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ORIGINAL ARTICLE

Comet and micronucleus assays do not correlate extent of DNA damage with clinical staging of oral cavity squamous cell carcinoma

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The study was carried out at Centro de Estudos em Célula Tronco, Terapia Celular e Genética Toxicológica, Hospital Universitário Maria Aparecida Pedrossian, Empresa Brasileira de Serviços Hospitalares, Universidade Federal de Mato Grosso do Sul (UFMS), Campo Grande, MS, Brasil.

Abstract

Introduction: Oral cavity squamous cell carcinoma is the sixth most frequent malignant neoplasm worldwide. Its main etiological factor is smoking associated with alcohol consumption. **Objective:** To evaluate the applicability of the comet and micronucleus assays and the relationship between cell damage intensity and oral cavity squamous cell carcinoma (OCSCC) clinical stages. **Methods:** A total of 44 individuals, 24 oral cancer patients and 20 healthy volunteers, participated in the study. Exfoliated cells were collected from the oral cavity using a wooden spatula. All samples were analyzed by the comet (CA), micronucleus (MNA) and cell death (CDA) assays. **Results:** The diagnosis of OCSCC increases the frequency of genotoxic damage (CA – 3.21x; MNA – 3.93x); however, it was not possible to establish a correlation between frequency of DNA damage and disease staging. The CDA was not effective for the proposed biomonitoring. **Conclusion:** The CA and MNA were considered adequate for biomonitoring DNA damage. With the current design, the applicability of these assays to correlate DNA/cell damage extent and OCSCC clinical staging could not be demonstrated. Nevertheless, further studies should be conducted with larger sample sizes to increase the statistical power of these findings.

Keywords: genotoxicity; comet assay; micronucleus assay; apoptosis; biomonitoring; oral cancer.

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Introduction

Oral cavity squamous cell carcinoma (OCSCC) is the sixth most frequent malignant neoplasm worldwide[1]. Its main etiological factor is smoking associated with alcohol consumption[2-5].

Genetic changes are the primary basis of carcinogenesis, and this process slowly continues until a cell becomes mutated and gives rise to a clinically detectable tumor[6].

Some genotoxic methods are able to identify the extent of damage to the DNA integrity of cells[7], among them, the comet (CA), micronucleus (MNA) and apoptosis cell death (CDA) assays are often applied[8-10].

The CA is based on the assessment of DNA damage and repair in individual cells. Cells are subjected to cell membrane lysis solution, followed by induction of electrophoretic migration of released DNA in an agarose matrix. When viewed under a microscope, the migrated cell takes on the apparent shape of a comet. Comet analysis is based on the degree of DNA fragmentation and its migration by electrophoresis. Measures such as total "tail" length and DNA density provide indirect data on the state of the sample DNA[7].

The micronucleus, when present in the cell, is a biomarker of genotoxicity formed by chromosome fragments, or whole chromosomes, that delay migration during anaphase and eventually form small nuclei[8-11].

Apoptosis, a process of programmed cell death to cells no longer needed by the body, is characterized by the presence of nucleus fragmentation, chromatin condensation, and pyknosis[9]. Increased apoptosis-related changes may occur as a result of genotoxic damage and can be related to the process of malignant transformation[12].

Prognosis for patients with OCSCC is poor, and survival rate remains approximately 50%[13] depending on the stage of the compromised oral cavity area and on the TNM staging system[14]. Therefore, the identification of specific methods that could advance the diagnosis and screening measures can improve outcome in these patients[15].

In this context, this study evaluated the applicability of these assays and the relationship between cell damage intensity and the OCSCC clinical stages.

Methods

A total of 44 individuals, 24 patients who were seen during their first visit to the outpatient clinic of the Head and Neck Surgery Department at the Campo Grande Alfredo Abrão Cancer Hospital between August 2016 and August 2017 and 20 healthy volunteers, participated in this study. The healthy volunteer group consisted of 10 men (5 nonsmokers and 5 smokers) and 10 women (5 nonsmokers and 5 smokers). The volunteers were divided into a nonsmoker control group (NSC; n=10) and a smoker control group (SC; n=10).

