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Alteration on redox status in saliva of microcephaly children

Alteração do estado redox na saliva de crianças com microcefalia

Alteración del estado redox en la saliva de niños con microcefalia

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Abstract
Microcephaly is described as a reduction of the head circumference, due to the premature fusion of the bones of the skull, preventing the brain from growing normally and reaching its maximum development. This condition may result in neurological disorders, phonation and chewing dysfunction, dysphagia and risk of malnutrition. This alteration contributes to oral hygiene impairment, and continuous uses of the antipsychotic and anticonvulsant medication. Thus, the purpose of this study was to evaluate if microcephaly modified redox balance in saliva. Our hypothesis is that in the microcephalic patient's salivary oxidative stress is lower because of the increase in antioxidant defenses. The study included 13 patients with microcephaly (microcephalic group – MC) and 12 patients without neurological disorders (normocephalic group – NC), from zero to ten years old, no edentulous. Saliva was collected using a cotton wool swab, placing it on the child's mouth floor. After centrifugation, supernatants were fractionated and stored at -80 °C for analyses. Lipid oxidative was evaluated by TBARS methods, total antioxidant capacity by the ferric reducing ability (FRAP) assay, uric acid (UA) was quantified by modified Trinder reaction, and superoxide dismutase activity (SOD) by inhibition of the pyrogallol auto-oxidation. Total protein was measured using the method of Lowry. Compared to NC group, TBARS was significantly lower in MC group, while FRAP, UA and SOD were higher. Our hypothesis was confirmed. MC patients have lower salivary oxidative stress, due to increased oxidant defenses.
Keywords: Microcephaly; Saliva; Oxidative stress; Oxidative damage; Dysphagia.

Resumo

A microcefalia é descrita como uma redução da circunferência cefálica, devido a fusão prematura dos ossos do crânio, impedindo que o cérebro cresça normalmente e atinja seu máximo desenvolvimento. Pode resultar em desordens neurológicas, disfunção fonatória e mastigatória, disfagia e aumento do risco de desnutrição. Pode comprometer a qualidade da higiene bucal e favorecer o uso contínuo de medicação antipsicótica e anticonvulsivante. Assim, o objetivo deste estudo foi avaliar se a microcefalia modifica o equilíbrio redox na saliva. Nossa hipótese é que na saliva do paciente microcefálico o estresse oxidativo é menor devido ao aumento das defesas antioxidantes. O estudo incluiu 13 pacientes com microcefalia (MC) e 12 pacientes sem alterações neurológicas (grupo NC), de zero a dez anos, sem edêntulos. A saliva foi coletada utilizando rolete de algodão, colocando-o no assoalho bucal da criança. Após a centrifugação, os sobrenadantes foram fracionados e armazenados a -80°C para análises. A oxidação lipídica foi avaliada pelo método TBARS, a capacidade antioxidante total pela capacidade de redução do ferro (FRAP), o ácido úrico (UA) foi quantificado pela reação de Trinder modificada e a atividade da enzima superóxido dismutase (SOD) pela inibição da auto-oxidação do pirogalol. A proteína total foi medida utilizando o método de Lowry. Em comparação com o grupo NC, TBARS foi significativamente menor no grupo MC, enquanto FRAP, UA e SOD foram maiores. Nossa hipótese foi confirmada. Os pacientes com MC apresentam menor estresse oxidativo salivar, devido ao aumento das defesas antioxidantes.

Palavras-chave: Microcefalia; Saliva; Estresse oxidativo; Dano oxidativo; Disfagia.

Resumen

La microcefalia es una reducción de la circunferencia de la cabeza, debido a la fusión prematura de los huesos del cráneo, impidiendo que el cerebro crezca con normalidad y alcance su máximo desarrollo. Puede resultar en trastornos neurológicos, disfunción de la fonación y masticación, disfagia y riesgo de desnutrición. Contribuye al deterioro de la higiene bucal y al uso continuo de la medicación antipsicótica y anticonvulsiva. Este estudio evaluado si la microcefalia modifica el equilibrio redox en la saliva. Nuestra hipótesis es que en el paciente microcefálico el estrés oxidativo salival es menor por el aumento de las defensas antioxidantes. El estudio incluyó a 13 pacientes con microcefalia (MC) y 12 pacientes sin alteraciones neurológicas (NC), de cero a diez años, no desdentados. La saliva se recogió con un hisopo de algodón en el suelo de la boca. Después de la centrifugación, los sobrenadantes se almacenaron a -80°C. El oxidativo lipídico se evaluó mediante métodos TBARS, la capacidad antioxidante total mediante el ensayo de capacidad reductora férrica (FRAP), el ácido úrico (UA) cuantificó mediante la reacción de Trinder modificada y la actividad superóxido dismutasa (SOD) mediante la inhibición de la autooxidación del pirogalol. La proteína total se midió utilizando el método de Lowry. En comparación con el grupo NC, TBARS fue significativamente menor en el grupo MC, mientras que FRAP, UA y SOD fueron mayores. Nuestra hipótesis fue confirmada. Los pacientes con MC tienen menor estrés oxidativo salival, debido al aumento de las defensas antioxidantes.

