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HEALTH SCIENCES

Yerba mate (*Ilex paraguariensis* St. Hil.) extract inhibits hand-rolled cornhusk cigarette smoke-induced oxidative pulmonary damage

FERNANDA D.M. CAMARA, GIULIA S. PEDROSO, SILVANE S. ROMAN, ROGÉRIO M. DALLAGO, ALICE T. VALDUGA, BRUNA B. FERNANDES, EDUARDO B.B. CUNHA, PAULO CESAR L. SILVEIRA, RENATA T. NESI & RICARDO A. PINHO

Abstract: The aim of this study was to investigate the effects of yerba mate *(Ilex paraguariensis* St. Hil.) extract (YME) on oxidative stress parameters and pathological changes in the lungs of mice chronically exposed to hand-rolled cornhusk cigarette (HRC) smoke. Twenty-four male Swiss mice were divided into four groups exposed to the following treatments: control (ambient air), HRC, YME, and HRC plus YME. The animals were exposed to four HRCs per session, with 3 sessions/day, every day for 30 days. Twenty-four hours after the last inhalation, the mice were killed, and the left lungs were removed. The results showed that HRC contains elevated levels of tin and carbon oxide, but less arsenic, cobalt, manganese, and selenium than commercial cigarettes. YME administration reversed fibrosis, alveolar enlargement, and hemorrhage induced by HRC smoke. In addition, the YME and HRC significantly reduced the production of oxidants, oxidative damage and promoted a significant increase in total thiol. In conclusion, exposure to HRC smoke compromised pulmonary histoarchitecture by promoting structural changes and increasing oxidative stress in tissues. However, concomitant treatment with YME regulated the redox state and reduced the harmful effects of HRC smoke exposure in the lungs.

Key words: Yerba mate, hand-rolled cornhusk cigarettes, lungs, oxidative stress, inflammation.

INTRODUCTION

Smoking is considered the single most important risk factor for the development of approximately 50 tobacco-related diseases, including cancer, cardiovascular diseases, and lung emphysema (Thun et al. 2013). According to the World Health Organization (WHO 2013) the estimated number of deaths related to the consumption of tobacco is 6 million/year worldwide but may reach 8 million/year in 2030. The effects induced by tobacco are closely related to several disorders, affecting the health of the user and those passively exposed to smoke. However, the severity of disease has been suggested to be directly related to the amount and duration of exposure, and possibly the type and form of tobacco (Viegas 2008).

Among the different types of cigarettes available, hand-rolled cornhusk cigarettes (HRC) are produced by hand and consist of a small amount of macerated tobacco wrapped in cornhusk. The potential biological effects or consequences of HRC use on health remain to be elucidated. On the other hand, commercially manufactured cigarettes have been investigated in several studies aiming to characterize the

physicochemical properties of tobacco and its harmful effects on health (Javadian et al. 2015), as this is the most prevalent form of tobacco used. Its inhalation promotes changes in cell structure and lung redox state, resulting in inflammation and mutagenic/carcinogenic effects in the respiratory system (Lanzetti et al. 2008).

Antioxidant supplementation has been strongly recommended for the treatment of tobacco-related diseases (Al-Awaida et al. 2014, Lanzetti et al. 2008). Yerba mate has been identified as a potent antioxidant with anti-inflammatory effects (Lanzetti et al. 2008). Previous studies have shown its antioxidant effects in both animals and humans exposed to manufactured cigarette smoke (Gupta et al. 2016) but additional studies regarding the biological effects of exposure to HRC smoke are necessary. Thus, the aim of this study was to investigate the effects of yerba mate extract (YME, *Ilex paraguariensis* St. Hil.) on oxidative stress parameters and pathological changes in the lungs of mice chronically exposed to HRC smoke.

MATERIALS AND METHODS

HRC and commercial cigarettes

Tobacco and cornhusks were purchased from farmers in the city of Severino, RS, Brazil. The tobacco leaves were stripped, macerated, and indirectly exposed to the sun for 48 h. To prepare HRC, 0.8 g of dry tobacco was uniformly wrapped in cornhusk. The amount of tobacco used in each cigarette was equivalent to that present in a commercial cigarette. The commercial cigarettes used in present study were Marlboro Red cigarettes, manufactured by Philip Morris International in Brazil. The Malboro brand have been used extensively by different researchers to verify the biological effects of industrial cigarettes (Lanzetti et al. 2008, Menegali et al. 2009, Nesi et al. 2016, Valença et al. 2004). All procedures were performed in accordance with law no. 11794/08 (DOU 196, section 1, October/2008) and approved by the Ethics Committee on the Use of Animals of the Universidade do Extremo Sul Catarinense (protocol number 080-2014-01).

