Interval training improves right ventricular function in a mouse model of cigarette smoke induced-chronic obstructive pulmonary disease

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Summary

Patients with chronic obstructive pulmonary disease (COPD) have impaired lung function and exercise tolerance reducing their quality of life and increasing mortality**.** In recent years, there has been a growing consensus that exercise has beneficial effects for COPD patients, even for those with severely impaired pulmonary function. Even though COPD primarily affects the lungs, it has several systemic comorbid conditions including cardiac dysfunction with right sided heart failure, which is an adverse prognostic factor in COPD. While several clinical studies have investigated the relationship between exercise and COPD and found that exercise increases muscular strength and quality of life in these patients, there are limited data regarding the effect of exercise training on their cardiac function. **Objective:** To study whether interval training would improve cardiac function in a mice model of COPD. **Method:** 42 mice were randomized into cigarette smoke (CS) exposure or fresh air (FA) group. Following 14 weeks of CS and FA exposure, the two groups were again randomized into exercise training or sedentary group. The training group exercised one hour every day, five times a week for four weeks. Cardiac function was assessed by echocardiography. **Results:** In the CS exposed animals, delta peak oxygen uptake increased by 9.2% in CS and by 11 % in FA trained animals. Right ventricular (RV) systolic function was depressed by CS. Four weeks of interval training restored the depressed function of RV, while no changes were observed in the left ventricle function. **Conclusion:** Taken together, results from the present study support the hypothesis that CS decreased RV function and that the adaptations to interval training exercise were accompanied by enhanced systolic and diastolic RV global function. A reduction in vascular remodeling was suggested in the present study as a potential mechanism in which exercise training reduces RV afterload and dysfunction.

Key words: COPD, left ventricle, right ventricle, systolic and diastolic function, interval training, VO_{2peak}, cigarette smoke, pulmonary hypertension.

Contents

Abbreviations

COPD - Chronic Obstructive Pulmonary Disease

PH - Pulmonary Hypertension

CS - Cigarette Smoke

RV - Right Ventricle

LV - Left Ventricle

FA - Fresh Air

Sed - Sedentary

VO_{2max} - Maximum Oxygen Consumption

VO_{2peak} – Peak Oxygen consumption

IT-Interval Training

TAPSE - Tricuspid Annular Plane Systolic Excursion

EF - Ejection Fraction

FS - Fractional Shortening

E - Early Filling Mitral Inflow

E'- Early Filling Tissue Doppler

A – Late Filing Mitral Inflow

A'- Late Filing Tissue Doppler

SD – Standard Deviation

MLI – Mean Linear Intercept

1. Introduction

Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is a term for many chronic lung diseases characterized by expiratory airflow obstruction that interferes with normal respiration. The disease is manifested by emphysema and/or chronic bronchitis but the relative extent of them vary between individuals[1]. Common symptoms of COPD include; dyspnea, reduced exercise capacity, breathlessness, tiredness, cough, and sputum production. Although there is no cure for COPD, treatments as well as lifestyle changes such as cigarette smoke (CS) cessation and physical activity play an important role reducing symptoms and improving quality of life.

Risk factors and prevalence

Worldwide, COPD is a leading cause of morbidity and mortality and its prevalence has increased in the last two decades [1]. COPD is the only common disease where the incidence is still increasing, and it has been predicted that by year 2030 COPD will be the fourth leading cause of death from the sixth leading cause in 1990 [1]. In the European Union, as much as 6 % of the total health care budget is related to respiratory diseases, whereas COPD accounts for approximately 56 %[1]. The onset of the disease is triggered by chronic exposure to toxic gases and particles such as indoor and outdoor air pollution, occupational dusts, and chemicals. In the third U.S. National Health and Nutrition Examination Survey, airflow obstruction was found in about 14 % of white male smokers while in white nonsmokers the prevalence was only 3 %, making CS the number one risk factor for COPD[1]. It has been demonstrated that long time exposure to CS eventually leads to chronic inflammatory responses in the airways and lungs, consisting of two distinct processes, namely emphysema and chronic bronchitis[1].

Emphysema

Alveolar walls are the tissue surrounding the alveoli supplying them with oxygen. It normally holds the small airways called bronchioles open, allowing air to leave the lungs on exhalation. The lungs, which are stretched during inhalation, return to original shape during relaxation due to the elastic properties of the tissue. When the alveolar walls and the elastic components are damaged, relaxation back to resting state is impaired and the bronchioles collapse. Consequently, it is difficult for the lungs to empty causing increased resistance in the small

airways. Eventually this can cause gases to be "trapped" in the alveoli, inhibiting expiration and may result in hyperinflated lungs[2]. Emphysema is an irreversible disease of the lungs that does not only destroy the elastic components and small airways of the lung, but all alveolar wall structures are affected, including the small blood vessels (capillaries of the lung) that run throughout the lung and the pulmonary arteries (Figure 1.1). Thus, not only is airflow affected, but so is the blood flow, which reduces the efficiency of the gas exchange and increases the resistance in the pulmonary circulation. This has an impact on the ability for the lung not only to empty the alveoli but also for the blood to flow through the lungs to receive oxygen. Consequently this this process might contribute to pulmonary hypertension PH [3].

Chronic bronchitis

Chronic bronchitis is clinically defined as a persistent cough that produces sputum and mucus for at least three months per year in two consecutive years [1]. Chronic cough and mucus production is often seen together with metaplasia of the epithelium of the airways, causing normal cells in the epithelium to be replaced by goblet cells. Presence of pathological abnormalities in early stage COPD patients were first shown by[4]. They demonstrated a denuded epithelium with goblet cells and an increased number of inflammatory cells as well as fibrosis and epithelial hyperplasia in the walls of alveoli and bronchioles. A denuded epithelium can increase the amount of fluid and mucus and facilitate colonization of bacteria. Further, this may trigger inflammation and thickening of bronchial walls, making the airway passage narrower [5].

Figure 1-1. Pulmonary airway remodeling in emphysema and chronic bronchitis. Modified from http://www.earthtimes.org

Cigarette smoke-induced COPD

Active exposure to CS accounts for approximately 80 – 90 % of COPD cases in the United States with an increased incidence of related pulmonary diseases [6]. A lit cigarette burns at a temperature of approximately 800°C and when air is inhaled through the cigarette, temperature increases by an additional 100-200°C. Approximately 250 carcinogenic and noxious chemicals have been measured in CS [7]and as the heated air, mixed with the combustion products, are inhaled over the unburned tobacco, it rapidly cools, causing the noxious chemicals in the unburned tobacco to volatilize. These substances subsequently condense forming smoke, which is an aerosol of liquid particles, and easily absorbed into the lungs [8, 9]. This vapor rapidly enters the lower airways of the respiratory system and eventually the circulatory and lymphatic systems [10]. Although the mechanisms by which CS causes diseases are not fully understood, it is well known that many of the components present in CS are potent oxidants and oxidant injury is believed to be the major mechanism for CS-induced toxicity. [11].

Animal models are used to improve knowledge of the effects of CS. There are two main systems of CS exposure in animal models of emphysema.1). The nose-only smoke exposure machines offer a controlled dosage and exposure, as well as smoke delivery only to the respiratory system. The disadvantage with this method is that animals require restraint; 2). The whole body smoke exposure systems do not require restraint of the animals for smoking, and more animals are allowed to undergo simultaneous CS exposure, with free access to food and water. The disadvantage is a less controlled system compared to the nose-only smoke exposure system. However, in both methods quality of the CS exposure can be measured through carboxyhemoglobin levels [12].

Pulmonary hypertension associated with COPD

PH is a serious complication and a poor prognostic sign of COPD [13], defined as an elevation in the pressure in the pulmonary arteries of the lungs of ≥ 25 mm Hg at rest, assessed by right heart catheterization [14]. In addition, it has been reported that an increase in the mean pressure of 10 mm Hg at rest is associated with a 4-fold increase in mortality in COPD patients[15, 16]. The prevalence of pulmonary hypertension in COPD varies from 20 % to 91 % depending on definitions used for PH, severity of lung disease in the studied population, and the method used for assessing pulmonary artery pressure[16]. However, Hilde *et. al* [15] have demonstrated a prevalence of PH of 5 %, 27 %, and 53 % in Global Initiative for COPD stages II, III, and IV,

respectively; when using right heart catheterization in 98 patients. According to these findings, a significant number of patients with COPD develop PH over the course of their disease.

