DOI: 10.1590/2317-1782/201420140489

Original Article

Artigo Original

Otoacoustic emissions and biomarkers of oxidative stress in students of a tobacco-producing region

Emissões otoacústicas e biomarcadores do estresse oxidativo em escolares de região fumicultora

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Keywords

Pesticides
Tobacco
Hearing
Oxidative Stress
Child

ABSTRACT

Purpose: To verify the association between the amplitude of distortion-product otoacoustic emissions (DPOAE) and biomarkers of oxidative stress (OS) in resident students of the tobacco-producing region. **Methods:** Participated in the study group (SG) 21 normal-hearing students from the tobacco-producing region, and in the control group (CG) 25 normal-hearing students who did not live in the countryside. The auditory system was assessed by DPOAE and the following biomarkers: dichlorofluorescein diacetate (DCFH-DA) and micronucleus test (MN). **Results:** Both groups showed DPOAE present in both ears. Significant difference was detected between groups — in the right ear in the frequency of 4.000 Hz and in the left ear in the frequency of 2.000 Hz — with the mean amplitude of the DPOAE of the SG lower than the one found in the CG. Considering both ears, the SG presented lower mean across all frequencies and it was found a significant difference in the frequencies of 2.000 and 4.000 Hz. The overall mean of DPOAE, by ear, no significant differences were observed. In relation to the rate of production of free radicals, the mean of the SG was significantly higher than that of the mean of the CG. For the frequency of abnormal cells in the MN test, the mean of the SG was also considerate significantly higher than the mean of the CG. **Conclusion:** The SG showed a lower response level of DPOAE at all frequencies and high levels of biomarkers of EO, however there was no association between assessments.

Descritores

Praguicidas
Tabaco
Audição
Estresse oxidativo
Criança

RESUMO

Objetivo: Verificar a associação entre a amplitude das Emissões Otoacústicas Produto de Distorção (EOAPD) e biomarcadores do estresse oxidativo (EO) em escolares residentes de região fumicultora. **Métodos:** Participaram do grupo estudo (GE) 21 escolares normo-ouvintes residentes de região fumicultora, e do grupo controle (GC), 25 escolares normo-ouvintes que não residiam na zona rural. O sistema auditivo foi avaliado por meio das EOAPD, e os biomarcadores do EO foram: Diclorofluoresceína diacetato (DCFH-DA) e teste de micronúcleos (MN). **Resultados:** Os dois grupos apresentaram EOAPD presentes em ambas as orelhas. Detectou-se diferença significativa entre os grupos — na orelha direita, a frequência foi 4.000 Hz, e na esquerda, 2.000 Hz —, sendo a média de amplitude das EOAPD do GE menor que a do GC. Quanto às duas orelhas, o GE apresentou média inferior em todas as frequências, verificando-se diferença significativa em 2.000 e 4.000 Hz. Na média geral das EOAPD por orelha não foi observada diferença significativa. Na taxa de produção de radicais livres, a média do GE mostrou-se significativamente mais elevada que a do GC. Quanto à frequência de células alteradas no teste de MN, a média do GE também se apresentou significativamente mais elevada que a do GC. **Conclusão:** O GE apresentou nível de resposta das EOAPD menor em todas as frequências e índices elevados dos biomarcadores do EO, porém não foi verificada associação entre as avaliações.

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Received: 04/10/2013

Accepted: 03/25/2014

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Conflict of interests: nothing to declare.

INTRODUCTION

Over the last years, the concern about the consequences and risks of exposure to pesticides on human health has grown. It is important to remember that not only rural workers but also their entire families are exposed to pesticides, and their children may be susceptible since the prenatal period, thus forming a special risk group.

The effects of continuous exposure to pesticides on children and youth are little known. Because these individuals are still growing and developing, their organisms are more vulnerable to the action of chemical products. Moreover, they present recurrent habits, such as putting their hands in their mouths, which increases the chances of ingestion of pesticides found in the water, soil, and household dust[1].

In rural environments, the use of pesticides is still the farmers' main strategy to combat and prevent agricultural pests, and also to ensure that the crops are more productive[2]. Although the harms caused by these products are known, the farmers use them indiscriminately and without proper individual protection, which results in significant increases in the number of acute and chronic intoxications by these type of products[3].