The study sample was composed of adult patients without any previous treatment, diagnosed with OCSCC, who agreed to take part in the research. Exclusion criteria comprised patients diagnosed with other histological cancer types, or with previous history of oncological treatments. The control group included healthy adult patients, with no previous cancer treatments, who were categorized into smokers and nonsmokers.

The project was approved by the Human Research Ethics Committee of the Federal University of Mato Grosso do Sul under protocol no. 1.671.940. All participants signed an Informed Consent Form prior to study commencement. Each individual rinsed their mouth with drinking water, and the exfoliated cells were collected from the oral cavity using a wooden spatula.

The exfoliated cells were placed in 0.9% saline solution (5 mL), centrifuged at 2000 rpm for 10 min, and the supernatant was discarded. The individuals

in the OCSCC group were submitted to new collection of material for analysis 1-3 months after cancer treatment. This time was allowed in order to decrease the effects of radiotherapy on the cells.

Comet Assay (CA)

The CA was performed according to Singh et al.[16], with modifications by Navarro et al.[17] (Figure 1). The slides were stained *a posteriori* (100 µL ethidium bromide – $20x10^{-3}$ mg/mL), and 100 cells were analyzed using an epifluorescence microscope (Motic® - BA410). Cell damage analysis and classification were based on Kobayashi et al.[18]. The total score was calculated by adding the values resulting from the multiplication of the total number of observed cells from each lesion category by the value of the category[17].

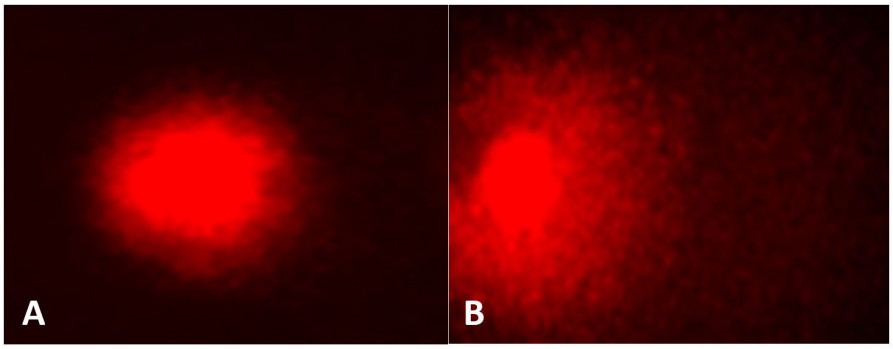

Figure 1. Comet assay images of epidermal mouth cells, ethidium bromide staining. **A** - Normal cell untreated with no signs of damage. **B** - Cell showing significant damage, with score IV in the comet assay classification.

Micronucleus Assay (MNA)

A volume of 100 µL of cell suspension were used to prepare the smears in glass slides. Next, the slides were stained with 10% Giemsa for 5 min and analyzed under a brightfield microscope (Nikon® – Eclipse E200). A total of 1000 cells per individual were analyzed and the number of micronuclei was determined[19].

Cell Death Assay (CDA)

The CDA was performed according to the description by Oliveira et al.[20]. A volume of 100 µL of cell suspension were used to prepare a smear on a glass slide. The slide was fixed in Carnoy's solution for 5 min, quickly dipped into plates containing decreasing concentrations of ethanol (95-25%), washed with McIlvaine buffer for 5 min, stained with Acridine Orange (0.01%, 5 min), and washed again with buffer solution. The analysis standard described by Carvalho et al.[21] was adopted.

Statistical analysis

The comet, micronucleus and cell death assays were evaluated using ANOVA/Tukey-Kramer tests according to a parametric distribution of the data processed in GraphPad Prism 7.00 software. Linear Pearson correlation coefficients were obtained using the SigmaPlot 12.5 software. A significance level of 5% ($p<0.05$) was adopted for all statistical analyses.

Results

Oral cavity squamous cell carcinoma (OCSCC) was more prevalent among men (83.33%), and the mean age of individuals for both sexes was 64.5 ±24.74 years. Individuals in the nonsmoker control (NSC) and smoker control (SC) groups presented mean ages of 41.5 ±28.99 and 51.5 ±20.50 years, respectively (Table 1).

