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Deoxyribonucleic acid damage and repair response in the chemotherapy of lung cancer: cross-sectional study

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Abstract: This study evaluated 24 patients with lung cancer (CA) and 23 individuals with no smoking history or cancer in the family and without respiratory disease in childhood (CO). Peripheral blood lymphocytes was used to perform alkaline comet assay and to assess DNA damage as well as to evaluate methyl methane sulfonate (MMS) DNA repair after one hour and three hours at 37 ºC. The percentage of residual damage (RD) after three hours of MMS treatment, for each patient was assessed. The majority of patients were in the CA group, male patients, former smokers, with a history of smoking for 15 years and without associated comorbidities. Alkaline and residual damages were higher in the CA group when compared to controls (alkaline damage P = 0.015 and RD P = 0.05). After one hour of MMS treatment the DNA damage of the CA increased indicating failure to repair it, compared to the controls, and after three hours DNA repair was observed in both groups. Patients with lung cancer are mostly men, former smokers and with more than 15 years of tobacco consumption, undergoing chemotherapy, have high rates of DNA damage and deficiency in their ability to repair against induced damage when compared to controls.

Key words: comet assay, DNA damage, DNA repair, lung cancer.

INTRODUCTION

Lung cancer is a disease that affects men and women, mainly those aged 50 years or older, of which rate has been significantly increasing worldwide, with smoking being the main cause (INCA 2016, OMS 2015). The World Health Organization has stated that around 170.000 lung cancer deaths occur per year worldwide. In Brazil, in 2016, 28.220 new cases occurred, of which 17.330 patients were males, and 10.890 were females (INCA 2016, OMS 2015). Genetic and occupational factors are also responsible for the disease onset and evolution. With high morbidity and mortality, lung cancer has

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been the subject of innumerable studies, with the aim of improving the forms of prevention, early diagnosis and understanding of its pathophysiology (INCA 2016, OMS 2015).

Lung cancer treatment, however, continues to be palliative in most cases, considering the high rates of metastasis and recurrence in the 5-year period (Jiang et al. 2015). Resection surgeries and chemotherapy and radiation therapy-based treatments cause adverse reactions ranging from fever, hair loss, weakness, reduced immunity, and deoxyribonucleic acid (DNA) damage. DNA damage and repair can be identified by the comet assay, which is considered effective, low-cost, and easy to perform (Gunasekarana et al. 2015, Dusinska and Collins 2008, Collins et al. 2008, Agnoletto et al. 2007, Wasson et al. 2008).

DNA damage can be considered positive when one assesses the need to damage and/or destroy cancer cells. In that case, not only malignant cells are selectively affected but also healthy cells can be damaged during treatment (Walker et al. 2016). In the study Sham et al. (2003) evaluated DNA damage and repair in peripheral blood lymphocytes in lung cancer patients and healthy controls using the comet assay, however, no significant differences in DNA damage and repairing efficiency were shown before or after chemotherapy or in lung cancer or normal subjects. Orlow et al. (2008) highlight that DNA damage as measured by the comet assay is associated with the development of multiple primary tumors in individuals with non-small cell lung cancer. However, in these studies, did not evaluate the effect of chemotherapeutic classes in lung cancer patients. The aim was investigated DNA damage and repair in lymphocytes from lung cancer patients undergoing chemotherapy. We hypothesized that patients with lung cancer undergoing chemotherapy may show increased DNA damage in lymphocytes for both basal and residual indices.

MATERIALS AND METHODS

STUDY DESIGN

A cross-sectional study, carried out with a convenience and non-probabilistic sampling procedure in accordance to STROBE statement, complied with the ethical and legal aspects of research involving human subjects, with the approval of the Ethics and Research Committee of Universidade de Santa Cruz do Sul under protocol n. 346.984. The patients with lung cancer who comprised the case (CA) group were selected at the Integrated Oncology Center (IOC) of Ana Nery Hospital in the city of Santa Cruz do Sul, State of Rio Grande do Sul, Brazil. The subjects that comprised the control (CO) group were formally invited to participate in this study from the Association of Retirees of Santa Cruz do Sul (APOPESC). All individuals who participated in this study signed the Free and Informed Consent Form (FICF).

