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Original Paper

Evaluation of the Diaphragm Muscle Remodeling, Inflammation, Oxidative Stress and Vascularization in Smokers: An Autopsy Study

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Key Words
Autopsy study • Cigarette smoke • Diaphragm muscle • Histopathological analysis

Abstract
Background/Aims: Cigarette smoking is a key factor in systemic inflammation and oxidative stress, and it has also been associated with the loss of muscle strength and an elevated risk of pulmonary diseases. Thus, this study aimed to analyze the effects of cigarette smoking on the diaphragm muscle structure of postmortem samples. ***Methods:*** Immunohistochemical techniques were used for muscle remodeling (metalloproteinases 2 and 9), inflammation (cyclooxygenase-2), oxidative stress (8-hydroxy-2′-deoxyguanosine), and vascularization (vascular endothelial growth factor). Hematoxylin and eosin stain was used for histopathological analysis and Picrosirius stain was used to highlight the collagen fibers. ***Results:*** Cigarette smokers had an increase of diaphragm muscle remodeling, oxidative stress, inflammation, and vascularization compared to non-smokers. ***Conclusion:*** Diaphragm muscle structure may be negatively affected by cigarette smoking.

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Introduction

Cigarette smoking (CS) induces adverse effects on several physiological systems through oxidative damage [1-5]. Regarding oxidative stress, 8-hydroxy-2′-deoxyguanosine (8-OHdG) is one of the predominant forms of free radical-induced oxidative lesions, and has therefore been widely used as a biomarker for oxidative stress in tissues (e.g. muscle) [6]. Protein degradation mechanisms induced by metalloproteinases (MMPs) may indicate potential therapeutic and preventive strategies for retarding structural and physiological deterioration of skeletal muscle as occurs in aging and in various pathologies [7]. Metalloproteinase type 2 (MMP-2) is associated with muscle function, and metalloproteinase type 9 (MMP-9) is generally associated to inflammatory damage on skeletal muscle [7]. CS-induced activation of inflammatory markers as cyclooxygenases (COX), mainly COX-2, and inflammatory cells which may contribute to enhance the oxidant production in tissues leading to muscle injury [8, 9]. For instance, in the muscle, CS increases muscle remodeling through increased atrophy F-box (MAFBx) expression [10], abnormal fibers, vascularization, and collagen deposition [11]. Thus, to understand the effects of CS on muscle structure is a key factor to mitigate CS-exposure negative effects in later life.

The diaphragm muscle is the main inspiratory muscle, which is negatively affected by aging [12-14] and smoking [11]. Increased susceptibility to respiratory complications is attributed to diaphragm muscle sarcopenia [15, 16]. Although, it is suggested that smoking increases diaphragm muscle injury leading to sarcopenia [11, 17, 18], there is still unclear evidences about the effects of smoking on diaphragm muscle structure during aging. Therefore, this study aimed to analyze the effects of cigarette smoking on the diaphragm muscle structure of postmortem samples.

Materials and Methods

Study design and setting

Ethical approval was obtained from the review board for human studies at the University of São Paulo Medical School (São Paulo, Brazil). This study was consistent with the Helsinki Declaration. Consent was obtained from the next of kin. A semi-structured interview was applied to the deceased's next of kin, who had at least weekly contact with the participant during the six months prior to death.

This cross-sectional study includes participants recruited from January 2019 to February 2019. We included sedentary individuals aged 50 years or older at time of death without history of alcohol use. Exclusion criteria were postmortem interval > 24h, Hepatitis B and C, HIV, neuromuscular disease and myopathy [19-21]. Additionally, we excluded the subjects when the next-of-kin did not known how many years or packs the subject smoked during his life. Inclusion criterion for controls was never had smoked, and a lack of respiratory pathology (e.g. chronic obstructive pulmonary disease – COPD) in the history and at autopsy.

Variables of interest included age, sex, weight (kg), body mass index (BMI), smoking status, muscle structure, adipocyte deposition, collagen deposit, muscle remodeling (MMP-2 and MMP-9), inflammation (COX-2), vascularization (vascular endothelial growth factor – VEGF), and oxidative stress (8-OHdG). Smoking status was acquired by interviewing the next-of-kin of the subjects included in the study. We used the period of smoking (years) and pack-years of smoking. We calculated pack-years as the number of cigarettes packs per day × years of smoking (one pack-year = 20 cigarettes per day for one year). Participants with a smoking consumption of over 30 pack-years have an increased risk to develop lung cancer and chronic obstructive pulmonary disease [22-25].