In regard to OCSCC staging, 25% of the patients presented stage I or II neoplasms, whereas 75% of them presented stage III or IV neoplasms. Among these individuals, 87.5% were smokers. Alcohol consumption was present in 75, 40, and 20% of the individuals in the OCSCC, SC and NSC, respectively (Table 1).

Concerning chronic disease, only chronic hypertension was mentioned by the individuals and confirmed, and it affected 12.5% of the individuals with OCSCC, 80% of those in the SC, and 10% of those in the NSC (Table 1).

As for the type of treatment, all patients with OCSCC were submitted to surgical interventions. Half of these patients were treated complementary with radiotherapy and 8.3% of them underwent concomitant chemotherapy and radiotherapy.

Table 1. Absolute and relative values and mean values ±standard error of the mean (SEM) according to sex, age, cancer staging and life habits of control individuals (NSC – Nonsmoker control group, SC – Smoker control group) and patients diagnosed with squamous cell carcinoma of the oral cavity (OCSCC).

Parameter	Groups studied		
	NSC (n=10)	SC (n=10)	OCSCC (n=24)
	Absolute value (%)		
Sex			
Female	5 (50%)	5 (50%)	4 (16.66%)
Male	5 (50%)	5 (50%)	20 (83.33%)
Age			
Minimum	21	37	47
Maximum	62	66	82
Mean value ±SEM	41.5 ± 28.99	51.5 ± 20.50	64.5 ± 24.74
TNM cancer staging			
I-II	-	-	6 (25%)
III-IV	-	-	18 (75%)
Chronic disease (Hypertension)			
Yes	1 (10%)	8 (80%)	3 (12.5%)
No	9 (90%)	2 (20%)	21 (87.5%)
Smoking			
Yes	0 (0%)	10 (100%)	21 (87.5%)
No	100 (100%)	0 (0%)	3 (12.5%)
Alcoholism			
Yes	2 (20%)	4 (40%)	18 (75%)
No	8 (80%)	6 (60%)	6 (25%)

The CA showed that smoking increases (p<0.05) the frequency of genomic damage and the lesion score by 2 and 3.1 times, respectively. Before therapy, patients with OCSCC presented a 3.21-fold greater genomic damage frequency and a 4.25-fold higher lesion score compared with those of individuals in the NSC (p<0.05). In the post-therapeutic intervention period, the frequency of DNA damage was reduced and did not differ from that for individuals in the NSC (p>0.05) (Figure 2A, 2B). Statistical relation with alcoholism was not analyzed owing to the small number of alcoholics in the control groups.