CRITERIA FOR INCLUSION AND EXCLUSION OF THE STUDY SUBJECTS

Twenty-three patients with lung cancer undergoing chemotherapy treatment (CA group), with diagnosis of lung cancer under treatment in IOC, with autonomy, > 18 years and agreeing to participate in the study through the FICF were included in the study. Twenty-four healthy subjects, with preserved pulmonary function, good cognition, adulthood, who signed FICF and without lung cancer were included in the study as CO group. Hospitalized patients and those with terminal lung cancer from the CA group were excluded; also, subjects with a history of smoking and family history of cancer were excluded, as well as those who reported recurrent respiratory diseases in childhood from the CO group (Figure 1).

CLINICAL AND EPIDEMIOLOGICAL DATA COLLECTION

On the beginning of the study, all participants answered a sociodemographic and clinical data

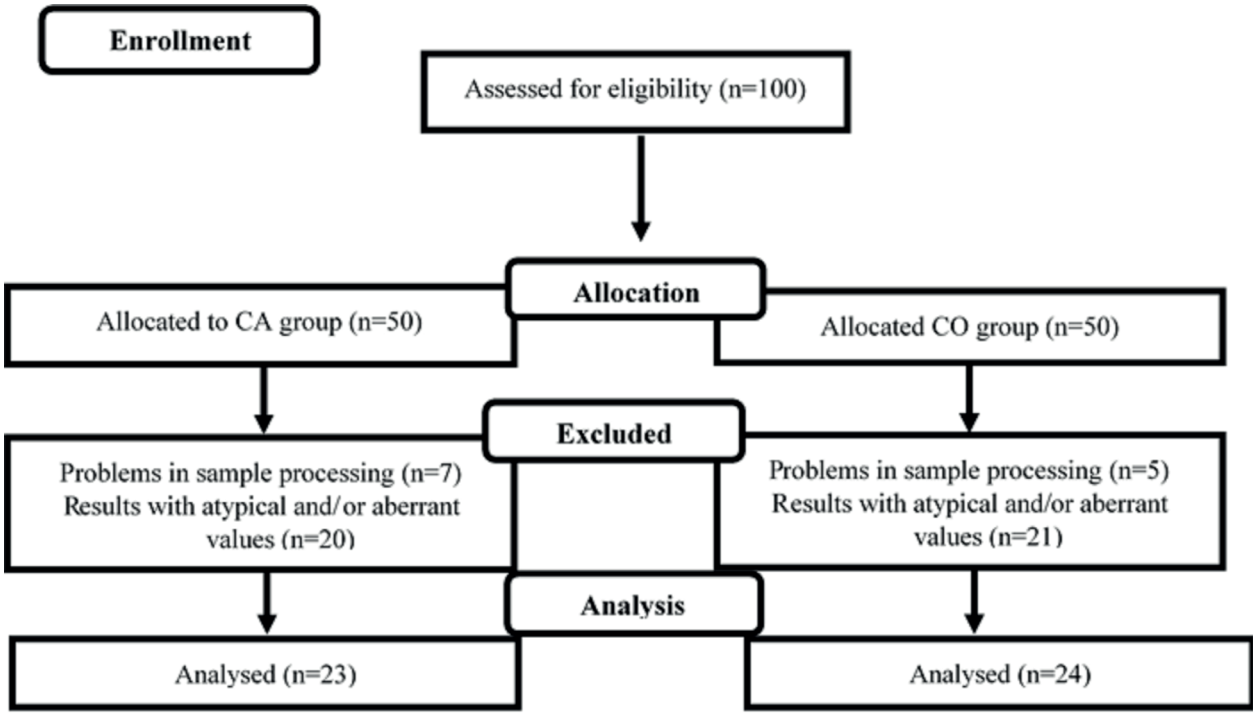

Figure 1 - Flow chart – final study sample to carry out the comet test. n = sample number; CA = group of cases with lung cancer; CO = control group.

collection questionnaire, to report on their health status. For the patients in the CA group, a medical file analysis was carried out with the purpose of gathering information on the type of chemotherapy used. All individuals in the CA group used one or more chemotherapeutic agents belonging to the following classes: platinum compounds, vinca alkaloids, taxanes, bisphosphonates, podophyllotoxins and pyrimidine analogs. After that, they were gathered according to the use of one or two classes of chemotherapeutic agents.