In tobacco farming, in addition to the risks posed by the exposure to pesticides, this population is subject to problems caused by contact with tobacco leaves. In this scenario, a large quantity of nicotine can be absorbed through the skin[4], which provokes intoxication. Nevertheless, the chronic effects on health, caused by dermal exposure to nicotine, as well as on the auditory system, remain unknown.

Over the last years, organophosphorus pesticides, more commonly used in tobacco farming, were introduced in high-priority groups for research on ototoxicity due to occupational exposure[5]. Ototoxicity is defined as any harm inflicted in the cochlear and/or vestibular system caused by exposure to chemical substances. In regard to nicotine ototoxicity, studies that relate nicotine exclusively to hearing loss are limited. Generally, the discussions pertain to the ototoxic effects of the nicotine consumed while smoking.

Humans are subject to oxidative stress (OS), which is defined as an imbalance between oxidant and antioxidant systems to the detriment of the latter. Innumerable reactive oxygen species (ROS) are generated, among them the free radicals, which, differently than the others, have one unpaired electron in their electronic layer, thus favoring oxidative harm[6]. These ROS can be formed from exogenous sources, such as tobacco, radiation, ultraviolet light, solvents, and some medicines[7] and pesticides[2], or from endogenous sources. The ROS are involved in an increasingly large number of pathologies, such as hearing loss (cochlear function alteration)[8,9].

One of the ways to proactively prevent the harms caused by exposure to toxic substances is through human biomonitoring, which allows for the identification of risk factors for the development of certain pathologies[10], such as hearing loss. The biological monitoring of an individual's exposure to chemical agents is conducted by measuring these substances or their metabolites in several biological environments, such as blood, urine, saliva, and exhaled air. These biological parameters are called biological indicators or biomarkers[11].

Biomarkers can be used to identify any harm to macromolecules (lipids, proteins, and DNA) caused by OS[12]. There are different biomarkers to assess OS; some verify specific results such as harm inflicted on DNA, which can be evaluated, for instance, through a micronucleus (MN) test, or quantified through the production levels of ROS in the organism by using fluorescent methods.

The MN test is used to monitor populations that have been occupationally exposed for many years[13]. MNs are acentric chromosome fragments or whole chromosomes left behind on the course of mitotic cellular division, which appear in the cell's cytoplasm during the interphase as a small additional nucleus[2]. Currently, this test, carried out in oral mucosa cells, has been used to analyze multiple factors, including environmental and occupational exposure (such as to pesticides), lifestyle (alcohol consumption, smoking, stress, etc.), cancer, and other diseases[14].

Recently, fluorescent methods have stood out due to their greater sensitivity, and have been used very frequently to monitor ROS production in the cells of different tissues. Another great advantage of these methods lies in their marked sensitivity in detecting species, which enables quantitative analyses[15]. Dichlorofluorescein diacetate (DCFH-DA) is one of the fluorescent methods, which pertains to the production of free radicals.

Cochlear function assessments can also be carried out through evoked otoacoustic emissions (EOAE), which is an efficient method for early detection of hearing alterations. According to Kemp[16], alterations in EOAE response are observed before alterations in the hearing threshold (minimum sound level perceived by the human ear) are registered. This type of assessment is also used to monitor hearing loss. EOAE monitoring is a specific and sensitive method that identifies alterations in external ciliate cells (ECCs), as these lesions decrease the amplitude of EOAEs and of the signal/noise relation, with possible absence of response in more severely harmed regions[17].

The integrity of the auditory, peripheral, and central systems is directly reflected on normal language, speech, reading, and writing development. For this reason, it is important that any auditory harm caused by exposure to several ototoxic substances on students should be identified early, so as to prevent possible effects of this exposure on their development and learning.

The purpose of this study was to verify the association between the amplitude of distortion-product otoacoustic emissions (DPOAEs) and OS biomarkers in students who reside in a tobacco-producing region, thereby alerting and preventing future harm in this population.

METHODS

This study was approved by the ethics committee of the Universidade Federal de Santa Maria (UFSM), Santa Maria (RS), Brazil (Protocol No. 0237.0.243.000-11). This research project was conducted in partnership with the Workers Health Reference Center (CEREST) of Santa Maria. All individuals

agreed to participate in the study. They received a copy of it and presented the informed consent form signed by their parents or legal guardians. This is an observational, prospective, and cross-sectional study.