Sample Collection and Histochemical Techniques

All samples were provided from the São Paulo Autopsy Service (SPAS) from University of São Paulo Medical School. Samples were collected, at approximately 5 cm from the central tendon (midcostal region), fixed in 10% buffered formalin and embedded in paraffin as previous described [11, 19, 26]. The blocks were cut with a microtome (6 μm – thickness section) [11, 19]. Transverse sections were stained with picrosirius red for collagen fibers and hematoxylin and eosin (H&E) for inflammatory cells, fat deposition, and blood vessels [11, 19, 26].

Immunohistochemical Techniques

Diaphragm samples were cut into non-serial 3-μm slices and mounted onto silanized slides. Each sample was deparaffinized and hydrated. Assay procedures were performed according to the manufacturer's instructions. The epitopes were blocked with peroxidase and incubated with a specific first antibody. In this study, we used the following primary antibodies for metalloproteinases: MMP-2 Antibody (Santa Cruz Biotechnology, sc-10736) and MMP-9 Antibody (Santa Cruz Biotechnology, sc-6840). Inflammation was evaluated with COX-2 (Santa Cruz Biotechnology, sc-1745). Oxidative stress and vascularization were evaluated with 8-OHdG (Santa Cruz Biotechnology, sc-66036) and VEGF (Santa Cruz Biotechnology, sc-7269), respectively. The sections were washed with a PBS-Tween solution and incubated with biotinylated secondary antibody, avidin-chain enzyme, stained with DAB (3,3′-diaminobenzidine), and lastly with hematoxylin (Supplementary Fig. 1 – for all supplemental material see www.cellphysiolbiochem.com). The sections were dehydrated conventionally with ethanol, cleared with xylene, and mounted with synthetic resin for light microscopy analysis.

Quantitative Analysis

Twenty randomly selected fields were analyzed with a light microscope (Zeiss, x100 magnifications) for each staining technique, i.e. a total of 140 scanned images per patient, using an image analysis program (Axio Vision Software, Zeiss). For volume density (Vv) of the collagen fibers (Picrosirius red), adipocyte deposition, metaloproteinases 2 and 9 (muscle remodeling), vascularization (blood vessels and VEGF), inflammatory marker (COX-2) and inflammatory cells (H&E), and oxidative stress (8-OHdG) the photomicrographs of the diaphragm were analyzed using the Image J software (version 1.47, National Institutes of Health) by a stereological test-system with 336 points and values were expressed as a percentage [11, 14, 19].

The Vv was estimated as: (Vv [structure] = PP [structure]/PT), where PP is the number of points that hit the structure, and PT is the total test-points [11, 19]. Additionally, we analyzed the cross-sectional area (μm^2) of 20 normal muscle fibers in each H&E staining scanned images using Axio Vision software (100x magnification), i.e. a total of 400 normal muscle fibers per patient. In Addition, in all the immunohistochemical techniques, a complementary quantitative analysis of the images was performed using the program ImageJ, where the imunostained areas (brown precipitates due to the DAB) were selected and the program quantified immunoexpression intensity using the "analyze particles" plugin. All analysis were conducted by experienced morphologists blinded to all clinical data to avoid bias [27].

Statistical Analysis

Data were expressed as mean ± standard error (SEM) for continuous variables. We initially conducted a two-tailed unpaired Student's t-test to examine whether the groups were different regarding demographics and pathological continuous variables. We used chi-square or Fisher exact tests when appropriate for categorical variables. Multivariate linear regression adjusted for age, sex, body mass index (BMI), cardiovascular cause of death, diabetes mellitus, and hypertension was used for the association between smoking and histopathological findings through the beta coefficient (β) and 95 % confidence interval (95 % CI). The statistical analyses were performed using SPSS software (IBM SPSS Statistics version 21.0). The alpha level was set at the 0.05 level.

