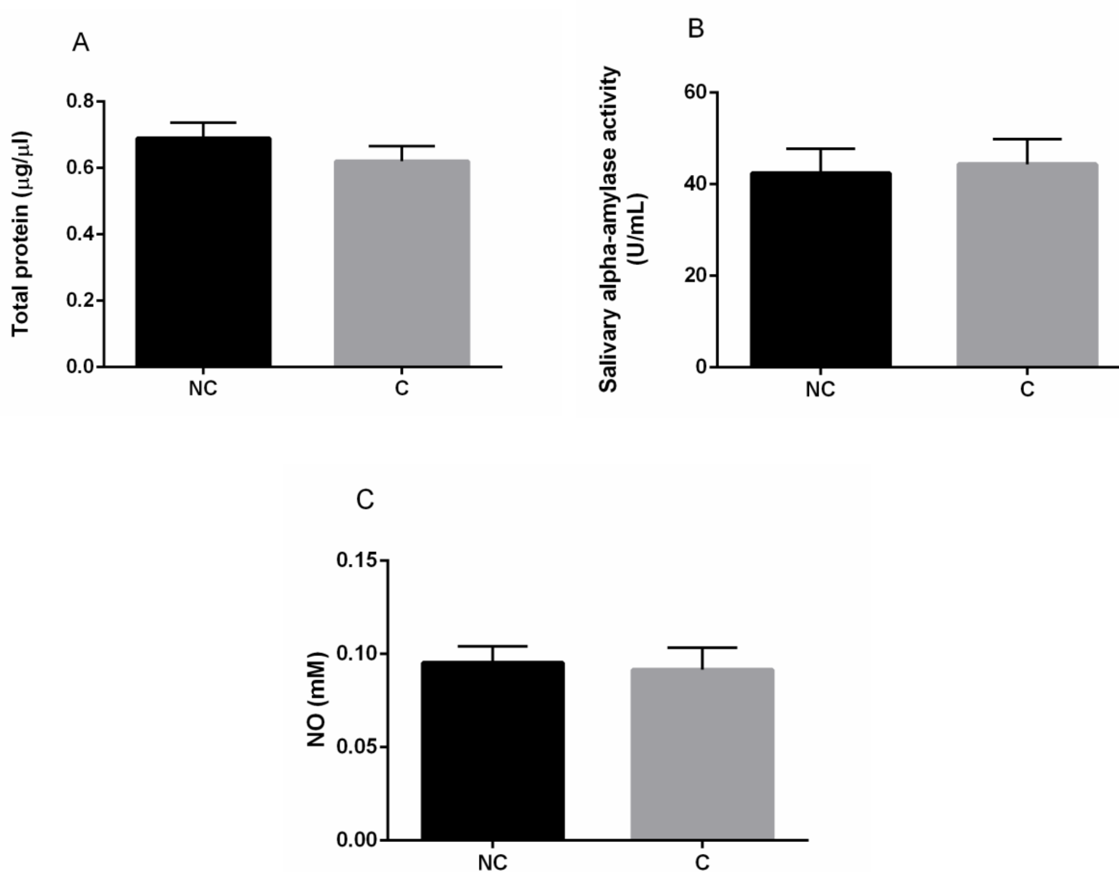
181 Table 1 shows the sample characterization of the caries (C) and non-caries (NC) groups.

Parameters	Non-caries (NC)	Caries (C)
Men (%)	51%	51%
Women (%)	49%	49%
Mean of caries lesions	-	4,17± 2,79
Salivary pH	6,83± 0,35	6,98± 0,48
Salivary flow (mL/min)	0,67± 0,36	0,66±0,52

182 Table 1. Sample characterization of the caries (C) and non-caries (NC) group. Note: Values of number
183 of caries lesions, salivary pH and flow are expressed as mean ± S.D. *p < 0. 05 vs NC group; Non-caries
184 group (NC) (n=30), Caries group (C) (n=30).

185 The percentage between genders was the same in both groups. The number of carious
186 lesions found in group C was on average 4.17 lesions per individual. Interestingly, there was
187 no difference between the groups in the salivary pH and flow analysis.

