Changes in diaphragm contractility in cigarette smoking-exposed and smoking cessation rats are associated with alterations in mitochondrial morphology and homeostasis

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Abstract
The effects of cigarette smoking (CS) cessation on the diaphragm are unknown, as are the CS-induced diaphragmatic mitochondrial changes. We examined the changes in diaphragm contractility, as well as alterations in mitochondrial morphology, function and homeostasis during CS exposure and after cessation. Rats were randomly divided into CS exposure and CS cessation groups: 3-month CS (S3), 6-month CS (S6), 6-month CS followed by 3-month cessation (S6N3). The changes in the diaphragm were investigated, including contractile properties, the ultrastructure, mitochondrial function and the expression of markers of mitochondrial homeostasis. CS exposure disrupted the diaphragmatic mitochondrial morphology and function (S6), which was significantly alleviated in the S6N3 group. The mitochondrial homeostasis was depressed (S6), as indicated by the downregulation of Pink1 and Mfn1. Interestingly, the Mfn1 level was recovered after smoking cessation (S6N3). In conclusion, smoking cessation eased CS-induced diaphragmatic dysfunction and mitochondrial deregulation, which are likely associated with deregulated mitochondrial homeostasis.

Keywords
autophagy, cigarette smoking, diaphragm, fission, fusion, mitochondrion

1 | BACKGROUND

Chronic obstructive pulmonary disease (COPD) is characterized by persistent respiratory symptoms and airflow limitations, which are due to airway and/or alveolar abnormalities.1 Skeletal muscle dysfunctions in both respiratory and limb muscles, as the extrapulmonary comorbidities of COPD, are associated with poor health status, exacerbation, mortality, and hospitalizations in COPD patients, and thus, the muscular dysfunction...
should be actively treated. Intriguingly, the diaphragm undergoes a positive adaptation (training-like effect) to protect against muscle dysfunction in the early stage of COPD. In addition, compared to other skeletal muscle, diaphragm muscle shows more resistance to muscular atrophy in COPD, suggesting a diaphragm-specific pathophysiological mechanism in muscle dysfunction. However, a recent study shows maladaptive changes in diaphragm muscle, including impaired cytoplasm integrity with enhanced proteolysis, during early COPD caused by cigarette smoking (CS). This contradiction is likely due to the CS-induced adverse effects on respiratory muscle disrupting this adaptive transition.

CS is one of the greatest risk factors for the pathogenesis and progression of COPD. The CS-induced deleterious effects on skeletal muscles, other than the respiratory system, are also significantly involved in the pathogenesis of COPD. Both clinical studies on smokers and CS-exposed animal models have shown impaired skeletal muscle function before the development of respiratory symptoms, suggesting a direct effect of CS on muscles. A study showed that CS-induced skeletal muscle deregulation, including mitochondrial dysfunction and diaphragm muscle atrophy, was significantly recovered by CS cessation in a mouse model. Although this study showed the beneficial effects of CS cessation on diaphragm, it is largely unknown if the long-term duration of CS exposure would lead to different effects of CS cessation.

CS-induced oxidative stress is an important mechanism of muscle dysfunction in COPD. The oxidative stress-induced changes of mitochondrial structure and function have been highlighted in locomotor muscles in COPD, mainly characterized by decreased oxidation capacity, increased reactive oxygen species (ROS) and increased autophagy and apoptosis, while CS cessation obviously alleviates mitochondrial dysfunction. Our previous transcriptome profiling study showed that metabolic gene expression was deregulated along with mitochondrial morphological changes in the diaphragm muscle from CS-exposed rats. Of note, the precise role of mitophagy and its relationship to respiratory muscle dysfunction are unclear because few studies have been conducted.

Previous studies have indicated that CS exposure disrupts mitophagy and results in the accumulation of damaged mitochondria. The imbalance of mitochondrial fusion/fission caused by CS exposure increases the susceptibility of the lung to cellular stress and senescence, which contributes to age-related COPD pathogenesis. However, these previous studies focused on the mitochondrial changes in pulmonary epithelial cells, bronchial smooth muscle cells and skeletal muscle of the limbs in the pathogenesis of COPD, and the mitochondrial autophagy and fusion/fission in the diaphragm during the development of COPD and after CS cessation is largely unknown. In this study, we explored the association between CS-induced changes in diaphragm contractility and mitochondrial alterations in morphology and homeostasis using a rat model in response to CS exposure and CS cessation.