Palabras clave: Microcefalia; Saliva; Estrés oxidativo; Daño oxidativo; Disfagia.

1. Introduction

Microcephaly is described by the World Health Organization - WHO (2018) as a reduction of the head circumference due to the premature fusion of the bones of the skull, preventing the brain from growing normally without reaching its maximum development. Thus, its functions are compromised, reflecting on the functioning of the human body. This condition may result in cerebral palsy, seizures, mental retardation and other neurological alterations (Ashwal, Michelson, Plawner, & Dobyns, 2009). The condition may have unknown etiology, or may be caused by innumerable genetic and environmental factors (Dumars, Williams, & Steele-Sandlin, 1980), like viral infections (Ferreira et al., 2021). It can be present at birth (congenital) or developed after birth (acquired) (Rump et al., 2016). Microcephalic patients are highly dependent on their families or caregivers to perform their daily activities due to their limitations.

Neurological disorders can result in dysfunctions on phonation and chewing, common on microcephaly and can affect the normal reflexes of swallowing, causing dysphagia (Marques, Vasconcelos, Andrade, & Hora, 2018). Furthermore, this alteration contributes to a worse outcome, most notably increased risk of malnutrition, pneumonia and a higher mortality (Serel Arslan, Demir, İnal, & Karaduman, 2018; Warnecke, Dziewas, Wirth, Bauer, & Prell, 2019). Besides that, this alteration contributed to oral hygiene impairment and continuous uses of the antipsychotic and anticonvulsant medication (Dougall & Fiske, 2008).

Saliva is a multifunctional fluid, essential for the maintenance of oral health and homeostasis. It is responsible for lubrication, elimination of undesirable substances, digestion, neutralization of acids and bases, protection against

demineralization and also antimicrobial function (Pannunzio et al., 2010). This fluid comes from the salivary glands, crevicular fluid and tissue transudate, is composed of water, electrolytes, proteins, enzymes, volatile compounds, hormones of endogenous origin and cellular components such as desquamated epithelial mucosa cells and microorganisms (Humphrey & Williamson, 2001). Studies have reported the physical chemical characteristics of saliva or the concentration of its components, as some show the differences related to the oxidative stress of this fluid in neurologically compromised patients (Cunha-Correia, Neto, Pereira, Aguiar, & Nakamune, 2014; de Sousa et al., 2015).

In addition, saliva also plays an important role in the control of oxidative stress through its enzymatic and non-enzymatic antioxidant systems. The oxidative stress reflects the imbalance between the generation of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and hydroxyl radical, and the ability to scavenge such harmful substances or repair resulting damage through enzymatic and non-enzymatic antioxidant defense systems (Xing et al., 2018). Enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are part of the enzyme system (Giuca et al., 2010), while uric acid (UA), glutathione (GSH), sialic acid and albumin make up the non-enzymatic system (Diab-Ladki, Pellat, & Chahine, 2003; Leite et al., 2012). Previous study from our group (Cunha-Correia et al., 2014) have already shown significant differences in oxidative stress in saliva of patients with neurological disorders, but no studies on antioxidant defense and oxidative damage in microcephalic children have been found in the literature.

Therefore, the purpose of this study was to evaluate redox balance in saliva of the microcephaly patients. To this end, enzymatic (SOD) and non-enzymatic (FRAP, UA) antioxidant defenses and lipid oxidative damage marker (TBARS) were studied. Our hypothesis is that the salivary oxidative stress is lower in microcephalic patients due to the increase in antioxidant defenses.

