

<b>Original Article</b>	<b>The protective role of the exercise on the remote lung damage following ischemia/reperfusion injury in the hind-limb of the adult male albino rat: A histological and a morphometric study</b> <i>Rasha I. Anwar, Reneah R. Bushra and Hala Z.E. Mohamed</i> <i>Department of Human Anatomy and Embryology, Faculty of Medicine, Assiut University</i>
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### ABSTRACT

**Introduction:** Reperfusion of tissues following a prolonged period of an acute-onset ischemia causes injury to distant organs such as the lungs, kidneys, heart and liver through mediators released from the ischemic tissue entering the systemic circulation. Aerobic physical training of a moderate intensity has been recognized to improve the cardiorespiratory function.

**Aim of the work:** This study was designed to investigate the influence of physical training on the remote lung damage induced by rat hind-limb I/R injury.

**Material and Methods:** 30 adult male rats, weighing (200-250) gm were used in this study. The rats were divided into three equal groups: the control group, I/R group (underwent limb ischemia for 3 hours followed by 3 hours of reperfusion) and exercise + I/R group (trained rats for 4 weeks were subjected to limb ischemia for 3 hours and then 3 hours of reperfusion. At the end of the 3 hours of reperfusion, the rats were sacrificed and the specimens from the lung were taken. The specimens were processed for light and electron microscopic study. The area percentage of collagen fiber content was measured and the results were statistically analyzed.

**Results:** Light microscopic examination of I/R group showed thickened interalveolar septa with massive interstitial cellular infiltration, loss of normal architecture of the lung and hypertrophied arterial wall. The ultrastructure showed pneumocytes with rarified, vacuolated cytoplasm and destructed organelles. Type II pneumocytes were characterized by the presence of large vacuoles and few lamellar bodies. Alveolar macrophage showed numerous dense bodies, autophagic vacuoles and inclusion vacuoles. The exercise + I/R group showed marked improvement in the histological and ultrastructural alterations of I/R induced lung injury. The means of area percent of collagen fibers showed high significant increase in I/R group when compared to control and exercise + I/R groups.

**Conclusion:** The exercise may protect against the remote lung injury caused by the oxidative damage following the hind-limb I/R injury.

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**Key Words:** Exercise, hind-limb, Ischemia/reperfusion, lung, rat.

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### INTRODUCTION

The limb ischemia-reperfusion (I/R) injury is a common clinical event which has been observed in thrombosis, atherosclerosis and during surgery (Zhang *et al.* 2011).

Reperfusion of tissues following a prolonged period of an acute-onset ischemia causes injury to the tissue involved and to distant organs (Ho *et al.*, 2009). This damage occurs through mediators released from the ischemic tissue entering the systemic circulation and causes damage to re-

remote organs such as the lungs, kidneys, heart and liver. This can result in the development of systemic inflammatory response syndrome (SIRS) (Kamal, 2014).

SIRS involves an accumulation of immunocytes such as macrophages and neutrophils in major organs, release of cytotoxic substances such as proinflammatory cytokines, reactive oxygen species and neutrophil elastase and enhancement of vascular permeability. The acute pulmonary edema caused by enhanced vascular permeability impairing the respiratory function, results in

a condition called an acute lung injury (ALI) (Kitamura et al., 2010).

A healthy lifestyle has been strongly associated with the practice of regular physical activity. The physical exercise prevents or reduces the deleterious effects of pathological conditions, such as arterial hypertension, coronary artery disease and diabetes mellitus (Zanesco and Antunes, 2007). The aerobic physical training of moderate intensity has been recognized to improve the cardiorespiratory function (Kamal and Anwar, 2013).

Furthermore, the regular exercise exerts protective effects against diseases associated with systemic inflammation (Mussi et al. 2008).

### AIM OF THE WORK

This study was aimed to investigate the influence of physical training on the remote lung damage and the inflammatory responses induced by the rat hind-limb I/R injury.

### MATERIAL AND METHODS

#### Animals:

This study was carried out on 30 adult male albino rats weighing 200-250 gm. They were obtained from Animal House, Faculty of Medicine, Assiut University. Animals were maintained under 12/12 hours light/dark cycle with suitable room temperature  $23 \pm 5$  C°. They had free access to food and water. This study was in accordance with the Guide Lines of Animal Ethics Committee that were established with the internationally accepted principles for the laboratory animal use and care.

#### Training Program:

The exercise training comprised swimming, which was accomplished between 8:00 and 9:00 a.m. The swimming period was initially for 15 min/ day and was gradually increased. Thus, the rats were able to perform the exercise for 60 min/day. After 1 week of this training period, the rats were made to swim for 1 hour, five times a week, for 4 weeks (Aydin et al., 2009 and Teixeira et al., 2009).