Metal analysis

Sample aliquots were milled in a cryogenic mill (Spex 6750 Freezer Mill, USA) and acid-digested as follows: 200 mg of the sample was transferred to a polytetrafluorethylene flask (Tecnal, Brazil) to which 3 mL HNO_3 was added, and the mixture was allowed to incubate overnight. Subsequently, the flask was closed with a screw cap and placed on a heated block (Tecnal) for 4 h at 90 °C. Next, the mixture was allowed to cool to room temperature. Thereafter, 1.5 mL hydrogen peroxide was added, and the mixture was further heated on the heating block for 4 h at 160 °C. The solution obtained was cooled to room temperature, transferred to a volumetric polypropylene vial, and its volume was adjusted to 20 mL with water.

Carbon monoxide (CO) analysis

The amount of monoxide produced during the combustion of the cigarettes (commercial and HRC) was determined using a portable digital CO gauge (C-3000, Instrutherm, Brazil). The cigarette smoke was collected and stored in a 2-L polyethylene terephthalate (PET) bottle. The internal volume of the bottle (measured with water) was approximately 1.3 L. A 10-mL syringe was attached to the bottom of the bottle where the cigarettes were burned. After four draws, the CO content was measured using a CO analyzer attached to the inside of the bottle.

Total phenolic and flavonoid content analysis

The concentration of phenolic compounds in the plant extract was determined via spectrophotometry using Folin-Ciocalteu reagent. *I. paraguariensis* extract (0.5 mL, 1 mg/mL) was diluted with 5 mL Folin-Ciocalteu reagent (1:10). Sodium carbonate solution (4 mL, 1 M) was added after 3 min and stirred. The absorbance was measured at a wavelength of 765 nm. Gallic acid was used as the standard in the preparation of a calibration curve. Measurements were performed in triplicate. Total flavonoid content was estimated via colorimetry using aluminum chloride. *Ilex paraguariensis St. Hil.* extract (0.5 mL, 1 mg/mL) was diluted with 1.5 mL methanol, 0.1 mL 1 M potassium acetate, and 2.8 mL water, stirred, and allowed to stand for 3 min before the addition of 0.1 mL 10% aluminum chloride solution. The absorbance was measured at a wavelength of 415 nm. Quercetin was used as the standard in the preparation of a calibration curve. Measurements were done in triplicate.

Experimental procedures

Samples*:* Twenty-four male Swiss mice weighing approximately 30-50 g were divided into four groups (n = 6): control (mice exposed to ambient air), mice exposed to HRC smoke, mice treated with YME, and mice exposed to HRC smoke and treated with YME. Animals were kept in specific cages in a controlled environment (22 °C, 12-h light-dark cycles) with free access to food and water.

Exposure to cigarette smoke*:* The mice were exposed to smoke from four HRC per session, with three sessions per day, every day for a total of 30 days. The animals were placed in an acrylic inhalation chamber (40 × 30 cm, 25 cm in height) inside a fume hood. The cigarettes were coupled to a 60-mL plastic syringe to collect the smoke, which was immediately injected into the chamber. The animals were exposed to cigarette smoke for 6 min, followed by removal of the cover of the inhalation chamber. The smoke was eliminated for 1 min using an exhaust system, followed by re-exposure to the cigarette smoke. According to previously published methods, animals in the control group were kept inside an inhalation chamber but in the absence of cigarette smoke. Twenty-four hours after the last inhalation, the mice were killed, and the left lungs were removed for histological analyses whereas the right lungs were processed and stored at -80 °C for biochemical analyses.

YME administration*:* During the 30 days of exposure to cigarette smoke, the animals concomitantly received 1% YME via oral gavage (Table I). YME was prepared fresh weekly in water, and 0.5 mL was administered per animal daily at room temperature.

