Nature and Science

Websites:
http://www.sciencepub.net/nature
http://www.sciencepub.net

Emails:
naturesciencej@gmail.com
editor@sciencepub.net

MARSLAND PRESS
Multidisciplinary Academic Journal Publisher

Study of the Effect of Naltrexone and Silymarin on Amiodarone Induced Pulmonary Fibrosis in Albino Rats

AsmaaFawzy El-Tantawy, Samia Hussein Abo Al-Soud, Ahmed Ismaeil Yassin, AmanyAbd El-Rahim Abdin

Medical Pharmacology Department, Faculty of Medicine, Tanta University, Egypt
E-mail:asmaa.fawzy55@gmail.com.

Abstract: Pulmonary fibrosis (PF) is a progressive interstitial lung disease that is characterized by increased deposition of extracellular matrix proteins. Amiodarone-induced PF model shows the main fundamental features of pulmonary fibrosis, including the same morphological pattern that mimics PF in humans. Naltrexone acts as a pure non-specific opioid receptor competitive antagonist that blocks all opioid receptors, it can decrease tissue inflammation and fibrosis through potential mechanisms including removal of the free radicals. Silymarin has reported over the last decade as a herbal remedy for hepatoprotection due to its antioxidant properties.The aim of this study was to evaluate the possible anti-fibrotic, anti-inflammatory and anti-oxidant effects of naltrexone and silymarin in amiodarone induced-PF in albino rats.This experiment was performed on 48 male albino rats divided into 6 equal groups; normal control group, untreated amiodarone induced PF group, amiodarone induced PF group protected by naltrexone, amiodarone induced PF group protected by silymarin, amiodarone induced PF group treated by naltrexone, amiodarone induced PF group treated by silymarin. The results showed that the untreated induced-PF group showed a significant increase in transforming growth factor β_1 (TGF-β_1), hydroxyproline (Hyp), tumor necrosis factor-α (TNF-α), malondialdehyde (MDA) levels, and decrease in Superoxide dismutase (SOD) activity, when compared to normal control group, also the present study showed that prophylaxis and treatment with naltrexone and silymarin produce significant decrease in all parameters except for SOD activity which showed significant increase. There was a non-significant difference between naltrexone and silymarin groups regarding all measured parameters except for SOD activity where there was a significant increase in SOD activity with silymarin compared to naltrexone. Also histopathological examination of lung tissue stained with Mallory stain showed improvement of fibrosis score in protected and treated groups when compared to untreated group. These findings suggest that naltrexone and silymarin have a significant role either in protection or treatment of amiodarone induced-PF, in regard to improvement of inflammation, anti-fibrotic and antioxidant effects. It could be recommended to verify these results in further clinical studies.

[Asmaa Fawzy El-Tantawy, Samia Hussein Abo Al-Soud, Ahmed Ismaeil Yassin, Amany Abd El-Rahim Abdin.**Study of the Effect of Naltrexone and Silymarin on Amiodarone Induced Pulmonary Fibrosis in Albino Rats.***Nat Sci***2020;18(2):117-130]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 18. doi:10.7537/marsnsj180220.18.

Keywords: Pulmonary fibrosis (PF), Amiodarone, Naltrexone, Silymarin.

Abbreviations: (PF) Pulmonary fibrosis, (ECM) Extracellular matrix, (AIPF) Amiodarone Induced PF, (IL) Interleukins, (TNF-α) Tumor necrosis factor-α (TGF-β) Transforming growth factor-β. (ET-1) Endothelin 1. (PG) Prostaglandin. (IL-6) Interleukin 6. (ROS) Reactive oxygen species. (CMC) Carboxy methyl cellulose.

1. Introduction

Pulmonary fibrosis (PF) is an interstitial lung disease that is induced by different factors. There are several causes contribute to the pathogenesis of pulmonary fibrosis including oxidative stress, inflammation, epithelial-mesenchymal transition, and immune disorders, all these factors lead to alveolar epithelial cell injury and fibroblast proliferation that consequently result in abnormal deposition of the extracellular matrix (ECM) components (Li et al., 2015).