#### Limb ischemia-reperfusion model:

The animals were anesthetized by an intraperitoneal injection of urethane (600 mg/kg). Bilateral rubber bands were ligated above the greater trochanter for 3 hours to induce ischemia. Then reperfusion was initiated by removing the bands for

another 3 hours. Occlusion and reperfusion were confirmed by Doppler ultrasound probe, which was placed distal to the site at which the rubber band had applied (Kamal, 2014).

#### Experimental Design:

The rats were divided into 3 groups (10 rats each):

Group I: The rats in this group were used as control.

Group II (I/R): The sedentary rats underwent limb ischemia for 3 hours followed by 3 hours of reperfusion.

Group III (exercise + I/R): The trained rats (for 4 weeks) were subjected to limb ischemia for 3 hours and then 3 hours of reperfusion.

#### Tissue Processing and Sampling:

At the end of the 3 hours of reperfusion, all the animals were sacrificed after anesthesia by using ethyl ether. Lung specimens were taken from the rats in all groups.

For light microscopic study, lung specimens were fixed in 10% formalin, prepared for paraffin sections (8  $\mu$ m) and stained with hematoxylin and eosin (H&E) and Masson's trichrome (Bancroft and Gamble, 2008).

Ultrastructural study of the lung in all groups was done. The specimens were fixed in 2.5% phosphate buffered glutaraldehyde solution and then processed for obtaining semithin sections (1  $\mu$ m) which stained with toluidine blue. Ultrathin sections (50 nm) were cut with ultramicrotome, stained with uranyl acetate and lead citrate to be examined and photographed with the transmission electron microscope (Jeol-JEM-100 CXII; Jeol, Tokyo, Japan) in the Electron Microscopic Unit of Assiut University.

#### Morphometric Procedure and Statistical Analysis:

The area percent of fibrous tissue in the interalveolar septa, around the bronchioles and blood vessels was measured from the slides stained with Masson's trichrome using image analysis system (Leica Q500). Five square fields (x400) were taken from each slide in the three groups. Area measurement was 73473.00  $\mu$ m<sup>2</sup>.

The obtained data were expressed as mean  $\pm$  SD. Statistical analysis was done using SPSS software, version 16.00 (Chicago, Illinois, USA). One way analysis of variance (ANOVA) was carried out for intergroup comparisons.  $P \leq 0.05$  and  $P \leq 0.01$  were considered a significant and a highly significant, respectively.

## RESULTS

### A. Histological Results:

#### I. Light microscopic results:

##### 1- Hematoxylin and Eosin stain (H&E):

In group I (control) lung sections showed the normal architecture with the bronchioles, blood vessels and thin interalveolar septa surrounding the irregular clear alveoli and alveolar sacs. The spaces were lined by both flattened type I and cuboidal type II pneumocytes (Fig. 1). While the sections of group II showed thickened interalveolar septa with massive interstitial cellular infiltration and loss of normal architecture of the lung. Hypertrophied arterial wall could be also seen (Fig. 2). In group III, the architecture of the lung appeared more or less normal with thin interalveolar septa in some areas and thickened in others. Mild inflammatory cellular infiltration and thickened arterial wall were seen (Fig. 3).

##### 2- Masson's trichrome stain:

Sections of group I showed a little amount of collagen fibers in the interalveolar septa, around the bronchioles and blood vessels (Fig. 4). There was massive collagen fibers deposition in group II as compared to group I condensed around bronchioles, blood vessels and in the interalveolar septa and obliterating some alveoli (Fig. 5). In group III, there was few collagen fibers deposition nearly similar to group I (Fig. 6).

##### 3- Toluidine blue stain:

Group I the sections showed flattened nuclei in type I pneumocytes and rounded nuclei in type II pneumocytes. Thin interalveolar septa and an apparent little amount of macrophages were observed (Fig.7).

In group II, the pneumocytes appeared vacuolated, desquamated cells in alveolar lumen and macrophages in the thickened interalveolar septa could be seen (Fig. 8).

The sections in group III showed marked improvement of lung architecture but with focal thickening of the interalveolar septa. Some vacuolated pneumocytes and macrophages could be seen (Fig. 9).