The respiratory system

The function of the respiratory system is to supply the blood with oxygen. The oxygen rich blood is delivered to all parts of the body. Shortly, the deoxygenated blood from the right ventricle (RV) is pumped through the pulmonary artery to the lungs. In the lungs oxygen diffuses from the alveoli to the blood. Oxygen-rich blood returns to the heart via the pulmonary veins in order to be pumped back into systemic circulation. If the respiratory system loses its ability to provide an adequate gas exchange, the oxygen deficit consequently causes cellular activity to slow down, having an important impact in the whole body.

In PH, several mechanisms increase the pressure within the pulmonary circulation. The main mechanisms are suggested to be the destruction of the pulmonary vascular bed, remodeling of the lung vasculature (pulmonary artery, pulmonary capillaries and pulmonary vein), and hypoxic vasoconstriction [17].

Destruction of pulmonary vascular bed

In emphysema the total cross-sectional area of the pulmonary circulation is reduced, causing fewer and enlarged alveoli together with a reduced vascular bed. The structural changes in the alveoli decrease the gas exchange surface and interfere with normal gas exchange, while a reduction in the vascular bed may increase resistance in the remaining pulmonary vasculature [17]. Emphysematous destruction of the pulmonary vascular bed was considered as one of the main mechanisms underlying PH[18]. However, there is a lack of evidences supporting this, and in the 1960s it was already suggested that destruction of the pulmonary bed alone was not the main contributor to PH in COPD[19, 20].

Hypoxic vasoconstriction and pulmonary vascular remodeling

In order to maintain balance between ventilation and perfusion, blood is diverted away from the poorly ventilated alveoli by vasoconstriction. This response is termed hypoxic pulmonary vasoconstriction. During hypoxia, voltage-gated potassium channels are inhibited, causing cytosolic calcium influx and depolarization of pulmonary artery smooth muscle cells

membrane, which leads to pulmonary vasoconstriction[21]. For a long time, it was believed that vasoconstriction as a consequence of hypoxia was the main reason underlying PH. This view has been challenged by the finding that oxygen therapy does not completely reverse PH associated with COPD[22, 23]. Muscularization, intimal thickening and medial hypertrophy in all layers of the vessels have been found in COPD patients. Such changes have also been found in COPD patients with only mild airflow obstructions and no hypoxemia [24]. Together, these results indicate that remodeling of the pulmonary vasculature may have other causes than hypoxia[25]. As mention above, hypoxia inhibits voltage gated potassium channels in the pulmonary artery smooth muscle cells. This causes a reduction in the potassium current hence favoring depolarization and raises cytosolic calcium concentration. Further, this stimulates cell growth and inhibits apoptosis, resulting in fibrosis, cell growth of smooth muscle cell and hypertrophy of the small pulmonary arteries [17]. Eventually, the pulmonary arteries become more muscular, narrower, thicker, and less compliant offering higher resistance to flow [26].

Another factor contributing to the vascular remodeling is the endothelial cells, which are the major components contributing to homeostasis in the vascular system [27]. These cells have several important tasks in the pulmonary vessels such as reducing vascular tone, regulating the vessels response to flow as well as the vasoconstriction in hypoxic state[28]. Several molecular mediators keep vasoconstriction and dilatation in balance. One of the most important vasodilators is nitric oxide (NO), which is mainly produced by endothelial NO synthase. In addition, NO is an anti-proliferative and protects against vascular remodeling. Prostacyclin is another important vasodilator while one of the main mediators of vasoconstriction is endothelin-1. Endothelin-1 may also contribute to the vascular abnormalities associated with PH[18]. Several studies have investigated the balance between vasoconstrictors and vasodilators in pulmonary arteries in COPD patients with PH. It has been demonstrated a reduction in synthesis and release of NO from the lungs [29-31], reduction in expression of prostacyclin synthase mRNA [32], and an abnormal expression of endothelin-1 [33]. In addition, COPD patients have an increased amount of inflammatory cells present in the connective tissue covering the pulmonary muscular arteries when compared to nonsmokers [1]. Due to the fact that COPD is an inflammatory disease, it is likely that also these inflammatory cells contributes to remodeling of pulmonary vessels [34]. This is supported by studies showing that the extent of pulmonary remodeling correlates with the degree of inflammatory cell presence in small airways[35, 36].

Management of pulmonary hypertension

To date there is no specific treatments for PH associated with COPD, although in hypoxic patients, long term oxygen therapy is the most common treatment [37]. It has been demonstrated that long term oxygen therapy reduces progression of PH, but it rarely improves or normalizes pulmonary pressure or the structural remodeling of the pulmonary vasculature [37]. Furthermore, despite the fact that inhalation of conventional vasodilators, like calcium channel blockers, have shown acute reversal of hypoxic vasoconstriction, chronic use of vasodilators are not recommended since inhibition of hypoxic vasoconstriction can worsen gas exchange [14]. Prostanoids, endothelin receptor antagonist and phosphodiesterase inhibitors can be used in order to induce pulmonary vasodilation and reverse pulmonary vascular remodeling[38]. However, there are limited data confirming the effectiveness and safety of such pharmacological treatments. Most of the studies have investigated acute effects in a low number of patients or uncontrolled case studies. Furthermore, it has been suggested that long term use of these drugs can induce negative RV adaptations (cardiac specific effects), which not necessarily would be explained by pressure overload [39]. The acute effects of pharmalogical treatments have been evaluated in a meta-analysis. The authors described that the primary outcome (mostly short time exercise capacity) in the included studies did not predict survival[40]. Therefore, the European Respiratory Society has recommended that pharmalogical treatments in these patients in general are not recommended[14]. Considering the impact of PH on patients prognosis, the lack of documented effective treatments is still concerning. The fact that there are no treatment strategies known for reducing resistance in the pulmonary vasculature and improving RV function, highlights the need for new treatment strategies.

Cardiac implication of pulmonary hypertension

Cardiovascular diseases such as stroke, ischemic heart diseases and right-sided heart failure are important causes of mortality in COPD patients, with PH as the main factor linked to right sided heart failure [35]. The prevalence of deaths caused by cardiovascular disease in COPD has shown to be 20 % compared to 9 % in subjects without COPD[41]. Despite the treatments used in the management of vascular remodeling, pulmonary vascular obstruction usually progresses and causes an increase in afterload on the RV[38]. In patients suffering increased RV afterload, the RV may become hypertrophied with a reduction in the cavity size (Figure 1.2). Over time server RV pressure overload will cause a reduction in cardiac contractile force and eventually RV dilatation. Patients with decreased RV function suffer a progressively

decreasing clinical course due to the combination of both a decreased RV function and the ventilatory handicap [42].

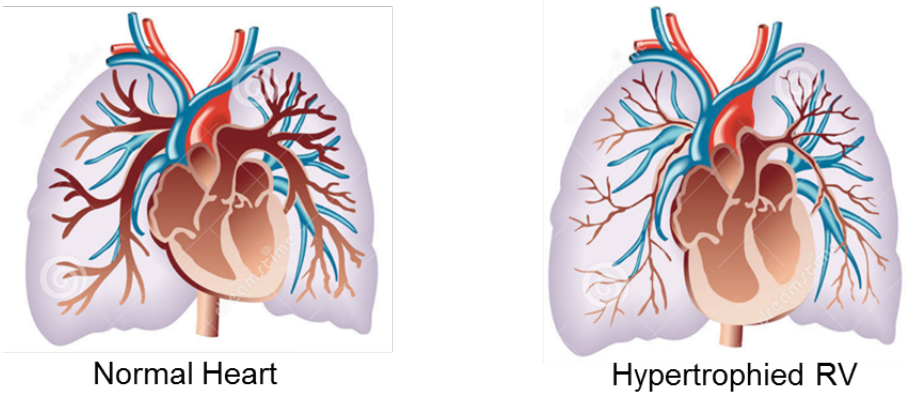

Figure 1-2. Right ventricular remodeling in pulmonary hypertension. Borrowed and modified from http://www.cdc.gov.