Histology*:* Twenty-four hours after the last exposure to cigarette smoke, the leftlung was removed and immersed in a fixative solution of 10% paraformaldehyde solution for 24 h for subsequent histological processing. Cleavage of the material was performed using a longitudinal cut. The tissue was embedded in paraffin and sliced using a microtome to obtain sections of 4-mm thickness. The slides were stained with hematoxylin and eosin (H&E stain, 6 and 2%, respectively), and slides were prepared for image acquisition and analysis of tissue histology.

Oxidative stress parameters*:* Oxidized intracellular 2',7'-dichlorofluorescein (DCF) levels were monitored in samples incubated with 2',7'-dichlorodihydrofluorescein (DCFH). The formation of the oxidized fluorescent derivative was monitored at excitation and emission wavelengths of 488 and 525 nm, respectively, using a fluorescence spectrophotometer instruments (Cai et al 2007). The malondialdehyde (MDA) concentrations in serum samples were determined via reverse-phase high-performance liquid chromatography (HPLC; Agilent

Table I. Concentration of phenolic compounds and flavonoid in the *Ilex paraguariensis* extract.

Compounds	R^2	mg/g
Total phenolic*	0.9916	6.9
Flavonoid**	0.9930	0.5

*** Folin-ciocalteu method.**
**** Aluminium chloride method.**

Technologies 1200 series; Santa Clara, CA, USA) according to the method described by Grotto et al. 2007 using thiobarbituric acid derivatization. A standard curve was prepared using MDA tetrabutylammonium salt at concentrations ranging from 0.5 to 5.0 µM. Results are expressed as µmol/L MDA. Protein carbonyl content was measured using labeled protein hydrazone derivatives and 2,4-dinitrophenylhydrazide at 370 nm according to the method described by Levine et al. 1990. Total thiol content (sulfhydryl) was determined spectrophotometrically at a wavelength of 412 nm using 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) oxidation as reference. The amount of 2-nitro-5-thiobenzoic acid (TNB) formed was calculated (as thiol group equivalents), according to the method previously described by Ellman 1959.

Statistical analysis

Statistical analysis was performed using the Kruskal-Wallis non-parametric distribution test, followed by the Student-Newman-Keuls test using BioEstat. Differences were considered significant if $p < 0.05$. All data are presented as means ± standard error of the mean (SEM). The groups were compared via one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. Statistical difference between groups was considered significant if $p \leq 0.05$ and the analyses were performed using 16.0 GraphPad Prism (GraphPad Software, San Diego, CA, USA).

RESULTS

Metal content

The metal contents in HRC and commercial cigarettes are shown in Table II. Both types of cigarettes contained significant amounts of metal. The metal content in HRC was lower than that in commercial cigarettes, especially with regard to arsenic (50%), cobalt (51%), manganese (70%), and selenium (90%). However, the tin content in HRC was 290% greater than that in commercial cigarettes.

CO levels

The levels of CO produced by the combustion of different types of cigarettes (commercial and HRC) are shown in Table III. Commercial cigarettes showed a lower CO content (539 ± 11 mg/L) than that observed in HRC (1945 ± 27 mg/L).

Histology results

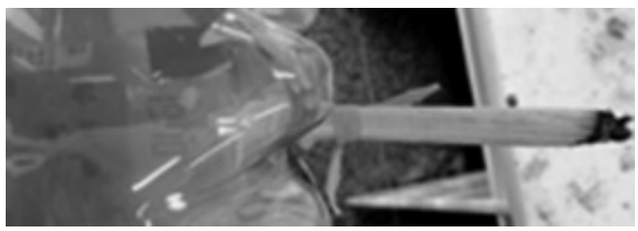
Images of longitudinal sections of lung tissue stained with H&E stain are shown in Figure 1. In Figure 1a, the lung tissue of animals exposed to ambient air (control) presents normal histoarchitecture with no histological changes. However, the image of the pulmonary parenchyma from mice in the HRC group (Figure 1b) shows fibrotic areas (black star), areas of alveolar enlargement (AE), and hemorrhage (black arrow). Animals that received YME showed

Table II. Metal analysis.