### II. Ultrastructural results:

Examination of the lung sections in group I showed type I pneumocyte with nearly rounded nucleus surrounded by a thin layer of cytoplasm containing mitochondria, rough endoplasmic

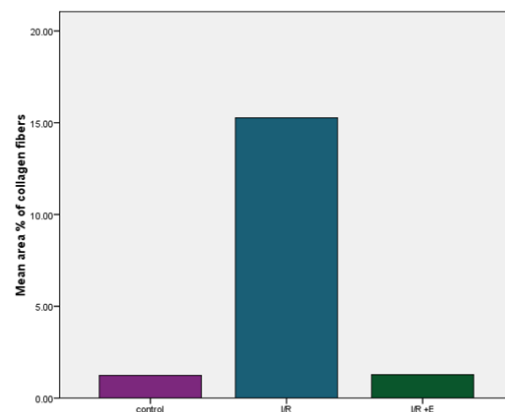
reticulum and free ribosomes (Fig. 10). Type II pneumocyte characterized by the presence of short microvilli and lamellar bodies in the cytoplasm (Fig. 11). Alveolar macrophage appeared with its irregular outline and kidney shaped nucleus. The cytoplasm contained mitochondria, free ribosomes and rough endoplasmic reticulum (Fig. 12).

In group II, type I the pneumocyte showed a dispersed chromatin in the nucleus. The cytoplasm was rarified and contained dilated rough endoplasmic reticulum and destructed mitochondria (Fig. 13). The cytoplasm of type II pneumocyte was rarified and characterized by the presence of large vacuoles, few mitochondria and lamellar bodies. The microvilli were not obvious (Fig. 14). The alveolar macrophage showed numerous dense bodies, autophagic vacuoles and inclusion vacuoles (Fig. 15).

The lung sections in group III showed type I pneumocyte with nucleus more or less similar to control, the cytoplasm was free from large vacuoles (Fig. 16). Type II pneumocyte showed lamellar bodies and short microvilli similar to control. At the same time some vacuoles were still seen (Fig. 17). Alveolar macrophage with kidney shaped nucleus appeared similar to control to a great extent (Fig. 18).

### B- Morphometric and statistical results:

The means of area percent of collagen fibers in group (I), group (II) and group (III) were 1.23, 15.27 and 1.27, respectively, with highly significant increase ( $P < 0.01$ ) in group II when compared with group I & group III and non-significant difference ( $P > 0.05$ ) between group I & group III (Histogram 1, Table 1).

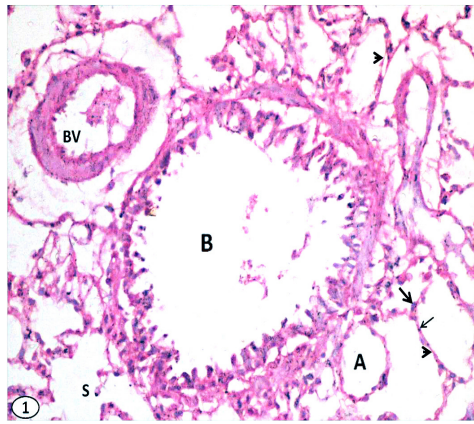


**Histogram 1:** The mean values of the area percentage of collagen fibers in the lung of the studied groups.

I/R: ischemia/reperfusion

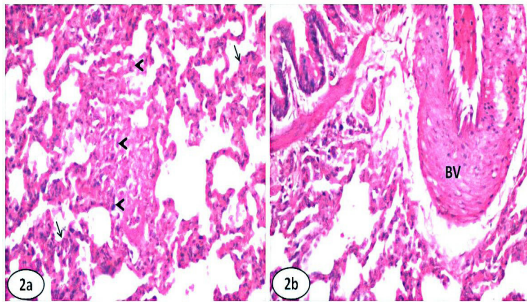
I/R + E: ischemia/reperfusion + exercise





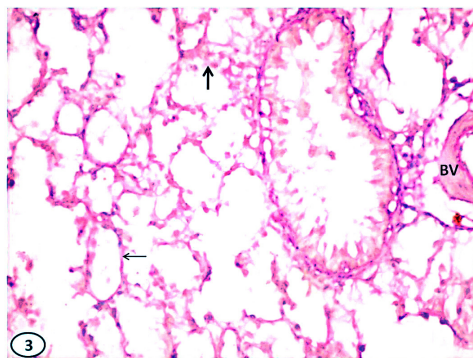
**Fig. 1:** A photomicrograph of a section in the lung of group I (control) showing normal lung architecture with clear alveoli (A), alveolar sacs (S), thin interalveolar septa (arrow heads), bronchioles (B) and blood vessels (BV). Type I flattened pneumocyte (thin arrow) and type II cuboidal pneumocyte (thick arrow) can be seen lining the spaces.