Results

During the study period, 18 subjects were eligible to participate in this study. However, six subjects had inconsistent data from the next-of-kin regarding the period of smoking. Twelve subjects met the inclusion and exclusion criteria for the Smoker and Control groups (Table 1). Regarding the characteristics, the smoker group was significantly younger than the control group (60.67±1.47 versus 80.00±0.73 years, $p < 0.0001$). Additionally, the control group has four diabetic subjects, in contrast, the smoker group had one diabetic subject ($p = 0.0790$).

Table 1. Patient characteristics. BMI: body mass index. M: male. F: female. NA: not applicable. H: hypertension. DM: diabetes mellitus

Subject	Age (years)	Sex	BMI (kg/m^2)	Smoking (years)	Pack-years of smoking	Cause of death	Pathologies
1	81	M	24,13	NA	NA	Pulmonary edema	H
2	80	M	22,02	NA	NA	Myocardial Infarction	DM, H
3	79	M	19,74	NA	NA	Myocardial Infarction	DM, H
4	83	F	18,14	NA	NA	Myocardial Infarction	DM, H
5	79	F	29,36	NA	NA	Pulmonary edema	DM, H
6	78	F	19,78	NA	NA	Sepsis	NA
7	61	M	19,40	46	46	Pulmonary edema	NA
8	59	M	31,35	42	84	Myocardial Infarction	H
9	61	M	29,63	21	42	Pulmonary edema	DM, H
10	62	F	25,22	40	40	Myocardial Infarction	NA
11	66	F	21,83	41	82	Myocardial Infarction	H
12	55	F	18,59	35	70	Myocardial Infarction	NA

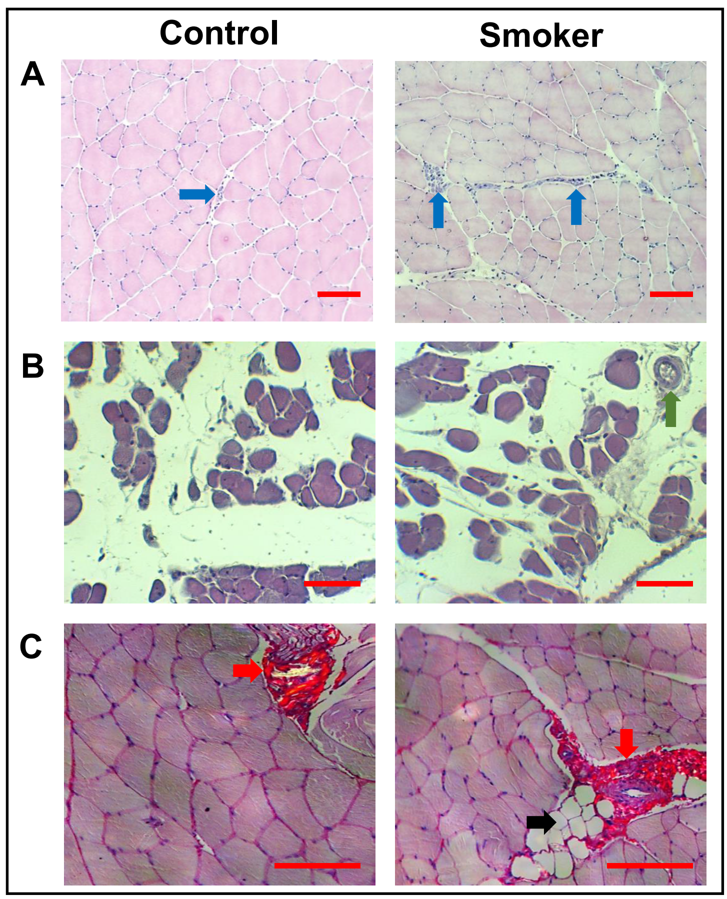

Fig. 1. Representative photomicrographs for the control and smoker groups stained with hematoxylin and eosin (A and B) and picrosirius red (C) showing: (A) inflammatory cells; (B) blood vessels; and (C) collagen and fat deposition. Blue arrows: inflammatory cells. Green arrow: blood vessel. Red arrows: collagen deposit. Black arrow: adipocyte deposition. Scale bars = 50 μm.