188 Figure 1 shows the total protein (A), the salivary alpha-amylase activity (B) and NO
189 concentration in saliva (C). The salivary total protein concentration and alpha-amylase activity
190 showed no difference between the NC and C groups. Similarly, there was no difference between
191 the groups regarding the concentration of NO.



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199 Fig. 1. Salivary total protein (A), salivary alpha-amylase activity (B), salivary nitric oxide (NO) in non-
200 caries (NC) and caries (C) group. Values expressed as mean ± S.E.M. *p < 0. 05 vs NC group; Student's
201 t-test; NC: n=30; C: n=30).

202

203 Non-enzymatic salivary biomarkers of oxidative stress are shown in figure 2. It was
204 observed that there was no difference between the NC and C groups, regarding the levels of
205 lipid peroxidation (TBARS) (Figure 2-A), total antioxidant capacity (FRAP) (Figure 2-B) and
206 salivary GSH levels (Figure 2-C).
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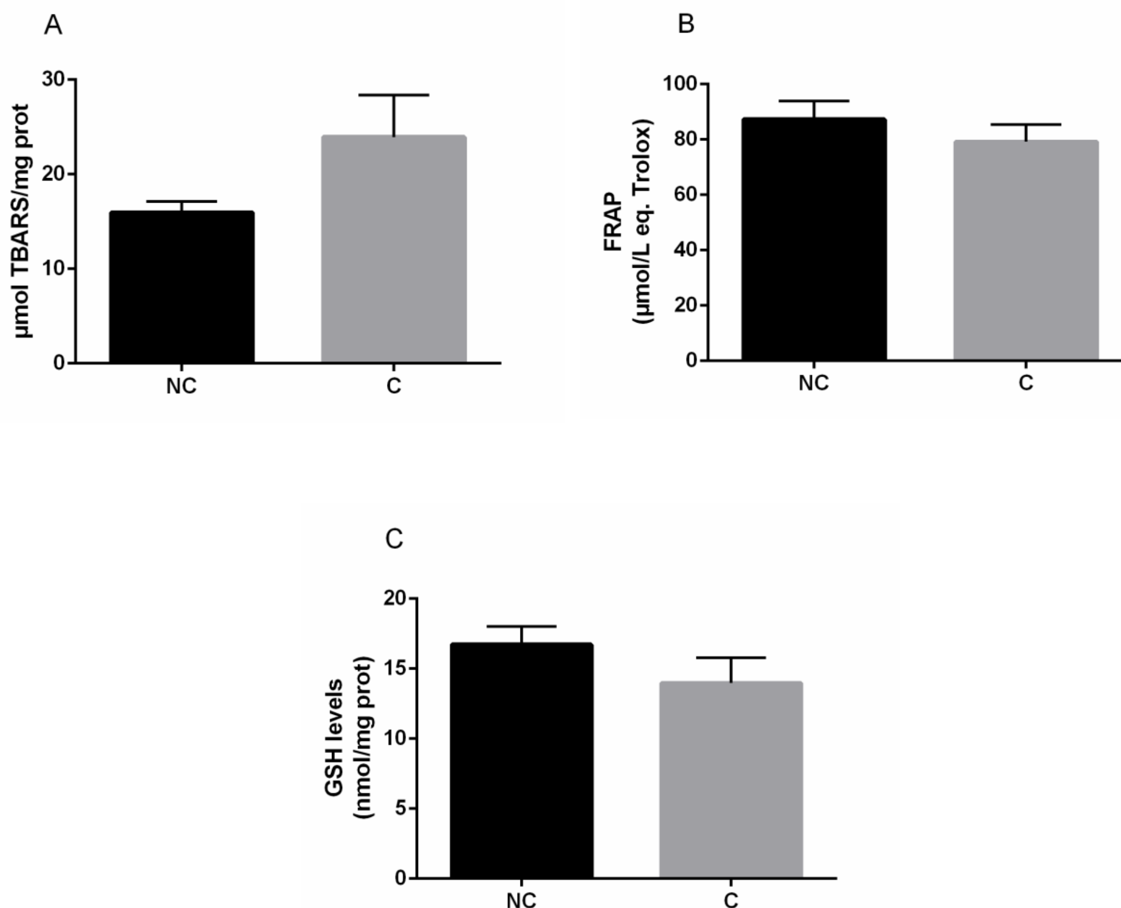

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215
216 Fig. 2. Non-enzymatic salivary biomarkers of oxidative stress analysis in saliva of NC and C groups.
217 Levels of lipid peroxidation (TBARS) (A); Total antioxidant capacity by ferric reducing antioxidant
218 power (FRAP) (B) and levels of reduced glutathione (GSH) (C). Values expressed as mean ± S.E.M. *p
219 < 0. 05 vs NC group; Student's t-test; NC: n=30; C: n=30).

