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Dry tobacco leaves: an *in vivo* and *in silico* approach to the consequences of occupational exposure

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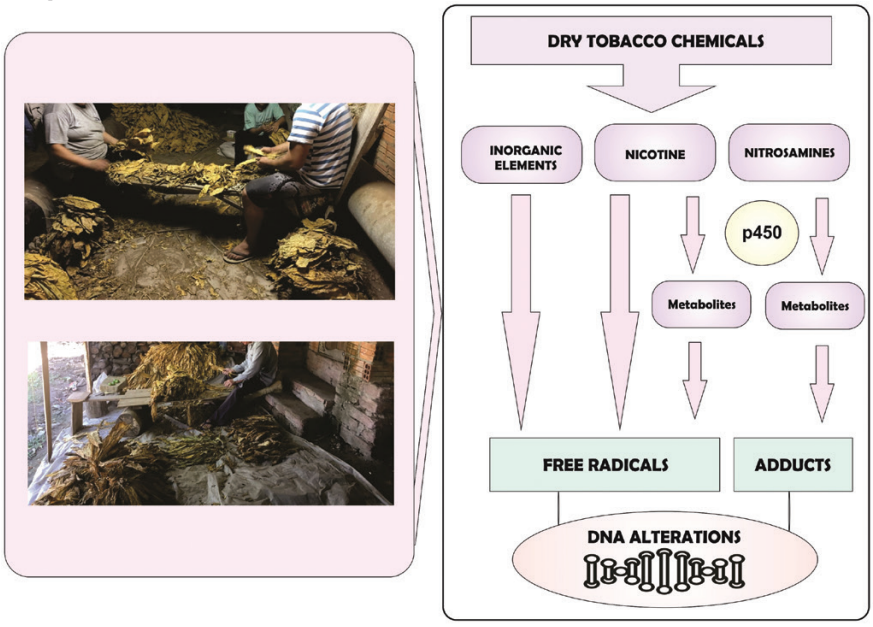
Abstract

Exposure of tobacco workers handling dried tobacco leaves has been linked to an increased risk of toxicity and respiratory illness due to the presence of nicotine and other chemicals. This study aimed to evaluate the DNA damage caused by the exposure of tobacco growers during the dry leaf classification process and the relation to cellular mechanisms. A total of 86 individuals participated in the study, divided into a group exposed to dry tobacco (n = 44) and a control group (n = 42). Genotoxicity was evaluated using the alkaline comet assay and lymphocyte micronucleus (MN) assay (CBMN-Cyt), and measurement of telomere length. The levels of oxidative and nitrosative stress were evaluated through the formation of thiobarbituric acid reactive species, and nitric oxide levels, respectively. The inorganic elements were measured in the samples using particle-induced X-ray emission method. The combination of variables was demonstrated through principal component analysis and the interactions were expanded through systems biology. Comet assay, MN, death cells, thiobarbituric acid reactive species, and nitrosative stress showed a significant increase for all exposed groups in relation to the control. Telomere length showed a significant decrease for exposed women and total exposed group in relation to men and control groups, respectively. Bromine (Br) and rubidium (Rb) in the exposed group presented higher levels than control groups. Correlations between nitrate and apoptosis; Br and MN and necrosis; and Rb and telomeres; besides age and DNA damage and death cells were observed. The systems biology analysis demonstrated that tobacco elements can increase the nuclear translocation of NFKB dimers inducing HDAC2 expression, which, associated with BRCA1 protein, can potentially repress transcription of genes that promote DNA repair. Dry tobacco workers exposed to dry leaves and their different agents showed DNA damage by different mechanisms, including redox imbalance.

Graphical Abstract



Keywords: occupational exposure; dry tobacco; nicotine; tobacco-specific nitrosamines; DNA damage; grading

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Introduction

Tobacco is grown in several countries [1, 2], being considered a crop of great socioeconomic importance, as more than 30 million rural workers may be involved in production worldwide. In the southern region of Brazil, tobacco is highly representative. In the 2020/2021 harvest, tobacco was grown by 138 thousand integrated producers, representing approximately 585 thousand people participating in this production cycle. The robust production of tobacco in Brazil keeps it in the spotlight on the world stage. It has occupied the position of second largest producer worldwide and, since 1993, the largest tobacco exporter in the world [2–5].

Tobacco cultivation takes approximately 10 months, from seedling production to harvest. In Brazil, the harvest usually takes place from January to March, with intensive use of labor. After harvesting, the tobacco goes through the drying process [6–8]. To maintain plant quality, different pesticides are used for the maintenance and cultivation of tobacco [7, 9]. In addition to the use of pesticides, tobacco contains nicotine, a natural compound present in the plant. Nicotine is known to be a toxic and potent substance i.e. easily and quickly absorbed by the skin and mucous membranes [10, 11]. It can cause acute toxicity, in addition to offering mainly long-term health risks, such as chromosomal damage and genomic instability [12–16].

Nicotine represents about 90% of the alkaloids found in the tobacco plant, while the smaller alkaloids, nornicotine, anatabine, and anabasine, represent 10% [11, 17, 18]. It is believed that these alkaloids arise during tobacco processing due to bacterial action or the oxidation process, and that they are responsible for the formation of tobacco-specific nitrosamines (TSNAs), due to their reaction with nitrosing agents [11]. The main nitrosamines are NNK [4-(methylnitrosamino)-abutanoal], NNN [*N*′-nitrosonornicotine], NAT [*N*′-nitrosoanatabine], NAB [*N*′-nitrosoanabasin], NNAL [4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol], iso-NNAl, and iso-NNAC [10, 11]. Nitrosamines have biological effects such as carcinogenicity, mutagenicity, embryopathy, and teratogenic actions [11], e.g. NNK and NNN are classified in Group 1 as carcinogens in humans by the International Agency for Research on Cancer [10, 19, 20].