H&E X 400



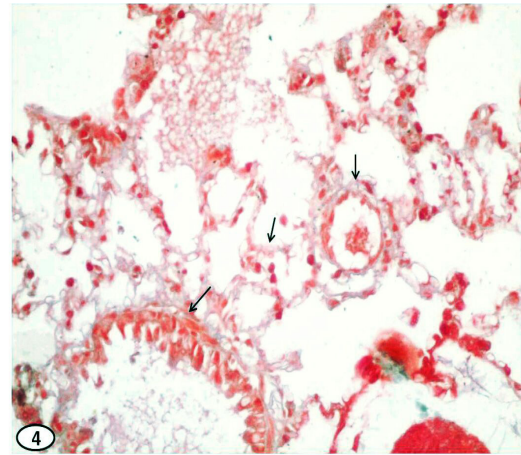
**Fig. 2:** (2a & 2b): A photomicrograph of a section in the lung of group II (I/R) showing; a: thickened interalveolar septa (arrows) with massive interstitial cellular infiltration (arrow heads) and loss of normal architecture. b: Hypertrophied arterial wall is noticed (BV).

H&E X 400



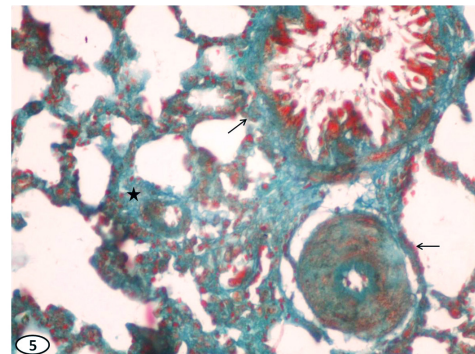
**Fig. 3:** A photomicrograph of a section in the lung of group III (exercise + I/R) showing that the lung architecture appears more or less normal with thin interalveolar septa in some areas (thin arrow) and thickened infiltrated with inflammatory cells in other areas (thick arrow). Thickened arterial wall is still seen (BV).

H&E X 400



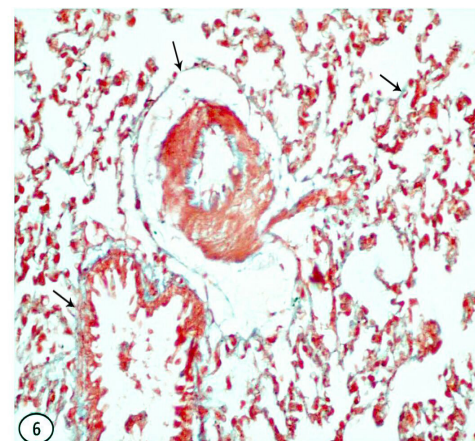
**Fig. 4:** A photomicrograph of a section in the lung of group I (control) showing little amount of collagen fibers in interalveolar septa, around bronchioles and blood vessels (arrows).

Masson's trichrome X400



**Fig. 5:** A photomicrograph of a section in the lung of group II (I/R) showing collagen fibers deposition around bronchioles, blood vessels and in the interalveolar septa (arrows) which is obliterating some alveoli (star).

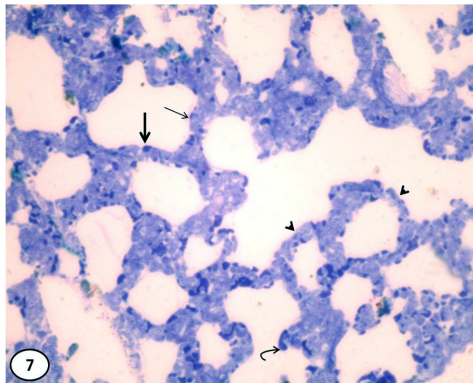
Masson's trichrome X400



**Fig. 6:** A photomicrograph of a section in the lung of group III (exercise + I/R) showing that there is few collagen fibers deposition (arrows) nearly similar to control group.