The study group (SG) comprised students who resided in the rural tobacco-producing area of a municipality in the central region of Rio Grande do Sul. For the control group (CG), we recruited students who lived in the urban area of another municipality in the State's central region where tobacco was not produced. We opted for selecting CG students from another municipality to ensure that they were free from exposure to pesticides and nicotine that is derived from tobacco leaves.

The inclusion criteria adopted in this study were the following: age between 7 and 14 years, normal hearing, and not being continuously exposed to intense noise and cigarette smoke. The exclusion criteria were having a history of otologic alterations and audiological alterations, having chronic diseases, and/or using continued medication.

During the selection process for the SG, 103 students who lived in the tobacco-producing region in question were invited to participate in the study. However, only 25 showed interest, and 22 participated. To recruit participants for the CG, we visited three public schools. About 250 students were invited, but only 57 showed interest, of which 26 took part in the study.

The convenience sample initially counted 48 volunteers. Of this number, two presented alterations on a basic audiological assessment. They were excluded from the study and referred to the necessary care services. The final sample comprised 46 students aged between 7 and 14 years, which were divided into two groups:

- Study group (SG): 21 students (12 females and 9 males), aged between 7 and 14 years, with normal hearing, residing in a tobacco-producing region
- Control group (CG): 25 students (17 females and 8 males), aged between 7 and 14 years, with normal hearing, who did not live in the rural area in question

The audiological assessments and the collection of biological materials were carried out at CEREST.

With the purpose of identifying few of the inclusion and/or exclusion criteria of the study, we applied a questionnaire to the parents and/or legal guardians and to the participants, which pertained to personal data, otologic history and overall health, auditory complaints, and socioenvironmental aspects, such as continuous exposure to cigarettes and/or intense noise, and place and years of residence. We highlight that none of the individuals, in both groups, was exposed to cigarette smoke and/or intense noise on a daily basis. In other words, only the CG participants were exposed to ototoxic substances, exclusively to organophosphorus pesticides and to the nicotine released by tobacco leaves. Therefore, all of them met the inclusion criteria.

To verify their auditory condition, we advised the students to undergo a visual inspection of their external acoustic meatus, using a Welch Allyn Klinic otoscope, followed by a tone threshold audiometry (TTA).

The TTA was carried out in an acoustically treated booth with an Interacoustics audiometer (model AC40) and TDH-39 earplugs. As this procedure was only an audiological triage, we assessed airway thresholds at the frequencies of 500, 1,000, 2,000, and 4,000 Hz. We used the descending–ascending technique. The individuals were considered as having normal hearing when their three-tone threshold average (500, 1,000, and 2,000 Hz) was lower than or equal to 25 dB HL.

Acoustic immittance was measured using an Interacoustics middle ear analyzer (model AT 235) and a 226-Hz sounder tone, with the purpose of assessing tympanometric curves and acoustic reflexes at the frequencies of 500–4,000 Hz bilaterally, in the contralateral mode. Only the children with type A tympanogram and presence of acoustic reflexes were included in the sample.

Later, the participants were assessed by means of DPOAEs in both ears. We investigated the DPOAEs because they evaluate a broader range of frequencies, including high ones, which are important when assessing individuals exposed to noise and/or toxic substances.

The DPOAEs were recorded with an Interacoustics cochlear analyzer (OtoRead Screening). To obtain the DPOAEs (2F1-F2), we used two pure tones at a ratio of F2/F1=1.22, in which F1 was presented at an intensity of L1=65 dB SPL and L2=55 dB SPL. To measure the DPOAEs, we tested the frequencies of 1,500, 2,000, 3,000, 4,000, 5,000, and 6,000 Hz. We considered the DPOAEs to be present when the signal/noise ratio was equal to or higher than 6 dB SPL in at least three frequencies[18].

The DPOAEs were also evaluated by comparing the average values of the signal/noise ratio per frequency between the SG and the CG.

Moreover, we collected biological materials. However, only 18 individuals in the SG and 18 in the CG underwent this procedure, thus reducing the sample for these assessments.

To evaluate OS parameters, we carried out the following experimental tests: DCFH-DA, which refers to free radical rates, and the MN test to assess possible meta-nuclear alterations.