220

221 Enzymatic salivary biomarkers of oxidative stress are shown in figure 3. An increase in
222 SOD activity was observed in the C group compared to NC group ($p<0.05$) (Figure 3-A). Such
223 increase was also verified regarding the activity of CAT ($p<0.05$) (Figure 3-B).

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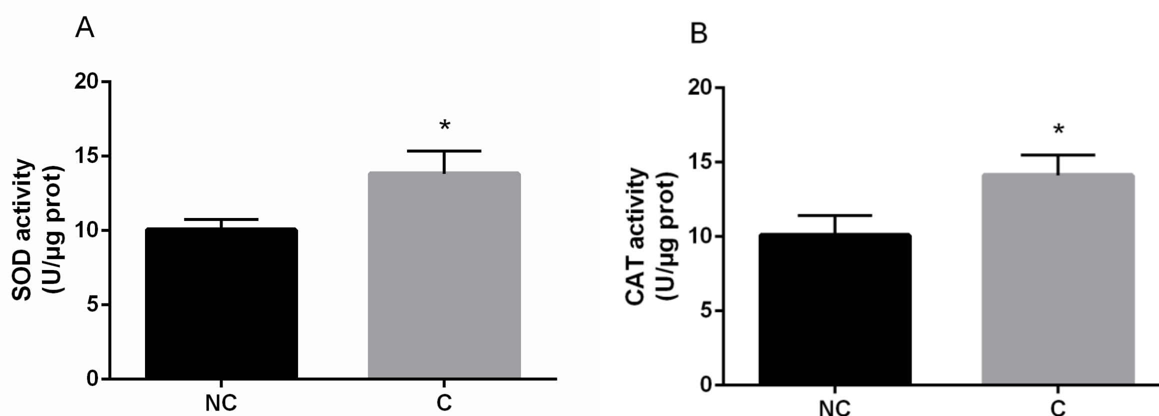

225

226 Fig. 3. Enzymatic salivary biomarkers of oxidative stress analysis in saliva of NC and C groups.
227 Superoxide dismutase activity (SOD) (A); Catalase activity (CAT) (B). Values expressed as mean ±
228 S.E.M. *$p < 0.05$ vs NC group; Student's t-test; NC: n=30; C: n=30).

229

230 **Discussion**

231 The development and progression of dental caries can be attributed to damage caused
232 by oxidative stress and the lack of compensation of the body's antioxidant system. In our study,
233 we analyzed salivary samples of adults with and without caries, evaluating the amount of total
234 protein, alpha-amylase activity and NO concentration of the groups. In addition, we analyzed
235 enzymatic salivary biomarkers (SOD and CAT activity) and non-enzymatic biomarkers
236 (TBARS, FRAP and GSH levels) of oxidative stress. Our results showed increased biomarkers
237 of oxidative stress (SOD and CAT), as possible compensatory mechanism against oxidative
238 damage.

239 Regarding salivary flow, there was no significant difference between the NC and C
240 groups. These data corroborate findings of other study in which the association of the salivary
241 flow rate with the development of caries was not established after analysis of the groups (Öztürk
242 *et al.*, 2008). On the other hand, a study conducted by Pyati et al. (2018) showed a significant
243 difference in salivary flow between groups of children with and without caries. Some studies
244 state that the low salivary flow acts as a risk factor for the appearance of caries, since a higher
245 flow can promote the dilution of the bacterial substrate and can also act as a carrier mechanism
246 of protective components for the dental surface, preventing demineralization (Pyati *et al.*,
247 2018).

248 In addition, studies that also found no difference in salivary flow in the group of carious
249 individuals suggest that the results that associate the decrease in salivary flow with the
250 development of caries are inconsistent and a high rate of salivary flow may not interfere in the
251 individual's resistance to caries (Farsi, 2008; Cunha-Cruz *et al.*, 2013).