Representative images (Fig. 1 and Fig. 2) and graphics (Fig. 3 and Fig. 4) for the quantitative analysis are shown below. The diaphragm of smokers had a significant increase of inflammatory cells (3.09±0.14 versus 1.46±0.08%) and inflammatory mediator (COX-2) (14.56±0.29 versus 8.28±0.23%) when compared to the control group. In addition, cigarette smoking significant increased oxidative stress (8-OHdG) (15.14±0.19 versus 6.87±0.14%) when compared to the control group.

Muscle remodeling was observed with picrosirius red staining for collagen fibers and MMP-2 and MMP-9. Smokers had a significant higher percentage of collagen deposit (16.62±0.10 versus 11.92±0.09%), MMP-2 (11.49±0.17 versus 5.88±0.20%), and MMP-9 (10.72±0.25 versus 7.60±0.28%) when compared to non-smokers.

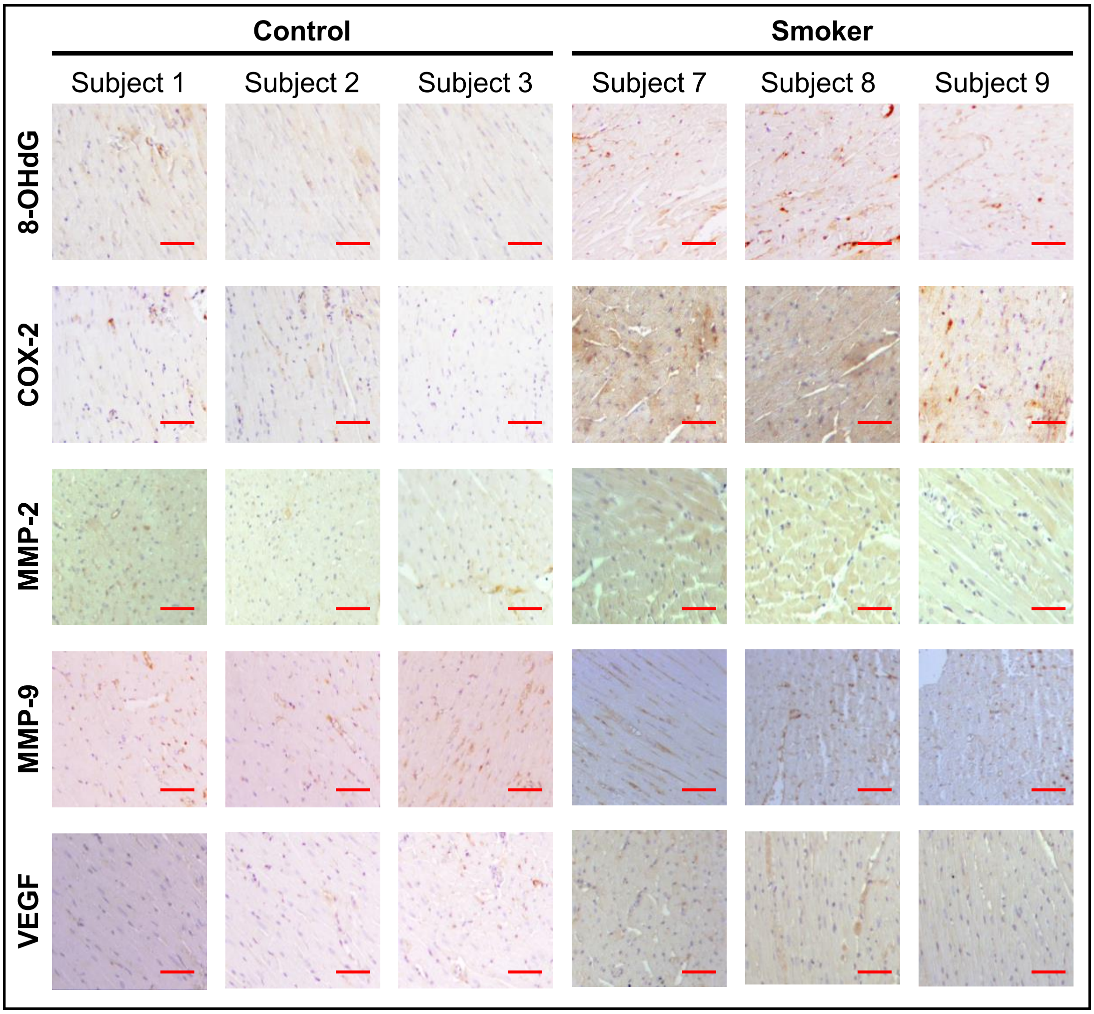

Fig. 2. Representative photomicrographs of three independent individuals for the control and smoker groups stained with 8-hydroxy-2′-deoxyguanosine (8-OHdG), ciclooxigenase-2 (COX-2), metalloproteinases (MMP) 2 and 9, and vascular endothelial growth factor (VEGF). Scale bars = 50 µm.