Idiopathic pulmonary fibrosis (IPF) is a progressive life-threatening disease and it is the most common type of interstitial lung disease. It is characterized by scarring of the lungs, that leads to organ malfunction, disruption of gas exchange, and respiratory failure (Baddini-Martinez et al., 2015).

Causes of PF are multifactorial including exogenous factors as long-term exposure to occupational agents such as asbestos, silica, and irradiation (Arizmendi et al., 2014). Some chemotherapeutic agents as methotrexate and bleomycin, antiarrhythmic drugs as amiodarone and propranolol, and specific antibiotics as ethambutol, also can play a role in the etiology of PF (Molyneaux and Maher, 2013). In addition, oxidative stress and

inflammation have also a great role in pulmonary damage. Studies have demonstrated that cytokines including interleukins (IL), tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) effectively take part in collagen deposition (Razavi-Azarkhiavi et al., 2014). Idiopathic pulmonary fibrosis, formally known as "cryptogenic fibrosing alveolitis", is caused by repeated injury to small areas of the lungs resulting in inflammation and then scarring of the lungs. Repetitive injuries to the alveolar epithelium or endothelium lead to PF through triggering of the immune system. Inflammatory mediators such as the profibrotic cytokine, transforming growth factor-beta (TGF-β) activate angiogenesis and myofibroblasts to produce extracellular matrix (ECM) components such as collagen and fibronectin (Fernandez and Eickelberg, 2012).Damaged tissues repair is a core biological mechanism allowing the regulated replacement of dead or damaged cells after injury, this process is very important for survival. If this process becomes dysregulated, it leads to the evolution of a permanent fibrotic scar. This scar is characterized by the excessive accumulation of ECM components as hyaluronic acid, fibronectin, proteoglycans and interstitial collagens at the site of tissue injury and development of fibrogenesis.

Numerous cytokines and growth factors have been implicated as mediators in the pathogenesis of pulmonary fibrosis. One such mediator, transforming growth factor beta (TGF-β), is a key regulator of both normal wound repair and the aberrant repair mechanisms characteristic of many fibrotic diseases, including pulmonary fibrosis. It is believed that TGF-β is a central regulator of the recruitment, activation, and differentiation of myofibroblasts during the early phases of tissue repair (Kosmidis et al., 2016). In addition to direct injury of lung cells and matrix, oxidants may also take part in the development of PF by their direct effects on cytokines and growth factors. Examples of these mediators are TGF-β, endothelin 1 (ET-1), prostaglandin (PG) E_2 and interleukin 6 (IL-6) (Ahn et al., 2011). TGF-β is believed to be a central regulator of the activation and differentiation of myofibroblasts that occur in the early stages of tissue repair. The persistence of the myofibroblastic phenotype in the areas of active fibrosis is a characteristic finding in fibrotic lung disease. There is currently no cure for IPF. The main aim of treatment is to relieve the symptoms as much as possible and slow down its progression. As the condition becomes more advanced, end of life (palliative) care will be offered.

Amiodarone is a commonly prescribed broad-spectrum antiarrhythmic drug. It is a highly lipophilic iodinated benzofuran derivative (Joukar et al., 2014). Amiodarone has severe adverse effects on many organs involving the cornea, liver, lung, neuromuscular system, skin and thyroid, which often limit its use (Nasri et al., 2015). An important feature of amiodarone is its high lipid solubility. It is mainly accumulated in adipose tissue and highly perfused organs as liver, lungs, and spleen (Lafuente-Lafuente et al., 2009).