252 In our study, no significant differences were found in the analysis of salivary pH
253 between the groups. This result corroborates the one found by ÖZTüRK et al. (2008) in which
254 there was no difference in salivary pH between the groups with and without caries (Öztürk *et*
255 *al.*, 2008). This result, however, was not the expected since the pH tends to decrease with the
256 proliferation of bacteria and the increase of their metabolites (Kawashima *et al.*, 2003; Shi *et*
257 *al.*, 2007; Seki *et al.*, 2006). The lack of difference observed in salivary pH may be due to
258 different hygiene habits, diet and severity of caries lesions, parameters not taken in
259 consideration here.

260 The concentration of total protein in the saliva of the patients analyzed did not show
261 significant difference between the NC and C groups. This data corroborates the study conducted
262 by Rao et al. (2008) in which no differences were observed between the groups (Roa *et al.*,

263 2008). On the other hand, other studies report a difference in the concentration of total protein
264 in the saliva of patients with and without caries, as presented in the study of Vibhakar et al.
265 (2013) (Vibhakar *et al.*, 2013). The study conducted by Hemadi et al.(2017), suggests that
266 further studies should be done to determine the value of total protein concentration as a
267 predictor of tooth decay in children (Hemadi *et al.*, 2017). In addition, a systematic review
268 carried out by Martins et al.(2013), concluded that there is insufficient evidence to establish
269 salivary proteins as a biomarker for dental caries (Martins *et al.*, 2013).

270 The analysis of salivary amylase activity showed no difference between groups. This
271 study corroborates the one conducted by De Farias et al. (2003) in which they observed similar
272 amylase activity in children with and without caries (de Farias and Bezerra, 2003). On the other
273 hand, our results differs from that found by Singh et al. (2015) in which the concentration of
274 alpha amylase was higher in the group with caries compared to the group without the disease
275 (Singh *et al.*, 2015). The study conducted by Borghi et al. (2017), showed that alpha amylase
276 activity was significantly higher in the saliva of children who did not have the disease (Borghi
277 *et al.*, 2017). This shows the controversy regarding the influence of alpha amylase on the
278 development of caries.

279 The concentration of NO showed no differences between groups. Our results
280 corroborate the study conducted by Aksit-Bicak et al. (2019) (Aksit-Bicak *et al.*, 2019).
281 According to the study, the association of NO levels and the presence of caries cannot be
282 considered specific. On the other hand, a study conducted for Bayindir et al. (2005) showed
283 that NO levels were significantly higher in the group of individuals with poor oral hygiene and
284 with more caries lesions (Bayindir, Polat and Seven, 2005). This may have contributed to our
285 results, since most individuals in group C brushed their teeth before collection (data not
286 showed) and presented a variable number of lesions.

287 TBARS method is a potential biomarker of oxidative stress in the oral cavity (Behuliak
288 *et al.*, 2009). In our study, although TBARS levels were higher in the caries group, there were
289 no statistical difference. Another study by Öztürk et al. (2008) also reported similar levels of
290 lipid peroxidation in saliva of the groups analyzed (Öztürk *et al.*, 2008). In contrast to our
291 results, some studies found significantly higher levels of malondialdehyde in the saliva of caries
292 patients compared to the control group (Ahmadi-Motamayel *et al.*, 2018; Sarode *et al.*, 2012).
293 Besides that, oral hygiene, by tooth-brushing, reduces salivary TBARS (Hodosy and Celec,
294 2005). This may have contributed to the results of our study, since most of the group C
295 individuals reported that they brushed their teeth before collection (data not showed).

296 Regarding total antioxidant capacity, there was no difference between groups NC and
297 C. This result is in contrast to that obtained in the study by Muchandi, et al. (2015), in which it
298 was demonstrated that the total antioxidant capacity in the saliva of patients with caries was
299 higher compared to the saliva of patients that did not present the lesions (Muchandi *et al.*,
300 2015). According to the study by Patel et al. (2015), this increase in the total antioxidant
301 capacity observed in individuals with caries may be related to inadequate oral cavity hygiene
302 and also to increased age. In addition, the study suggests that there is a linear correlation
303 between the total antioxidant capacity in saliva and the presence of caries (Patel and Pujara,
304 2015).