2 | METHODS

2.1 | Establishment of the COPD rat model

Seven-week-old SPF male Sprague–Dawley (SD) rats (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were housed under conditions of 12-hour light/dark cycle, 25–26°C, and 50%–60% humidity at the animal facility, Beijing Chaoyang Hospital. Rats were given ad libitum access to food and water. All animal procedures approved by the Animal Care and Ethics Committee of Beijing Chaoyang Hospital were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Beijing Chaoyang Hospital, Capital Medical University. After a 1-week adaptation, the rats were randomly divided into three CS-exposed groups and the respective control groups according to the duration of CS exposure: a 3-month CS group (S3, n = 10) with a matched no-smoking control group (C3, n = 14); a 6-month CS group (S6, n = 9) with a matched control group (C6, n = 15); and a 6-month CS group followed by 3-month cessation (S6N3, n = 9) with a matched control group (C9, n = 7). The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.

The CS group rats were exposed to CS with Baisha Filter cigarettes (tobacco type: tar, 10 mg; nicotine content, 1.0 mg; and carbon monoxide, 13 mg) for 1 h twice a day. The SIBATA SG-300 oro-nasal cigarette suction system (Sibata Scientific Technology Ltd., Tokyo, Japan) and a CS exposure chamber were used. The exposure lasted for 3 min/cigarette, and 20 cigarettes were used for each exposure procedure, with a maintained level of total particulate matter of 993.6 ± 125.7 mg/m³. The control rats were housed under the same conditions without CS exposure. The COPD model was established by evaluating the pulmonary functional changes and lung tissue histological changes for further experiments. The rats were excluded if the rats died prematurely, if the rats did not meet the following experimental schedules and...
criteria or if the examples failed to meet quality control standards described below.

### 2.2 Pulmonary function test and histopathological analyses

Pulmonary function was assessed using an AniRes 2005 animal lung function analysis system (Beijing Beilanbo Technology). The forced expiratory volume in 0.3 s (FEV0.3), forced vital capacity (FVC) and the ratio of FEV0.3/FVC were calculated.

Following the pulmonary functional assay, the rats were killed by exsanguination from the abdominal aorta. The lungs were inflated with 4% paraformaldehyde solution through the endotracheal intubation at a constant pressure of 25 cmH2O for 10 min. Then, the right lower lobe was removed from the rats after ligating the bronchi, fixed in 4% paraformaldehyde solution for 48 h and embedded in paraffin. These paraffin-embedded tissues were cut into 5-μm sections and stained with haematoxylin and eosin (H&E, ScyTek Laboratories).

Emphysema, reflecting pulmonary injury, was assessed by measuring the mean linear intercept (MLI) and the mean alveolar number (MAN), as previously described. Ten fields (100×) for the MLI and 20 fields (200×) for the MAN (without the large trachea and blood vessels) were randomly selected, and a cross was drawn through the centre of each field. The number of alveolar intervals (Ni) lying on the cross, the total length (L) of the cross, the area of the field (S) and the number of alveoli (Na) in each field was determined (MLI = L/Ni and MAN = Na/S) using Adobe Photoshop CS5.

Semi-quantitative histological assessment of H&E sections was performed blindly using an ordinal scoring system designed to distinguish the degree of lung inflammation. Severity scores ranging from 0 to 4 were used, and severity was assessed by noting the most advanced grade present within the specific sample irrespective of its horizontal extent. The extent was defined as the horizontal distribution of pathology, where a score of 0, 1, 2 or 3 meant that none of the lung was involved, ≤1/3 involvement, 1/2 involvement or ≥2/3 involvement, respectively. The overall score was defined as a combined assessment of severity and extent (overall score = severity × extent).

### 2.3 Contractile function of the diaphragm in vitro

Diaphragmatic contractile properties were measured using the RM6240 biological signal acquisition and processing system (Chengdu Instrument Factory, Sichuan, China). The diaphragm was quickly removed and immediately immersed in Krebs solution containing the following (mmol/L): 118NaCl, 4.7KCl, 1.25 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3 and 11 glucose; pH value, 7.3–7.4. A rectangular muscle bundle, ~5 mm wide, parallel to the long axis of the fibres, was cut in the upper region of the right hemi-diaphragm with the central tendon and rib reserved. The diaphragm bundle was placed vertically into the glass bath filled with Krebs solution at 37°C and perfused with a mixture of 95% O2 and 5% CO2.