A blood sample was collected to determine the rate of free radical production. The biological material was obtained by means of venipuncture, performed by a qualified nursing technician. The syringe and needle seals were removed in the volunteer's presence and attached in succession. The blood was immediately stored in a tube with sodium heparin anticoagulant. Then, the syringe and the needle were discarded in containers suitable for perforating and infectious/contagious materials.

This study is based on the use of a nonfluorescent 2¢,7¢-dichlorofluorescein diacetate (DCFH-DA) catheter, which easily penetrates cellular membranes and is deacetylated by intracellular esterase enzymes, thus forming 2¢,7¢-dichlorodihydrofluorescein (DCFH). In the presence of ROS, this nonfluorescent metabolite, in turn, is transformed into highly fluorescent dichlorofluorescein (DCF)[19]. Thus, the basis for this study is the florescence emission that is directly and proportionately related to the presence of ROS[20]. Therefore, the greater the fluorescence emitted, the larger the presence of oxidant compounds. This evaluation was carried out in accordance with that described by Costa Krewer et al.[21]. In this experimental

practice, we used black 96-well ELISA plates, and the determination was based on four replicates for the entire sample tested. In each plate well, we added 50 µL sample under analysis, along with 65 µL HCl, 10 mM Tris buffer (pH 7.4), and 10 µL DCFH-DA reagent (0.1 mM). After 1 hour of incubation at room temperature and protection from direct light, the samples were analyzed in regard to their fluorescence at 488-nm excitation and 525-nm emission using a SpectraMax Plus 384 fluorescence spectroscope.

The MN test was conducted in accordance with that described by Fronza et al.[9]. For this test, we collected oral mucosa epithelial cells by scraping the mucosa of the cheeks. The material was then deposited into a Falcon conical tube with 2 mL physiological solution or phosphate-buffered saline (pH 7.4) solution and refrigerated in a Styrofoam box until the processing stage. The samples were centrifuged at 1,000–1,500 RPM for 10 minutes at room temperature. We then discarded the supernatant substance using individual Pasteur pipettes. Next, we added 1.5 mL fixative solution and centrifuged the material one more time at 1,000–1,500 RPM for 1–2 minutes. We discarded the supernatant substance again, maintaining a bit of the fixative solution in the tube so as to homogenize the contents, resuspend the cells, and place them on clean and previously labeled blades. After they dried, we colored the cells with panoptic stains. To complete this stage, we washed the blades with distillated water to remove the excess of stain, and kept them at room temperature to dry.

After the material was dry, it was observed in an Olympus® binocular optical microscope (model CX40), with 400´ magnification for cell counting and for subsequent data analysis. We counted 1,000 cells, which were classified as normal cells (no alteration), cells with MN, binucleated cells, cells with nuclear bridges, and cells with nuclear buds or broken eggs, as shown in Figure 1. For comparative analysis, we used the sum of abnormal cells of all types in the total 1,000 cells evaluated.

The data collected were distributed on an Excel 2007 electronic spreadsheet to be statistically analyzed later. The statistical analyses were carried out using Statistical Package for the Social Sciences (SPSS), version 17.0. We used Kolmogorov–Smirnov test to verify the normality of the variables.

In all comparative analyses, we used Student's *t*-test for independent samples. To evaluate the association between the variables (DPOAE and OS biomarkers), we used Pearson's correlation coefficient. A significance level of 5% was adopted.

RESULTS

All individuals, both in the SG and in the CG, presented DPOAEs in both ears.

Comparing the response amplitude of the DPOAEs between the groups per frequency, we detected statistically significant differences in the right ear (RE) at 4,000 Hz ($p<0.05$), which indicates that the mean of the SG (18.0±6.3) was lower than that of the CG (21.7±5.6). In the left ear (LE), significant differences were found at 2,000 Hz, which once again indicates that the mean of the SG (21.0±7.1) was lower than that of the CG (25.3±5.1) (Table 1).