305 Our study did not find a significant difference between the NC and C groups in relation
306 to the levels of GSH. The role of GSH as an antioxidant in relation to caries was first studied
307 by Öztürk et al. (2008) that found lower levels of GSH in the group of individuals with caries
308 compared to the non-caries group (Öztürk *et al.*, 2008). On the other hand, one study conducted
309 with children found higher levels of GSH in the group of individuals with the disease (Han *et*
310 *al.*, 2013). Therefore, these results may suggest that the influence of GSH on the caries
311 development process is not completely explained yet.

In our study there was a significant increase in SOD activity in group C compared to NC. This result corroborates with others that showed an increase in the activity of SOD in the saliva of patients with caries, compared to patients that do not have the disease (Halliwell, 1999; da Silva *et al.*, 2016). Other study suggests that the increase in SOD activity may be a result of a compensatory mechanism to reduce oxidative damage, showing the protective function of SOD against the deleterious effects of reactive oxygen and nitrogen species (da Silva *et al.*, 2016).

In addition to SOD, there was also an increase in the activity of CAT in the saliva of individuals in group C. This result corroborates with Al-Souz et al. (2015), that demonstrated the activity of the CAT is higher in individuals with caries, compared to those who do not have the disease (Al-Souz and Al-Obaidi, 2015) . The study presents the increased activity of CAT as a defense mechanism against oxidative stress.

The significantly increased activity of SOD and CAT enzymes in carious individuals may have occurred as an initial defense mechanism against oxidative stress, preventing the alteration of other biomarkers and oxidative damage, since these enzymes act through mechanisms that prevent and/or control the formation of free radicals and non-radical species that are involved with the initiation of chain reactions (Barbosa *et al.*, 2010). Therefore, this may have prevented more damage, such as the lipid peroxidation (TBARS levels). To our knowledge, this study was the first to analyze all these parameters together (enzymatic and non-enzymatic biomarkers of oxidative stress, nitric oxide level and alpha amylase activity) in a significant number of salivary samples.

Another possible explanation for the lack of differences in the others biomarkers analyzed is the heterogeneity of the samples, since group C included individuals who had different stages of caries, and ~~the groups~~ included individuals of different sexes, who had

336 distinct eating and hygiene habits. For future perspectives, we suggest a next study
337 differentiating the biochemical mechanisms in the different clinical stages of caries for
338 monitoring the onset and progression of the disease. And in this way, to establish oral conditions
339 that can enable the appearance of caries and consequently in its development, interfering in its
340 prevention and treatment.

341 In conclusion, it was observed that individuals with caries had greater activity of
342 oxidative stress enzymes (CAT and SOD), which may have played a role as an initial defense
343 mechanism against oxidative stress, preventing the alteration of other biomarkers and oxidative
344 damage. In this way, this study contributed to the better understanding of the biochemical
345 mechanisms involved in this disease, highlighting the relationship between the enzymatic
346 imbalance linked to oxidative stress.

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350 **Statement of Ethics**

351 All experimental procedures were carried out in accordance with The Code of Ethics of
352 the World Medical Association (Declaration of Helsinki) and were approved by the Institutional
353 Review Board of the Federal University of Uberlandia (nº090690). The subjects were given
354 informational briefings, and they provided voluntary, written informed consent for
355 participation.

356 **Disclosure Statement**

357 The authors declare that there is no conflict of interest regarding the publication of this
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Author Contributions

JRD performed sample collection, the biochemical analysis and the discussion of the manuscript; AVS performed the biochemical analysis and discussion; RRT designed the study; LGP performed the biochemical and data analysis; DCC performed the biochemical analysis and discussion of the article; FSE designed the study and helped in the discussion of the article.

References

Ahmadi-Motamayel, F., Goodarzi, M. T., Mahdavinezhad, A., Jamshidi, Z. and Darvishi, M. (2018) 'Salivary and Serum Antioxidant and Oxidative Stress Markers in Dental Caries', *Caries Res,* 52(6), pp. 565-569.

Aksit-Bicak, D., Emekli-Alturfan, E., Ustundag, U. V. and Akyuz, S. (2019) 'Assessment of dental caries and salivary nitric oxide levels in children with dyspepsia', *BMC Oral Health,* 19(1), pp. 11.