OBTAINING THE BIOLOGICAL MATERIAL

Blood collection was done by trained person and it followed WHO guidelines on drawing blood (WHO 2010). A single peripheral blood sample was collected through venipuncture and stored in an ethylenediaminetetraacetic acid (EDTA) tube protected from light. An aliquot of this blood sample was used for lymphocyte extraction and subsequent performance of the alkaline version of the comet assay and repair kinetics, and another aliquot was stored in an ultra-freezer if sample replication was required.

COMET ASSAY

The comet assay was performed according to Collins et al. (2008). The interpretation of the comet assay results considered 5 levels, from zero damage (DNA is intact) to the presence of a tail (like a comet), which can vary from 1 to 4, depending on the size of the tail (Gunasekarana et al. 2015).

The comet assay was performed under alkaline conditions: 20 µL aliquots of lymphocytes were mixed with 200 µL of low melting point agarose (0.7% in phosphate buffer) and placed under microscope slides pre-coated with 1.5% agarose. Subsequently, the slides were incubated in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 20 mM of NaOH, pH 10.2, 1% Triton X-100, and 10% DMSO). After 24 hours, the slides were removed from the lysis solution and placed in

an electrophoresis unit, covered with an alkaline buffer, and refrigerated at 4 °C. In this alkaline version of the comet assay (10M NaOH, 1mM EDTA, and pH > 13), 20 minutes of denaturation and 15 minutes of electrophoresis were performed. For DNA damage assessment, 100 cells per sample were analyzed by light microscopy (100x). Cells were visually classified into the five classes. After that, the damage index (DI) was calculated for each sample, ranging from 0 to 400.

REPAIR KINETICS OF DNA DAMAGE

To assess DNA repair capacity, lymphocytes were treated with methyl methanesulfonate (MMS), an alkylating agent, (8 x 10^{-5} mM) for five minutes at 37 °C. After the treatment, the cells were washed in phosphate buffered saline (PBS) (centrifuged for five minutes at 3500 rpm) twice. Suspension aliquots (20 μL) were processed immediately (time zero minutes), and after 60 and 180 minutes post-incubation with MMS, using the alkaline comet assay. The results were analyzed at different times post-treatment with MMS and the percentage of residual damage (RD) in the DNA after T180' was calculated using the DI value at T0' for each subject as 100% of induced damage (Agnoletto et al. 2007).

STATISTICAL ANALYSIS

The experimental results and epidemiological data were analyzed using the Statistical Package for Social Sciences Program, version 23.0 (SPSS 23.0). Descriptive analysis was carried out to delineate the epidemiological and pathophysiological profiles of lung cancer patients, quantifying the damage index and repair kinetics of the DNA. Subsequently, Student's *t*-test for continuous variables and chi-square for categorical variables. Pearson correlation was applied. The results were evaluated according to the assumption of normality, constant variance, and independence. A P-value < 0.05 was considered significant.

RESULTS

The clinical characteristics of the CA and CO groups are described in Table I, where a higher frequency of males, smoking history and damage index in the CA group compared to the CO group can be observed, but without statistical significance.

Although both groups had high levels of DNA damage, patients from the CA group showed a significant increase when compared to controls (Figure 2a). In the analyses involving the repair of MMS-induced damage, it was observed that the repair index showed a differentiated profile between the groups at different times (T0', T60' and T180'), being significantly higher in the CA than in CO group (Figure 2b). It was also observed that in the CA group the DNA breakage was the highest at T60', when compared to T0', while the CO group showed a decrease in the induced damage, demonstrating signs of damage repair.

The estimated RD, after DNA repair kinetics, also differed significantly (P = 0.050) between the groups, suggesting that lung cancer patients had a delay in DNA repair (Figure 2c).