The rib end of the diaphragm was tied to an ‘L’-shaped metal bar at the bottom of the bath, and the other end was connected to the tension transducer. Two silver-stimulating electrodes separated by a distance of 1 cm were inserted into the bundle. After a 30-min thermo-equilibration period, the bundle was extended to its optimal length (Lo, the length with peak twitch force), and stimulations were delivered through an electronic stimulator. Subsequently, the threshold voltage at Lo was determined, defined as the minimum stimulus intensity that caused the maximum contraction of the diaphragm bundle. The voltage was then increased by 20% to ensure supramaximal stimulation, and this voltage was used during the subsequent experiments.

#### 2.3.1 Twitch characteristics

Two twitches were recorded at Lo to determine the twitch tension (Pt), the maximum tension rise rate (+dT/dtmax) and the maximum tension fall rate (−dT/dtmax). The average value for the two twitches was used for further analysis.

#### 2.3.2 Maximal tetanic tension (Po)

The bundle was stimulated twice tetanically at 160 Hz with a 0.2 ms wave width for 250 ms to obtain a clear plateau in force generation. The Po was recorded as the plateau tension, and the average value was used.

#### 2.3.3 Force–frequency curve

The bundle was stimulated at the following frequencies: 10, 20, 40, 60, 80, 100 and 150 Hz. Each stimulus was separated by a 2-min interval. The curve was plotted with stimulation frequency on the X axis and force generation and relative force to the maximum force during the sequence of stimuli on the Y axis.
2.3.4 | Fatigue index (FI)

The fatigue index (FI) was assessed with a low-frequency protocol in accordance with the modified Burke method. The muscle bundle was fatigued by using 330-ms stimulations repeated at 40 Hz and applied every second for 2 min. The FI was calculated as the force at the end of the 2-min stimulation divided by the maximum force during the initial stimulation.

2.3.5 | Standardization of values

The values of force were corrected using the cross-sectional area (CSA) of the muscle bundle. The CSA (cm²) was calculated by dividing the weight by the specific density (1.056 g/cm³) and muscle length.

2.4 | Electron microscopy

Samples of the diaphragm were processed for electron microscopy according to the standard methods at the Electron Microscope Laboratory of Peking University People’s Hospital. Briefly, each diaphragm sample was immediately fixed with 3% glutaraldehyde solution and 1% osmic acid solution and then dehydrated with different concentrations of ethanol. The samples were embedded in epoxy resin. Ultrathin sections were obtained along the long axis of muscle fibres and stained using uranyl acetate and lead citrate. The ultrastructure of the diaphragm cells, such as the muscle fibre arrangement, ‘Z-line’ and mitochondrial morphology, was observed and photographed in three copper nets for each sample using an FEI Tecnai Spirit Transmission Electron Microscope at constant calibrated magnifications of 6000× and 11 500×. Ten randomly selected fields from each copper net were analysed (6000×). The mitochondrial number density (NA) and mitochondrial volume density (Vv) were established and calculated by ImageScope image analysis software. Ten horizontal and 10 vertical lines were cross-drawn equidistant through the space. NA (/μm²) = mitochondrial number of reference space/whole space area; Vv (%) = number of points that hit mitochondria/number of points that hit reference space.

2.5 | Adenosine 5'-triphosphate (ATP) content measurement

Adenosine 5'-triphosphate (ATP) content was detected using an ATP assay kit (Beyotime, China). Briefly, the diaphragm tissues were lysed by ATP releasing reagent. After mixing with ATP detection solution containing luciferase, the bioluminescence was measured by Synergy HT luminescence plate reader. A fresh standard curve was prepared each time, and ATP content was estimated according to the curve. Results were normalized to tissue protein concentration, which was determined by a BCA protein assay kit (Beyotime, China).

2.6 | Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, USA). First-strand cDNA synthesis was performed using Prime Script RT Master Mix (TaKaRa Biotech, Dalian, China). The mRNA levels of target genes were measured by quantitative real-time polymerase chain reaction (qPCR) using SYBR Green Master Mix (TaKaRa Biotech, Dalian, China), in summary for mitochondrial respiratory complex content: NADH:ubiquinone oxidoreductase subunit B8 (Ndufb8), succinate dehydrogenase complex iron sulfur subunit B (Sdhb) and cytochrome c oxidase subunit 4I1 (Cox4I1) and for mitophagy and fusion/fission: PTEN induced putative kinase 1 (Pink1), BCL2 interacting protein 3 (Bnip3), mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), optic atrophy 1 (Opa1) and dynamin-related protein 1 (Drp1). The total amount of mRNA was normalized to Actin levels.

The primers needed are listed in Table 1.