It has also been demonstrated that COPD influences the left side of the heart. In one population-based study, LV structure and function was measured in 2816 subjects where 38 % were former smokers, 13 % current smokers, and 49 % never-smokers. From this study it was concluded that the degree of emphysema correlated with reduction in LV diastolic volume, cardiac mass, stroke volume, and cardiac output[2]. In another study, Watz et al,[43] measured chamber size, LV filling and relaxation, lung function, and 6-min walk distance in 138 patients with mild to severe COPD. Subjects with low scores on pulmonary testing (total lung capacity and inspiratory lung capacity) had impaired LV diastolic function together with impaired RV function. The reduced LV function is suggested to be a result of the hypertrophied RV with a pressure exceeding the pressure in LV. The relatively high RV pressure causes the intraventricular septum to bow towards left. Since the two ventricles are sharing the same intraventricular septum, and also the fact that compliance of the pericardial sack is low, bowing of the intraventricular septum can eventually compress the LV and restrict diastolic filling lowering LV stroke volume[44]. Another explanation for LV dysfunction is that hyperinflated lungs increases the intra thoracic pressure and thereby impairing LV filling [45].

Possible Cellular and Molecular Mechanisms

Afterload-stimulated RV hypertrophy is mainly a result of increased wall stress leading to protein synthesis and hypertrophied cardiomyocytes. This wall stress is sensed by stress

activated ion channels in the cardiomyocytes. Hence mechanical stress is transduced into intracellular chemical proteins involved in contractile protein synthesis. This process is termed pressure-induced growth [39] . Mechanisms underlying transition from RV hypertrophy to dilatation and failure is not well investigated in the RV. However, one of the main mechanisms is changes in the thick myosin filaments in the cardiomyocytes. In failing hearts there is a switch from α- to β- myosin filament. The β- myosin has lower adenosine triphosphate, which results in lower systolic function [39]. The systolic dysfunction is not only associated with alterations in the contractile proteins, but also with alterations in ionic channels and enzymes involved in excitation-contraction coupling in cardiomyocytes [39].

Effect of exercise training in COPD and pulmonary hypertension

The ultimate measure of a treatment for any group of patients is improved quality of life and reduced mortality. Physical inactivity is a major independent risk factor for hospitalization and mortality in COPD patients[46], and exercise capacity a better predictor for mortality than the commonly used maximal expiratory flow during forced exhalation[47]. Therefore, reduced exercise tolerance seen in patients with COPD has an important impact on prognosis, disability and their ability to perform daily activities, and consequently decreasing the patients' quality of life[1] .

Exercise training at higher intensities has proven more beneficial in improving maximum oxygen consumption both in healthy and in diseased subjects[48-50]. High intensity interval training is together with moderate continuous training the two most common exercise intensities studied in COPD patients[51]. High intensity interval training consists of short bouts of high intensity exercise separated by active or passive breaks in between [52]. The duration in High intensity training protocols varies between 30 - 60 seconds or even up to several minutes, and the intensity is ranging from 85 - 95 % of peak heart rate. Moderate continuous training consists of exercise at intensities between 64 – 76 % of peak heart rate[52]. The recovery periods in high intensity training allow longer total time spent at high intensity when compared to moderate continuous training, which result in greater training stimulation and possible better exercise adaptations. Several studies have shown superior exercise adaptation regarding VO_{2max} and global heart function following high intensity protocols compared to moderate continuous protocols [49, 53, 54].

In COPD patients, it is believed that the ventilatory limitations make them unable to exercise at higher levels for a sustained period and thereby preventing the cardiovascular system from being taxed [55]. Several studies have therefore investigated the effects of exercise on aerobic capacity in COPD patients using different training protocols. A meta-analysis by Beauchamp *et al.*[51] compared interval training and continuous training in eight randomized controlled trials, addressing peak oxygen uptake (VO_{2peak}), peak power, 6-min walk test distance, and health related quality of life. A total of 388 COPD patients were randomized to either interval or continuous training. Even though interval training resulted in significant improvements in exercise capacity and health related quality of life, this meta-analysis found no differences between the two training strategies. One important confounding factor was that in the studies included there was almost no difference in exercise intensity between the high intensity phases of the interval training and the intensity performed in the continuous exercise program (80 % (40 Watt) and 70 % (35 Watt) of $Power_{peak}$, respectively). Another relevant confounding factor was that in the included studies, the period of exercise training varied from three to 16 weeks, although the guidelines for pulmonary rehabilitation recommend at least 12 weeks [56]. Other factors that make comparisons between these studies difficult are the differences in the duration of the interval phase in the high intensity group, which differed from 30 seconds to 3 minutes; and the training frequencies, that also ranged from 2- 5 times per week. These differences in exercise protocols make comparison between studies included in this meta-analyze difficult and are a major limitation for the study.

Despite the prognostic value of exercise capacity and the impact of cardiac dysfunction on diagnosis and survival[2, 57, 58], evidences of exercise training effect on cardiac function in COPD patients are still sparse. To our knowledge, there is currently only one clinical study investigating left and RV function following exercise in COPD patients. This study reported a depressed LV and RV function in COPD patients compared to healthy controls, which was reverted by 10 weeks of exercise training (high intensity training and moderate continuous training), and accompanied by increased exercise tolerance. Furthermore, they found no difference in exercise adaptations between the two exercise training protocols[54]. Most studies have only investigated 6-min walk distance following exercise intervention without any cardiac effects. However, in a study performed by Mereles *et.al* [59], effects of exercise training in patients with severe PH was investigated. Exercise training in combination with respiratory training improved 6-min walk distance, exercise capacity, quality of life, world health organization functional class, and peak oxygen consumption. The 6-min walk distance

improved by 22 %, which is higher compared to other studies using pharmalogical interventions. RV cardiac index and dimensions was measured by echocardiography but no significant differences were found between the training and the control group. However, due to a small number of subjects with different training interventions (exercise training and respiratory training) enrolled in the study it is difficult to address the improvements to one single component.

Exercise training is seen as the cornerstone of COPD rehabilitation and has proven to improve quality of life and reduce hospitalization and mortality in patients with COPD [60]. These improvements have traditionally been related to the adaptations in peripheral skeletal muscles and less is known about the effects on the cardiovascular system. Considering the prevalence of cardiac dysfunction in COPD, its impact on patients' health and diagnosis, and also the fact that several studies on different pharmacological treatments have shown disappointing results in improving the long term survival in COPD patients, more studies should be conducted in order to determine the effects of exercise training on cardiac function in COPD condition.

Aims and hypothesis

The aim of this study was to examine the effect of IT on cardiac function in an animal model of COPD. Further we wanted to study the effect of IT on pulmonary blood vessel remodeling. We hypothesized that 14 weeks of exposure to CS would induce pulmonary emphysema, decreased cardiac function and also induce pulmonary vasculature remodeling. Further we hypothesized that four weeks of IT would attenuate the detrimental effects of CS exposure on both cardiac function and pulmonary vasculature remodeling in mice.

2. Material and Methods

Animals and Cigarette Smoke Exposure

42 female A/J-OlaHsd mice were exposed to either fresh air (FA) or CS for six hours/day for five days/week during 14 weeks. 3R4F research cigarettes were used for CS exposure and the procedure was generated by a smoking apparatus previously used by Nilsson *et al.*[61] (Figure 2.1). A. CS concentration was monitored by Casella micro dust aerosol monitor and puff intervals in the smoking protocol were adjusted in order to achieve a concentration of 100-200 mg /m^3 total particulate matter.

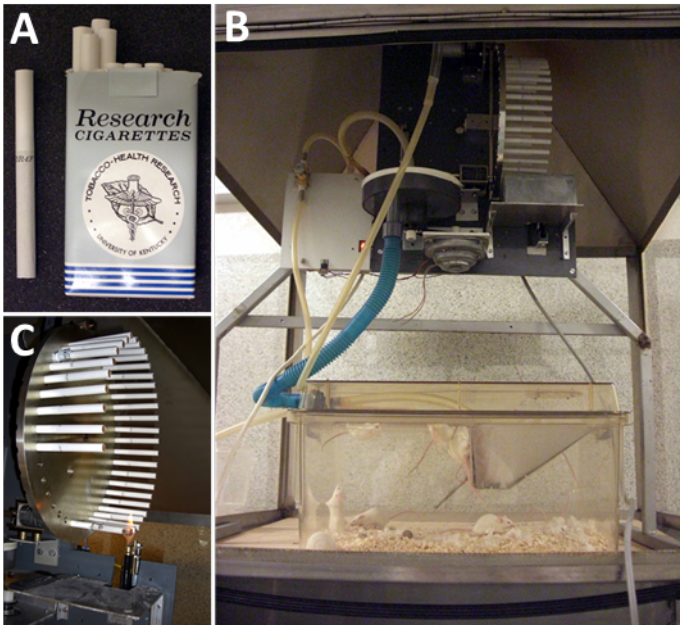

Figure 2-1. Schematic cigarette smoke (CS) apparatus used for 14 weeks of CS exposure. A. 3R4F research cigarettes. B. The setup used for CS exposure, C. a carousel that holds the cigarettes.