During the processing of dry tobacco, a large amount of dust is generated, a characteristic of the leaf itself, which when dry ends up breaking/crumbling, releasing volatile tobacco components into the ambient air [5, 16, 21, 22]. This tobacco dust can affect the respiratory tract, causing allergies, rashes, wheezing, shortness of breath, dyspnea, rhinitis, nausea, dizziness, and vomiting in exposed workers. Studies have indicated high frequencies of respiratory disorders and the occurrence of allergic alveolitis and decreased lung function in tobacco, cigar, and cigarette factory workers [17, 23, 24]. Studies from India indicated that the inspired concentration of dust was 150 times higher in tobacco processing plants than in the general environment and reported an increased incidence of chronic bronchitis among tobacco processors [17, 25].

Studies involving occupational exposure to dry tobacco are still scarce. The literature has different studies evaluating the problem of pesticides, nicotine, green tobacco disease, but little is known about dry tobacco and its effects on the workers' health. In view of the knowledge regarding the possible harm that the compounds present in tobacco can cause to health, this study aimed to evaluate DNA damage, oxidative and nitrosative stress and the relation with cellular mechanisms and their associations through systems biology.

Methodology

Individuals and sampling

This study was approved by the ULBRA Research Ethics Committee (CAAE/CONEP 13898619.0.0000.5349). The study took place in the cities of Santa Cruz do Sul and Sobradinho, in the state of Rio Grande do Sul (Brazil). The samples were collected between January and March 2020. The estimated population of these cities, according to IBGE [26] data, is approximately 147,312 inhabitants. Eighty-six (86) individuals participated in the study, distributed among 44 exposed to dry tobacco, who were working with the classification of dry leaves, and 42 belonging to the control group, in the same region as the exposed group, but who perform activities that are not influenced by harmful agents (mainly school and offices employees). All individuals participating in the study were invited to answer a version of the Informed Consent Form (ICF) and a Portuguese version adapted from the questionnaire of the Commission for Protection Against Environmental Mutagens and Carcinogens [27], and a Nutritional 24-hour recall questionnaire (R24 h) [28] providing them with information about their lifestyle patterns. Blood samples were collected by venipuncture, with vacutainers containing heparin, collected by a qualified professional. The samples were stored and transported under controlled temperature until processed at ULBRA's Toxicological Genetics Laboratory.

An assessment of the obesity factor was performed using the body mass index (BMI), as follows: below 18.5 = underweight; between 18.5 and 24.9 = ideal weight; between 25.0 and 29.9 = overweight; above 30.0 = obesity [29]; and waist-to-height ratio (WHtR), being considered high risk (>0.5) and low risk (<0.5) for cardiovascular diseases and diabetes [30, 31].

Alkaline comet assay

The alkaline comet assay (CA) followed the recommendations of Tice *et al.* [32] with some modifications. Blood samples were placed on slides containing low-melting point agarose, placed in lysis buffer, and subsequently subjected to DNA electrophoresis. Then, DNA slides were stained with silver nitrate. One hundred cells per subject were counted using an optical microscope (Zeiss Primo Star). To calculate the visual score (0–400), the evaluation followed the recommendations of Collins [33], considering each cell an attribution of damage, distributed in five classes (no damage = 0 to maximum damage = 4) according to the tail size and shape. The visual confirmation score is a well-validated assessment method [33].

Cytokinesis-block micronucleus assay

The performance of the cytokinesis-block micronucleus (CBMN-Cyt) assay test followed the recommendations described by Fenech [34]. Lymphocyte cell cultures were established for each blood sample collected with the use of cytochalasin B. After the incubation period, lymphocytes were collected, fixed, and placed on microscopic slides through a cytocentrifuge and later stained with Panotic reagent

(Instant-Prov). For everyone, 1000 binucleated cells were evaluated, scoring to determine the frequency of micronuclei (MN), nuclear plasmatic bridges (NPB), and nuclear buds (NBUD). To assess the frequency of necrotic (NECR) and apoptotic (APOP) cells, 500 cells were randomly scored using optical microscopy (1000×).

Telomere length measurement

Telomere length (TL) measurement followed the quantitative polymerase chain reaction (qPCR) protocol described by Cawthon [35] with minor modifications by Kahl *et al.* [36]. Isolated genomic DNA samples were quantified in a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Australia), and only samples between the range 1.8–2.0 (OD260/OD280) were used. Each sample was then diluted at the time of use as per experimental requirement (5 ng/µl). A standard curve was established by serially diluting known amounts of a DNA pool. In each run, pooled genomic DNA generated within the laboratory was also included. Standard DNA and reference sample were the same for all experiments. A melting curve analysis was performed to verify the specificity and identity of the products. The 36B4 single copy gene was used to control amplification. The samples were analyzed in triplicate, with negative and lymphoblastic T-leukemia cell line 1301 as positive control and standard curve on each plate, using the Step One Plus™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The following was used for the preparation of the master mix solution: SYBR Green Power (Applied Biosystems, Foster City, CA, USA), 20 ng of DNA, injection water, 0.2 µmol of telomere primers (forward: 5′-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3′; reverse: 5′-CCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3′) and 0.2 µmol of 36B4 primers (forward: 5′-CAGCAAGTGGGAAGGTGTAATCC-3′; reverse: 5′-CCCATTCTATCATCAACGGGTACAA-3′). The qPCR followed the conditions (for telomere and 36B4 amplicons): Activation of Taq polymerase for 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min. The cycle limit (Ct) of each sample was used to calculate the total telomeres in kb per human diploid genome.

Thiobarbituric acid reactive species

The determination of the lipid peroxidation index was evaluated through the formation of thiobarbituric acid reactive species during a heating reaction. This assay is considered a sensitive method for measuring lipid peroxidation, as indicated by Wills [37] with modifications. From each sample, 400 µl were taken and combined with 600 µl of 15% trichloroacetic acid and 0.67% thiobarbituric acid. Then the mixture was heated to 100°C for 15 min. After cooling to room temperature, they were centrifuged at 5200 × *g* for 5 min. Once the supernatants had been obtained, the absorbance at 530 nm was measured. The hydrolyzed malondialdehyde (MDA) was used as a standard and the results were expressed as nmol MDA/ml.

Evaluation of nitrosative stress

The production of nitric oxide was measured indirectly through a quantitative colorimetric test by the Griess reaction. This test is based on the enzymatic reduction of nitrates to nitrites in the presence of nitrate reductase and nicotinamide adenine dinucleotide phosphate, and subsequent reaction of the formed nitrites (or initially present in the samples) with the Griess reagent (mixture of sulfanilamide and naphthylethylenediamine, specific for NO_2^-). The reading will be performed in a microplate reader at 540 nm, and the results expressed in mmol of NO_2^- NO_3^-, according to Granger *et al.* [38].