Al-Souz, B. T. and Al-Obaidi, W. A. (2015) 'Salivary antioxidants in relation to dental caries among a group of lead-acid batteries factory workers', *Journal of Baghdad College of Dentistry,* 325(2218), pp. 1-10.

Barbosa, K. B. F., Costa, N. M. B., Alfenas, R. d. C. G., De Paula, S. O., Minim, V. P. R. and Bressan, J. (2010) 'Oxidative stress: concept, implications and modulating factors', *Revista de Nutrição,* 23(4), pp. 629-643.

Battino, M., Ferreiro, M. S., Gallardo, I., Newman, H. N. and Bullon, P. (2002) 'The antioxidant capacity of saliva', *Journal of Clinical Periodontology: Review article,* 29(3), pp. 189-194.

Bayindir, Y. Z., Polat, M. F. and Seven, N. (2005) 'Nitric oxide concentrations in saliva and dental plaque in relation to caries experience and oral hygiene', *Caries research,* 39(2), pp. 130-133.

Behuliak, M., Pálffy, R., Gardlík, R., Hodosy, J., Halčák, L. and Celec, P. (2009) 'Variability of thiobarbituric acid reacting substances in saliva', *Disease markers,* 26(2), pp. 49-53.

Borghi, G. N., Rodrigues, L. P., Lopes, L. M., Parisotto, T. M., Steiner-Oliveira, C. and Nobre-dos-Santos, M. (2017) 'Relationship among alpha amylase and carbonic anhydrase VI in saliva, visible biofilm, and early childhood caries: a longitudinal study', *International journal of paediatric dentistry,* 27(3), pp. 174-182.

Bradford, M. M. (1976) 'A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding', *Anal Biochem,* 72, pp. 248-54.

Bryan, N. S. and Grisham, M. B. (2007) 'Methods to detect nitric oxide and its metabolites in biological samples', *Free Radical Biology and Medicine,* 43(5), pp. 645-657.