Metals	Detection limit	Manufactured Cigarettes (µg/g)	Hand-rolled Cornhusk cigarette (µg/g)	Percentage of increase or decrease (compared to manufactured cigarettes)
As	0.02	0.16 ± 0.03	0.08 ± 0.02	↓50%
Cd	0.00	0.59 ± 0.05	0.73 ± 0.25	↑23%
Co	0.00	0.969 ± 0.09	0.47 ± 0.16	↓51%
Cr	0.38	1.80 ± 0.69	0.95 ±0.32	↓47%
Cu	0.03	13.9 ± 1.1	15.9 ± 5.1	↑14%
Fe	4.05	490.2 ± 131.2	486.7 ± 84.3	↓0.7%
Mn	0.06	212.0 ± 16.1	62.8 ± 23.1	↓70%
Ni	0.03	2.22 ± 0.10	1.74 ± 0.54	↓21%
Se	0.31	0.82 ± 0.03	0.08 ± 0.02	↓90%
Sn	0.15	2.95 ± 0.35	11.6 ± 0.3	↑293%

Presence of metals in hand-rolled cornhusk and manufactured cigarettes. AS=arsenic; Cd=cadmium; Co=cobalt; Cr=chromium; Cu=Copper; Fe=iron; Mn=manganese; Ni=nickel; Se=selenium; Sn=tin.

Table III. Presence of carbon monoxide.

Samples	CO (mg/L)	CO analyzer*
Commercial cigarette	539 ± 11	
Cornhusk cigarette**	1945 ± 24	

Presence of carbon monoxide (CO) during the combustion of commercial and cornhusk cigarettes. Data are presented as means ± standard deviation (n=3).

*** Equipament produced manually using a CO analyzer inside the plastic bottle.**

**** The cornhusk mass (0.369 ± 0.065 g) - corresponds ≅ 30% of cigarette mass.**

intact alveolar septa (asterisk, Figure 1c) similar to those of mice in the control group. Similar results were observed in mice in the HRC + YME group with preserved alveolar duct (Figure 1d). In addition, stereological assessment of alveolar diameter suggests the development of emphysema in animals exposed to HRC, which was significantly reduced by treatment with YME (Figure 1e).

Oxidative stress parameters

Indicators of cellular oxidant production and oxidative damage in lipids and proteins are shown in Figure 2. As noted in Figure 2a, the production of DCF was significantly increased in HRC group mice, and this was significantly reduced by concomitant treatment with YME. As expected, exposure to HRC smoke also elevated lipoperoxidation levels (Figure 2b) and increased carbonylation of proteins (Figure 2c). However, the concomitant use of YME and HRC caused a significant reduction in these values. Increased levels of total thiols were observed from YEM supplementation, but in the presence of HRC the values remained like the control group. (Figure 2d).

DISCUSSION

HRC is one of several forms of tobacco consumption but its effects on human health remain largely unexplored. Because they are not processed commercially and are produced manually, their effects have been deemed less severe. However, our results indicated the high susceptibility of mouse lungs to HRC smoke exposure. Metals are important in several vital metabolic pathways but certain metals which exist in cigarette smoke and thus are inhaled during smoking can cause serious diseases (Stavrides 2006). For example, metals such as lead, and cadmium are particularly toxic. Cobalt, copper, and nickel can be highly toxic when inhaled via smoking, as they are inhaled as carbon compounds (Iwegbue et al. 2009). The potentially toxic inorganic constituents found in both types of cigarettes probably originated from the tobacco plant itself and they could be absorbed from soil as a consequence of pesticide and fertilizer use, as well as environmental factors. In addition, the cigarette manufacturing process contributes significantly to the high concentration of metals found in manufactured cigarettes (Halstead et al. 2015). Inhaled metals remain for long periods in various areas of the pulmonary tissue and some toxic metals are able of causing serious malfunctions to different vital cellular activities (Stavrides 2006).

Depending on the tissue targeted, the fixation of inorganic ions can induce different effects. For example, studies have shown that cadmium accumulates in various organs of the body and directly interferes in the chemical reactions of the cells (Johnson 2001). Lead toxicity produces severe effects on the brain, kidney, nervous system, and red blood cells, and has been associated with fetal and brain impairment (Ashraf 2012). Tobacco plants that absorb nickel present in the soil have demonstrated genotoxic potential associated with lung cancer and nasal cavity cancer (Das et al. 2008). Furthermore, tin was the chemical that stood out among the metals investigated and showed a concentration 290% greater in HRC than that in industrial cigarettes. According to Jiang and colleagues (Jiang et al. 2012), tin causes significant adverse effects to organisms, such as the formation of tumors observed in the lungs of mice that inhaled tin-containing dust. Other studies have also revealed the harmful effects of tin on other tissues such as the brain, kidneys, and skin (Roney et al. 2011).