2.7 | Western blot

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was run on 8% or 10% gradient gels with equal amounts 50 μg protein loaded. Relative amounts of Pink1, Bnip3, Mfn1, Opa1 and Tubulin were assessed using the following primary antibodies: (1) Pink1 (product no. ab23707, Abcam, Cambridge, MA, USA); (2) Bnip3 (product no. ab109326, Abcam, Cambridge, MA, USA); (3) Mfn1 (product no. ab104274, Abcam, Cambridge, MA, USA); (4) Opa1 (product no. ab157457, Abcam, Cambridge, MA, USA); and (5) Tubulin (product no. ab108342, Abcam, Cambridge, MA, USA). Secondary antibodies (Zhongshan Golden Bridge, Beijing, China) were applied at room temperature for 2 h. Protein bands were visualized using ECL reagent. ImageJ software was used for subsequent quantification and statistical analysis.

2.8 | Statistical analysis

For normally distributed continuous variables, the data are presented as the mean ± SD, unless stated otherwise.
To compare continuous variables, the Kolmogorov–Smirnov test was used to test the normality of the data. Statistical comparisons between two groups were performed via two-tailed Student’s t-test for normally distributed continuous variables or the Mann–Whitney U test for non-normally distributed continuous variables. Statistical comparisons of three or more groups were performed using one-way ANOVA (least significant difference post hoc test) for normally distributed variables and one-way Kruskal–Wallis tests (Dunn’s post hoc test) for non-normally distributed variables. Upon considering age-related changes in lung morphology and muscle strength, two-way ANOVA for normally distributed variables (least significant difference post-hoc test) and two-way Kruskal–Wallis tests for non-normally distributed variables were performed. Statistical significance was set at \( p < 0.05 \). The data were analysed with SPSS 17.0 software.

| Gene | Forward primer | Reverse primer |
|------|----------------|----------------|
| Actin | GCAGGAGTACGATGAGTCCG | ACGCAGCTCAAGTACAGTCC |
| Ndufb8 | AGGCCGTTACCTCCTCCAAAG | GAGTCCCATCCAGAGGGCA |
| Sdhb | CGATGTTGCTGAGTGCCTCTCAATC | ACCGTTGTTCCCTGATTG |
| Cox4i1 | TCTACTCCGCTTGGCTTCG | CCACTACGGCAAGGGGTA |
| Pink1 | GCTTCCGGCCTTGGAGATTAT | GGGATGTTGCTTCCATAC |
| Bnip3 | ATTGGAAGTCTCAGAGTCAAA | GAGATGCTTGGTGGCTTCG |
| Mfn1 | TGCATGGACTACTGCTCCG | GACTTGGCTGCTGAGTT |
| Mfn2 | GCCAGAGAAGACACATCAGAAG | AAGTGGTGGAGAGGGAGA |
| Opa1 | CGACGGAGACACCTCGGCA | CAGGTGACCCGGAGTGA |
| Drp1 | TGGAAAAGACGTAGTGCTGG | CAACCTCAGTTTCTTCTGTTT |

### RESULTS

#### 3.1 | CS exposure caused marked structural and functional disruptions, and CS cessation failed to lead to a significant recovery

Compared to the control rats, all the CS-exposed groups had thinner bodies and significantly lower body weight (Figure 1A), accompanied with marked decline of lung function indicated by decreased FEV\(_{0.3}/\)FVC and disruptions of alveolar structure and formation of emphysema indicated by enlarged MLI and decreased MAN, along with enhanced infiltration of inflammatory cells within the alveolar cavity and around the bronchi (Figure 1B–G). The longer CS exposure led to more significant distention of the air spaces and the destruction of the alveolar septa in the S6 and S6N3 rats and the highest histopathology score in the S6 rats (Figure 1C,D,G). S6 rats also showed an enhanced decrease in MAN (Figure 1F). The results confirmed the progressive adverse structural change in response to CS exposure.

Additionally, the CS cessation group (S6N3) exhibited a decrease in pulmonary inflammatory infiltration, thinner alveolar septum and a significant increase in MAN versus S6 (Figure 1C,D,F). However, compared to the age-matched controls (C9), MAN, MLI and histological score measurements in the S6N3 group showed significant changes, suggesting that smoking cessation did not significantly alleviate the alveolar complications induced by CS (Figure 1E–G).