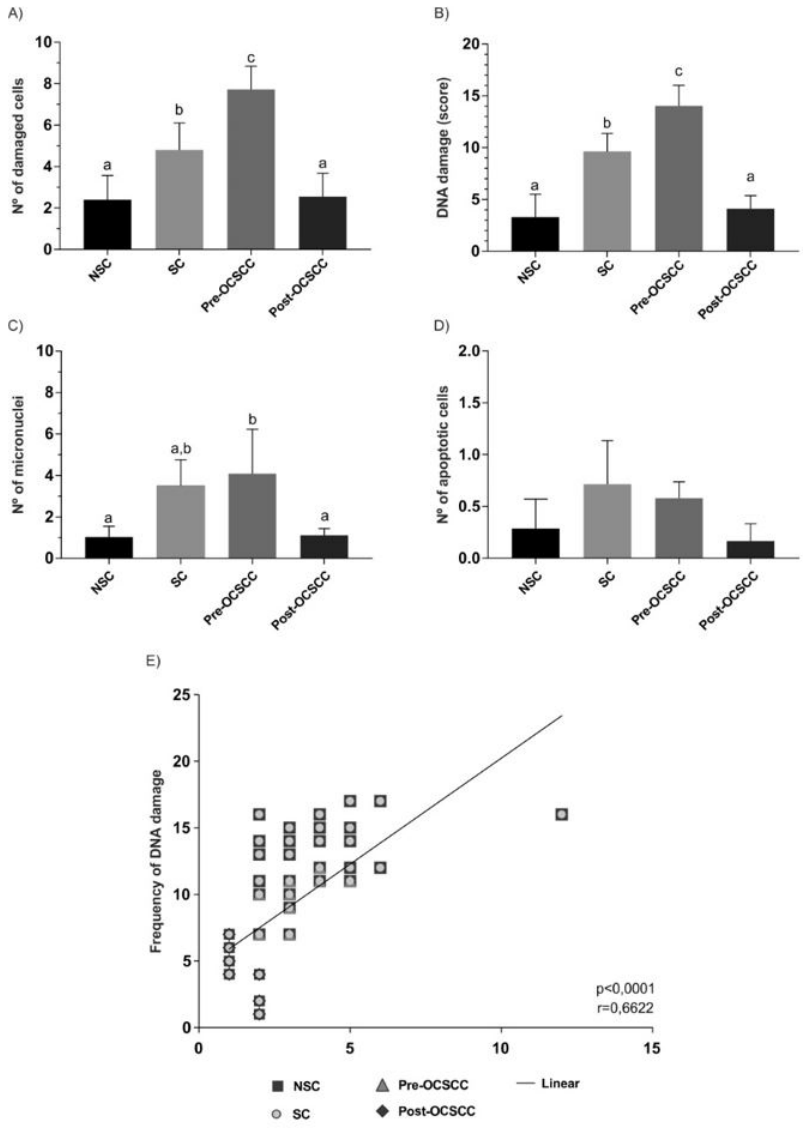

Figure 2. A - Mean ±standard error of the mean (SEM) of the frequency of cells with lesions. **B** - Comet assay score. **C** - Frequency of micronuclei. **D** - frequency of apoptotic cells. **E** - Scatter plot showing the linear Pearson correlation between frequency of genotoxic damage (comet assay score × micronuclei frequency). Each symbol represents the frequency of both variables for a single individual. The continuous line represents the linear regression line. NSC – nonsmoker control group, SC – smoker control group, OCSCC – oral cavity squamous cell carcinoma group, Pre – pre-therapeutic intervention, Post – post-therapeutic intervention. Statistical test: **A**, **B**, C, and **D** - ANOVA/Tukey; different letters indicate statistically significant differences (p<0.05); linear Pearson correlation (p<0.05).

Results of the MNA showed that individuals with a diagnosis of OCSCC present a higher frequency of chromosomal damage (Figure 3). In regard to the NSC, there was a 3.93-fold increase ($p<0.05$) in the frequency of micronucleated cells at the pretreatment evaluation. After treatment, this frequency was reduced and was not different from that of individuals in the NSC ($p>0.05$) (Figure 2C).

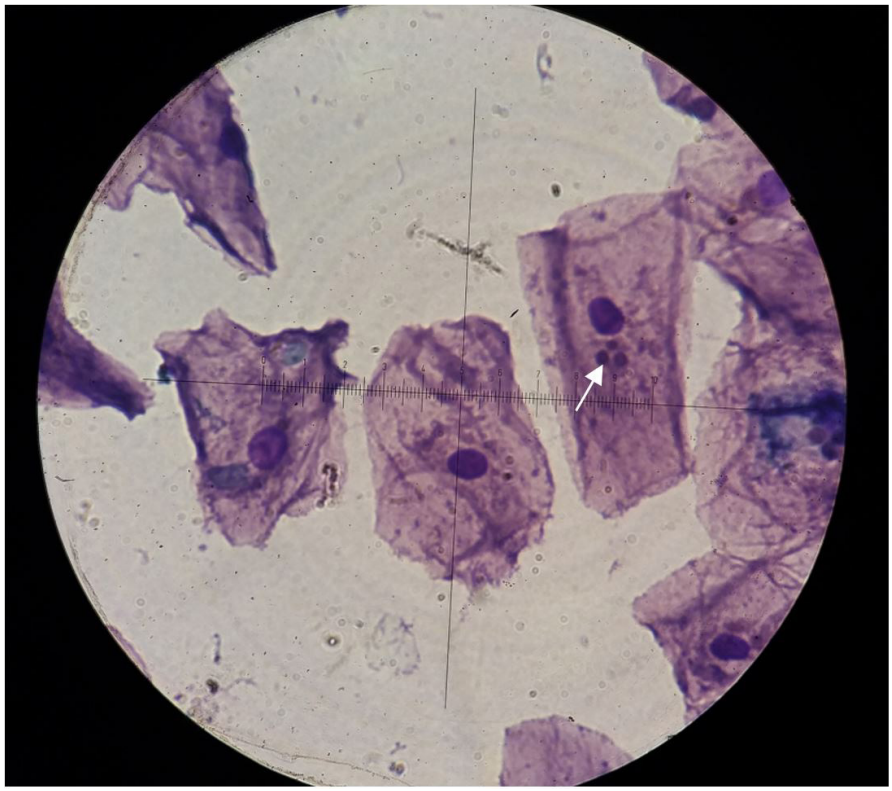

Figure 3. Oral mucosae of patients with oral cancer stained with Giemsa 10%. White arrow shows presence of micronucleus.