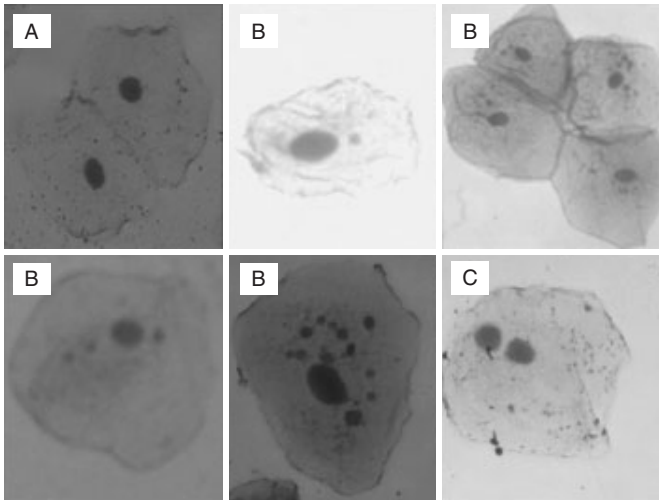

Figure 1. Classification of the cells according to the micronucleus test: (A) unaltered cell; (B) cell with micronucleus; (C) binucleated cell[9]

In regard to the comparisons pertaining to the averages of the other frequencies, both in relation to the RE and the LE, the differences observed between the SG and the CG were not significant ($p>0.05$), although we observed that, in general, the averages of the SG were lower than those of the CG.

Considering the RE and the LE, the SG presented lower averages than the CG in all frequencies. Regarding the 2,000-Hz frequency mean, we detected significant differences ($p<0.05$), as the SG presented a lower average (21.2±6.7) than the CG (25.4±4.4). Another significant difference ($p<0.05$) was evidenced by the 4,000-Hz frequency mean, in which the average of the SG (18.2±4.9) was again lower than that of the CG (21.1±4.9) (Table 1).

Regarding the overall DPOAE average by ear, we did not observe any statistically significant differences (Table 1).

In regard to the comparison of the production rate of free radicals (DCFH-DA) between the groups, we observed that the average of the SG (23144.2±2726.5) was significantly ($p<0.01$) higher than that of the CG (20.5329±2.4471) (Table 2). These results indicate that the group of students exposed to these elements presented a larger number of ROS.

In respect to the results pertaining to the comparison of averages of frequency of abnormal cells on the MN test, we detected a statistically significant difference ($p<0.01$), as the mean of the SG (24.9±9.8) was higher than that of the CG (14.9±7.8) (Table 2). This shows that the group of students exposed to the elements in question presented a higher rate of harms that were inflicted at a cellular level (altered cells) than those of the group that was not exposed.

In the analysis that involved comparisons between the amplitudes of the DPOAEs, by frequency and by ear, and of OS biomarkers (frequency of altered cells on the MN test, and the production rate of free radicals), we did not detect any significant linear relations ($p>0.05$).

Table 1. Comparative analysis of the average values (mean and standard deviation) of the amplitude of response of the distortion-product otoacoustic emissions per frequency

DPOAE (Hz)	Groups						p-value
	Study (n=21)			Control (n=25)			
	Mean	Standard deviation	Amplitude (Minimum–Maximum)	Mean	Standard deviation	Amplitude (Minimum–Maximum)	
Right ear							
1,500	21.4	6.8	8–32	21.4	7.0	3–30	0.993¶
2,000	21.4	7.6	10–37	25.4	5.9	11–35	0.051¶
3,000	22.4	6.2	8–31	22.4	5.1	13–31	0.990¶
4,000	18.0	6.3	0–27	21.7	5.6	11–32	0.043¶*
5,000	21.8	9.7	0–34	22.3	7.0	9–34	0.837¶
6,000	20.8	10.5	0–34	23.7	6.7	9–36	0.288§
Overall mean	20.9	6.7	5–31	22.8	4.7	12–32	0.251¶
Left ear							
1,500	20.1	6.2	6–28	20.6	7.7	-5–33	0.814¶
2,000	21.0	7.1	6–34	25.3	5.1	17–36	0.020¶*
3,000	21.0	7.9	1–33	22.9	3.6	16–34	0.322§
4,000	18.3	6.4	0–27	20.4	5.5	4–29	0.224¶
5,000	22.6	7.6	2–32	24.4	5.5	9–35	0.362¶
6,000	24.0	8.3	0–35	25.2	5.9	12–36	0.584¶
Overall mean	21.2	5.8	6–29	23.2	4.0	12–32	0.169¶
Left ear – right ear mean							
1,500	20.8	5.8	7.5–29.0	21.0	6.7	1.0–30.0	0.891¶
2,000	21.2	6.7	8.0–34.5	25.4	4.4	17.5–35.0	0.019§*
3,000	21.7	6.4	4.5–31.0	22.6	3.7	14.5–31.0	0.561§
4,000	18.2	4.9	6.5–27.0	21.1	4.9	10.0–30.5	0.049¶*
5,000	22.2	7.6	1.0–32.5	23.3	5.3	9.0–34.5	0.563§
6,000	22.4	7.8	0.0–33.0	24.4	5.6	10.5–36.0	0.331§
Overall mean	21.0	5.6	5.9–30.7	23.0	4.1	12.3–31.9	0.171¶