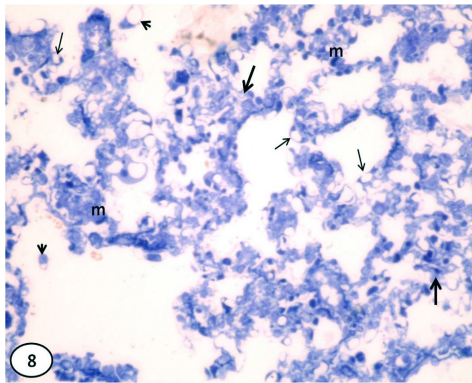
Masson's trichrome X400





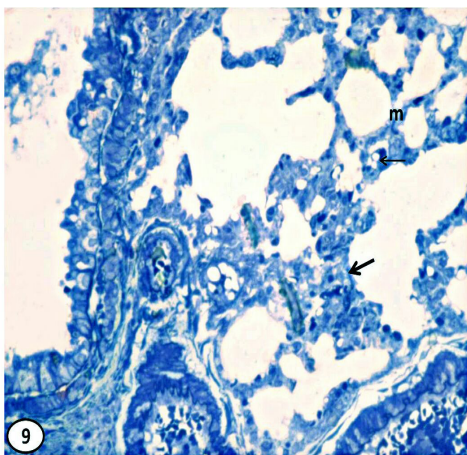
**Fig. 7:** A photomicrograph of a section in the lung of group I (control) showing flattened nuclei in type I pneumocytes (thin arrow) and rounded nuclei in type II pneumocytes (thick arrow). The interalveolar septa appear thin (arrow heads) and macrophage can be seen (curved arrow).

Toluidine blue X 400



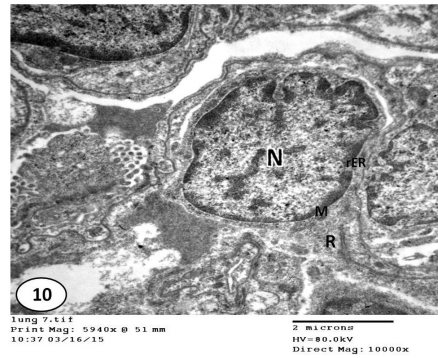
**Fig. 8:** A photomicrograph of a section in the lung of group II (I/R) showing vacuolated pneumocytes (thin arrows) and desquamated cells in alveolar lumen (arrow heads). Macrophages (m) in the thickened interalveolar septa (thick arrows) can be seen.

Toluidine blue X 40



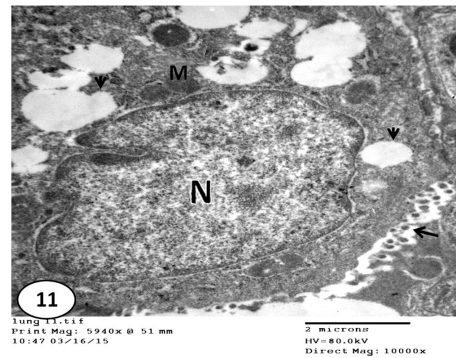
**Fig. 9:** A photomicrograph of a section in the lung of group III (exercise + I/R) showing that the interalveolar septa are thickened in some parts (thick arrow). Some vacuolated pneumocytes (thin arrow) and macrophages (m) are seen.

Toluidine blue X 400



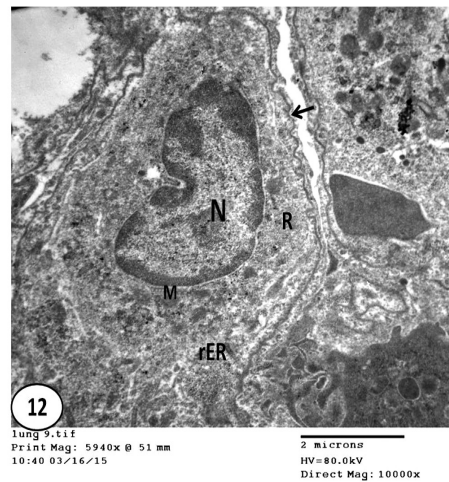
**Fig. 10:** An electron micrograph of a section in the lung of group I (control) showing type I pneumocyte with nearly rounded nucleus (N) surrounded by a thin layer of cytoplasm containing mitochondria (M), rough endoplasmic reticulum (rER) and free ribosomes (R).

X10000



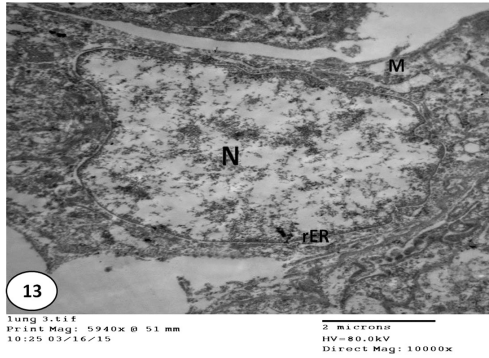
**Fig. 11:** An electron micrograph of a section in the lung of group I (control) showing type II pneumocyte with its short microvilli (arrow) and nucleus (N). Notice the presence of lamellar bodies (arrow heads) and mitochondria (M) in the cytoplasm.