We observed that smoking increased blood vessels (2.72±0.12 versus 0.87±0.04%) and vascularization marker (VEGF) (8.29±0.25 versus 5.89±0.29%) in the diaphragm muscle when compared to the control group. In addition, we observed an increased deposition of adipocytes in smokers when compared to non-smokers (10.58±0.14 versus 5.77±0.12%). On the other hand, total cross-sectional area decreased in smokers when compared to non-smokers (1,035±9.03 versus 1,316±13.51%), indicating muscle loss.

The immunohistochemical intensity evaluation showed a slight significant increase in all techniques ($p < 0.05$), which suggests an accumulation of the analyzed parameters in certain areas of the images (Fig. 3). Multivariate linear regression analyses were performed to examine the association between smoking status and histopathological findings, adjusting for age, sex, body mass index, cardiovascular cause of death, diabetes mellitus, and hypertension (Table 2). Therefore, in the full model, we observed an increased association between all histopathological findings, mainly COX-2 ($\beta = 9.9582$, $p<0.0001$), collagen fibers ($\beta = 5.5960$, $p<0.0001$), and adipocyte deposition ($\beta = 5.1634$, $p<0.0001$).

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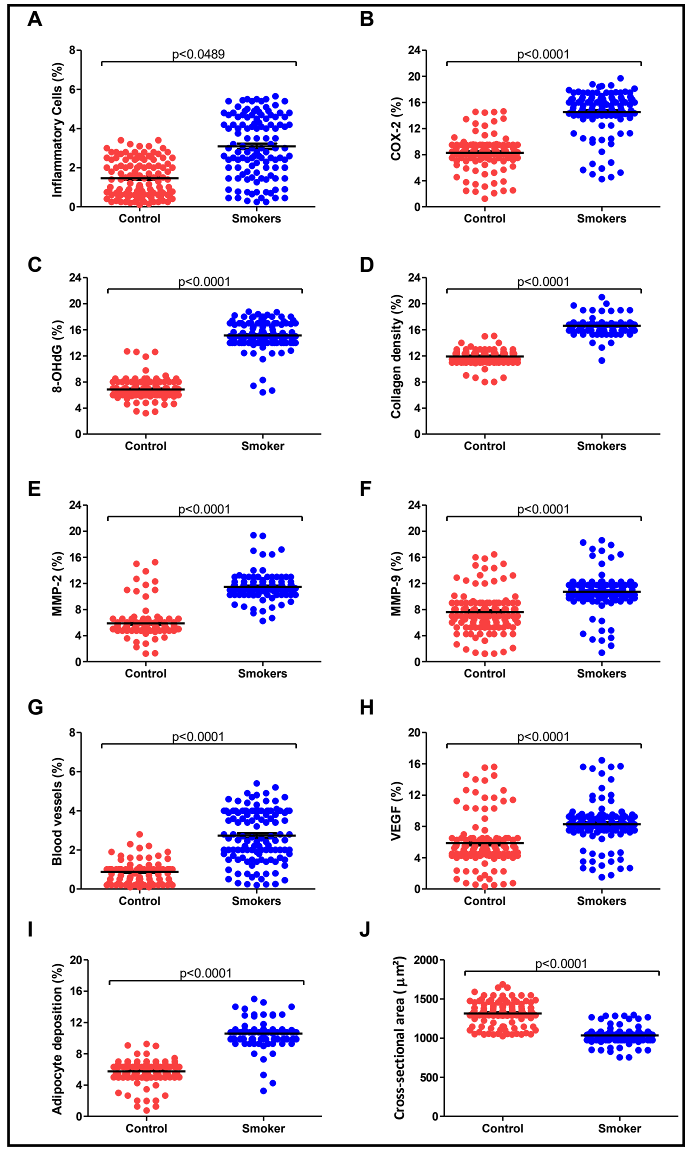