Overall average of the right ear and left ear; and average of the right ear and left ear between the study and control groups (n=46)
*p<0.05; ¶Student's *t*-test for independent groups considering variance homogeneity; §Student's *t*-test for independent groups considering variance heterogeneity
Caption: DPOAE = distortion-product otoacoustic emissions

Table 2. Comparative analysis of the average values (mean and standard deviation) of the dichlorofluorescein diacetate rate of free radical production, and of the frequency of abnormal cells in the micronucleus test between the study group and the control group (n=36)

Variable	Groups						p-value
	Study (n=18)			Control (n=18)			
	Mean	Standard deviation	Amplitude (Minimum–Maximum)	Mean	Standard deviation	Amplitude (Minimum–Maximum)	
Rate of free radical production	23,144.2	2,726.5	16,899.5–27.850.5	20,532.9	2,447.1	16,525.3–25,236.6	0.005¶*
Frequency of abnormal cells (MN test)	24.9	9.8	3.0–42.0	14.9	7.8	7.0–40.0	0.002§*

*p<0.05; ¶Student's *t*-test for independent groups considering variance homogeneity; §Student's *t*-test for independent groups considering variance heterogeneity

DISCUSSION

The children of tobacco-growing farmers constitute a special group at risk for adverse effects to their health caused by continuous exposure to pesticides and nicotine. While conducting this study, we believed that the group of students exposed to pesticides and nicotine would present indicators of possible cochlear and OS alterations, such as reduced DPOAE amplitude, high rates of free radical production, and high frequency of altered cells on the MN test, respectively. All these hypotheses were verified. Some were statistically significant, others were not. We proceed to analyze them in light of the literature available on the topic.

Keeping in mind the scarcity of studies on the effects of the exposure to pesticides and nicotine (tobacco leaf) on the auditory system and its association with OS biomarkers, our results were compared to similar studies whenever possible. In their absence, the results were compared to studies carried out with

individuals exposed to noise, ototoxic medication, smoke, and others that presented any possible correlation.

OAE capture cochlear functioning by means of ECC responses, and the test can reveal either the integrity or alteration of these structures before any signs of irregularity are detected through TTA[22].

Hearing loss associated with exposure to pesticides presents a similar configuration to that caused by ototoxic medication and noise; that is, it is characterized by being a neurosensory alteration that tackles high frequencies first[23]. Many authors have showed the importance of OAE in the audiological monitoring of occupationally exposed individuals, as they are useful in early diagnoses of hearing impairments in initial stages. Among the OAE types are the transient otacoustic emissions (TOAEs) and DPOAEs. These two techniques have proved effective for early assessments of the cochlear functioning of occupationally exposed populations (noise and/or chemical substances). The disadvantage of TOAEs is that this technique does not reach frequencies higher than 4,000 Hz, differently than DPOAEs, which are more efficient to detect higher frequencies, important elements in the assessment of individuals occupationally exposed to noise, pesticides, and/or solvent because they impact higher frequencies first[22]. In this study, we opted for DPOAEs so as to encompass a larger number of frequencies. Consequently, we might not have captured alterations that could have already been in the cochlea (ECC), because TOAEs are more sensitive in capturing ECC alterations than DPOAEs.

As mentioned earlier, audiological alterations caused by exposure to pesticides are similar to those caused by noise. According to Frota and Iório[24], cochlear alterations caused by exposure to high levels of sound pressure prompt early changes in OAE amplitude. Thus, it is believed that cochlear alterations caused by exposure to pesticides and nicotine can generate alterations in OAE amplitude. In this study, the students exposed to pesticides and nicotine presented precocious alterations in OAE amplitude.