A moderate and significant correlation was found between DNA DI at T0' and DI at T60', only for the CA group, i.e., lung cancer patients are more susceptible to MMS-induced damage and/or DNA repair delay (Figure 3).

The chemotherapeutic agents used in the treatment of the CA group belonged to the classes of platinum compounds, vinca alkaloids, taxanes, bisphosphonates, podophyllotoxins and pyrimidine analogs, and all patients with lung cancer underwent treatment with one, two or three drugs, but up to no more than two classes [n = 5 (21%) used one chemotherapeutic class; n = 19 (79%) used two classes].

TABLE I
Clinical characteristics of CA and CO groups.

Variables	CA (n = 24)	CO (n = 23)	P
Gender, n (%)			0.30
Male	13 (54.2)	9 (39.1)	
Female	11 (45.8)	14 (60.9)	
Age in years (mean ± sd)	67.4 ± 7.0	63.3 ± 7.4	0.06
Ethnicity, n (%)			0.10
Caucasian	19 (79.2)	22 (95.7)	
Non-Caucasian	5 (20.8)	1 (4.3)	
Smoking history. n (%)			
Smoker	7 (29.2)	-	-
Ex-smoker	15 (62.5)	-	-
Non-smoker	2 (8.3)	23 (100.0)	-
Time of smoking, n (%)			
10-15 years	1 (4.5)	-	-
More than 15 years	21 (95.5)	-	-
Comorbidities. n (%)			0.78
Yes	11 (45.8)	14 (60.8)	
SAH	7 (29.1)	7 (30.4)	
Diabetes	2 (8.0)	-	
Others	2 (8.0)	7 (30.4)	
No	13 (54.2)	9 (39.1)	
Cigarettes smoked/years (median)	7300 (1460-21900)	-	-
Education. n (%)			0.14
Non-literate	2 (8.3)	-	
Incomplete basic school	12 (50.0)	7 (38.8)	
Complete basic school	10 (41.6)	1 (5.5)	
Complete high school	-	2 (11.1)	
Incomplete high school	-	2 (11.1)	
Complete higher education	-	6 (33.3)	
DNA Damage Index			
Alkaline DI (mean ± sd)	291.1 ± 53.1	261.6 ± 32.4	0.013

Data expressed as ±: mean and standard deviation (sd); n (%): sample number and percentage; CA: group with lung cancer; CO: Control; SAH: systemic arterial hypertension; Student's *t*-test for continuous variables and chi-square for categorical variables.