397 Cunha-Cruz, J., Scott, J., Rothen, M., Mancl, L., Lawhorn, T., Brossel, K. and Berg, J. (2013)
398 'Salivary characteristics and dental caries: evidence from general dental practices', *J Am Dent*
399 *Assoc,* 144(5), pp. e31-40.
400 da Silva, P. V., Troiano, J. A., Nakamune, A. C. M. S., Pessan, J. P. and Antoniali, C. (2016)
401 'Increased activity of the antioxidants systems modulate the oxidative stress in saliva of toddlers
402 with early childhood caries', *Archives of oral biology,* 70, pp. 62-66.
403 de Farias, D. G. and Bezerra, A. C. (2003) 'Salivary antibodies, amylase and protein from
404 children with early childhood caries', *Clin Oral Investig,* 7(3), pp. 154-7.
405 Doel, J. J., Hector, M. P., Amirtham, C. V., Al-Anzan, L. A., Benjamin, N. and Allaker, R. P.
406 (2004) 'Protective effect of salivary nitrate and microbial nitrate reductase activity against
407 caries', *European journal of oral sciences,* 112(5), pp. 424-428.
408 Dringen, R. (2000) 'Metabolism and functions of glutathione in brain', *Progress in*
409 *neurobiology,* 62(6), pp. 649-671.
410 Eagappan, A. R. S., Rao, V. A. P., Sujatha, S., Senthil, D., Sathiyajeeva, J. and Rajaraman, G.
411 (2016) 'Evaluation of salivary nitric oxide level in children with early childhood caries', *Dental*
412 *research journal,* 13(4), pp. 338.
413 Farsi, N. (2008) 'Dental caries in relation to salivary factors in Saudi population groups', *J*
414 *Contemp Dent Pract,* 9(3), pp. 16-23.
415 Gilgun-Sherki, Y., Melamed, E. and Offen, D. (2001) 'Oxidative stress induced-
416 neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier',
417 *Neuropharmacology,* 40(8), pp. 959-975.
418 Granger, D. A., Kivlighan, K. T., El-Sheikh, M., Gordis, E. B. and Stroud, L. R. (2007) 'Salivary
419 alpha-Amylase in Biobehavioral Research: Recent Developments and Applications', *Annals of*
420 *the New York Academy of sciences,* 1098(1), pp. 122-144.
421 Gutteridge, J. M. C. and Halliwell, B. (2018) 'Mini-Review: Oxidative stress, redox stress or
422 redox success?', *Biochemical and biophysical research communications,* 502(2), pp. 183-186.
423 Halliwell, B. (1999) 'Antioxidant defence mechanisms: from the beginning to the end (of the
424 beginning)', *Free radical research,* 31(4), pp. 261-272.
425 Han, D. H., Kim, M. J., Jun, E. J. and Kim, J. B. (2013) 'The role of glutathione metabolism in
426 cariogenic bacterial growth and caries in Korean children', *Arch Oral Biol,* 58(5), pp. 493-9.
427 Hemadi, A. S., Huang, R., Zhou, Y. and Zou, J. (2017) 'Salivary proteins and microbiota as
428 biomarkers for early childhood caries risk assessment', *Int J Oral Sci,* 9(11), pp. e1.
429 Hodosy, J. and Celec, P. (2005) 'Daytime of sampling, tooth-brushing and ascorbic acid
430 influence salivary thiobarbituric acid reacting substances–a potential clinical marker of gingival
431 status', *Disease markers,* 21(4), pp. 203-207.
432 Justino, A. B., Pereira, M. N., Peixoto, L. G., Vilela, D. D., Caixeta, D. C., de Souza, A. V.,
433 Teixeira, R. R., Silva, H. C. G., de Moura, F. B. R. and Moraes, I. B. (2017) 'Hepatoprotective
434 properties of a polyphenol-enriched fraction from Annona crassiflora Mart. fruit peel against
435 diabetes-induced oxidative and nitrosative stress', *Journal of agricultural and food chemistry,*
436 65(22), pp. 4428-4438.
437 Kawashima, M., Hanada, N., Hamada, T., Tagami, J. and Senpuku, H. (2003) 'Real-time
438 interaction of oral streptococci with human salivary components', *Oral microbiology and*
439 *immunology,* 18(4), pp. 220-225.
440 Leites, A., Pinto, M. B. and Sousa, E. R. d. (2006) 'Aspectos microbiológicos da cárie dental',
441 *Salusvita,* 25(2), pp. 239-52.
442 Malamud, D. (2011) 'Saliva as a diagnostic fluid', *Dental Clinics,* 55(1), pp. 159-178.
443 Martins, C., Buczynski, A. K., Maia, L. C., Siqueira, W. L. and Castro, G. F. (2013) 'Salivary
444 proteins as a biomarker for dental caries--a systematic review', *J Dent,* 41(1), pp. 2-8.