Körbes et al.[25] investigated the effect of organophosphates on the hearing of animals. The authors did not observe any functional cochlear alterations evidenced by the presence of DPOAEs but only verify that both groups that had received pesticides presented alterations in the cytoarchitecture of the ECCs, with more significant harm experienced by the animals that received high dosages. The results of this study are similar to the findings of the aforementioned authors — although their study was carried out with animals, they did not find functional cochlear alterations either, highlighted by the presence of DPOAEs.

Regarding the action of nicotine over the auditory system, the studies found in the literature are relatively limited, and the majority in which nicotine is one of the main substances studied focuses on the effects of cigarettes on hearing. In a study that evaluated the effect of cigarettes on the auditory system, the authors compared TOAE results, among other tests, between smokers and nonsmokers. They verified that the group of smokers presented lower TOAE response levels at 1,000 Hz in both ears and at 4,000 Hz in the LE, and concluded that cigarettes have a harmful effect on the auditory system[26]. The results in this study agree with the findings of the aforementioned authors, despite the use of DPOAEs. We observed that the SG presented DPOAE response levels that were lower than or equal to those of the CG in all frequencies, in both ears. Significant differences were found at 4,000 Hz in the RE, and at 2,000 Hz in the LE. Considering the average of both ears, the SG indicated lower DPOAE response levels in all frequencies, with significant differences at 2,000 and 4,000 Hz (Table 1).

It is possible to find several studies on human biomonitoring in populations exposed to pesticides, including children[1,27,28]. The studies in question are important tools to estimate risks, as well as to prevent and diagnose future alterations early on, caused by exposure to several toxic substances, such as pesticides and nicotine.

In this study, we detected statistically significant differences in the MN test when comparing the sum of abnormal cells between the groups. The average of the SG was higher than that of the CG. These findings agree with the results of other studies in which the authors also found significant differences in all abnormal cell types when comparing the exposed group to the CG[27,28]. Other authors specifically studied children exposed to pesticides and also observed a significant increase in the frequency of cells with MN in the exposed group[1]. Differently than the other authors, Pastor et al.[29] did not find any distinction between individuals who were exposed and not exposed to pesticides in regard to the frequency of MN.

Another biomarker used in this study was the rate of production of free radicals, a fluorescent method that detects ROS. The biological and toxicological effects of ROS have increasingly become an object of interest for researchers in different areas, especially because of their relation to several pathological conditions. However, ROS have a few characteristics that make their detection difficult, such as high reactivity and short half-life[30], which hinder their determination *in vivo*. Thus, the fluorescent catheters that detect ROS are promising tools and excellent ROS sensors due to their high sensitivity and simple data collection[31].

In spite of this, as observed in the literature, the technique has not yet been frequently used in studies on human biomonitoring. In this study, it proved to be highly sensitive in detecting ROS and a good biomarker of OS in the population studied. The results of our research show that, on comparison of the rates of free radical production, the average of the SG was significantly higher ($p<0.01$) than that of the CG.

In this study, we aimed at verifying an association between DPOAE amplitude and OS biomarkers in students exposed to pesticides and nicotine. For this result to have clinical significance, it must occur inversely; that is, a reduction in DPOAE amplitude must be related to an increase in OS biomarkers, and vice versa. In our research, we observed this inverse association, as expected for both groups, in a qualitative analysis. Our results reveal that the SG presented lower averages of DPOAE amplitude, a large number of altered cells in the MN test, and a high rate of free radical production in comparison to the CG. However, in the statistical analysis, no significant correlation occurred between DPOAE frequency and OS biomarkers, thus indicating that these

two variables were independently related in both groups. We believe a significant association was not observed due to the small sample. Thus, it is important to conduct other studies with a larger number of individuals to confirm these findings.

CONCLUSION

In this study, the students in both groups presented DPOAEs. However, the SG showed lower averages of DPOAE amplitude at all frequencies.

The SG registered a significantly higher rate of free radical production and a marked presence of altered cells in the MN test. The high rates of OS biomarkers may also be associated with the exposure of these individuals to pesticides and nicotine. However, no significant associations were verified between DPOAE response levels and OS biomarker results.

**LRK is the author of the dissertation on which this article is based. She was responsible for data collection, tabulation and analysis, as well as manuscript elaboration; AKM and FM contributed with data collection and analysis; MVG and AFS contributed with project elaboration, dissertation supervision, and manuscript elaboration.*

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