Blood samples

Blood samples were taken every second week in order to estimate the amount of carbon monoxide bound to hemoglobin (COBh) in the CS exposed mice. Blood samples were also taken from one mice not included in the study, which was exposed for one day together with the CS exposed animals and immediately after exposure blood samples was taken from aorta under isoflurane anesthesia. Thereafter the mouse was sacrificed. Blood samples were analyzed on a Radiometer ABL800-machine. COHb at various time points in CS exposed animals was 25.8 ± 2.3 %.

Emphysema Quantification

A 21 gauge butterfly needle fixed with 15 cm H_20 pressure of 4 % buffered formalin solution for > 2 hours, was used to cannulate the left lobe and the diaphragmatic lobe of the lungs. After cannulation, the lung lobes were kept in formalin solution. Following fixation in formalin, the lung samples were processed, paraffin embedded, sectioned and stained with hematoxylin, eosin and saffron. The samples were then analyzed using an Olympus light microscope with digital recorder. For further investigation, the lobe with the least artifacts and the most uniform expansion was chosen. An enumerated grid with each cell corresponding to a visual field under 200 x magnifications was applied and 10 fields chosen randomly and photographed for further investigation. A 100 um x 100 um grid was superimposed on the images and the number of transitions from air space to tissue was counted. Areas with vessels or airways > 50 um, or with obvious artifacts were excluded. Thereafter airspace enlargement was calculated by using the mean linear intercept (MLI).

Quantification of Pulmonary Vessel Muscularization

The sections of lung tissue were deparaffinized, rehydrated and then demasked for 20 minutes at 97°C with PT Link/Target Retrival Solution (K8004, Dako, Glostrup, Denmark), followed by immunostaining performed with Dako Autostainer Plus (Dako). First step in the staining procedure was blocking for endogenous alkaline phosphatase and peroxidase with Dual Endogenous Enzyme Block (S2003, Dako) for five minutes. Then the sections were incubated with antibodies targeting actin (smooth muscle) (SMA, M0851, Dako 1:25) and von Willebrand factor (vWF, A0082, Dako 1:300) for 40 minutes. The antibodies used were diluted with antibody diluent (S2023, Dako). Secondary step was performed by applying Biotinylated goat-anti-rabbit (E0432, 1:800, Dako) for 15 minutes before incubation in 1:1 UltraVision Quanto Mouse on Mouse (TL-QHDM, Thermo Fischer Scientific, Waltham, MA, US) and streptavidin alkaline phosphatase (K5005, Dako) for 30 minutes. For visualization, the samples were incubated with DAB+ (1:50, K4007 Dako) for two times five minutes and then Ferangi Blue (1:100, FB813, Biocare Medical, Concorde, CA, US) for five minutes were applied for visualization. Wash buffer (S3006, Dako) was used for rinsing between the steps in the staining protocol. At the end the sections were rinsed with water and dried 10 minutes at 60°C and mounted. Sections were then examined (BX40CY microscope, Olympus) and positive staining was used to identify pulmonary blood vessels, and staining with SMA was used to assess muscularization of the vessels. Vessels 30 - 125 µm were scored for SMA-staining. A score from 1 - 5 was used to score actin staining: 1 = No actin staining; 2 = Faint

actin staining; 3 = distinct staining ≤ ½ of the circumference; 4 = distinct staining ≤ ¾ of the circumference; and 5 = distinct staining of the entire circumference. An average of 65 ± 21 vessels per animal was scored and the mean score per animal was used in the statistical analysis. All histomorphometric analyses were done in a blinded manner.

Aerobic Capacity Test

Oxygen uptake was tested during treadmill running in a metabolic chamber with ambient air leading through the chamber. Samples of gas were extracted and analyzed in the O_2 and CO_2 analyzer. Following 10 - 15 minutes warm up at intensities between 40 - 50 % of VO_{2peak}, treadmill band speed was increased by 0.03 m/s every minute until the mice were not able to continue running. To keep the animal running, electrical stimulus was given from a stainless steel grid at the end of lane. Brushes at the end also provided the animals from pinching feet and tail between the lane and steel grid. In order to compare aerobic capacity in mice with different body weights, VO_{2peak} was expressed in relation to the body weights raised to the power of 0.75. The trained animals were tested every week in order to see the development over time of the VO_{2peak} and also they were used for adjusting IT intensities as described under. Sedentary animals were tested only at pre and post period.

Exercise Training

Mice in the exercise training group performed uphill treadmill running at 25° for one hour a day, five days a week for four weeks. Training sessions consisted of a 10 minute warm up period at 50 – 60 % of $VO2_{peak}$ followed by high intensity running in a four times four interval format at intensity similar to 85 – 95 % of estimated VO_{2peak}. Between each interval subjects performed active breaks of two minutes at intensity similar to the warm up. Intensity level was adjusted when exercise tolerance improved to ensure that the high intensity was achieved thought the whole training intervention period. IT intensity was increased by increasing the band speed. It was adjusted based on the values obtained from the VO_2 tests every week in order to achieve the right intensity.

Echocardiography

Global heart function was evaluated with echocardiography using the Vevo 770 Visual Sonics (Toronto, Canada). The probe used was 250 Hz. Animals were anesthetized with 2.5 % isoflurane which was adjusted to keep the heart rate and respiration approximately similar

between animals. In B-mode apical four chamber images, the M-mode cursor was oriented to the junction of the tricuspid valve plane in the right ventricular free wall, and through the apex (Figure 2.2A). Movement of the RV base generates echoes that were received and registered. Tricuspidal annular plane systolic excursion (TAPSE) was defined as the differences in systolic and diastolic displacement of the RV base (Figure 2.2B). TAPSE, LV ejection fraction and LV fractional shortening were calculated from M-mode images. Early and late peak mitral annular diastole (e`) and systole (S`) velocity were measured by pulsed wave tissue Doppler. Doppler velocities were measured in the intraventricular septum in LV and from the lateral tricuspid annulus free wall in RV. LV Peak early (E) and late (A) mitral inflow velocities and isovolumetric relaxation time (IVRT) were obtained in the apical four chamber view. In all calculations three measurements were obtained and the mean was used for analyzes. IVRT in the LV was obtained from the mitral inflow pattern while IVRT in the RV was obtained from a regional TD trace from the lateral tricuspid valve ring. RV IVRT was defined as the time interval between the end of systolic motion and the beginning of the diastolic motion.

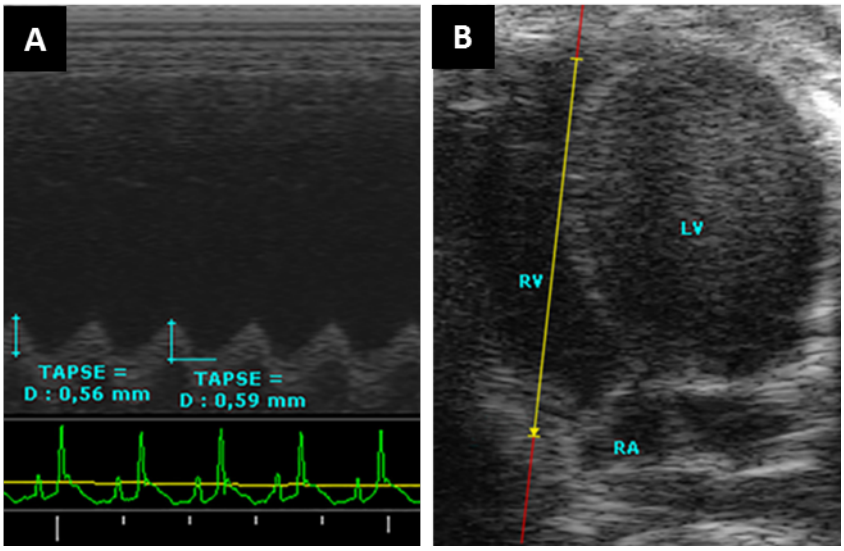

Figure 2-2. Standard technique for measuring tricuspid annular plane systolic excursion in M-mode (A) obtained in the apical four chamber view (B).