X1000



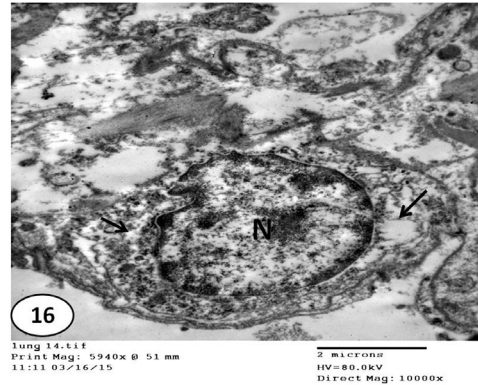
**Fig. 12:** An electron micrograph of a section in the lung of group I (control) showing alveolar macrophage with its irregular outline (arrow) and kidney shaped nucleus (N). The cytoplasm contains mitochondria (M), free ribosomes (R) and rough endoplasmic reticulum (rER).

X10000



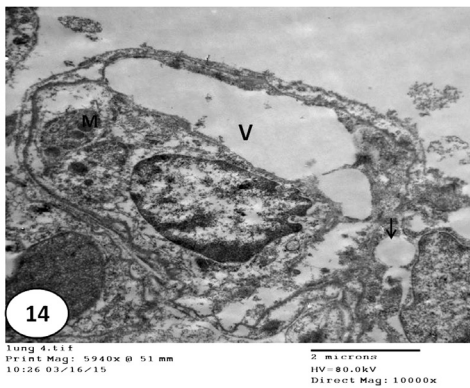
**Fig. 13:** An electron micrograph of a section in the lung of group II (I/R) showing type I pneumocyte with dispersed chromatin in the nucleus (N). The cytoplasm is rarified and contains dilated rough endoplasmic reticulum (rER) and destroyed mitochondria (M).

X10000



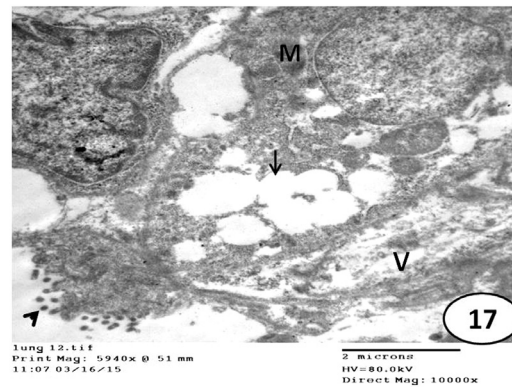
**Fig. 16:** An electron micrograph of a section in the lung of group III (exercise + I/R) showing type I pneumocyte with more or less normal nucleus (N). The cytoplasm is free of the large vacuoles but still rarified in some areas (arrows).

X10000



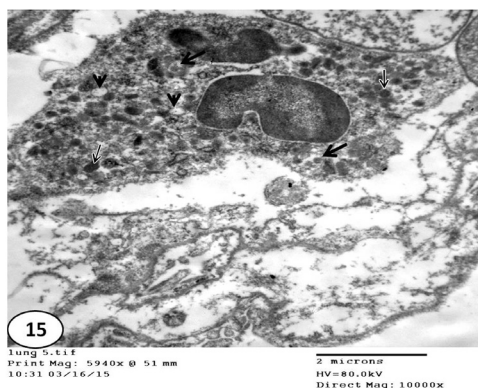
**Fig. 14:** An electron micrograph of a section in the lung of group II (I/R) showing type II pneumocyte with rarified cytoplasm containing large vacuole (V), few mitochondria (M) and lamellar bodies (arrow). The microvilli are not obvious.

X10000



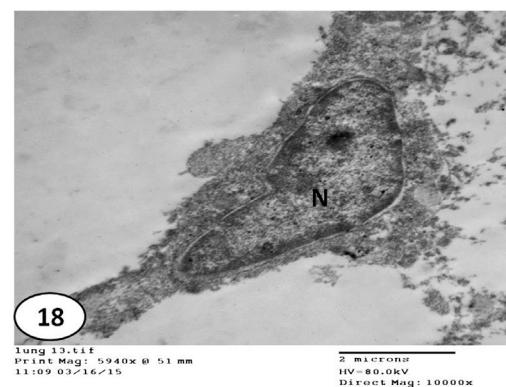
**Fig. 17:** An electron micrograph of a section in the lung of group III (exercise + I/R) showing type II pneumocyte with lamellar bodies (arrow), short microvilli (arrow head), mitochondria (M) and some vacuoles are still seen (V).

X10000



**Fig. 15:** An electron micrograph of a section in the lung of group II (I/R) showing alveolar macrophage with numerous dense bodies (thin arrows), autophagic vacuoles (thick arrows) and inclusion vacuoles (arrow heads).

X10000



**Fig. 18:** An electron micrograph of a section in the lung of group III (exercise + I/R) showing alveolar macrophage that are similar to control with its kidney shaped nucleus (N)

X10000.