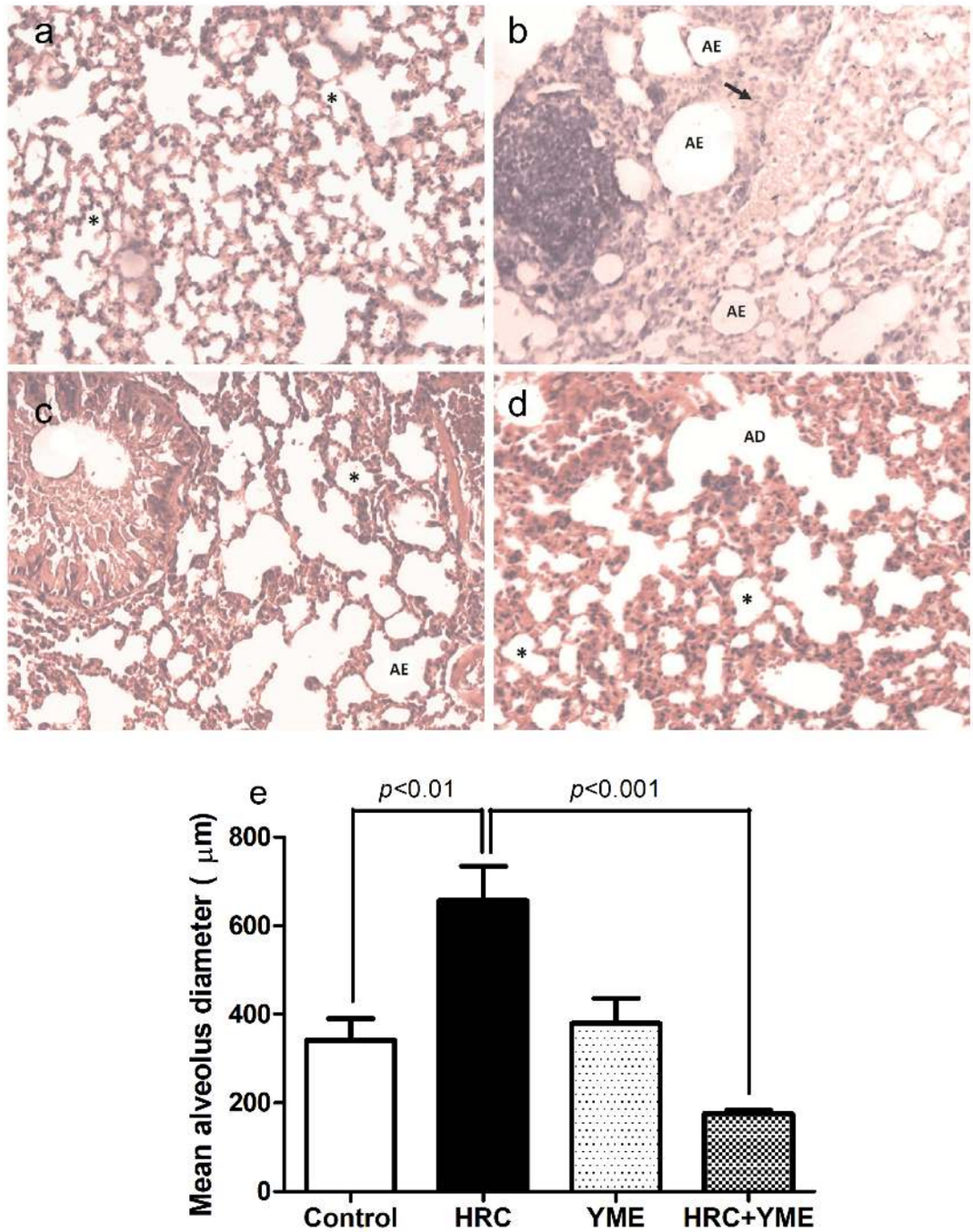


Figure 1. Pathological changes in the lung tissue of mice chronically exposed to hand-rolled cornhusk cigarette (HRC) smoke and treated with *Ilex paraguariensis* extract (YME). Photomicrograph of lung parenchyma areas showing respectively: (a) lung tissue exposed to ambient air showing normal alveolar ducts (back star); (b) lung parenchyma exposed to HRC, showing alveolar enlargement areas (EA) with septal thickening, presence of cellular infiltration and intense areas of atelectasis (black arrow); (c) lung tissue of mice exposed to ambient air and received YME showing normal alveolar areas (black star), septal integrity and low areas of alveolar enlargement. (d) areas of lung tissue exposed to HRC smoke and treated with YME showing presence of cellular infiltration and normal alveolar areas of the lung parenchyma (black star) with areas of intact alveolar ducts (AD) (Images: H.E, 25x objective). (e) represent represents the mean alveolar diameter in control, HRC, YME and HRC+YME.