#### 3.2 | CS exposure depressed diaphragmatic contractile function, which was eased by 3-month CS cessation

To evaluate the diaphragmatic contractile function, we measured Pt/CSA, \(+dT/dt_{max} - dt/dt_{max}\), Po/CSA and the force–frequency curve to evaluate the contractile properties and force generation capacity at increasing stimulation frequencies and FI to evaluate the fatigue resistance. Compared to controls, the diaphragmatic function in S3 rats exhibited significant decreases in Pt/CSA, \(+dT/dt_{max}\) and \(-dT/dt_{max}\), without changes in Po/CSA (Figure 2A–D). As shown in the force–frequency curve, the relative force at low frequency stimulation tended to decrease in the S3 group, but the differences did not reach the statistical significance (Figure 2F), and the force generation between the S3 and C3 group similar (Figure S2). Interestingly, the FI of the diaphragms of S3 rats was significantly increased versus that in the control group, suggesting that the endurance of the diaphragm was enhanced after short-term CS exposure (Figure 2E).
FIGURE 1  CS exposure caused marked structural and functional disruptions and CS cessation failed to cause a significant recovery. (A) CS-exposed rats showed decreased body weight versus the controls. (B) CS exposure caused a significant decrease in FEV_{0.3}/FVC, and the S6 and S6N3 groups exhibited severe decreases compared to the S3 group; there was no significant difference in FEV_{0.3}/FVC between the S6 and S6N3 groups. (C,D) Representative H&E-stained images of lung tissue. Significant distention of the alveolar cavity with destruction of the alveolar septa and reduced alveolar units were observed in the lungs of S3, S6 and S6N3 rats compared to the control rats (100×) (C). The S6 lungs showed more severe changes than S3 and S6N3 lungs. Increased inflammatory cell infiltration within the alveolar cavity and around the bronchi in the lungs from S3, S6 and S6N3 rats compared to the control rats (200×) (D). (E–G) Compared to the control rats, in all CS groups (S3, S6 and S6N3), the MLI and the histopathology scores significantly increased, and MAN decreased. The S6 rats showed enhanced decrease in MAN versus the S3 rats, while the CS cessation group (S6N3) exhibited a significant increase in MAN versus S6, rather than MLI and the histopathology scores. Data are presented as the mean ± SD, n = 6–15 per group. Scale bar: 100 μm for (C); 50 μm for (D). CS, cigarette smoking; FEV_{0.3}, forced expiratory volume in 0.3 s; FVC, forced vital capacity; H&E, haematoxylin and eosin; MAN, mean alveolar number; MLI, mean linear intercept.
CS exposure depressed diaphragmatic contractile function, and CS cessation eased CS-induced contractile decline.

(A) Compared to controls, the Pt/CSA was significantly decreased in S3 and S6 rats, but not in S6N3 rats. Upon the prolongation of CS exposure, the Pt/CSA of the S6 rats was worse than that of the S3 rats. (B) The Po/CSA exhibited a significant decrease in S6 rats, but not in S3 and S6N3 rats. (C,D) The +dT/dtmax and -dT/dtmax were significantly decreased in S3 and S6 rats, but not in S6N3 rats. Upon the prolongation of CS exposure, S6 rats showed worse declines of -dT/dtmax versus the S3 rats. After smoking cessation for 3 months (S6N3), the +dT/dtmax and -dT/dtmax were significantly improved compared to those in the S6 rats. (E) The FI of the diaphragm from S3 rats was significantly increased compared to that in the control group. (F–H) Force–frequency curve of diaphragm for the CS groups and the controls. The relative force at low-frequency stimulation tended to decrease in the S3 group, but the differences did not reach the statistical significance (F). In the S6 diaphragm, the force–frequency curve indicated a rightward shift, and the relative force increase was significantly weaker with the increase of stimulation frequency (G). After smoking cessation for 3 months, the S6N3 rat and age-matched controls (C9) exhibited similar in force–frequency curve (H). Data are presented as the mean ± SD, n = 6–8 per group, *p < 0.05 versus control group. +dT/dtmax, maximum tension rise rate; CS, cigarette smoking; CSA, cross-sectional area; -dT/dtmax, maximum tension fall rate; FI, fatigue index; Po, maximal tetanic tension; Pt, twitch tension.
These findings suggested that short-term CS exposure promoted an adaptive transition in diaphragmatic function to maintain respiratory function. However, 3-month CS exposure significantly repressed the contractility of the extensor digitorum longus (EDL) muscle both in Pt/CSA, Po/CSA, dT/dt\textsubscript{max} and force–frequency relationship (Figures S1 and S3), suggesting that EDL muscles were more vulnerable to CS exposure. This discrepancy also indicated a distinct regulation between diaphragm and limb muscle during CS-induced COPD.