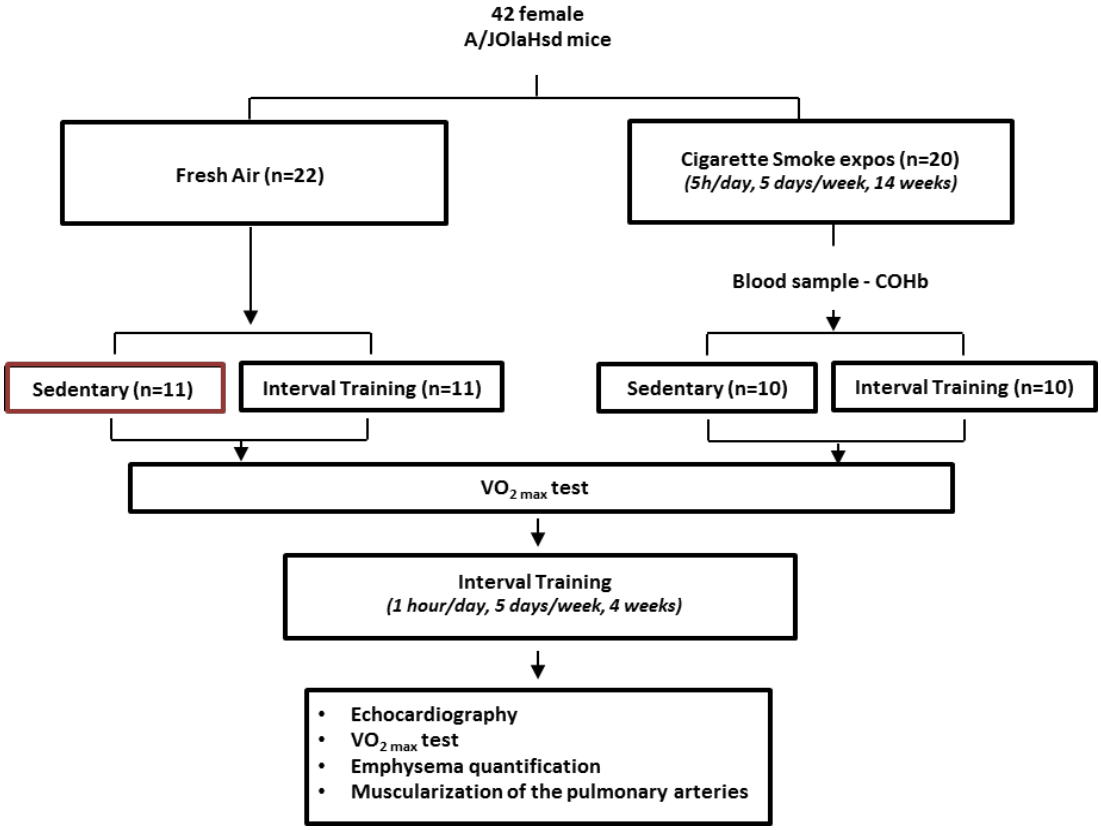

Figure 2-3. Schematic diagram of experimental design.

Statistical Analysis

All values are expressed as mean ± standard deviations (SD). Comparisons of parameters were performed by two-way ANOVA with Tukey's post-hoc test. Results were considered significant at P values < 0.05.

4. Results

Lung Emphysema

Morphological changes in lungs of mice exposed to CS were measured by MLI and related to emphysema. Figure 3.1A shows representative images of lung sections of mice exposed for 14 weeks to FA or CS. The alveolar wall destruction and airspace enlargement observed in mice exposed to CS compared to FA mice suggest that 14 weeks to CS exposure induced emphysema in female A/J-OlaHsd mice. The MLI in the lungs of mice exposed to smoke for 14 weeks was larger than in those exposed to FA ($P < 0.05$, Figure 3.1B). IT did not change the MLI neither in FA nor CS exposed mice (Figure 3.1B).

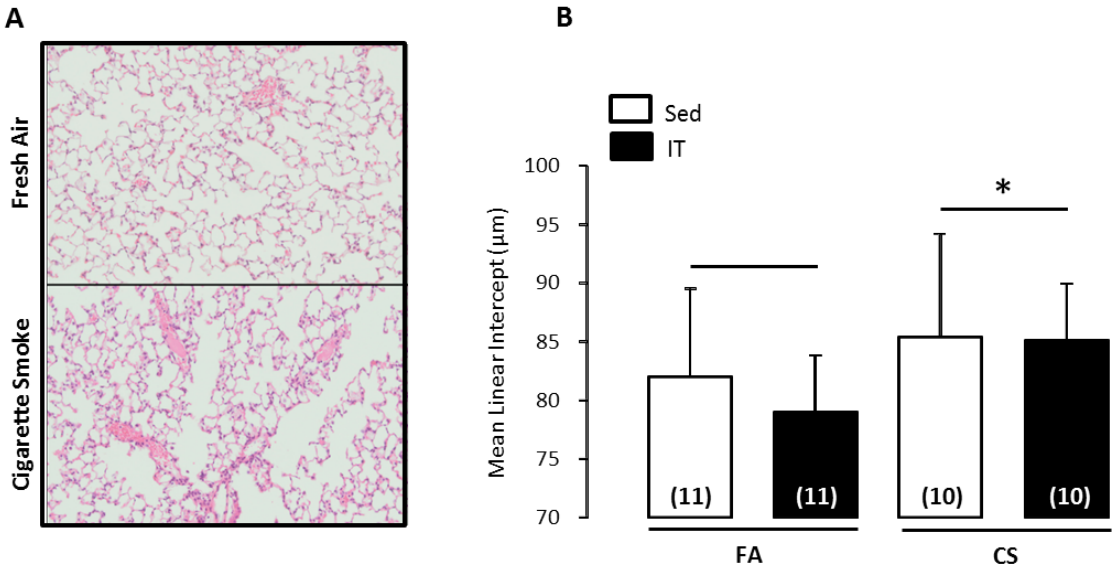

Figure 4-1. Cigarette smoke-induced emphysema in lungs of mice. A. Representative images of lung sections of mice exposed to fresh air or cigarette smoke for 14 weeks. B. Comparison in the mean linear intercept in the lung sections between sedentary and exercised mice exposed to fresh air and sedentary and exercised mice exposed to cigarette smoke for 14 weeks. Data are presented as mean ± SD. * $P < 0.05$ vs. fresh air mice. Sed: sedentary; IT: interval training; FA: fresh air; CS: cigarette smoke.

Muscular Pulmonary Artery Structure

The effect of CS and exercise on pulmonary vascular remodeling was measured by assessing the degree of muscularization of pulmonary vessels. CS exposure did not induce muscularization of pulmonary vessels in mice compared with those exposed to FA. However, IT intervention reduced muscularization of pulmonary vessels compared with sedentary groups ($P < 0.05$, Figure 3.2A and 3.2B).

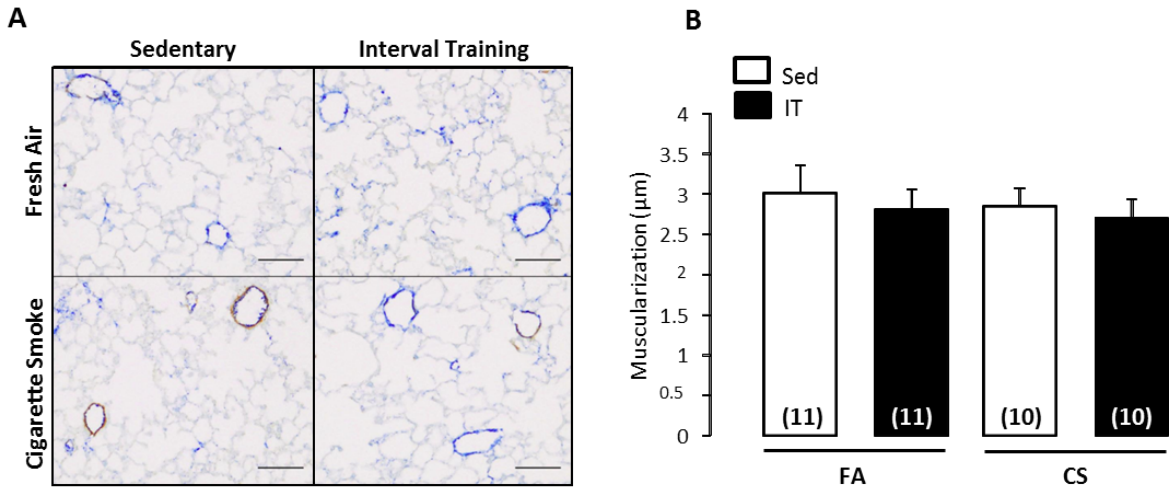

Figure 4-2. Muscularization of pulmonary vessels in mice exposed to fresh air and cigarette smoke for 14 weeks, and from trained and untrained mice. A. Sample images showing different degrees of vessel muscularization in lung vessels. Scale bars 100 µm. B. Muscularization of pulmonary vessel in the lung sections in mice exposed to fresh air and mice exposed to cigarette smoke for 14 weeks. Data are presented as mean ± SD. Sed: sedentary; IT: interval training; FA: fresh air; CS: cigarette smoke.