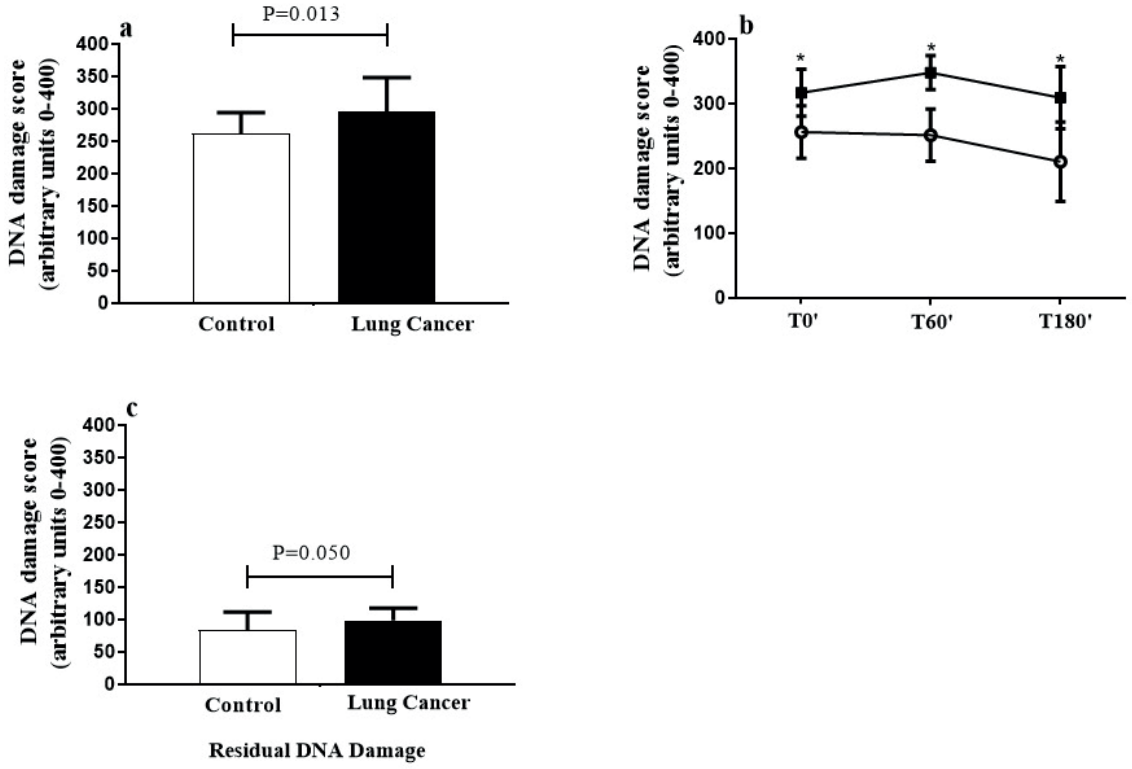

Figure 2 - DNA Damage Index and repair kinetics post-incubation with methyl methanesulfonate in the CO and CA groups through the comet assay. **a**: Frequency of baseline damage index; **b**: repair kinetics: frequency of DNA damage index at different times [time zero (T0') *P < 0.050; Time 60 minutes (T60') *P < 0.050; Time 180 minutes (T180') *P < 0.050 post-incubation with MMS; **c**: Frequency of residual damage after incubation with MMS. CO: control group; CA: group with lung cancer; Student's *t*-test was performed to compare the alkaline damage index and the residual damage between the groups and the analysis of variance (ANOVA) test with Tukey post-hoc.

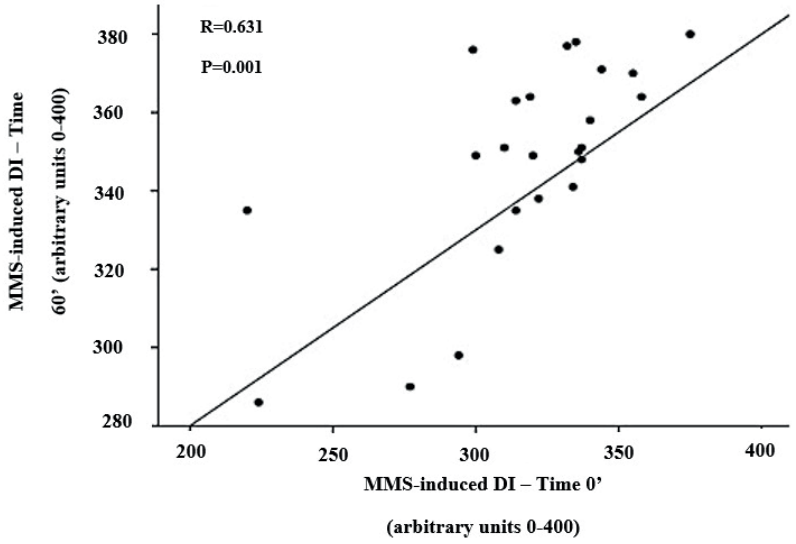

Figure 3 - Association between MMS-induced DI in DNA, at times 0 and 60 minutes, in patients with lung cancer. DI = DAMAGE index; MMS = methyl methanesulfonate; Spearman's Correlation was used.