2. Methodology

2.1 Patient selection

The Research Protocol was approved by the Human Ethics Committee of the São Paulo State University (Unesp), School of Dentistry, Araçatuba (CAAE: 81055517.1.0000.5420). This cross-sectional study included 13 patients with microcephaly (microcephalic group – MC) and 12 patients without neurological disorders (normocephalic group – NC), from zero to ten years old, no edentulous. MC patients were selected based on a population-based convenience sample from Dental Assistance Center for Disabled Persons (CAOE) of the School of Dentistry, Araçatuba. Subsequently, based on the age range of these patients, NC children were selected from the baby clinic of the School of Dentistry of Araçatuba-UNESP and from school Leonísia de Castro, in Araçatuba (SP, Brazil). The selection criteria in both groups were age and good oral health attested by one dentist. Besides, a survey was made in the medical records of patients with microcephaly, considering gender, medications in use, etiology of the microcephaly, feeding type, mastication quality and presence of dysphagia. The convenience sampling method and the sample number were certified (https://www.openepi.com/Menu/OE_Menu.htm) based on FRAP values determined in saliva in the MC and NC groups, to the test power greater than 90%.

2.2 Saliva collection

Saliva collection was only performed after the parents, or those legally responsible for the children, signed the informed consent. For children aged seven years and over, the child's informed consent was delivered, and the collection of the saliva was only carried out after the signing of both terms. To minimize possible variations resulting from circadian rhythms, unstimulated whole saliva was collected between 8:00 and 10:00 a.m.

Saliva samples were collected using a cotton wool swab, placed on the child's mouth floor. The procedure was interrupted if the patient had any discomfort, and it wasn't resumed. The cotton was put into a specific tube system, taken to the

laboratory and centrifuged at 3,500 x g for 10 min at 4°C to remove cellular and food debris, squamous cells, and insoluble contaminants. The supernatants were fractionated and stored at -80 °C for analyses (dos Santos et al., 2012).

2.3 Analysis of oxidative status

The salivary analyses were performed by the same professional and simultaneously throughout the study. To evaluate lipid oxidative damage, TBARS were analyzed. An aliquot of the saliva was added to the solution of thiobarbituric acid (0.67%, m / v) in strongly acid medium [15% (w / v) trichloroacetic acid and 0.25 mol / L hydrochloric acid], and incubated at 90 °C for 45 minutes (Bird & Draper, 1984). Subsequently, the centrifugation was performed for 20 minutes at 3,000 x g and the supernatants were collected for absorbance reading at 532 nm. The TBARS concentration was calculated using the molar extinction coefficient of 1,55 x 105 M-1cm-1 (Bose, Sutherland, & Pinsky, 1989) and the results expressed as μmol / mg protein.

The total antioxidant capacity was measured by the ferric reducing ability (FRAP) assay and results were expressed as Fe2+ μmol / mg protein (Benzie & Strain, 1996). Salivary UA was quantified by spectrophotometry using the modified Trinder reaction (Labtest Diagnostic SA, SP, Brazil).

SOD was estimated by the method of Marklund & Marklund (1974), based on the inhibition of the superoxide radical reaction with pyrogallol. The rate of inhibition of pyrogallol autoxidation after the addition of enzyme extract was noted, and the amount of enzyme required to give 50% inhibition of pyrogallol autoxidation was considered as one unit of enzyme activity. The enzymatic activity was expressed as U / mL and U / mg protein. Total protein was measured using the method of Lowry, Rosebrough, Farr, & Randall (1951), with bovine serum albumin used as the standard.

2.4 Statistical analysis

Data were expressed as mean ± standard deviation of the mean. Data normality was determined using the Shapiro-Wilk test and analyzed using a t test of Student. To verify correlation between parameters Pearson Test was used. For all analyzes, the level of significance was set at 5% ($p < 0.05$).

3. Results

The study included 25 subjects from 2 to 10 years (average of 6.5 years). Table 1 shows number of patients, mem/women relation, cause of microcephaly according to the International Classification of Diseases for Neurology (World Health Organization, 1997), and use and type of medication.

Table 1. Number of patients, men/women relation, disease classification, etiology of microcephaly and use of the medication in the normocephalic (NC, n=12) and microcephalic (MC, n=13) groups.