Quantification of inorganic elements (PIXE)

The inorganic elements were determined using the PIXE (particle-induced X-ray emission) technique. Briefly, the dried blood samples were pressed into pellets and placed inside the reaction chamber for analysis. Energetic protons reaching the samples inside the reaction chamber cause the ionization of inner layers of atoms present in each sample. This ionization process can induce the production of characteristic X-rays emitted by the atoms in the sample. The X-rays are then collected by the Si (Li) detector placed at −135° in relation to the beam direction. Data analysis took place using the GUPIXWIN software developed at the University of Guelph [39]. Samples were analyzed in triplicate.

Systems biology

The interaction network was constructed including the compounds found in tobacco, nicotine, the nitrosamines NNA, NNK, and NNAL and the inorganic elements detected by the PIXE technique. The subnetworks were built evaluating compound–protein and protein–protein interactions using the STITCH v.5.0 database (http://stitch.embl.de) [40] and the STRING search tool 11.0 (http://string-db.org) [41–43], respectively. In these two online search tools, the interaction network was downloaded based on the following parameters: no more than 50 interactions, a high confidence score (0.400), and a network depth equal to 2. All of the active prediction methods were enabled, excluding text mining. The predicted interactions were extracted using *Homo sapiens* as model organism. Different subnetworks generated were combined into a single compound–protein interaction network through the Cytoscape 3.8.0 Advanced Merge Network plugin [44]. Using the Molecular Complex Detection (MCODE) plugin [45] in Cytoscape 3.8.0, the clusters were analyzed proposing to search for functional protein complexes. For this, the parameters used were: loops included; degree cutoff 2; deletion of single connected nodes from cluster (haircut option enabled); expansion of cluster by one neighbor shell allowed (fluff option enabled); node density cutoff 0.1; node score cutoff 0.2; kcore 2; and maximum depth of network 100. To as cluster, Biological Network Gene Ontology (BiNGO) [46] plugin was used to analyze the genetic ontology. The degree of functional enrichment for a given cluster and category was quantitatively computed (*P* value) by hypergeometric distribution, and multiple test correction was also assessed by applying the false discovery rate algorithm which was fully implemented using the BiNGO software with a significance level of $P < .05$. To characterize the node importance in network interaction, global properties such as betweenness and node degree centralities were computed using Centiscape 2.2 plugin [47]. Nodes with a relatively higher degree were called hubs and nodes with high betweenness were called bottlenecks [48, 49]. A hub-bottleneck (HB) node can be a key regulator of biological processes, being considered indispensable for the result of network communication. Queries on the

human gene database (GeneCards—http://www.genecards.org/) were made to understand the function of each HB, as this database contains extensive information about human genes [50].

Statistical analysis

The normality of variables was evaluated by the Kolmogorov–Smirnov test. Student's unpaired *t* test was used to compare groups (control and exposed). The Spearman's test correlation was performed to assess the relationship between independent variables. Statistical analysis was performed using GraphPad PRISM software, version 5.01 (GraphPad Inc., San Diego, CA). Principal component analysis based on descriptive data analysis was applied to damaged marker parameters to assess the relationships between variables and the influence of these parameters on the overall results, using the XLSTAT® 2020.3.1 program (ADDINSOFT SARL, Paris, France) [51].

Results

Eighty-six subjects were considered in this study. The study groups were formed by 44 subjects exposed to dry tobacco during the leaf classification period and 42 control subjects, 23 exposed men and 20 controls, and 21 exposed women and 22 controls. The mean age of the groups was 39.65 ± 12.55 years for the control group and 39.87 ± 10.23 for the exposed group (Table 1). BMI and WHtR were calculated for the groups (Table 1). Other participant characteristics data are also described in Table 1.

Visual score (CA) determined by alkaline CA showed significantly increased values for all exposed groups (men, women, and all groups = men + women) in relation to control groups (Fig. 1). Table 2 presents CBMN-Cyt test results. This table shows a significant increase of MN for all exposed groups when compared with the control group, and in addition women higher than men in the exposed group. For the NBUD parameter, a significant increase in relation to the control group was only observed when women and men were evaluated together (all exposed). In the assessment of cell death, the parameters of apoptosis and necrosis showed a significant increase in all exposed groups when compared with their control groups. The assessment of TL showed a significant decrease for exposed women, when compared with the exposed group, and in the group of all exposed in relation to all controls (Fig. 2).

The determination of thiobarbituric acid reactive species and the production of nitrates showed significantly higher values for all exposed groups than their control groups (Figs 3 and 4). The inorganic elements detected by the PIXE technique can be seen in Table 3. Seventeen (17) inorganic elements were detected, including bromine (Br) and rubidium (Rb), which were significantly different for the exposed group when compared with their control groups (Table 3).

Correlations were performed between the parameters studied, and only the significant results are presented in the sequence. Different correlations in relation to age were performed demonstrating significant results for exposed men in relation to necrosis ($r = 0.3876$; $P = .0284$), BMI ($r = 0.4354$; $P = .0063$), and WHtR ($r = 0.5362$; $P = .0006$). In addition, all exposed groups (men + women) in relation to necrosis ($r = 0.3228$; $P = .0174$) and WHtR ($r = 0.2924$; $P = .0200$) presented a significant correlation with age. Other significant correlations for the exposed group were observed in relation to Br and MN (all groups: $r = 0.3346$; $P = .0303$; female group: $r = 0.6605$; $P = .0021$); Br and necrosis (all groups: $r =$

Table 1. Main characteristics of the control group and the group occupationally exposed to dry tobacco; and observed parameters for BMI and person's waist/height, related to cardiovascular disease (WHtR).