Aerobic capacity

Oxygen uptake measurement before four weeks of exercise training was not different between FA and CS groups (Figure 3.3A), while following aerobic IT, delta peakVO_2 increased by 9.2 % in CS mice and by 11 % in FA mice ($P < 0.05$, Figure 3.3B). No significant difference in delta peakVO_2 was found between the sedentary groups (Figure 3.3B).

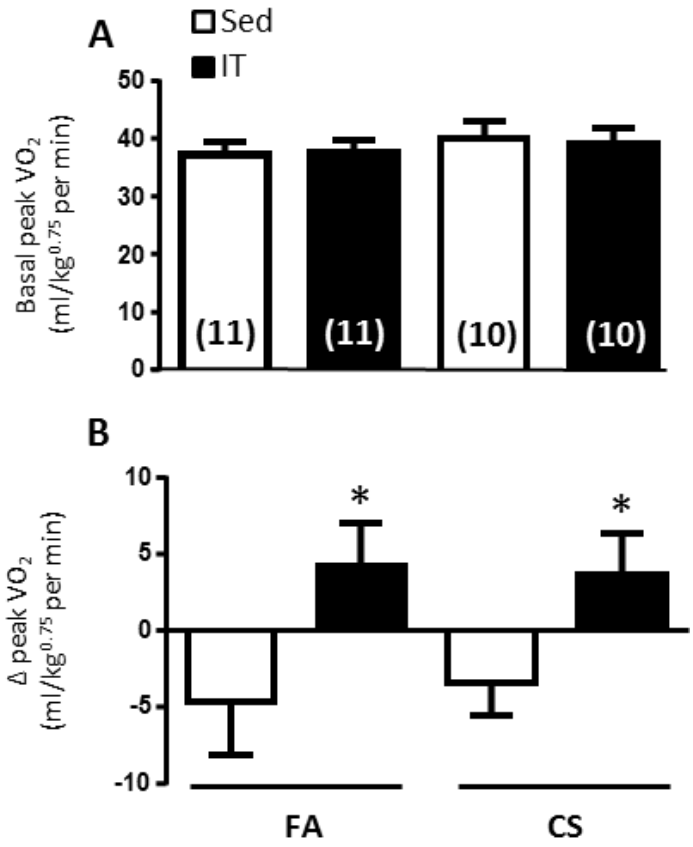

Figure 4-3. A. Basal peakVO_2 measured before four weeks of exercise training intervention. B. delta peakVO_2 determined between pre and post four weeks of exercise training intervention. Data are presented as mean ± SD. * $P < 0.05$ vs. sedentary groups. Sed = sedentary, IT = interval training, FA = fresh air, and CS = cigarette smoke.

Cardiac function

Mice exposed to CS presented impaired RV function compared to those exposed to FA (Figure 3.4). RV systolic dysfunction was associated with a reduction in TAPSE in CS mice compared to FA mice ($P < 0.05$, Figure 3.4A and 3.4B), although S' from RV did not show significant difference between sedentary mice exposed to FA and CS (Figure 3.4C). Diastolic function in the RV was measured as e' and did not show any difference between CS exposed mice and FA mice (Figure 3.4D). Four weeks of IT increased TAPSE and S' in the RV in CS exposed mice compared to those CS sedentary, and improved RV systolic function in CS mice compared to FA sedentary mice ($P < 0.05$, Figure 3.4A and 3.4C), suggesting that IT is efficient in restoring RV function in CS exposed mice.

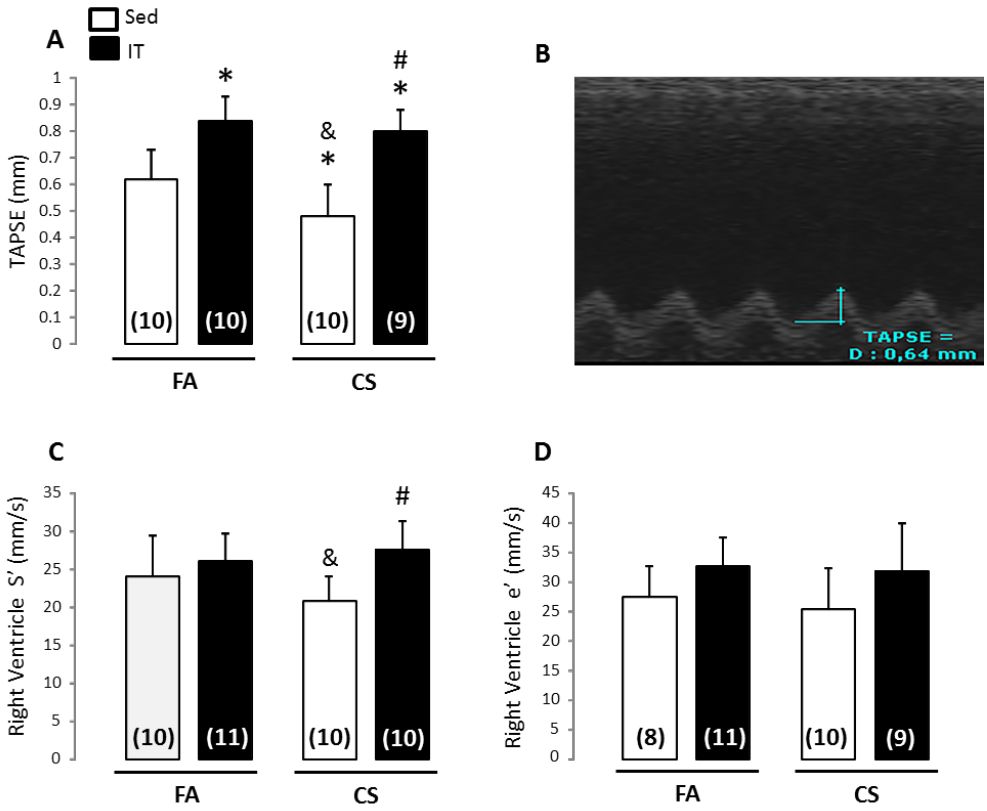

Figure 4-4. Right ventricle systolic and diastolic function in mice exposed to fresh air and cigarette smoke for 14 weeks. A.Tricuspid annular plane systolic excursion (TAPSE). B. Myocardial segmental systolic velocity (S'). C. Peak myocardial early diastolic velocity (e'). Data are presented as mean ± SD. * $P < 0.05$ vs. Fresh Air mice; # $P < 0.05$ vs. CS sedentary mice; and & $P < 0.05$ vs. FA trained mice. Sed = sedentary, IT = interval training, FA = fresh air, and CS = cigarette smoke.

LV systolic and diastolic function measured as fractional shortening and S' and e', respectively, were not impaired after 14 weeks of CS exposure when compared to FA sedentary mice (Figure 3.5A - 3.5D). Four weeks of IT did not improve LV function in neither FA nor CS exposed mice (Figure 3.5A – 3.5D).