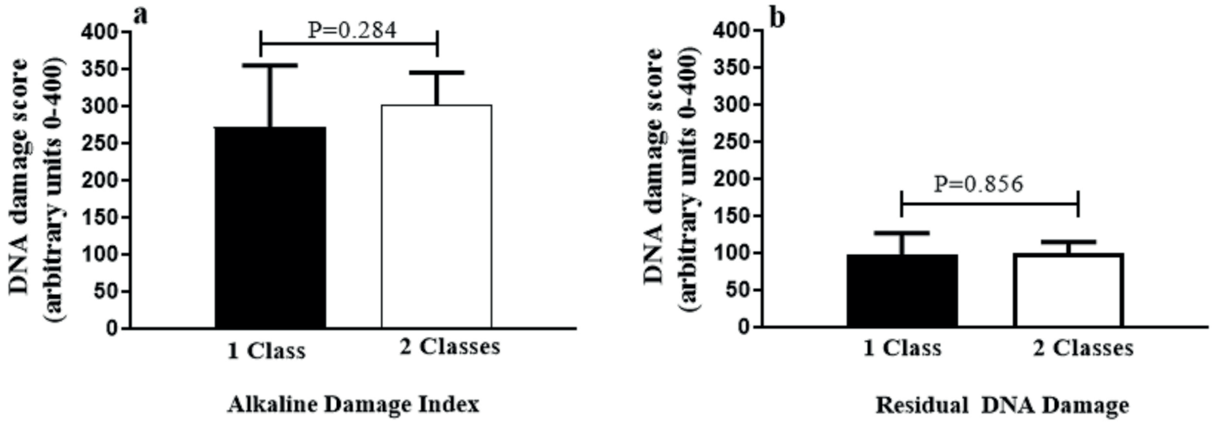

Figure 4 - Alkaline damage index and residual damage of lung cancer patients stratified by groups undergoing treatment with 1 or 2 classes of chemotherapeutic RDugs. 1 class: number of subjects = 5; 2 classes: number of subjects =19; DI: Damage index. **a**: Frequency of baseline damage index; **b**: Frequency of residual damage after incubation with MMS. Student's *t*-test was performed to compare the alkaline damage index and residual damage between the groups.

We stratified the results of the CA group comet assay for the classes of chemotherapy agents used in the treatment and no significant differences were observed. Nevertheless, patients with lung cancer undergoing treatment with 2 chemotherapeutic classes had higher baseline DI and RD values (Alkaline DI: 301.2 ± 43.8; RD: 99.2 ± 15.5) when compared to patients receiving treatment with one class (DI 272.0 ± 82.6; RD 97.5 ± 29.4), however, the values were not statistically different between groups (Figure 4).

DISCUSSION

The main findings of the present study can be summarized in three points: 1) Patients with lung cancer show high DNA damage index; 2) these patients have a higher alkylating agent-induced damage; 3) it is not possible to state that they have DNA repair deficiency at T60' post-incubation with MMS, or if they have an accumulation of DNA damage.

As previously described in the literature, lung cancer patients are expected to be more prone to DNA damage, both due to the pathological process and the radiochemotherapy (Li et al. 2016). All types of lung tissue injury may cause DNA damage (Rennard et al. 2006), which is considered the main cause of lung cancer development and is usually recognized and repaired by intrinsic factors (Orlow et al. 2008, Li et al. 2016, Loeb et al. 2003). DNA damage also induces several cell responses, which allow cells to eliminate and/or manage these changes physiologically (Sancar et al. 2004). In our study, a high DNA damage index was found in both groups (Figure 2a). It is necessary to consider that, when assessing DNA damage in older patients undergoing cancer treatment, aspects such as the natural aging process and/or previous pathologies (Zhou et al. 2011, Subash et al. 2010) can influence the outcome of the damage index (Walker et al. 2016).

The high standard for the DNA damage values in the comet assay found could reflect the great genetic heterogeneity of the groups, as found in previous studies, but with other populations (da Silva et al. 2013a, b). It is a critical aspect in human biomonitoring studies associated with physiological state and life style (Maluf et al. 2007). DNA damage and DNA repair seem to be closely associated with aging as a result of endogenous factors, lifestyle, occupational or environmental exposure (Mergener et al. 2009).

In our study, an enhanced susceptibility to the alkylating agent MMS that induced DNA damage

was observed in lung cancer patients (Figure 2b and 2c). In our opinion, the increment of DI in the DNA at T60' in the CA group, when compared to controls, is more characterized by an accumulation of DNA damage resulting from chemotherapy than a deficiency or delay in the damaged DNA repair. This may be corroborated by the positive correlation found between T0' and T60' (Figure 3) as well as the high damage in the CA group undergoing chemotherapy with two classes of drugs (Figure 4a).