	NC	MC
Number of patients	12	13
Men/women	9/3	5/8
Disease (ICD-10)		
Q02 Microcephaly	–	13
Etiology of microcephaly		
Not diagnosed	–	9
Environmental factors	–	4
Use of medication		
None	12	7
Antipsychotic	–	5
Anticonvulsant	–	3
Antidopaminergic	–	1
Antispastic	–	1

Source: Authors.

The type of feeding, chewing efficiency and presence of dysphagia were showed in table 2. Approximately 54% patients in the MC group do not have full feeding, 62% have chewing problems and dysphagia.

Table 2. Type of feeding, chewing efficiency and dysphagia in the normocephalic (NC, n=12) and microcephalic (MC, n=13) groups.

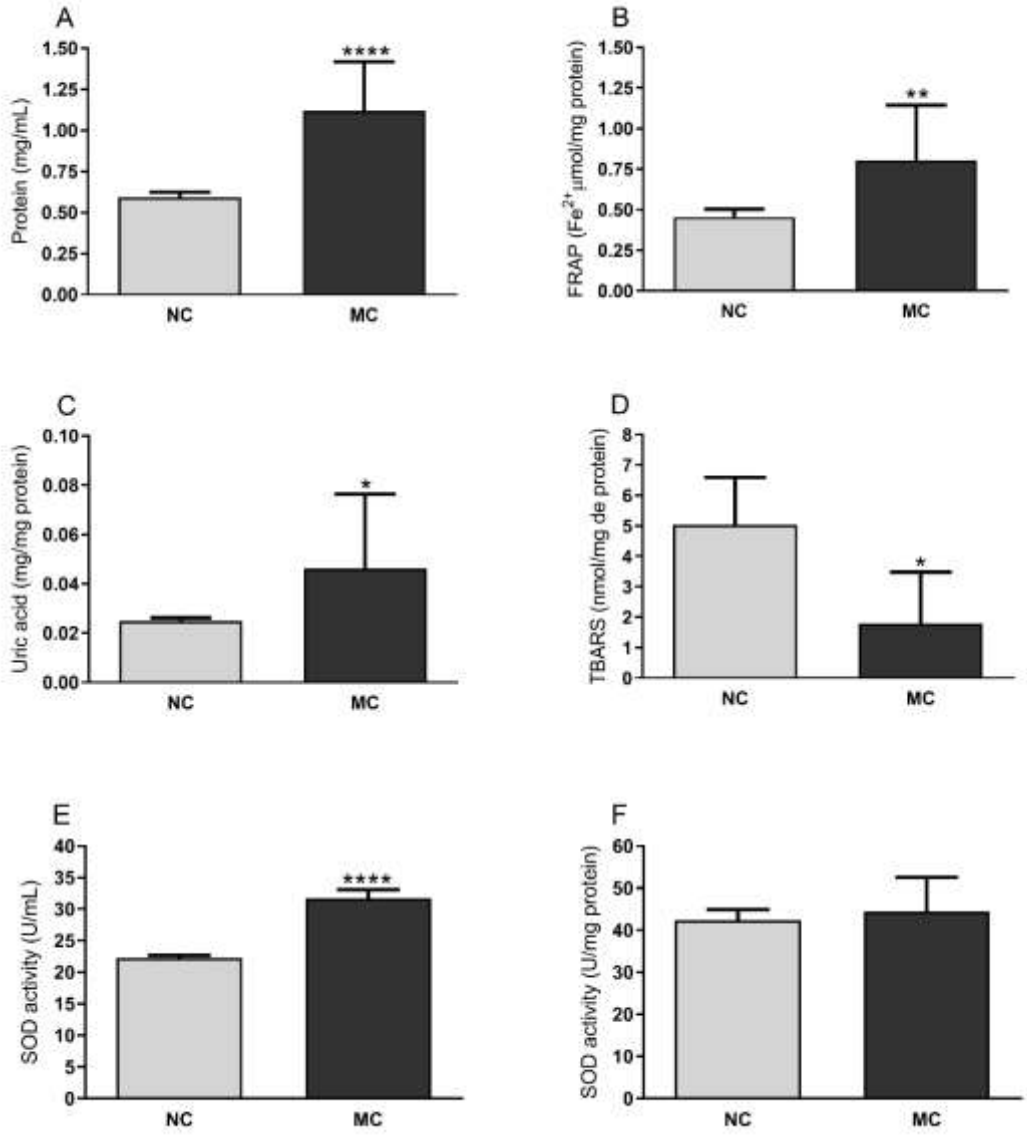
	NC	MC
Type of feeding		
Full	12	6
Pasty	-	6
Enteral	–	1
Chewing efficiency		
Efficient	12	5
Inefficient	–	5
Do not chew	-	3
Dysphagia		
Absent	12	5
Present	–	8

Source: Authors.