Population characteristics	Control		Exposed	
	Male	Female	Male	Female
Individuals (*n*)	20	22	23	21
Age (years; mean ± SD)	35.45 ± 12.41	37.73 ± 13.98	43.09 ± 13.02	43.23 ± 12.55
Alcohol consumption (%)[a]				
Yes	50	50	61	24
No	50	50	39	76
Meat consumption (%)[b]				
Yes	100	100	100	100
No	0	0	0	0
Consumption of fruits and vegetables (%)[b]				
Yes	65	86	100	91
No	35	14	0	9
BMI (%)				
Under weight	0	3.57	0	3.70
Ideal weight	57.14	46.42	55.26	33.33
Overweight + obesity	42.86	50.01	44.74	62.97
WHtR (%)				
Low risk	0	0	0	4
High risk	100	100	100	96

aAlcoholic drink at least once a week.
bConsumption at least once a week. Study participants do not supplement with vitamins. Smokers were not considered in this study.

0.4951; $P = .0009$; male group: $r = 0.4522$; $P = .0303$; female group: $r = 0.5422$; $P = .0165$); Br and NPB (male group: $r = 0.4423$; $P = .0346$); Rb and DNA damage observed using CA for the exposed group ($r = 0.3560$; $P = .0358$); and Rb and TL (all groups: $r = 0.5072$; $P = .0069$; male group: $r = 0.7410$; $P = .0016$). The principal component analysis results show a spatial distribution of the results found and the inference of these data in each sample. Two significant principal components are demonstrated through the scores and the position of each sample in the ordering plane (F1 and F2), explaining, respectively, 25.08% and 15.47% of the total variation. Thus, two components responsible for the data structure were contacted by the factor analysis, explaining 40.55% of the total variance. F1 highlighted the exposed individuals and was positively charged by damage parameters such as telomeres, apoptosis, necrosis, NPB, and MN, by the Rb element, and by the nitrate parameter. F1 discriminated the association of the parameters of BMI and WHtR, Br, MN, and NBUD. F2 demonstrates that the control subjects were separated from the exposed subjects, and that most of the exposed subjects are in the fraction of the graph where the associations with the damage found in the study occur (Fig. 4).

A network of 1712 node was grouped in which the interaction of 1695 proteins and 17 chemical compounds related to dry tobacco farmer exposure were identified. The DTCPI (Dry Tobacco Chemicals—Protein Interaction) network (Supplementary Fig. 1) was named to assemble the interactome of these protein and chemical compounds. The topological properties of the DTCPI network were analyzed in relation to clusterization, gene ontology, and centralities (node degree and betweenness). Using the MCODE plugin in Cytoscape, the global network was partitioned into ten clusters (score >10), which were regarded as the network core in terms of functionality (data not shown). To determine the biological function of each cluster, pathway enrichment from

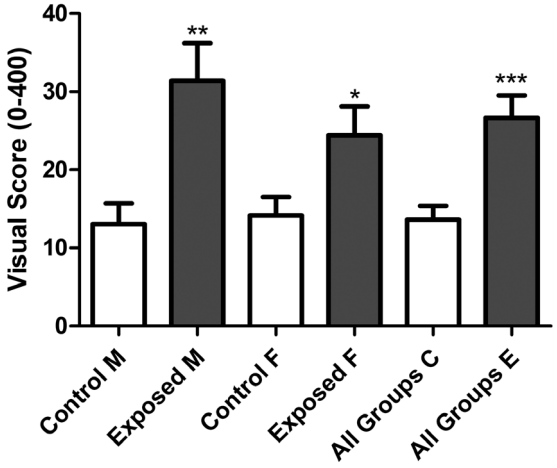

Figure 1. Mean ± standard deviation of the visual score (0–400) assessed by the CA in cells from the exposed group compared with the control group. Male (M), Female (F), All groups C (control), and All groups E (exposed). ***Significant at $P < .001$; **significant at $P < .01$; *significant at $P < .05$; unpaired *t* test.

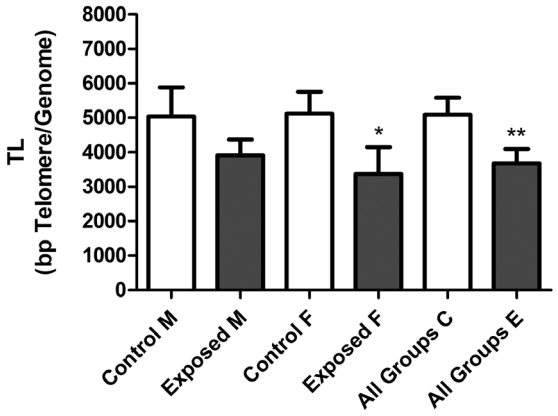

Figure 2. TL using qPCR comparing groups exposed to dry tobacco with control groups. Male (M), Female (F), All groups C (control), and All groups E (exposed). Data were expressed in base pairs (bp) (mean ± standard deviation). **Significant for $P < .01$; *significant for $P < .05$; unpaired *t* test.

Table 2. Analysis of the different parameters of the CBMN-Cyt assay of individuals exposed to dry tobacco.

Parameters	Male		Female		All groups	
	Control	Exposed	Control	Exposed	Control	Exposed
Cell proliferation (1000 cells)						
NDI	125.8 ± 122.3	126.8 ± 120.1	124.5 ± 126.3	126.6 ± 130.3	125.1 ± 123.9	126.7 ± 124.1
BN	235.8 ± 60.26	224.8 ± 67.47	205.2 ± 63.89	205.8 ± 76.97	219.2 ± 63.26	217.0 ± 71.41
DNA damage (1000 BN)						
MN	1.75 ± 1.39	4.47 ± 3.31**	2.56 ± 2.50	8.32 ± 9.42*,a	2.16 ± 2.03	6.04 ± 6.72**
NPB	0.75 ± 1.00	0.91 ± 1.14	1.25 ± 1.61	0.64 ± 0.85	1.00 ± 1.34	0.79 ± 1.03
NBUD	4.12 ± 3.42	6.09 ± 4.57	4.87 ± 4.27	7.54 ± 6.17	4.50 ± 3.83	6.68 ± 5.27*
Cell death (1000 cells)						
Apoptotic	2.0 ± 2.08	4.65 ± 3.50**	0.87 ± 1.02	4.23 ± 3.24***	1.47 ± 1.76	4.48 ± 3.37***
Necrotic	0.31 ± 0.79	1.25 ± 1.34*	0.06 ± 0.25	1.32 ± 1.32***	0.18 ± 0.59	1.28 ± 1.32***

Comparison of the exposed group with the control group. BN, binucleated cell.
aSignificant in relation to exposed male group ($P < .01$), unpaired *t* test.
*Significant for $P < .05$ (test *t*).
**Significant for $P < .01$.
***Significant for $P < .001$.