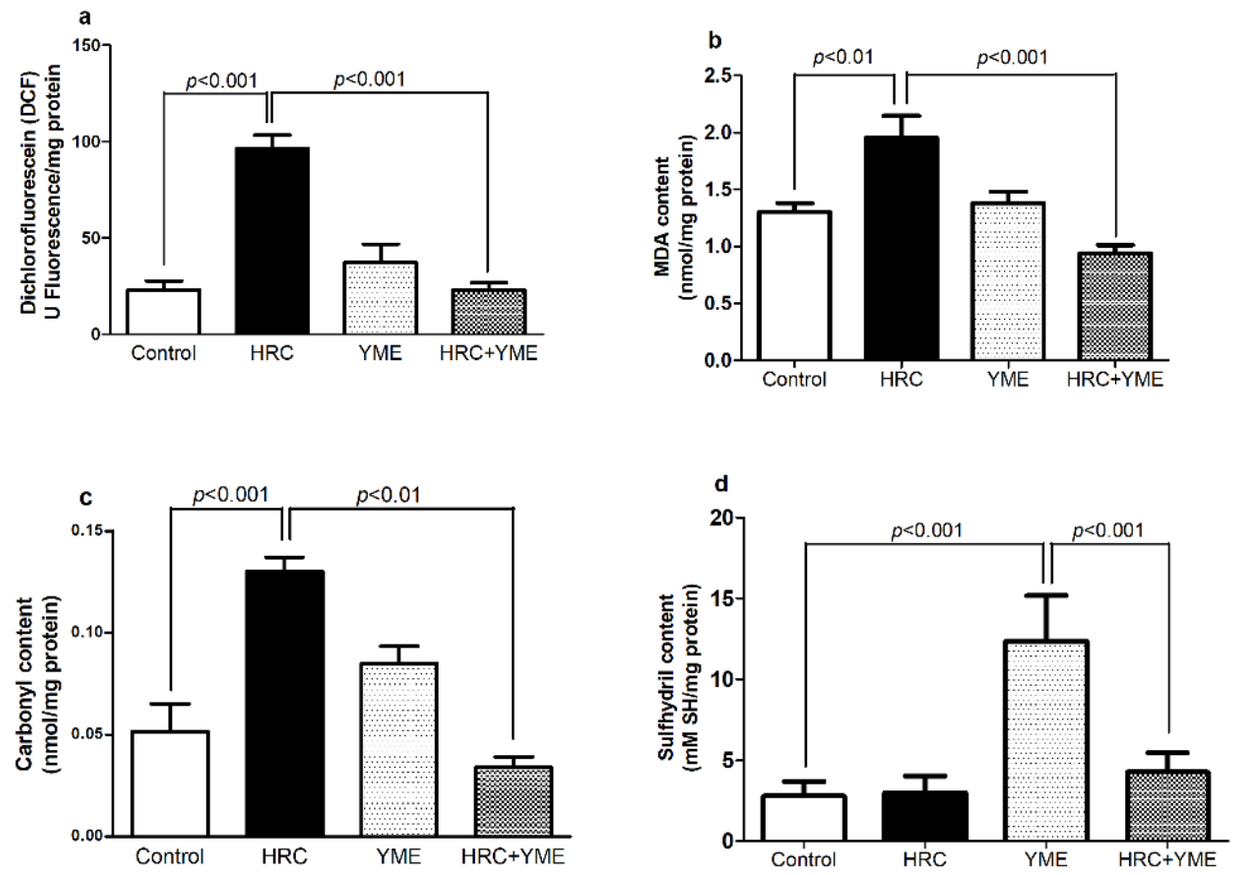


Figure 2. Oxidative stress parameters in the lungs of mice chronically exposed to hand-rolled cornhusk cigarette (HRC) smoke and treated with *Ilex paraguariensis* extract. The groups were compared via one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. Statistical difference between groups was considered significant if $p \leq 0.05$ and the analyses were performed using 16.0 GraphPad Prism (GraphPad Software, San Diego, CA, USA). a= DCFH oxidation; b= lipoperoxidation levels; c= carbonylation of proteins; c= total thiol content.

Besides increasing metal levels, HRC smoke also promotes possibly elevated levels of CO. As seen in Table II, elevated levels of CO were observed after the combustion of HRC, and this can lead to an increase in carboxyhemoglobin, which directly affects the transfer of oxygen to tissues and may lead to hypoxia in different organs (Jardim et al. 2010). Furthermore, CO can modulate the activities of several cellular signaling molecules and result in the alteration of mitochondrial function, reactive oxygen species (ROS) production, and release of proapoptotic and proinflammatory mediators (Ryter et al. 2018).

The exorbitant CO levels measured after HRC combustion relative to that in commercial cigarettes observed in the present study can be related to the way in which the cigarette is made. The confection of HRC is done manually and the tobacco is compacted inside the cigarette, possibly reducing the cigarette's porosity and limiting access to oxygen. In this scenario, it is possible to speculate that the high presence of CO in cigarette combustion is partially absorbed by the smokers and alters cellular redox metabolism.

Considering the scientific evidence indicating that some of the symptoms or

pathophysiology of CO (Kavakli et al. 2011) and metal toxicity may result from increased free radical-mediated lung injury, we investigated whether the use of YME *(I. paraguariensis* St. Hil.), recognized as a potent antioxidant (Panza et al. 2018), modulates HRC-induced pulmonary injury and oxidative stress. Our results indicated a significant