The use of chemotherapeutic agents seems to influence the increase in the DNA damage index (Li et al. 2016, Choi et al. 2015) but in our study, our results could prove this assumption. Cytotoxic chemotherapeutic agents may cause secondary neoplasms and tumor cell evolution, contributing to the aggravation of DNA damage (Muenzi et al. 2015, Szikriszt et al. 2016). DNA repair is critical for protection against lung cancer, as it removes unwanted mutations present in the DNA strand (Dusinska and Collins 2008). Therefore, tumor cell repair capacity is one of the main determinants of the efficacy and resistance to DNA-damaging chemotherapeutics, such as cisplatin, used in more than 90% of lung cancer chemotherapy treatments (Choi et al. 2015).

The main limitation of the study was the strict inclusion/exclusion criteria that limited our sample size, which did not allow us to make inferences about DNA damage and chemotherapeutic treatment. Although the main age of the groups was heterogeneous, we evaluated only adult volunteers who agreed to participate in the study.

The present study showed an increase in DNA damage in lymphocytes from lung cancer patients, both for baseline and residual indices. The conclusion also suggest an accumulation of DNA damage in these patients due to the association between induced damage immediately after incubation with the MMS alkylating agent and 1 hour later. Our study emphasizes the use of the comet assay as a biomonitoring method for patients with lung cancer undergoing chemotherapy treatment, aiming to better understand genomic instability alterations in clinical practice.

AUTHOR CONTRIBUTIONS

Marcia Raquel Schneider contributed to the planning of the study, its execution, statistical analysis, data interpretation and writing of the manuscript. Andréa Lúcia Gonçalves da Silva and Augusto Ferreira Weber contributed to the statistical analysis, data collection, and writing of the manuscript.

Cássia da Luz Goulart and Paloma de Borba Schneiders contributed to execution of the study and revision of the manuscript. Lia Gonçalves Possuelo contributed to the planning of the study and revision of the manuscript. Andréia Rosane de Moura Valim contributed to the planning of the study, its execution, and revision of the manuscript.

REFERENCES

AGNOLETTO MH, GUECHEVA TN, DONDÉ F, DE OLIVEIRA AF, FRANKE F, CASSINI C, SALVADOR M, HENRIQUES JA AND SAFFI J. 2007. Association of low repair efficiency with high hormone receptors expression and SOD activity in breast cancer patients. Clin Biochem 40(16-17): 1252-1258.

CHOI YJ, PARK JM, YI JM, LEEM SH AND KANG TH. 2015. Enhanced nucleotide excision repair capacity in lung cancer cells by preconditioning with DNA-damaging agents. Oncotarget 6(26): 22575-22586.

COLLINS AR, OSCOZ AA, BRUNBORG G, GAIVÃO I, GIOVANNELLI L, KRUSZEWSKI M, SMITH CC AND STETINA R. 2008. The comet assay: topical issues. Mutagenesis 23(3): 143-151.

DA SILVA AL, DA ROSA HT, KARNOPP TE, CHARLIER CF, ELLWANGER JH, MOURA DJ, POSSUELO LG, VALIM AR, GUECHEVA TN AND HENRIQUES JA. 2013b. Evaluation of DNA damage in COPD patients and its correlation with polymorphisms in repair genes. BMC Med Genet 14: 93.

DA SILVA ALG, ROSA HT, CHARLIER CF, SALVADOR M, MOURA DJ, VALIM ARM, GUECHEVA TN AND HENRIQUES JAP. 2013a. DNA Damage and Oxidative Stress in Patients with Chronic Obstructive Pulmonary Disease. J Open Biom 6: 1-8.

DUSINSKA M AND COLLINS AR. 2008. The comet assay in human biomonitoring: gene environment interactions. Mutagenesis 23(3): 191-205.

GUNASEKARANA V, RAJ GV AND CHAND P. 2015. A comprehensive review on clinical applications of comet assay. J Clin Diagn Res 9(3): GEO1-GEO5.