Fig. 3. Representative graphics for the quantitative analysis between the control and smoker groups. Scatter dot plot (each dot represents one field) showing the percentage (points that touched the studied structure divided by the total of points = percentage of the structure) of (A) inflammatory cells; (B) COX-2; (C) 8-OHdG; (D) collagen fibers; (E) MMP-2; (F) MMP-9; (G) blood vessels; (H) VEGF; and (I) adipocyte deposition. The total cross-sectional area (J) between the groups (each dot represents one fiber). Data are mean ± SEM values.

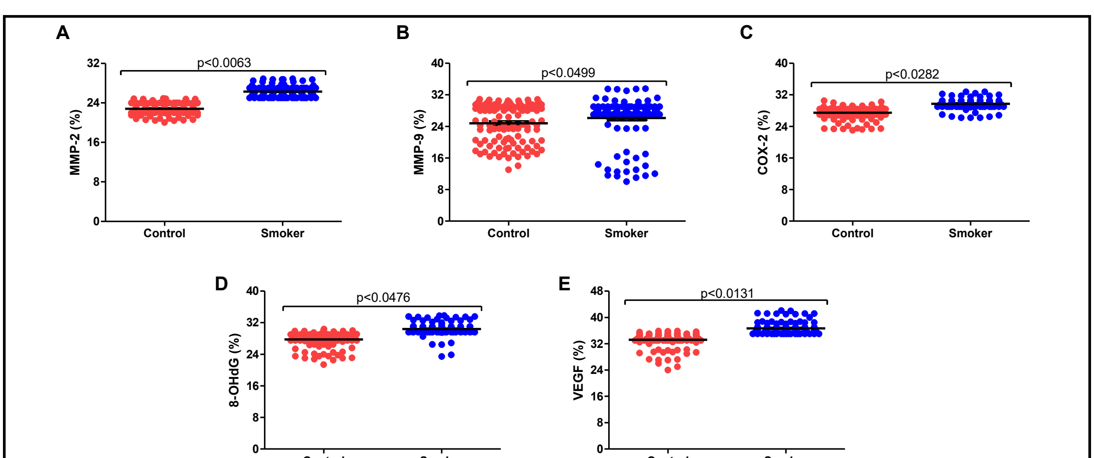

Fig. 4. Representative graphics for the quantitative analysis between the control and smoker groups. Scatter dot plot (each dot represents one field) showing the intensity of (A) MMP-2; (B) MMP-9; (C) COX-2; (D) 8-OHdG; (E) VEGF. Data are mean ± SEM values.

Discussion

In the present investigation, we highlight that cigarette smoking affects the diaphragm muscle structure by increasing its oxidative stress, inflammation, vasculature, and remodeling markers.

Cigarette smoking constitutes a risk factor for COPD [28], stroke [29], and coronary heart disease [30]. Systemic inflammation and oxidative stress are mechanisms related to negative muscle alterations in smokers, and the leading cause to the pathogenesis of COPD [9, 17, 31-34]. Our results are in accordance with previous investigations, as we observed significantly increase in both inflammatory cells and mediator (Cox-2), as well as, an increase of oxidative stress (8-OHdG) in the diaphragm of smokers. Additionally, sarcopenia and smoking had been related in previous clinical [35-37] and experimental [1-5, 9-11] studies by the increase of oxidative stress and inflammation, leading to the decrease of muscle cross-sectional area.