susceptibility of the lung to HRC smoke. We observed fibrotic areas, alveolar enlargement, and hemorrhage induced by HRC. Similar results were previously reported in several studies (Gan et al. 2011) investigating commercially manufactured cigarettes. These HRC-induced alterations result from toxic particles produced and released during cigarette combustion such as CO, metals, and possibly other toxic agents not analyzed in the present study, but which have been reported in previous studies with commercial cigarettes, such as polycyclic aromatic hydrocarbons, N-nitrosamines, and others. These toxic agents accelerate pulmonary aging, directly affecting the ventilatory capacity and leading to the destruction of the respiratory epithelium with consequent thickening of the septum and enlargement of the alveolar area (Vij et al. 2018).

Unlike, effects observed after exposure to HRC, animals that received concomitant treatment with YME showed intact alveolar septa similar to those of mice in the control group. In addition, stereological assessment of alveolar diameter suggested the development of emphysema in animals exposed to HRC, which was significantly reduced after treatment with YME. The positive effects of YME administration on lung histoarchitecture may be result of antioxidant-mediated cellular protection, as was suggested by Lanzetti and colleagues (Lanzetti et al. 2008).

In addition to containing several oxidants, cigarette smoke, when inhaled, can initiate a series of cellular reactions and lead to increased production of ROS (Wong et al. 2016). This situation may explain elevated DCF levels, an indicator of the production of hydrogen peroxide in the cell. Increased production of hydrogen peroxide triggers a cascade of cellular events that leads to oxidative damage in biomolecules, compromising pulmonary structure and function. In this sense, Gupta and collaborators (Gupta et al. 2016) suggested that oxidants present in tobacco smoke not only cause direct oxidative damage to lung proteins, contributing the most part to lung injury, but also activate proinflammatory factors involved in tobacco-induced lung injury.