Upon the prolongation of CS exposure, the diaphragm function declined, as indicated by the marked decreases in Pt/CSA, dT/dt\textsubscript{max}, Po/CSA and force generation in the S6 group, which was more serious versus the S3 group (Figures 2A–D and S2). Furthermore, in addition to the depressed force generation under each stimulus frequency in S6 rats, the force–frequency relationship curve exhibited a significant rightward shift versus age-paired controls (Figure 2G). The right-shifting force-frequency curve is commonly observed after fatigue, and the rightward displacement in S6 suggests the impairment of the excitation–contraction coupling mechanism.\textsuperscript{30} Additionally, the FI of S6 rats exhibited a trend of increase as that in the S3, although no statistical difference was achieved (Figure 2E). These observations suggested progressive disruption in diaphragmatic contractile properties and force generation capacity upon long-term CS exposure, rather than endurance.

Smoking cessation for 3 months likely eased the diaphragm contractile function, as evidenced by the observation that S6N3 rats and age-matched controls (C9) exhibited similar diaphragm function (Figure 2A–E, H). Consistently, S6N3 showed a significant increase in dT/dt\textsubscript{max} compared to S6 (Figure 2C,D), suggesting a beneficial effect of cessation on diaphragm function. The beneficial effects of CS cessation were also observed in EDL muscle (Figures S1 and S3). This interesting observation confirmed the benefits of smoking cessation for smoking patients with COPD.

### 3.3 Long-term CS exposure caused disruptions of mitochondrial morphology and function in diaphragm muscle, while CS cessation eased mitochondrial damage

We examined diaphragm myofibrils and mitochondrial morphological changes using electron microscopy. Compared to age-paired controls, S3, S6 and S6N3 diaphragms failed to show obvious changes in the morphology of diaphragm muscle cells, with clear Z-lines and sarcomeres arranged in order (Figure 3A). However, CS induced marked changes in diaphragmatic mitochondria. S3 rats displayed a slight change in the morphology of diaphragmatic mitochondria with increased mitochondrial number, especially in the perinuclear area (Figure 3A,C). These morphological changes were consistent with the enhanced endurance of diaphragm muscle in S3 rats.

Conversely, the S6 rats exhibited marked morphologic changes in diaphragmatic mitochondria, including obviously fuzzy outlines, vague or missing cristae and even vacuolar-like degeneration (Figure 3A). Moreover, the mitochondrial volume density and number density were significantly decreased (Figure 3B,C). To evaluate the mitochondrial energy production and oxidative phosphorylation capacity in diaphragmatic muscle, the ATP content and the mRNA levels of Ndufb8, Sdhb and Cox4i1 (the subunits of mitochondrial respiratory chain enzyme complexes I, II and IV, respectively) were examined. The results showed that the ATP contents and the mRNA levels of Ndufb8, Sdhb and Cox4i1 were significantly decreased in the S6 (Figure 3D–F), indicating a long-term CS exposure-induced mitochondrial dysfunction in diaphragms. Intriguingly, the morphology and function of the mitochondria in the S6N3 group seemed to be recovered, as indicated by clear outlines and cristae and a less vacuolar-like structure (Figure 3A) and by the comparable ATP content and mRNA levels of Ndufb8, Sdhb and Cox4i1 versus the C9 group (Figure 3D,F). Particularly, the mitochondrial volume density was significantly higher than that of the control (Figure 3B). The 6-month CS exposure caused remarkable disruption of diaphragm mitochondria, which was eased by cessation, likely contribute to the improved diaphragmatic function in S6N3 rats.

### 3.4 Long-term CS exposure induced deregulation of mitophagy and fusion/fission, which was alleviated by CS cessation

To examine whether mitochondrial morphology changes in the diaphragm were associated with alterations in mitophagy and mitochondrial fusion/fission, we measured the expression levels of their key regulators in the diaphragm, including Pink1, Bnip3, Mfn1, Mfn2, Opa1 and Drp1. There was no significant difference in these markers between the S3 and C3 groups at both the mRNA and protein levels (Figure 4A,B), whereas the S6 diaphragms showed significantly decreased expression of Pink1 and Mfn1 (Figure 4A,C). This finding accorded with the observations of mitochondrial morphology. Of note, both the mRNA and protein levels of Mfn1 were recovered in the CS cessation rat (S6N3) group, whereas Pink1 expression was significantly decreased compared
FIGURE 3  Legend on next page.
The lesions after CS exposure caused disruption of mitochondrial morphology and function in diaphragm muscle, and CS-cessation seemed to be recovered as indicated by clear outlines and cristae and a less vacuolar-like structure. The mitochondrial volume density (B) and number density (C) were measured. The S3 rats displayed an increased mitochondrial number density. Upon the prolongation of CS exposure, the S6 rats exhibited significant decreases in both mitochondrial volume density and number density. After smoking cessation for months (S6N3), both mitochondrial volume density and number density showed improved compared to the S6 rats, especially the mitochondrial volume density significantly increased versus the control (C9).