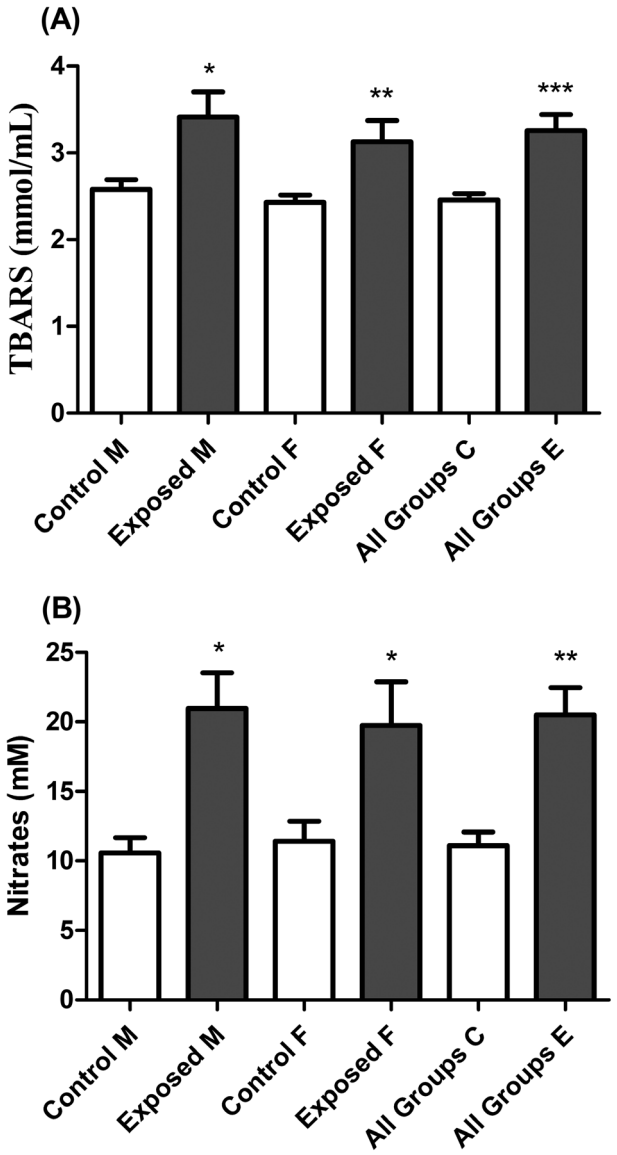

Figure 3. Mean and standard deviation obtained by thiobarbituric acid reactive substances (TBARS, mmol/ml), comparing the group exposed to dry tobacco with the control group (A); and nitrate concentrations (mM) measured in blood samples (serum) from growers exposed to dry tobacco compared with the control group (B). Male (M), Female (F), All groups C (control), and All groups E (exposed). ***Significant for $P <$ .001; **significant compared with control group with $P <$ .01; *significant compared with control group with $P <$ .05; unpaired *t* test.

the seven modules was processed using the BinGO plugin. Cluster 2 was demonstrated to be highly enriched and with GO bioprocesses highly relevant to this network such as DNA repair (P value = 4.61×10^{-81}), chromosome condensation (P value = 7.22×10^{-11}), telomere organization (P value = 6.17×10^{-10}), regulation of gene expression (P value = 8.90×10^{-7}), histone modification (P value = 3.55×10^{-5}), and response to oxidative stress (P value = 1.26×10^{-4}) (Supplementary Table 1). In line with this, node degree and betweenness were calculated to evaluate the importance of proteins in the CCPI network and the 20 highest HB nodes were: MAPK1, MAPK3, HSP90AA1, PRKACA, PRKACB, PCNA, AKT1, CREBBP, COX2, CFTR, TP53, JUN, EP300, SOD, F2, STAT1, GNB1, UBC, BRCA1, and CYP1A1 (Fig. 5).

Discussion

Given the data presented, there is no doubt regarding the importance of tobacco cultivation, especially in the south of Brazil. Each harvest involves a considerable number of workers, often entire families, who earn their livelihood by working with this crop [1, 5]. On the other hand, it is clear from existing studies that tobacco compounds cause harm to the health of these workers, as they undergo exposure to mixtures of pesticides, nicotine, and TSNAs for several months during the harvest [7, 9, 11–13, 15, 52].

In our study, evaluating workers exposed only to dry tobacco manipulation, we observed an increase in DNA damage through the CA (visual score) and CBMN-Cyt assay (MN and NBUD) for exposed groups. Other studies demonstrated genotoxicity through the CA in tobacco workers when compared with the control group, such as Da Silva *et al.* [9] who evaluated workers in different tobacco growing seasons. In studies in the same region, Alves *et al.* [15] also demonstrated DNA damage in the farmers' cells exposed to green tobacco leaves (nicotine) and Dalberto *et al.* [53] both in the harvest group (harvest) and in the classification group (TSNAs). Khanna *et al.* [54] found chromosomal aberrations in workers at a tobacco processing plant in India, associated with handling bidi tobacco and inhaling tobacco dust. Through the CA, it is possible to detect single breaks and double breaks in the DNA strand, formation of alkaline labile sites, incomplete base excision repair events, DNA–DNA crosslinks, and DNA–protein crosslinks [32, 55]. MN formation is associated with clastogenic and aneugenic effect, and NBUD is related to amplified DNA that might occur transiently after breakage of NPB [56]. We also observed damage by apoptosis and necrosis using the CBMN-Cyt assay.