**Table 1:** Mean area% of collagen fibers in the lung of the studied groups of animals. Data are presented as mean± standard deviation (SD).

Groups	Means ± SD
Control (group I)	1.23± 0.17
I/R (group II)	15.27± 5.77
I/R + E (group III)	1.27± 0.22
P (group I vs. group II)	0.003**
P (group II vs. group III)	0.003**
P (group I vs. group III)	0.787 <sup>Ns</sup>

\*\* : high significant ( $P < 0.01$ ).

Ns: non-significant ( $P > 0.05$ ).

## DISCUSSION

Interruption of blood flow to any tissue results in inadequate tissue oxygenation and an increase in cellular anaerobic pathways that cause cell dysfunction, which may progress to cell death. (Glantzounis *et al.*, 2009). Following reperfusion of ischemic limb mortality may happen due to multiple organ dysfunction caused by systemic inflammatory response (Yassin *et al.*, 2002). The removal of the toxic metabolites formed during ischemia through reperfusion is important, but their infusion of into systemic circulation may cause further tissue damage (Carden and Granger, 2000). The remote injury may occur in any organ; however death usually results from heart, lung, kidney, or liver failure (Wunder *et al.*, 2002).

Practicing of regular physical activity is very important in healthy life style. Physical exercise reduces the deleterious effects of cardiovascular and inflammatory disorders (Mussi *et al.*, 2008). So, the aim of this work was to investigate the influence of physical training on remote lung damage following I/R of hind limb in rats by histological and morphometric study.

I/R group showed thickened interalveolar septa with massive interstitial cellular infiltration, loss of normal architecture of the lung and hypertrophied arterial wall. Moreover, pneumocytes appeared vacuolated with dispersed chromatin, presence of large vacuoles and distorted organelles. The apoptotic changes observed, was proved by some investigators, they referred that injury of cells after reperfusion occurred in the form of apoptosis. Some apoptosis promoting substances were released in great amount which trigger apoptosis (Sun *et al.*, 2004). Apoptosis may be due to oxidative stress which is associated with increased production of oxidizing species or decreases the antioxidant defenses (Schafer and Buettner, 2001), which in turn can cause cell death, and even moderate oxidation can trigger apoptosis (Lennon *et al.* 1991). Oxidative stress resulted in respiratory distress syndrome which

manifested under microscope by epithelial injury in the alveoli, pulmonary edema and cellular infiltration (Soliman *et al.* 2009).

Cunha *et al.* (2013) reported that, the oxidative stress may be an important contributor to the development of lung injury by the disruption of protein and lipid integrity.

The I/R injury lead to changes in permeability and excessive production of oxygen free radicals (OFRs) and release of proteolytic lysosomal enzymes and mitochondrial matrix enzymes which caused cellular destruction (Zaglool *et al.* 2011)

Increased deposition of collagen fibers and marked cellular infiltration could explain the thickening of interalveolar septa (Gadalla, 2012). The inflammatory cellular infiltration was concomitant with the excessive amount of collagen fibers. This was explained by (Atiq *et al.*, 2009) who reported that reactive oxygen species might cause mitochondrial dysfunction which could lead to necrosis, fibrosis and collagen deposition (Kamal, 2014). Deposition of collagen fibers could progress into fibrosis which was a chronic and incurable. Gadalla (2012) explained that an injury to epithelium and basement membrane lead to an inflammatory and immune cells migration to the site of injury and the release of cytokines and the overproduction of collagen fibers and fibrosis.

The ultrastructural findings of type II might be due to effect of lipid peroxidation of membrane phospholipids and the impairment in surfactant secretion was supported by the presence of lamellar body residues which contributed to the functional effect on oxygen. The alteration in the air blood barrier might be due to lysis in type II pneumocytes (Soliman *et al.*, 2009).

This work revealed that the exercise reduced the remote lung damage following hindlimb I/R injury in rats. By light microscope, group III showed a marked improvement in the architecture of the lung with thin interalveolar septa in some areas and thickened in others. The mild inflammatory cellular infiltration and thickened arterial wall were still seen. The reduced fibrosis was confirmed by few collagen fibers. By electron microscope, pneumocytes appeared more or less similar to the normal. However, some pneumocytes were still vacuolated. In harmony with these results, French *et al.* (2008) and De Waard and Duncker (2009) proved that the exercise suppressed the apoptosis induced by ischemia. Moderate exercise could induce mild oxidative stress which stimulated the expression of antioxidant enzymes.