445 Michiels, C., Raes, M., Toussaint, O. and Remacle, J. (1994) 'Importance of Se-glutathione
446 peroxidase, catalase, and Cu/Zn-SOD for cell survival against oxidative stress', *Free radical*
447 *Biology and medicine,* 17(3), pp. 235-248.
448 Mobarak, E. H. and Abdallah, D. M. (2011) 'Saliva nitric oxide levels in relation to caries
449 experience and oral hygiene', *Journal of Advanced Research,* 2(4), pp. 357-362.
450 Muchandi, S., Walimbe, H., Bijle, M. N., Nankar, M., Chaturvedi, S. and Karekar, P. (2015)
451 'Comparative evaluation and correlation of salivary total antioxidant capacity and salivary pH
452 in caries-free and severe early childhood caries children', *J Contemp Dent Pract,* 16(3), pp. 234-
453 7.
454 Patel, M. and Pujara, P. 'Total Antioxidant Capacity of Saliva and Its Relationship with Early
455 Childhood Caries and Rampant Caries'.
456 Pinheiro, C. E. (1982) 'Curso de bioquímica da carie Dental', *Revista Paulista de Odontologia,*
457 4(3), pp. 40-47.
458 Pitts, N. B., Zero, D. T., Marsh, P. D., Ekstrand, K., Weintraub, J. A., Ramos-Gomez, F.,
459 Tagami, J., Twetman, S., Tsakos, G. and Ismail, A. (2017) 'Dental caries', *Nat Rev Dis Primers,*
460 3, pp. 17030.
461 Pyati, S. A., Naveen Kumar, R., Kumar, V., Praveen Kumar, N. H. and Parveen Reddy, K. M.
462 (2018) 'Salivary Flow Rate, pH, Buffering Capacity, Total Protein, Oxidative Stress and
463 Antioxidant Capacity in Children with and without Dental Caries', *J Clin Pediatr Dent,* 42(6),
464 pp. 445-449.
465 Roa, N. S., Chaves, M., Gomez, M. and Jaramillo, L. M. (2008) 'Association of salivary proteins
466 with dental caries in a Colombian population', *Acta Odontol Latinoam,* 21(1), pp. 69-75.
467 Sarode, G., Shelar, A., Sarode, S. and Bagul, N. (2012) 'Association between dental caries and
468 lipid peroxidation in saliva', *International Journal of Oral and Maxillofacial Pathology,* 3(2),
469 pp. 2-4.
470 Scannapieco, F. A., Torres, G. and Levine, M. J. (1993) 'Salivary alpha-amylase: role in dental
471 plaque and caries formation', *Critical Reviews in Oral Biology & Medicine,* 4(3), pp. 301-307.
472 Schanaider, T.-R. (2010) 'Atividade da catalase no pulmão, rim e intestino delgado não
473 isquemiado de ratos após reperfusão intestinal', *Rev. Col. Bras. Cir,* 37(1), pp. 031-038.
474 Schneider, C. D. and Oliveira, A. R. d. (2004) 'Oxygen free radicals and exercise: mechanisms
475 of synthesis and adaptation to the physical training', *Revista Brasileira de Medicina do Esporte,*
476 10(4), pp. 308-313.
477 Seki, M., Yamashita, Y., Shibata, Y., Torigoe, H., Tsuda, H. and Maeno, M. (2006) 'Effect of
478 mixed mutans streptococci colonization on caries development', *Oral microbiology and*
479 *immunology,* 21(1), pp. 47-52.
480 Shi, M., Wang, X., Yamanaka, T., Ogita, F., Nakatani, K. and Takeuchi, T. (2007) 'Effects of
481 anaerobic exercise and aerobic exercise on biomarkers of oxidative stress', *Environ Health Prev*
482 *Med,* 12(5), pp. 202-8.
483 Sies, H. (1997) 'Oxidative stress: oxidants and antioxidants', *Experimental Physiology:*
484 *Translation and Integration,* 82(2), pp. 291-295.
485 Singh, S., Sharma, A., Sood, P. B., Sood, A., Zaidi, I. and Sinha, A. (2015) 'Saliva as a
486 prediction tool for dental caries: An in vivo study', *Journal of oral biology and craniofacial*
487 *research,* 5(2), pp. 59-64.
488 Teixeira, R. R., de Souza, A. V., Peixoto, L. G., Machado, H. L., Caixeta, D. C., Vilela, D. D.,
489 Baptista, N. B., Franci, C. R. and Espindola, F. S. (2017) 'Royal jelly decreases corticosterone
490 levels and improves the brain antioxidant system in restraint and cold stressed rats',
491 *Neuroscience letters,* 655, pp. 179-185.
492 Tothova, L., Kamodyova, N., Cervenka, T. and Celec, P. (2015) 'Salivary markers of oxidative
493 stress in oral diseases', *Front Cell Infect Microbiol,* 5, pp. 73.

494 Twetman, S. 'The evidence base for professional and self-care prevention-caries, erosion and
495 sensitivity'. 2015: BioMed Central, S4.
496 Vibhakar, P. A., Patankar, S. R., Yadav, M. R. and Vibhakar, P. (2013) 'Salivary total protein
497 levels and their correlation to dental caries', *International journal of oral and maxillofacial*
498 *pathology,* 4(3), pp. 13-16.
499 Yagi, K. (1998) 'Simple assay for the level of total lipid peroxides in serum or plasma', *Methods*
500 *Mol Biol,* 108, pp. 101-6.
501 Öztürk, L. K., Furuncuoğlu, H., Atala, M. H., Uluköylü, O., Akyüz, S. and Yarat, A. (2008)
502 'Association between dental-oral health in young adults and salivary glutathione, lipid
503 peroxidation and sialic acid levels and carbonic anhydrase activity', *Brazilian Journal of*
504 *Medical and Biological Research,* 41(11), pp. 956-959.

505