The relation of DNA damage and chemical elements of tobacco leaves has been demonstrated, after detection by an increase in the frequency of MN related to serum cotinine levels in the harvest group [9, 15, 53], and in the dry tobacco classification group [53]. Our study demonstrated a correlation of genomic instability with nitrates, Br and Rb. Fischer *et al.* [57] demonstrated that the nitrate content of the tobacco has an enormous influence on the TSNA level (formed from nicotine). Nicotine has been found in tobacco powder in a processing environment [58]. A study carried out based on the exposure of V79 cells to aqueous extract of dry tobacco showed genotoxic damage to cells, and association with nicotine (1.56 mg/g of nicotine in dry tobacco leaf powder) [16]. Besides, during tobacco growing, workers are exposed to a wide range of pesticides, including organochlorines, organophosphates, carbamates, and pyrethroids [59, 60]. The composition of pesticides used in tobacco crops contains inorganic elements, detected also in our work in samples from exposed workers (significantly increased values only Br and Rb). Field-grown tobacco seedlings are produced in methyl bromide fumigated soil to manage pest problems [61, 62], and Rb is found in some fertilizers [63]. Some metals found in the tobacco plant can, through exposure, be deposited in the lungs. As they are not biodegradable, they can remain for long periods, causing changes in cellular functions through various devices. Associated with these data, it is worth noting that metals can act in the mechanism of carcinogenesis, through the inhibition of DNA repair, due to the generation of reactive oxygen species (ROS) [14, 64].

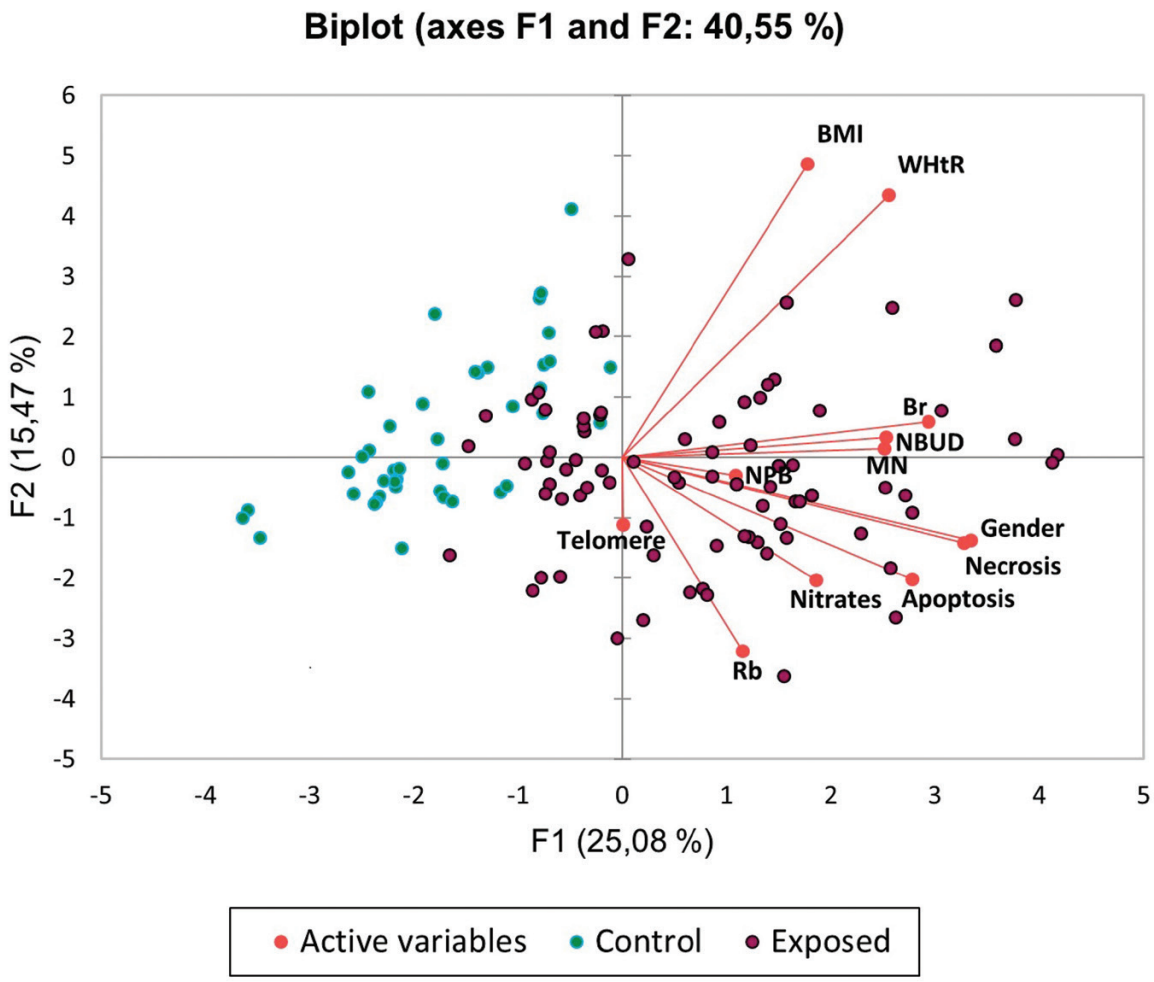

Figure 4. Principal component analysis (PCA) integrating DNA damage data and other parameters related to samples from individuals exposed to dry tobacco.

Table 3. Inorganic composition of whole blood samples from individuals exposed to dry tobacco compared with the control group (ppm ± standard error). ***Significant at $P < .001$ in relation to the control; **significant at $P < .01$; *significant at $P < .05$; unpaired *t* test.