A significant difference was observed between individuals in the NSC and SC: the latter group showed a 3.64-fold increase in chromosomal damage compared with the first group (Figure 2C).

The frequency of cell death ranged from 0.11 ±0.33 to 0.80 ±1.03 across the different study groups, and no statistically significant difference was found (Figure 2D).

No relationship was found when the correlation between DNA damage frequency (CA and MNA) and disease staging was evaluated. In addition, no relationships were observed between reduced frequency of DNA damage and treatments (chemotherapy, radiotherapy, and/or surgical intervention). However, when the frequency of genetic damage was correlated using the CA and MNA, significant positive and moderate correlations were found regardless of treatment ($r=0.662$; $p<0.001$) (Figure 2E).

Discussion

The first contact of potential genotoxic agents with the oral cavity occurs in the oral mucosa[22]. Genotoxic agents such as alcohol and cigarette components may trigger carcinogenesis[4,23], including OCSCC[5,24].

In general, there is correlation between the frequency of genotoxic damage and the occurrence of carcinogenesis[22]. For this reason, genotoxic tests are used by regulatory agencies to predict carcinogenic risk[25]. These assays are also applied in the clinical biomonitoring of periodontal diseases and viral infections[26,27]. However, the literature on the correlation between genotoxic data and OCSCC staging is still limited, which prompted this study.

Data showed that the frequencies of DNA damage assessed by the CA and MNA were increased in the SC. This result was expected, as induction of genomic/genetic damage through smoking has been widely reported in the literature[28,29].

In regard to OCSCC, the results showed that the patients presented increased comet positivity and micronucleus frequency before treatment. Despite this statistically significant difference compared with the NSC, it was not possible to identify a correlation between frequency of genotoxic damage and OCSCC staging.

After therapy, there was a reduction in genotoxic damage in all patients who were re-evaluated, regardless of medical strategy. Moreover, it was not possible to establish a correlation between frequency of DNA damage and disease staging or therapeutic intervention, but a significant reduction in damage frequency was observed after treatment.

Correlation between frequency of DNA damage evaluated by the CA and MNA showed significant positive and moderate relationships. This finding was expected because the literature indicates that damage evaluated by the CA is repairable genomic damage[30,31], whereas damage assessed by the MNA is irreparable genetic damage that has been introduced in the cell genome[32,33]; i.e., damage identified by the CA may become incorporated into the genome and transform into chromosomal/genetic damage[34].

The CDA did not present applicability for the proposed monitoring, as the frequency of the evaluated cells was too low.

This study evidenced the highest rate of genetic damage in the normal cells of patients with OCSCC, which significantly regressed after treatment. In smoking volunteers, higher rates of genetic damage were also observed, although to a lesser extent.

Limitations to this study include the small number of alcoholics in the control groups, which did not allow a statistical correlation with this possible aggravating factor, and the overall sample size, which can be considered small. However, the results were very significant as they demonstrate an important lack of correlation between level of genetic damage and clinical stage of the disease.

This study may provide future evidence to assist with the daily practice of oncology by correlating the extent of damage pre- and post-treatment with patient survival rate, treatment modality used and possible sequelae, thus bringing benefits to patients.

Conclusions

The comet and micronucleus assays are adequate for biomonitoring DNA damage at different levels (genomic or genetic) induced by exposure to genotoxic agents and/or caused by endogenous changes (such as in the case of genetic instability induced by cancer). With the current experimental design, applicability of these assays to correlate DNA/cell damage extent with OCSCC clinical staging could not be demonstrated. Nevertheless, further studies should be conducted with larger sample sizes to increase the statistical power of these findings.

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