In this oxidative condition generated by HRC smoke exposure, supplementation with YME promoted a significant reduction in DCF levels. The ability of YME to reduce hydrogen peroxide levels may be associated with two factors: a) the presence of polyphenols (Anesini et al. 2016) and b) the ability to chelate iron (Colpo et al. 2016). The increase in oxidant levels (DCF) promotes oxidative modifications in lipids and structural proteins, which play an important role in the etiology and/or progression of various diseases. These modifications or oxidations can be induced directly or indirectly by the reactions of secondary byproducts produced by the lipoperoxidation and the formation of lipoprotein products (Curtis et al. 2012).

The present study demonstrated high levels of MDA and protein carbonilation in mice in the HRC group, which were significantly reduced by YME supplementation. The cigarette smoke, when inhaled, interacts directly with the lung epithelia, the elevated DCF levels may have contributed to the lipid peroxidation and oxidation/modification of protein. Unlike, the YME increases endogenous antioxidant molecules, in addition to its natural antioxidant properties. One of the mechanisms suggested proposes that, even at low concentrations,

phenolic compounds present in the YME may stimulate the expression of transcription factors such as nuclear factor erythroid 2-related factor 2 (Nrf2), which regulates genes involved in the synthesis of reduced glutathione (GSH) and other endogenous antioxidants in cells. GSH, for example, has the ability to conjugate with xenobiotic elements, such as those present in cigarette smoke (Moskaug et al. 2005). In this sense, possible antioxidants synthesis stimulated by phenolic compounds may be beneficial in HRC-induced redox changes in the lung oxidative damage induced by HRC. Interestingly, HRC smoke did not change the total thiols content and it needs to be further investigated in future studies. In additional, a significant increase in sulfhydryl levels was observed in the YME group. Previous studies have already reported similar results in brain (Branco et al. 2013), liver and serum (González Arbeláez et al. 2016) and heart (Pamplona et al. 2006) and it response can be related to the antioxidant capacity of phenolic compounds present in yerba mate.

CONCLUSION

In conclusion, exposure to HRC compromises pulmonary histoarchitecture by promoting structural changes and increasing oxidative stress in tissues. However, the concomitant consumption of yerba mate regulated the redox state and reduced the harmful effects of exposure to straw cigarette smoke in the lung by activating mechanisms of cellular protection or exerting previously proven intrinsic antioxidant effects.

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FERNANDA D.M. CAMARA[1]
https://orcid.org/0000-0001-5325-0298

GIULIA S. PEDROSO[2]
https://orcid.org/0000-0002-5868-7058

SILVANE S. ROMAN[1]
https://orcid.org/0000-0002-7769-9759

ROGÉRIO M. DALLAGO[1]
https://orcid.org/0000-0001-7366-5562

ALICE T. VALDUGA[1]
https://orcid.org/0000-0002-5832-2737

BRUNA B. FERNANDES[2]
https://orcid.org/0000-0002-1528-3221

EDUARDO B.B. CUNHA[3]
https://orcid.org/0000-0002-0677-4061

PAULO C.L. SILVEIRA[2]
https://orcid.org/0000-0003-4908-2257

RENATA T. NESI[3]
https://orcid.org/0000-0002-6774-0893

RICARDO A. PINHO[3]
https://orcid.org/0000-0003-3116-4553

[1]Universidade Regional Integrada do Alto Uruguai e das Missões, Av. Sete de Setembro, 1621, Erechim, Fátima, 99709-910 RS, Brazil

[2]Programa de Pósgraduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, Laboratório de Fisiopatologia Experimental, Av. Universitária, 1105, Universitário, Criciúma, 88806-000 SC, Brazil

[3]Programa de Pós-graduação em Ciências da Saúde, Pontifícia Universidade Católica do Paraná, Laboratório de Bioquímica do Exercício em Saúde, Escola de Medicina, Rua Imaculada Conceição, 1155, 80215-901 Prado Velho, Curitiba, PR, Brazil

Correspondence to: **Ricardo A. Pinho**
E-mail: rapinho12@gmail.com

Author contrbuitions

FDC and RAP designed and performed the study and wrote the paper. FDC, GSP and BBF participated in the data collection and experimental procedures. SSR, RMD and ATV contributed in the data analysis. PCLS and RTN contributed to the study design and in the FDC, EDBBC writing of the manuscript and RAP supervised and designed the study.