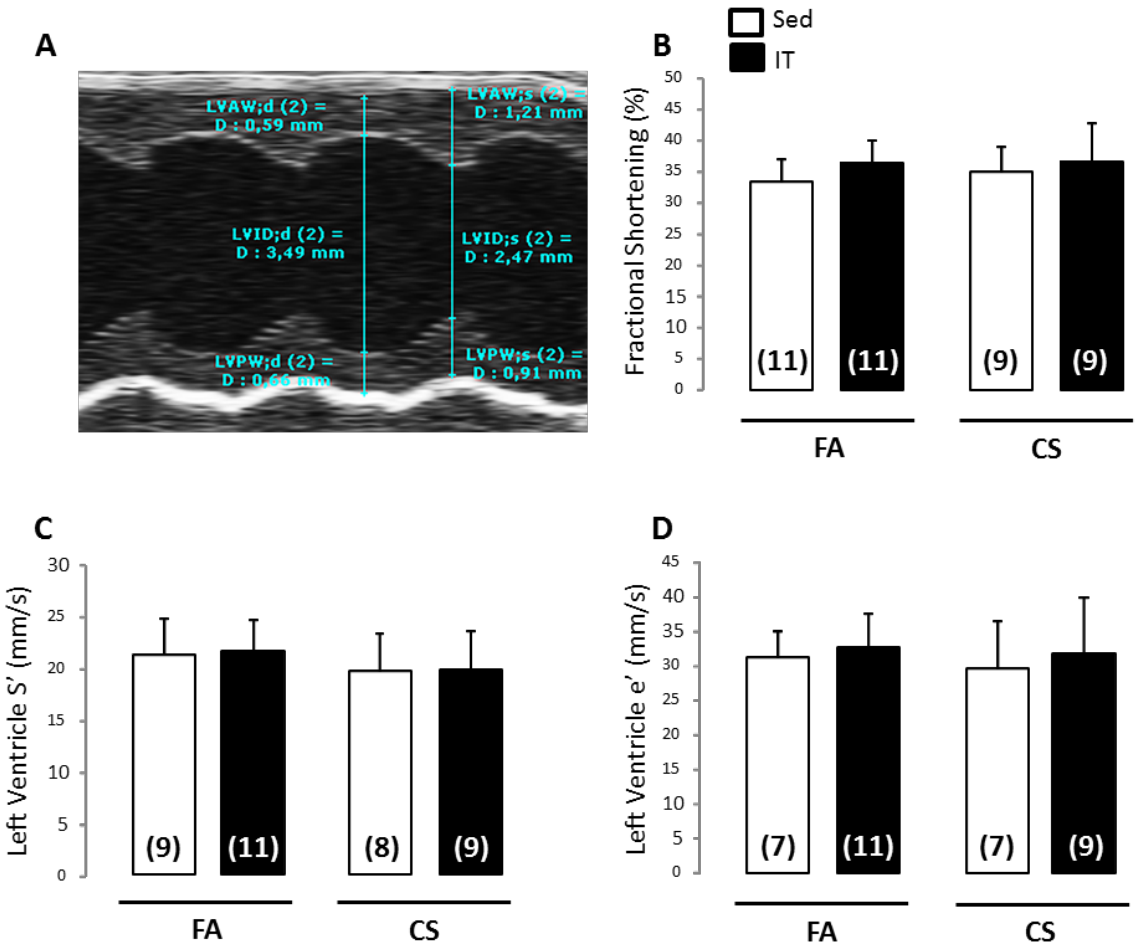

Figure 4-5. Left ventricle function in mice exposed to fresh air and cigarette smoke for 14 weeks. A. M-mode echocardiographic recordings. B. Fractional shortening. C. Myocardial segmental systolic velocity (S'). D. Peak myocardial early diastolic velocity (e'). Data are presented as mean ± SD. Sed = sedentary, IT = interval training, FA = fresh air, and CS = cigarette smoke.

CS exposure for 14 weeks did not affect LV structure in mice as shown by Figure 3.6. LV mass, LV internal dimensions and posterior wall thickness were not different between FA and CS exposure mice (Figure 3.6A-3.6D, respectively). Furthermore, IT did not induce LV remodeling neither in FA nor CS exposed mice (Figure 3.6).

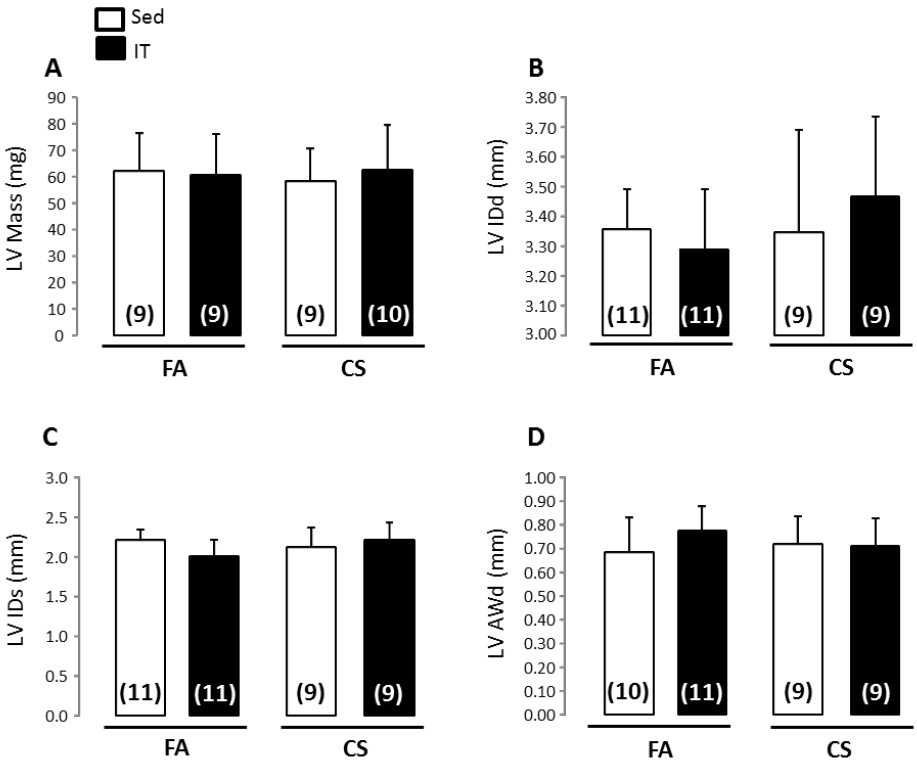

Figure 4-6. Left ventricular structure in mice exposed to fresh air and cigarette smoke for 14 weeks. A. Left ventricle mass. B. Left ventricle internal dimension during diastole. C. Left ventricle internal dimension during systole. D. Left ventricle posterior wall. Data are presented as mean ± SD.

5. Discussion

The main purpose of the present study was to investigate the effect of aerobic IT on cardiac function within an animal model of COPD. Our findings suggest that impaired RV systolic function following CS exposure can be restored by interval training exercise.

Several types of animal models have been carried out trying to simulate the human form of COPD [62]. However, due to the complexity of the disease with large subject to subject variations, animal models have problems in mimicking the human disease perfectly[62]. Nonetheless, in order to gain a totally understanding of the biomolecule, histological and molecular abnormalities underlying the disease, a systematic approach must be used. Mouse models exposing the whole body to CS are the most common approach used to describe the pathophysiology of COPD [62]. March et al., 2006 showed that female A/J mice developed emphysemic destruction and increased number of macrophages, neutrophils, and lymphocytes in a similar manner to that observed in human COPD, following 22 weeks of whole body exposure to CS. They used a concentration of 250 mg /m^3 total particulate matter similar to the concentration used in the present study. Their findings suggest that female A/J mice exposed to CS as a promising animal model to study disease pathogenesis and new therapeutic strategies in COPD.

Our data showed that at baseline (before exercise training intervention), animals exposed to CS surprisingly did not perform worse than those mice exposed to FA. Similar results have previously been reported by Toledo *et al.*[63], who reported that no significant differences in the physical test were observed between the groups following CS exposure for 30 min/day, five days/week for 24 weeks. The authors attributed this to the fact that their animal model only mimicked a mild form of COPD. In the present study, blood samples were taken during the CS exposure period and higher levels of COHb were found in CS animals. Since hemoglobin's (Hb) affinity for carbon monoxide is higher than for O_2, it occupyies binding sites on the Hb molecule when forming COHb. Therefore O_2 carrying capacity of the blood is reduced which leads to less O_2 offered to working muscles. Even though this effect is acute and may disappear shortly after hypoxic exposure, long time exposure will eventually lead to an increased production of red blood cells in the marrow of the long bones increasing O_2 carrying capacity of the blood. Therefore, in our study, we suggest the findings of baseline VO_{2peak} values to be a result of chronic exposure to high levels of carbon monoxide .

Due to its impact on patients prognosis and mortality, RV dysfunction is considered being the most important cardiac manifestation of COPD and several studies are in accordance with the findings in the present study, reporting depressed RV function in COPD [64-66]. It is clear that RV dysfunction must be explained by alterations in either the working conditions in which the heart has to work referred to as ventricular pre- and afterload, or abnormal ventricular contractility with, or without altered cardiac chronotropism [67].