Inorganic elements	Male		Female		All groups	
	Control	Exposed	Control	Exposed	Control	Exposed
Na	7930 ± 8307	8435 ± 1456	9281 ± 868	9224 ± 1082	8779 ± 1073	8786 ± 1354
Al	921 ± 646	899 ± 1252	990 ± 971	1159 ± 1254	964 ± 855	1034 ± 1247
P	1395 ± 115	1433 ± 99	1435 ± 78	1461 ± 124	1420 ± 94	1445 ± 111
Si	322 ± 117	434 ± 278	305 ± 104	385 ± 262	311 ± 107	404 ± 264
S	4553 ± 332	4564 ± 444	4700 ± 341	4477 ± 217	4645 ± 35	4525 ± 362
Cl	10 426 ± 958	10 758 ± 1357	11 632 ± 781	11 617 ± 1024	11 183 ± 1029	11 140 ± 1287
K	6536 ± 420	6404 ± 494	6823 ± 398	6426 ± 424	6536 ± 420	6404 ± 494
Ca	223 ± 53	280 ± 140	274 ± 70	298 ± 127	255 ± 68	288 ± 134
Ti	17.2 ± 11.2	25.6 ± 19.1	16.8 ± 11.0	38.3 ± 82.4	17.0 ± 10.9	32 ± 69
Cr	12.2 ± 12.5	20.7 ± 23.0	9.1 ± 7.8	10.6 ± 10.7	10.5 ± 10.2	15.4 ± 18.1
Mn	19.5 ± 21.1	64 ± 124	8.76 ± 3.86	13.1 ± 3.3	11.7 ± 11.2	44.4 ± 98.4
Fe	2603 ± 855	3514 ± 5955	2304 ± 225	2406 ± 289	2417 ± 567	3022 ± 4455
Cu	5.2 ± 1.4	6.05 ± 2.33	6.6 ± 2.0	6.8 ± 2.6	6.1 ± 1.9	6.4 ± 2.5
Zn	30.0 ± 5.6	28.2 ± 5.4	29.0 ± 4.0	24.8 ± 6.0	29.4 ± 4.6	28.0 ± 5.7
Br	12.5 ± 4.0	15.4 ± 4.6*	13.6 ± 5.3	18.6 ± 7.8**	13.2 ± 4.8	17.0 ± 6.5**
Rb	26.7 ± 8.9	41 ± 15**	27.4 ± 8.4	34.8 ± 15.0	27.1 ± 8.5	38.5 ± 15.1***
Ni	10.6 ± 7.2	13.6 ± 8.5	7.9 ± 4.6	8.5 ± 5.7	9.0 ± 5.7	11.6 ± 7.7

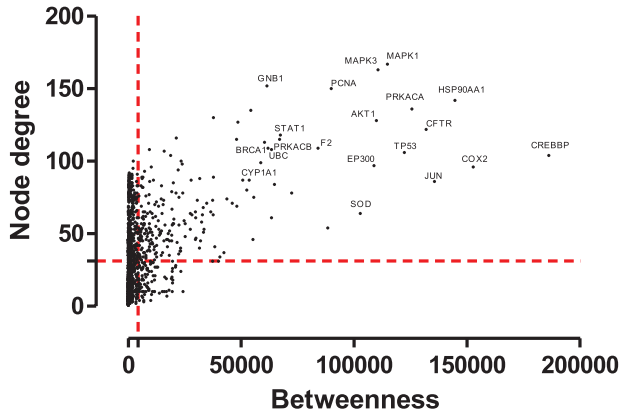

Figure 5. Centrality analysis of proteins and dry tobacco chemicals interaction networks. Dashed lines represent the threshold value calculated for each centrality. Proteins and chemicals are represented by circular dots. Only proteins with a high bottleneck and node degree score are indicated.

Regarding redox metabolism imbalance, our results showed that individuals involved in the dry tobacco grading showed an increase in both oxidative and nitrosative stress when compared with control groups. Increased protein oxidation, lipid peroxidation, nucleic acid oxidation (8-OHdG), and changes in antioxidant levels are related to oxidative stress. Continuous stress generates inflammation and subsequent cell death. Thus, it is possible that these increased results are associated with lipid peroxidation processes, membrane damage, or excessive DNA damage, which are activating cell death pathways [65]. In concordance [15] observed an oxidative imbalance in the group exposed to nicotine as well as in the studies of Kahl *et al.* [7] and Dalberto *et al.* [53] for both nicotine (the metabolite cotinine) and nitrates. Chemicals found in tobacco plants can induce oxidative stress, due to increased production of oxygen and nitrogen reactive species, which can damage lipids, proteins, and RNA and DNA [7, 66, 67], besides preventing repair proteins from exercising their functions [66, 68]. The result of this overproduction of reactive species contributing to a variety of pathological processes typical of different diseases, such as neurodegenerative, viral, toxic, or inflammatory diseases, contributing to the carcinogenicity process [68, 69].

Another way to assess the harm caused by tobacco exposure is the assessment of TL shortening, which is directly affected by ROS. Our study showed telomeric shortening in the exposed group (highlighting the female gender). Nicotine is associated with the regulation of the ubiquitin protein (UPS). Nicotine can activate UPS mechanisms and cause degradation of shelter complex proteins, leading to a variety of events that produce decreased telomerase activity, resulting in telomere shortening [14, 36]. Telomeres are protein complexes located at the ends of chromosomes that protect the chromosomes against genomic instability. Telomeres become shorter with biological aging, but inflammatory activities and increased oxidative damage can accelerate their shortening [70, 71]. When telomeres are extremely shortened, cells reach senescence or apoptosis [14]. The difference between genders observed for telomeres, as well as for MN, where women demonstrated a higher effect than men, can be associated with some factors such as: chemical composition of dry tobacco leaves and some difference in the exposure between genders (men exposed during all seasons and women mainly during grading); and body fat and relation with senescence, inflammatory process and oxidative unbalance (women typically present higher body fat/BMI compared with men) [72]. Different correlations in relation to age were showed, including BMI and WHtR, and in relation to chemical exposure during tobacco grading. We highlight Br and Rb that presented a difference between genders, where a correlation for women was observed between Br and MN and with necrosis; and Rb and TL were correlated for men.

Overweight and obesity are considered serious health problems, as they are related to increased risk, not only of chronic diseases such as heart disease and diabetes, but also related to several types of cancers [73]. Epidemiological evidence linking obesity to increased cancer risk is steadily growing. Obesity causes a physiological imbalance in tissue regulation and functionality, resulting in hyperglycemia, dyslipidemia, and inflammation. As a result of this situation, the generation of oxidative stress is exacerbated in obesity due to a decline in antioxidant defense systems. This oxidative stress can directly influence the DNA, producing mutagenic lesions that can be carcinogenic [74]. Changes in apoptotic and necrotic cells, MN, NPB, NBUD in lymphocytes from obese individuals showed significantly higher results than the values found in normal weight and overweight individuals [73].