INCA - INSTITUTO NACIONAL DE CÂNCER. 2016. Banco de dados do INCA. Disponível em: http://www2.inca.gov.br/wps/wcm/connect/inca/portal/home. Acessado em 2018 (Dia 10 de Maio).

JIANG T, ZHANG X, DING J, DUAN B AND LU S. 2015. Inflammation and cancer: inhibiting the progression of residual hepatic vx2 carcinoma by anti-inflammatory drug after incomplete radiofrequency ablation. Int J Clin Exp Pathol 8(11): 13945-13956.

LI L, ZHU T, GAO YF, ZHENG W, WANG CJ, XIAO L, HUANG MS, YIN JY, ZHOU HH AND LIU ZQ. 2016. Targeting DNA Damage Response in the Radio(Chemo) therapy of Non-Small Cell Lung Cancer. Int J Mol Sci 17(6): 1-15.

LOEB LA, LOEB KR AND ANDERSON JP. 2003. Multiple mutations and cancer. Proc Natl Acad Sci USA 100(3): 776-781.

MALUF SW, MERGENER M, DALCANALE L, COSTA CC, POLLO T, KAYSER M, DA SILVA LB, PRA D AND TEIXEIRA PJ. 2007. DNA damage in peripheral blood of patients with chronic obstructive pulmonary disease (COPD). Mutat Res 626(1): 180-184.

MERGENER M, MARTINS MR, ANTUNES MV, DA SILVA CC, LAZZARETTI C, FONTANIVE TO, SUYENAGA ES, ARDENGHI PG, MALUF SW AND GAMARO GD. 2009. Oxidative stress and DNA damage in older adults that do exercises regularly. Clin Biochem 42(16-17): 1648-1653.

MUENYI SC, LJUNGMAN M AND STATES JC. 2015. Arsenic disruption of DNA damage responses – potencial role in carcinogenesis and chemotherapy. Biomolecules 5(4): 2184-2193.

OMS - ORGANIZAÇÃO MUNDIAL DA SAÚDE. 2015. Banco de dados da OMS, 2015. Disponível em: http://www.paho.org/bra/. Acessado em 2018 (Dia 10 de Maio).

ORLOW ET AL. 2008. DNA damage and repair capacity in lung cancer patients: prediction of multiple primary tumors. J Clin Oncol 26(21): 3560-3566.

RENNARD SI, TOGO S AND HOLZ O. 2006. Cigarret smoke inhibits alveolar repair. A mechanism for the development of emphysema. Proc Am Thorac Soc 3(8): 703-708.

SANCAR A, LINDSEY-BOLTZ LA, UNSAL-KACMAZ K AND LINN S. 2004. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem 73: 39-85.

SHAM ASK, SZETO YT, BENZIE IFF AND TAN-UN KC. 2003. A Preliminary Study of DNA Damage in Peripheral Lymphocytes from Lung Cancer Patients and Healthy Subjects. Turk J Med Sci 33: 149-154.

SUBASH P, PREMAGURUMURTHY K, SARASABHARATHI A AND CHERIAN KM. 2010. Total antioxidant status and oxidative DNA damage in South Indian population of essential hypertensives. J Hum Hypertens 24(7): 475-482.

SZIKRISZT B ET AL. 2016. A compreehensive survey of the mutagenic impact of common cancer cytotoxics. Genome Biol 17: 99.

WALKER AM, STEVENS JJ, NDEBELE K AND TCHOUNWOU PB. 2016. Evaluation of arsenic trioxide potential for lung cancer treatment: Assessment of apoptotic mechanisms and oxidative damage. J Cancer Sci Ther 8(1): 1-9.

WASSON GR, MCKELVEY-MARTIN VJ AND DOWNES CS. 2008. The use of comet assay in the study of human nutrition and cancer. Mutagenesis 23(8): 153-162.

WHO. 2010. Guidelines Approved by the Guidelines Review Committee. WHO Guidelines on Drawing Blood: Best Practices in Phlebotomy. Geneva: World Health Organization; 2010.

ZHOU F, ONIZAWA S, NAGAI A AND AOSHIBA K. 2011. Epithelial cell senescence impairs repair process and exacerbates inflammation after airway injury. Respir Res 12: 78.