4 | DISCUSSION

As known, CS is one of the greatest risk factors for the pathogenesis COPD, and CS cessation is beneficial in preventing COPD progression, while the effects of cessation on the diaphragm are unknown. We hypothesized that cessation could ease CS-induced diaphragmatic dysfunction and mitochondrial changes would be involved. In the current study, we examined this hypothesis by use a CS exposure rat model. We found that CS exposure caused histological disruption and functional depression in the lungs, and CS cessation failed to result in a significant recovery. CS induced a significant decline in diaphragm muscle contractility, which was recovered after 3-month CS cessation. In parallel, the disruption of the mitochondrial morphology in diaphragm muscle was significantly alleviated after cessation. These diaphragm muscle changes are likely associated with deregulated mitochondrial homeostasis, including mitophagy and mitochondrial fusion/fission.

4.1 | CS exposure caused progressive irreversible disruption of lung structure

COPD, characterized by the airflow limitation, is a combination of airway inflammation and progressive loss of alveolar structure, resulting in significant decrease in pulmonary function. Smoking cessation has been shown to be the most effective intervention to slow the decline of lung function in COPD. However, an even longer duration of smoking cessation cannot fully reverse the lung pathology and inflammation. The lesions after smoking cessation may be attributed to the persistent inflammatory changes induced by the elevation of cytokines, such as interleukin-12 and matrix metalloproteinase 12. In this study, CS induced progressive disruption in lung morphology and function. CS cessation only partially reversed the adverse structural changes including constricted inflammation and clear alveolar structure, but failed to alleviate emphysema, as evidenced by the significant difference in MLI1 and MAN compared to the age-paired controls. This limited amelioration of emphysema reasons that smoking cessation failed to improve significant pulmonary functions.

4.2 | Mitochondrial alterations were essential for the diaphragmatic transition from adaptive to maladaptive in response to CS exposure

Although the mechanism of diaphragm dysfunction during COPD is not fully elucidated, it has been involved in several adverse alterations including oxidative stress, increased proteolysis, muscle atrophy, apoptosis and mitochondrial dysfunction. Notably, previous studies also indicate adaptive changes during COPD that are beneficial to diaphragm function, such as shorter sarcomere length, higher proportions of slow-twitch fibre, increased capillary contacts per fibre and increased mitochondrial density. The outcome of diaphragm function was dependent on the shift of the balance between adverse and adaptive alterations, which is associated with the progression of disease, acute exacerbation and treatment intervention. In our CS exposure rat model,
3-month CS exposure led to enhanced diaphragmatic endurance with increased mitochondrial density, whereas longer exposure caused depressed diaphragmatic contractility with disrupted mitochondrial morphology and function.

A recent published study by Zhang et al. showed the effects of short-term CS exposure (1, 2 and 3 months) on muscle fibre remodelling in rat diaphragms. Although they showed that endoplasmic reticulum stress-associated apoptosis caused by CS exposure contributed to the...

**FIGURE 4** Comparison of expression levels of key regulators related to mitophagy and fusion/fission in the rat diaphragm. There was no significant difference in these markers between the S3 and C3 groups at both the mRNA and protein levels (A,B). The S6 diaphragms showed significantly decreased expression of Pink1 and Mfn1 at both mRNA and protein levels (A,C). In the CS cessation (S6N3) group, both the mRNA and protein levels of Mfn1 were recovered, whereas Pink1 expression was significantly decreased versus C9 controls (A,D). Bnip3, BCL2 interacting protein 3; CS, cigarette smoking; Drp1, dynamin-related protein 1; Mfn1, mitofusin 1; Mfn2, mitofusin 2; Opa1, optic atrophy 1; Pink1, PTEN-induced putative kinase 1.
4.3 | CS cessation alleviated diaphragm dysfunction and mitochondrial damage induced by CS exposure

Therapeutic interventions such as smoking cessation, exercise and pharmacological interventions have been proved to play beneficial roles against diaphragm dysfunction in COPD, possibly by regulating oxidative stress, improving mitochondrial respiratory function and reversing muscle atrophy.14,15 Of note, the mechanism of smoking cessation on diaphragm and mitochondria is not clear because of limited research. In our rat model, diaphragmatic contractile impairment and mitochondrial destruction induced by 6-month CS exposure were eased after 3 months of smoking cessation under the persistent disruption in lung structure and function, suggesting the direct effect of CS cessation on the diaphragm. Our findings using this animal experimental model advanced the knowledge about the beneficial effect of smoking cessation in COPD.