Table 2. Association between smoking status and histopathological findings (n=12). Model I: Multivariate linear regression analysis between smoking status and histopathological findings adjusted for age, sex, and body mass index. Model II: Multivariate linear regression analysis between smoking status and histopathological findings adjusted for age, sex, body mass index, cardiovascular cause of death, diabetes mellitus, and hypertension. *p < 0.01

Parameters	Model I β Coefficient (95 % CI)	Model II β Coefficient (95 % CI)
Inflammatory cells	1.4868 (0.9841; 1.9894)*	1.4529 (0.7220; 2.1837)*
COX-2	9.8952 (8.7004; 11.0899)*	9.9582 (8.1918; 11.7245)*
8-OHdG	2.5280 (1.8233; 3.2326)*	2.4918 (1.3382; 3.6454)*
Collagen fibers	5.6296 (4.7834; 6.4759)*	5.5960 (4.2682; 6.9237)*
MMP-2	2.5629 (1.9374; 3.1945)*	2.5040 (1.5400; 3.4679)*
MMP-9	2.4218 (1.6441; 3.1995)*	2.4567 (1.2610; 3.6524)*
Blood vessels	1.5805 (0.3030; 2.8581)	1.8174 (0.2753; 3.3595)
VEGF	1.6426 (0.8943; 2.3910)*	1.9296 (1.1393; 2.7199)*
Adipocyte deposition	5.1503 (4.0032; 6.2975)*	5.1634 (3.8194; 6.5074)*
Cross-sectional area	-1,1619 (-2.0916;-0.2262)*	-1.5289 (-3.0293;-0.0286)*

Endothelial dysfunction has been observed in chronic smokers as well as after acute cigarette smoking [38-40]. Thus, the increase of microvasculature evaluated in our study by the volume density of blood vessels and VEGF staining may be an adaptative response to avoid an hypoxic state [41]. However, further studies should analyze the nitric oxide synthase as it has a major role on endothelial function [42].

Regarding muscle remodeling and injury, we observed increased deposit of collagen fibers, as wells as, a significantly increase of metaloproteinases 2 and 9 activity in smokers. A previous study of our group showed significantly increase of collagen fibers in the diaphragm of smokers without respiratory pathologies when compared to non-smokers [11]. Additionally, COPD patients had a significant increase of collagen deposit in the diaphragm musculature, which negatively affect its function [19]. However, in our study, none of the included patients had clinical evidence of respiratory pathologies.

However, our results should be examined considering the study limitations. We included individuals with a significant difference of age. According to previous studies, the diaphragm strength decreases with aging which is dependent of histological and neurotrophic alterations [12-14]. However, despite the age we observed significant alterations between the groups. Although we assessed the muscle histopathology with only histochemical and immunohistochemical techniques, our results were consistent with the literature. Further studies should address molecular techniques (muscle atrophy F-box, muscle-specific RING Finger 1, and myostatin) to corroborate with our findings [10]. We analyzed the total collagen deposit despite the type of fiber (I, intermediate, and III), and future studies are encouraged to elucidate the presence of each type of collagen fibers.

Although our study has limitations, we also have some advantages. We presented clinical and histopathological data from smokers who had not showed any evidence of respiratory pathology. We used a multivariate analysis to avoid bias from the confounding parameters. Regarding the postmortem period, our methodology was in accordance with previous studies [11, 14, 19, 20]. Additionally, we analyzed the diaphragm muscle structure with wellstablished markers for muscle remodeling [7], oxidative stress [6], inflammation [8], and vascularization [43].

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Conclusion

We described the negative effects of smoking on diaphragm muscle structure in a sample of Brazilian individuals. Accordingly, further studies are needed regarding the physiological and molecular mechanisms for a better understanding of our results.

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Author Contributions

W. J. F. is the guarantor of this study. R. A. B. N., R. R. S., and W. J. F. contributed to the study design. A. L. B., R. E. P. L., and R. D. R. participated in sample collection; R. R. S., C. K. S., and C. A. P. contributed to data analysis; R. A. B. N., L. B. M. M., and C. A. A. were responsible for histopathological assessment. R. A. B. N., R. R. S., C. K. S. and A. L. B. contributed to data interpretation and to the writing of the manuscript. L. B. M. M., C. A. P., C. A. A., R. E. P. L., R. D. R. and W. J. F. have reviewed and approved the final draft of the manuscript.

Statement of Ethics

Ethical approval was obtained from the review board for human studies at the University of São Paulo Medical School (São Paulo, Brazil). The study was consistent with the Helsinki Declaration. Consent was obtained from the next of kin.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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