Haemodynamics

In the present study we found emphysemic destruction of the alveolar bed following CS. The relationship between emphysema and increased RV afterload in patients with PH associated with COPD is well described [39]. The loss of alveolated lung tissue in emphysema raises the pressure in the remaining functional vessels causing PH and increased afterload on the RV. Consequently, the RV is forced to eject its blood into a lung circulation with increased resistance. If chronically pressure overloaded, the RV may undergo hypertrophy and dilatation which may result in systolic and diastolic dysfunction [68]. Our finding of emphysema further emphasizes the relationship of emphysema and PH with increased RV afterload and consequently RV systolic dysfunction and we suggest that the RV systolic dysfunction was caused by destruction of the lung tissue causing PH and increased afterload on the RV.

Knowledge about LV involvement in COPD is controversial and far less studied than the RV, and results have also been contradicting and inconclusive. Previous studies have shown preserved LV function in COPD patients. Davies and Overy [69] demonstrated that despite systemic venous hypertension, no signs of LV dysfunction were observed in COPD patients. Similar results were found by Willimas *et al.*[70]. They measured LV function by assessing LV response to an increased resistance to LV ejection by intravenous infusion of methoxamine, which is a α1-adrenergic receptor agonist. They found that patients with COPD responded normally to increased pressure load with preserved LV function. This was the case also in patients in whom the disease had led to RV failure. Also, in a study by Vizza *et al.*[71] it was found that RV dysfunction was present in 94 % in patients with pulmonary vascular disease while in contrast, LV dysfunction was present in only 19.6 %. However, contrary to these results, recent studies[2, 72, 73] have shown impaired LV function with depressed LV filling and stroke volume and it is now accepted that COPD patients without underlying ischemic disease may have impaired LV function.

In our study, we found that CS exposure followed by emphysema did not change LV function or structure. In line with our findings, Sussan *et al.*[74] reported that CS exposure for five days/ week for six months caused no significant alterations to the LV, but increased RV end-systolic pressure accompanied by impaired RV systolic ejection and prolonged IVRT. These findings corroborate ours suggesting both systolic and diastolic RV dysfunction with no changes in LV function in mice with emphysema following chronic exposure to CS.

The structural differences between LV and RV may explain why we found decreased RV function without changes in the LV. The RV has to pump the same volume as the LV, but due to the low resistance in the pulmonary circulation, the RV does so with only approximately 25 % of the stroke work. RV is a thinner and more compliant chamber unable to withstand pressure overload for a long time and therefore, it responds and adapts to hemodynamically (pre- and afterload) changes more easily than the LV [75]. In theory, this could cause the RV to (mal) adapt to pressure overload in a faster manner. If so, CS exposure period should be extended in order to investigate whether LV parameters would adapt to the volume/pressure changes at a later stage of the disease.

Effect of Exercise

Even though information regarding the effect of exercise on pulmonary structure and function in COPD is sparse, a few experimental studies have investigated the potential for exercise to inhibit development of pulmonary remodeling due to CS exposure. Toledo *et al.*[63] demonstrated that exercise protected against development of emphysema in active smokers. Using a mouse model of PH they found increased MLI of the alveoli wall in mice exposed to CS, but not in the mice exposed to exercise and FA. In addition to inhibiting structural damage, they also found that exercise restored the levels of pulmonary elastance and decreased the amount of inflammatory cells present in the lung tissue. These structural and molecular changes observed after exercise training period were attributed to reduced production of reactive oxygen species in the bronchoalveolar lavage together with increased production of antioxidants associated with exercise training. In line with these findings, Menegali *et al.*[76] have reported that mice that exercised while exposed to CS partially improved markers of pulmonary damage such as destruction of alveolar septum, increased amounts of macrophages, neutrophils, and collagen levels.

Exercise training used in a curative approach, looking into underlying mechanisms, has to our knowledge not been extensively studied in COPD. However, in a recent study Bronstad *et al*.[54] reported that unhealthy COPD patients improved both LV (EF and SV) and RV (S' and TAPSE) systolic function after 10 weeks of high intensity exercise training or MCT. They did not report changes in diastolic function. Findings from this study are in accordance with ours regarding the improvements observed in the RV function following four weeks of exercise training in a mouse model of COPD.

In addition to the improved RV systolic function, we also found that early diastolic relaxation measured as RV IVRT/RR was improved after exercise (data not shown). It is possible that improved RV systolic function could be a function of the improved early diastolic function observed after exercise intervention. Improved diastolic function would increase preload due to an increase in RV end-diastolic volume. According to the Frank-Starling mechanism, increased stretching of the cardiac fibers due to increased preload gives a more forceful contraction. However, to our knowledge there is no information regarding this mechanism in the RV in COPD.

Cardiomyocytes adaptations to exercise have been well studied in both health and disease and a regular program of exercise training has shown to restore cardiomyocyte contractility, relaxation and Ca^{2+} handling inducing cellular growth in length and width [53]. These structural cellular adaptations are followed by a proportional increase in ventricular cavities and LV thickness. In the present study, we did not detect structural changes in the LV measured by echocardiography following exercise training in neither CS exposed nor FA mice. This implies that structural cardiomyocyte adaptations may not have occurred and that improved RV function was caused by afterload changes induced by exercise.

Even though 14 weeks of exposure to CS did not increase muscularization, we found an effect of four weeks of exercise training decreasing muscularization in mice exposed to both FA and to CS. By reducing resistance in the pulmonary circulation, this mechanism may reduce afterload on the RV. However, the mechanisms underlying reduced muscularization following exercise are still unclear. In recent years there has been a growing interest in the molecular mechanisms involved in the pulmonary remodeling and one of the most promising mechanisms is related to the role of inducible nitric oxide synthase (iNOS) as a key molecular player in the development of COPD and pulmonary remodeling. In a study performed by Seimetz *et al.*[77], the role of iNOS in the development of PH was investigated and they found

that mice lacking iNOS (chimeric mice), were protected against vascular dysfunction, vascular remodeling and emphysema induced by three months of whole body exposure to CS. They also reported that wild type mice treated with iNOS inhibitors were protected from both structural and functional damage in the lung vasculature and alveoli. The main finding in the mentioned study was that treatment with iNOS inhibitors not only prevented, but also reversed already established disease in mice with emphysema and PH. These findings makes iNOS a potential target in the search for new treatment strategies in COPD.

It is well-established in the literature that iNOS is up regulated by pro-inflammatory cytokines including TNF-α, INF-ϒ and IL-β [78]. Furthermore, it is also known that exercise training reduces the levels of the these pro-inflammatory cytokines mention above [79, 80] and by this mechanism exercise training may prevent vascular remodeling modulating iNOS function by down regulating pro-inflammatory cytokines in mice exposed to CS.

Study limitations

In this study we had no measurement to conform that PH were established in the CS exposed animals. This could have been done by catheterization. On the other hand it was found that the mice had developed emphysema measured by MLI. Echocardiographic findings support this showing a depressed TAPSE. Even though the MLI method showed that the mice had developed emphysema, it gives no information about the severity of the disease.

We did not find a leveling off in the VO_{2peak} measurements which are in conflict with previous studies [81] which may indicate that the IT intervention period might have been too short. Hoydal *et al.*[81] demonstrated a levelling off on VO_{2max} following six weeks of IT. It is reasonable to believe that similar results with a further increase followed by levelling off of the $VO_{2peak,}$ may have taken place in the present study if the IT intervention period was extended. If so, this might also be the case regarding cardiac adaptations.

Due to anatomical challenges, RV measurements are difficult in small rodents and we did not obtain wall measurements or cavity sizes from the RV to confirm findings from TAPSE and tissue Doppler velocities. Also it would be easier to conclude if RV adaptations to exercise was due to the cardiac muscle or if it was more related to loading conditions (pre- and afterload). Also, structural and functional changes are developing and changing over time. We only obtained echocardiographic measurements at one single time point. Measurements of cardiac function should be obtained during CS exposure period as well as the exercise intervention period. This would have given information on cardiac function/structure development during the CS exposure and exercise intervention.
It is also a fact that echocardiographic parameters can change under stress. It would therefore be of interest to evaluate cardiac function obtaining stress echocardiography. This could be done by using dobutamine in order to raise the heart rate and mimic a form of stress echocardiography.

6. Conclusions

Taken together, the results from the present study support the hypothesis that CS decreased RV function and that was restored by IT in a mice model of COPD. On the other side it did not support the hypothesis that LV would be affected by CS. The novel finding in this study is that the adaptations to IT measured as VO_{2peak} were accompanied by enhanced systolic and diastolic RV global function. We also found a decreased muscularization of the pulmonary vessels following IT which has not been demonstrated before.

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