Systems biology is the field of research that aims to understand complex biological systems at the systems level, assessing cellular bioprocesses from a systematic rather than a reductionistic perspective [75]. In the present study, a systems biology approach was performed to identify the proteins associated with dry tobacco exposure and to predict the underlying molecular mechanisms. DTCPI network includes proteins linked to nicotine, nitrosamines, and inorganic elements. Nicotine and nitrosamines require metabolic activation through α-hydroxylation by several cytochrome P450 (CYP) enzymes. In this DTCPI network, CYP proteins are considered HB node. As results of metabolism, some metabolites can be electrophilic (have an electron-deficient center) and can react with DNA. The reaction of these metabolites with DNA can lead to the generation of DNA adducts, which are generally regarded as intermediaries in the mutagenesis process. Nitrosamines metabolites can generate DNA adducts [76]. Nicotine and its metabolite, cotinine, can increase ROS production, causing DNA damage [16]. Regarding protein interaction, dry tobacco chemicals, such as nitrosamines and nicotine, are described as activating the serine/threonine kinase AKT [36, 76, 77]. AKT1 is a protein with high node degree and betweenness values and, hence it corresponds to highly central proteins that connect several complexes. This protein phosphorylates numerous protein targets that control cell survival, proliferation, and motility. Previous studies suggest that Akt regulates transcriptional activity of the nuclear factor-κB (NFκB) by inducing phosphorylation [78]. Regulation of phosphorylation, transcription initiation from RNA polymerase II promoter and regulation of gene expression were significant bioprocesses in gene ontology analysis. Our results suggest that AKT1 causes nuclear translocation of NFκB and transcriptional activation of oncogenic HDAC2. Histone deacetylases act via the formation of large multiprotein complexes and are responsible for the deacetylation of lysine residues at the N-terminal regions of core histones (H2A, H2B, H3, and H4). This protein forms transcriptional repressor complexes by associating with many

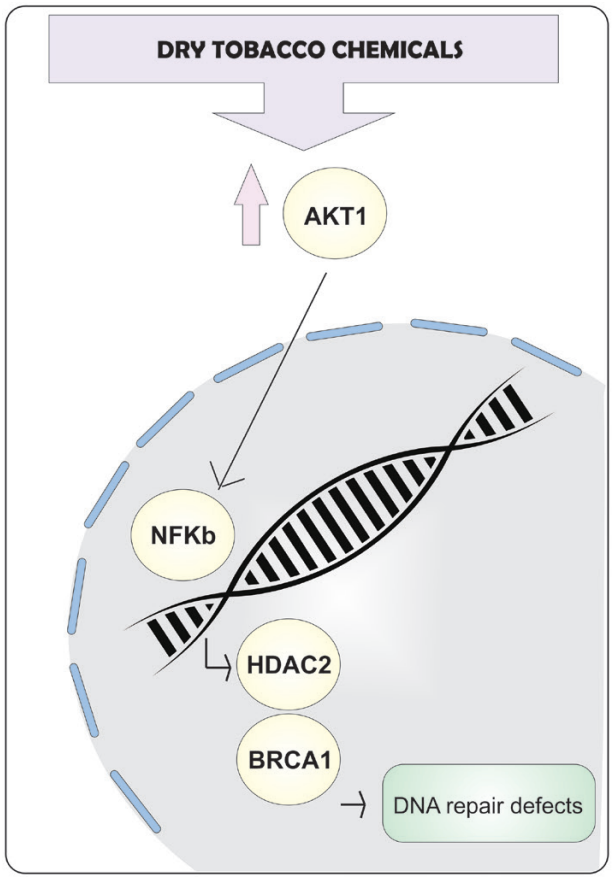

Figure 6. A molecular model illustrating how dry tobacco chemicals could cause DNA repair defects. DTC can increase the nuclear translocation of NFKB dimers inducing HDAC2 expression. HDAC2 associates with BRCA1 protein and potentially represses transcription of genes that promote DNA repair.

different proteins and often is associated with nucleosomal condensation and transcriptional repression. Thus, it plays an important role in transcriptional regulation, cell cycle progression, and developmental events (genecards.org). Bioprocesses such as chromosome organization, protein complex assembly, DNA packaging, chromosome condensation, and nucleosome assembly are present in cluster 2. Yarden *et al.* [79] suggest that HDAC2 associates with the carboxyl terminal domain (BRCT) of the BRCA1 protein, demonstrating that BRCA1 interacts with components of the histone deacetylase complex. Thus, BRCA1 may potentially repress transcription of genes that promote DNA repair. Many bioprocesses related to DNA repair were observed in Cluster 2 as nucleotide-excision repair, double-strand break repair, recombinational repair, and nonrecombinational repair. BRCA1 is recruited to DNA damage sites and acts in DNA repair [80]. In our analysis, BRCA1 interacts with many proteins from different repair pathways. Thus, DTC can increase the nuclear translocation of NFKB dimers inducing HDAC2 expression. HDAC2 associates with BRCA1 protein and potentially represses transcription of genes that promote DNA repair (Fig. 6).

The results found in our study agree with the existing results in the literature on occupational exposure to tobacco. It is evident that dry tobacco can causes damage to the health of exposed workers, as demonstrated by the different exposure routes. The main factors associated with genotoxicity in grading tobacco farmers were exposure to nicotine and TSNAs (nitrates) and inorganic elements (mainly Br and Rb). These cell insults may be associated with the increase in ROS and RNS, causing imbalance in the cell cycle, senescence, and cell death and, in addition, could be triggering carcinogenicity processes. Tobacco elements can increase the nuclear translocation of NFKB dimers inducing HDAC2 expression, which associated with BRCA1 protein can potentially repress transcription of genes that promote DNA repair. Further studies are needed to relate exposure to dry tobacco and the use of protective equipment, and the relation with genes polymorphisms and expression.

Supplementary data

Supplementary data is available at *Mutagenesis* online.

Supplementary Table 1. Specific gene ontology (GO) classes derived from chemical–protein interactions observed in cluster 2.

Supplementary Figure 1. The dry tobacco chemical–protein interacting (DTCPI) network with proteins of *Homo sapiens*. (a) Network and cluster 2; (b) proteins directly linked to BRCA1. BRCA1 is presented in lilac color node.

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