Mussi *et al.* (2008) reported that, prior physical training exerted a beneficial effect in remote

lung I/R injury in terms of significant attenuation of the resulting inflammatory oedema. Moreover, physical exercise at moderate intensity promoted upregulation of antioxidant enzyme expression in both humans and laboratory animals.

In agreement with the present results, previous studies showed that regular exercise had protective effects against diseases associated with systemic inflammation; this might be due to suppression of oxidative stress (Kamal, 2014).

Experimental evidence indicated that regular exercise was able to prevent the lipid peroxidation and oxidative damage to protein present in lung injury (Pinho et al., 2009). On the other hand, it had been suggested that the molecular basis for the beneficial effect of regular physical exercise may be related to the increase in reactive species (Radak et al., 2008; Powers et al., 2010, 2011 and Gomes et al., 2012). Physical exercise prevented some alterations in oxidative parameters, such as reactive species production, GPX activity, GSH content and nitrite levels, as well as the increase in NF- $\kappa$ B/p65 immunocent content caused by experimental lung injury. Lung protection induced by the physical training was effective in impeding the establishment of oxidative stress (Nesi et al., 2016).

In conclusion, the hind-limb I/R induced a remote lung injury. The exercise may protect against the oxidative damage caused by the lung injury. So, it is important to the individuals who undergone surgical operations which involve a prolonged ischemia to practice a regular exercise.

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## الدور الوقائي للرياضة علي تلف الرئة النائي بعد الإحتباس الدموي و إعادة تدفق الدم في الأطراف الخلفية للفأر الذكر البالغ: دراسة نسيجية وكمية

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### ملخص البحث

**الخلفية:** إن إعادة تدفق الدماء في الأنسجة بعد فترة طويلة من الإحتباس يسبب تلف للأعضاء النائية مثل الرئتين، الكليتين، القلب و الكبد و ذلك من خلال مواد وسيطة تخرج من الأنسجة المصابة و تدخل الدورة الدموية. إن التدريب البدني معتدل الشدة يعمل علي تحسين وظائف القلب و التنفس.

**الهدف من البحث:** معرفة تأثير التدريب البدني علي تلف الرئة النائي الناتج عن الإحتباس الدموي و إعادة تدفق الدم في الأطراف الخلفية للفأر الذكر البالغ.

**المواد وطرق البحث:** تم إستخدام ثلاثين من ذكور الفئران البالغين وزن (220-250 جم). تم تقسيم الفئران إلي 3 مجموعات متساوية: المجموعة الضابطة، المجموعة التي تم فيها إحداث إحتباس دموي لمدة 3 ساعات ثم إعادة تدفق الدم في الاطراف الخلفية لمدة 3 ساعات أخرى و المجموعة التي كانت تقوم بالتمارين لمدة 4 أسابيع ثم إحداث إحتباس دموي و إعادة تدفق كما في المجموعة الثانية. في نهاية مرحلة إعادة تدفق الدم تمت التضحية بالفئران و أخذ عينات من الرئة و تم تمريرها و تجهيزها للفحص بالميكروسكوب الضوئي و الإلكتروني. تم قياس نسبة مساحة ألياف الكولاجين و قد حلت النتائج إحصائيا.

**النتائج:** أظهرت نتائج الفحص بالميكروسكوب الضوئي لمجموعة الإحتباس ثم إعادة تدفق الدم زيادة سمك الحاجز بين الحويصلات الهوائية مع زيادة الخلايا الإلتهابية في النسيج البين خلوي، فقد للبناء الطبيعي للرئة و زيادة سمك جدار الأوعية الدموية. أظهر الفحص بالميكروسكوب الإلكتروني ان سيتوبلازم الخلايا الرئوية به تجاويف و عضيات تالفة. الخلايا الرئوية من النوع الثاني ظهر بها تجاويف كبيرة و قليل من الأجسام الرقائقية الخلايا الملتهمة الكبيرة الرئوية ظهر بها العديد من الأجسام الكثيفة , فجوات ذاتية البلعمة و فجوات مشتملة علي جسيمات. أما مجموعة التمرين فقد أظهرت تحسنا ملحوظا في التغيرات التي حدثت بسبب إحتباس و إعادة تدفق الدم. كما أظهر قياس نسبة مساحة ألياف الكولاجين زيادة ذات دلالة إحصائية في مجموعة الإحتباس و إعادة التدفق مقارنة بالمجموعتين الضابطة و التمرين.

**الأستنتاج:** إن ممارسة الرياضة من الممكن ان تحمي من الإصابة النائية للرئة و التي حدثت بسبب الضرر التأكسدي بعد الإحتباس الدموي و إعادة تدفق الدم في الأطراف الخلفية للفئران.