4.4 | Mitophagy and fusion/fission contribute to diaphragm dysfunction induced by CS exposure

Previous studies have shown a fundamental influence of CS-induced oxidative stress on mitochondrial quality control in COPD. Mitochondria dysfunction induced by deregulations of mitophagy and fusion/fission contribute to increased endogenous ROS production, decreased mitochondrial respiration, cell senescence and even muscular atrophy.14,19,21–24 However, the mitochondrial quality control in the diaphragm in response to CS exposure and CS cessation remains unclear. Here, we measured the expression levels of the key regulators in the diaphragm and found that long-term CS exposure caused significant decreases in Pink1 and Mfn1, accompanied by impaired mitochondria and decreased diaphragm dysfunction, suggesting deregulated mitochondrial quality control. Our results provide evidence for the importance of mitochondrial quality control deregulation in respiratory muscle dysfunction during CS-induced COPD.

Mitophagy, as an important mechanism of mitochondrial quality control, selectively eliminates damaged mitochondria via autophagy. Mitophagy is initiated via both Pink1-parkin RBR E3 ubiquitin protein ligase (PRKN)-dependent and PRKN-independent pathways. Upon being damaged, Pink1 recruits and activates PRKN to ubiquitinate mitochondrial outer membrane proteins, leading to the recruitment of autophagy receptors.36 The deregulation of Pink1-PRKN-mediated mitophagy is involved in mitochondrial ROS production in the lung and skeletal muscle in response to CS exposure.19,20 Pink1-PRKN-independent mitophagy is mediated directly by a mitochondrial outer membrane protein, such as Bnip3, which can recruit autophagy receptors after activation. Bnip3 can be activated by hypoxia-inducible factor 1 under hypoxic conditions and protects cells through reducing ROS.37,38 In our S6 rats, a decrease in Pink1, but not Bnip3, was observed in the diaphragm in response to CS exposure, suggesting that CS-induced mitophagy depression is likely associated with a Pink1-PRKN-dependent mechanism. Notably, CS cessation failed to significantly reverse Pink1 expression, suggesting that the CS-induced depression of mitochondrial mitophagy in the diaphragm was persistent. Given the slight recovery observed in diaphragm muscle strength after smoking cessation, perhaps a longer cessation time is necessary for recovery.

Previous studies have shown that airway epithelial cells from patients with COPD exhibit defective mitochondrial morphology, likely due to the impaired balance between mitochondrial fission and fusion.24 Mitochondrial fusion is associated with mitochondrial outer and inner membrane proteins, including Mfn1, Mfn2 and...
Opa1, while mitochondrial fission is associated with Drp1 and Fis1. Our results indicated a significantly decreased level of Mfn1 in the S6 group only, and this decrease was recovered in the CS cessation group at both the mRNA and protein level consistent with the changes in diaphragm muscle strength and mitochondrial morphology. It has been shown that mitochondrial fusion associated with Mfn1 helps to eliminate defective mitochondrial components to maintain normal function. Therefore, decreased Mfn1 expression herein suggested that the imbalance of mitochondrial quality control would be one of the critical causes of CS-induced diaphragmatic dys-function, and CS cessation eased such imbalance leading to the recovery of diaphragmatic function.

5 CONCLUSION

Our study revealed dynamic changes in the diaphragm contractile properties, in response to different durations of CS exposure and after 3-month smoking cessation. These dysfunctional changes were found together with damaged mitochondrial morphology and function, which was associated with deregulated mitochondrial homeostasis, including mitophagy and fusion/fission. Moreover, limb muscle was more vulnerable to CS exposure, suggesting a distinct regulation between diaphragm and limb muscle during CS-induced COPD. We also confirmed the inevitability of lung structural changes with persistent emphysema induced by CS exposure. Smoking cessation eased CS-induced dysfunction of rat diaphragms and mitochondria. All these diaphragm muscle changes are likely associated with the regulation of mitochondrial homeostasis, including mitophagy and fusion/fission. These findings advance the understanding of respiratory muscle transitions during the development of COPD, endorsing the importance of mitochondrial homeostasis in the pathogenesis of COPD.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest to disclose regarding the content of this article.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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