The Figure 1 show total proteina, antioxidant capacity, uric acid, 2-thiobarbituric acid-reactive substances and superoxide dismutase activity in saliva of normocephalic (NC group) and microcephalic (MC group). Lipid oxidative damage was lower in MC group, and this decrease was accompanied by greater non enzymatic and enzymatic antioxidant defenses. Total

protein (Figure 1A) was significantly higher (88.47%; $p < 0.0001$) in the MC group (1.118 ± 0.300 mg / mL) than the NC group (0.593 ± 0.096 mg / mL). The value of FRAP (Figure 1B) in the MC group (0.805 ± 0.340 Fe2+ µmol / mg protein) was higher (29.1%; $p < 0.01$) compared to the NC group (0.454 ± 0.154 µmol Fe2+ / mg protein). The salivary UA in the MC group (0.046 ± 0.030 mg / mg protein) was significantly higher (85.53 %; $p < 0.05$) than the NC (0.025 ± 0.004 mg / mg protein) as shown in Figure 1C. A positive correlation ($r = 0.503$, $p < 0.05$) was observed between FRAP and UA values. TBARS in saliva (Figure 1D) of the MC group (1.79 ± 1.68 nmol / mg protein) was lower (35.66 %; $p < 0.05$) than the values in the NC group (5.03 ± 4.70 nmol / mg protein). SOD activity (Figure 1E) was higher (42.51%; $p < 0.0001$) in the MC group (31.68 ± 1.38 U / mL) compared to the NC (22.23 ± 1.13 U / mL), however, the values expressed in specific activity (U / mg protein) were not different (NC group: 42.33 ± 6.27; MC group: 44.37 ± 8.16) as can be seen in Figure 1F.

Figure 1. Total protein (A); total antioxidant capacity - FRAP (B); uric acid - UA (C); 2-thiobarbituric acid-reactive substances - TBARS (D); superoxide dismutase activity expressed in U / mL (E) and in U / mg protein (F) in saliva of patients normocephalic (NC group) and microcephalic (MC group). Mean ± SD. *$p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ (Student's t-test).



Source: Authors.

4. Discussion

In the present study, we observed an imbalance antioxidant/oxidative balance in MC group, signaling reduced oxidative stress, by the higher enzymatic and non-enzymatic antioxidant defense and lower oxidative lipid damage marker in the saliva of patients with MC compared to NC. The increase of antioxidant defense may be a compensatory mechanism, particularly due deficient oral hygiene, use of the antipsychotic treatment, nutritional problems, and other dysfunction inherent to microcephaly.

Saliva plays an important role in monitoring general health and diseases in children, elderly people, and non-collaborative individuals (Kamodyová, Tóthová, & Celec, 2013). MC, corroborating Rump et al. (2016), has most of the time unknown etiology, since almost 70% of the MC group did not have a definite cause for the development of the disease.

Chewing problems observed by us in MC group is common condition in neurological diseases, such as amyotrophic lateral sclerosis and cerebral palsy (Cunha-Correia et al., 2014; Serel Arslan et al., 2018) and can result in a large and a poorly degraded bolus, which makes swallowing more difficult. Individuals with chewing problems were ten times more likely to have dysphagia, indicating that often the two conditions occur together or are at least perceived together (Streicher et al., 2018). Dysphagia is a physical disability that negatively affects a patient's ability to fulfill his or her nutritional needs and occurs in almost 62% of patients of the MC group. This condition complicates oral nutrition as normal foods and liquids (Simione, Wilson, Yunusova, & Green, 2016).

Lower liquid intake can result in reduced salivary flow and consequently greater protein concentration. In the present study increase of salivary protein concentration in MC patients it was found and may be related to hypohydration and decrease in the total volume of secreted saliva, as noted in patients with dementia, Down Syndrome and Cerebral Palsy (Choromańska et al., 2017; Cunha-Correia et al., 2014; de Sousa et al., 2015; Santos et al., 2011).

Saliva is the first line of defense against oxidative molecules presents in external substances ingested as food, drinks, and produced by microorganisms. It regulates redox status of oral cavity under physiological and pathological conditions. This fluid is rich in non-enzymatic antioxidants, like UA, albumin, ascorbic acid and glutathione (Ginsburg et al., 2013). In the present study, total antioxidant capacity was determined by the FRAP method, which allows the evaluation of the synergistic effect between the various non-enzymatic antioxidant components (Crews et al., 2001). Inverse relationship between oral hygiene and FRAP was observed in saliva of children (Araujo, Nakamune, Garcia, Pessan, & Antoniali, 2020) and cerebral palsy patients (Cunha-Correia et al., 2014). The MC patients in this paper are regularly assisted by the CAOE multidisciplinary team, furthermore their caregivers are guided as to the proper oral hygiene techniques, despite that hygiene difficulties are inherent to neurological conditions.

The greater antioxidant non-enzymatic defense, verified in the saliva of MC patients by FRAP values, can be attributed to a higher concentration of UA, like observed by correlation test. This compound is the final product of purine metabolism in the human body, formed from xanthine and hypoxanthine by reaction catalyzed with xanthine oxidoreductase (Varsha, Farah, & Suchetha, 2015) and representing more than 70% of the total antioxidant capacity in saliva (Moore, Calder, Miller, & Rice-Evans, 1994). The antioxidant UA's effect may be related to the scavenging of the ERO formed by the reaction of peroxynitrite, a strong oxidizing agent to interact with cell constituents (Whiteman, Ketsawatsakul, & Halliwell, 2002). In a recent literature review (Vernerová, Kujovská Krčmová, Melichar, & Švec, 2020) a linear relationship between serum and salivary UA is reported in different contexts, which leads to believe that the higher concentration of UA because of an innate error in the purine metabolism or even changes in the excretion process. Once this condition can be caused by innumerable genetic and environmental factors (Dumars et al., 1980) we cannot rule out a possible relationship between these factors and the increase in UA in the saliva of MC patients.

It is important to note that UA can also act as a pro-oxidant, when reacting with different oxidants producing ERO, which propagates radical chain reactions and causes oxidative damage (Varsha et al., 2015). Although the UA concentration in

MC group was remarkably high (85.53%) in relation to NC, less lipid oxidative damage measured by TBARS was observed, indicating that UA was acting as an antioxidant.

Lower lipid oxidation may be related to medication used by MC patients, who are usually treated with psychotropic drugs, which may include antipsychotics, antidepressants, mood stabilizers and anticonvulsants. In this context, antipsychotic treatment can be associated with a significant reduction of oxidative stress. According to Kriisa et al. (2016), antipsychotic medication in patients with episodes of psychosis interferes in the rates of serum lipid peroxidation and oxidative stress, showing a decrease in those when compared to patients with psychosis episodes who did not receive medication.

In addition to the non-enzymatic antioxidant defense, patients with MC showed greater enzymatic defense, as evidenced by the increase in SOD. In animal models, increased epithelial tissue SOD activity relative to the control group was found after anticonvulsant drug treatment (Wu et al., 2018), a kind of drug used by this group. Nutritional alterations resulted in chewing problems and dysphagia in MC groups; they may also be involved in the greater SOD activity observed in saliva, similarly to what has already been described with nutritional problems resulting from anorexia (Mascitti et al., 2019). When SOD was expressed in relation of total protein (U/mg) these increased of activity is not observed, because protein total is higher in saliva of the MC group than NC group.

We point out as a limitation of the work that we did not make the determination of salivary flow, due to the difficulty in collection, since patients with CM are more anxious (Camoin, Dany, Tardieu, Ruquet, & Le Coz, 2018; Davila, 1990) and we prioritize their comfort. To minimize salivary flow rate absence, all salivary parameters were expressed in protein concentration. Another choice was to evaluate only salivary UA over plasmatic, once the saliva demands a less invasive collection than blood, which is the ideal factor for this patient profile.

5. Conclusion

Our hypothesis was confirmed. MC patients have lower salivary oxidative stress, due increased oxidant defense. This response may be a compensatory mechanism, particularly due deficient oral hygiene, use of the antipsychotic treatment, nutritional problems, and other dysfunction inherent to microcephaly. New studies could be realized with the goal of evaluating whether the compensatory mechanism observed in saliva of microcephalic patients occurs in other body fluids or tissues, since this would confirm whether this is a characteristic factor of microcephaly.

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