Amiodarone induced pulmonary toxicity should be taken into consideration in patients under long-term amiodarone use, especially in elderly patients with pulmonary symptoms, functional and radiographic changes even if a low dose of the drug is administered for years (Hudzik and Polonski, 2012). The two most important risk factors for the amiodarone pulmonary toxicity are age and duration of therapy. Some researchers have suggested that the drug is directly toxic to the lung, others have reported that it has an indirect effect involving cell-mediated immune mechanisms (Mahavadi et al., 2014). The mechanism of pulmonary toxicity induced with amiodarone administration is multifactorial. Amiodarone and its metabolite cause damage to lung tissue either directly by a cytotoxic process or indirectly by immunologic reactions (Al-Shammari et al., 2016). Amphiphilic drugs especially produce a phospholipid storage disorder in the lungs of experimental animals and humans. The mechanism is currently believed to be the inhibition of lysosomal phospholipases and aggravation of oxidative stress through the production of reactive oxygen species (ROS), as reported by a study which revealed that AM is metabolized to an aryl radical that may give rise to other ROS (Nicolescu et al., 2008).

Amiodarone-induced lipid peroxidation (LPO) is an initiating event in AM-induced pulmonary fibrosis. Lipid peroxidation occurs when free radicals act on polyunsaturated fatty acids and cholesterol. LPO begins with a free radical donating an unpaired electron to a methylene carbon in a polyunsaturated fatty acid, which subsequently reacts with molecular oxygen to form a peroxyl radical. This peroxyl radical can then react with another peroxyl radical, attack membrane proteins, or abstract hydrogen atoms from adjacent fatty acid side chains leading to the production of a subsequent peroxyl radical. In this manner, the chain reaction of LPO is continued and can result in membrane damage, enzyme inhibition, release of lysosomal enzymes and protein-protein cross-linking, which can then induce cell death (Nicolescu et al., 2007).

Naltrexone is a non-specific opioid receptor antagonist, with the advantage over naloxone that it has greater oral bioavailability and a longer half-life, with a significantly longer receptor dissociation constant and is therefore effective in its oral form (Younger et al., 2014). Naltrexone HCl was approved

by FDA in 1984 for treatment of alcoholism and opioid detoxification (Kalueff, 2016).Low dose naltrexone is becoming commonly used as a long term treatment of autoimmune disorders (Bihari, 2013) and cocaine addiction (Sushchyk et al., 2016). The exact mechanisms of these actions are unknown, but it has been shown that it may upregulate endogenous opioid system through μ-opioid receptor antagonism (Ebrahimkhani et al., 2006).

It has been reported that opioids contribute to the process of fibrogenesis and naltrexone treatment had important protective effects by modulation of redox state and lipid peroxidation. The anti-fibrogenic roles for opioid antagonist have been also detected in liver cirrhosis models (Ebrahimkhani et al., 2008;Cohen-Naftaly and Friedman, 2011).In addition to the antagonist effect on mu-opioid and other opioid receptors, naltrexone simultaneously has an antagonist effect on non-opioid receptors (Toll-like receptor 4) that are found on macrophages such as microglia. It is via the non-opioid antagonist pathway that naltrexone is thought to exert its anti-inflammatory effects. Naltrexone reduces the production of reactive oxygen species and other potentially neuro-excitatory and neurotoxic chemicals by suppression of microglia activation. The anti-inflammatory effect of opioid antagonists may also extend to the periphery, as evidenced by suppressed TNF-alpha, IL-6, and other inflammatory agents in peripheral macrophages (De Minicis et al., 2008).

The flavonoid silymarin is a substance with documented hepatoprotective properties. Its mechanism of action is still poorly understood. However, the data in the literature indicate that silymarin act as an antioxidant, scavenger and regulator of the intracellular content of glutathione; stimulating liver regeneration; and as an inhibitor of the transformation of stellate hepatocytes into myofibroblasts (Abdel-Moneim et al., 2015). The key mechanism that ensures hepatoprotection appears to be free radical scavenging, it influences enzyme systems associated with glutathione and superoxide dismutase. Anti-inflammatory and anti-carcinogenic properties have also been documented in hepatotoxicity produced by toxins, ethanol and psychotropic drugs (Mata-Santos et al., 2014). Silymarin is a well-tolerated and effective for the treatment of many liver disorders characterized by degenerative necrosis and functional impairment and provides hepatoprotection against poisoning by galactosamine, thioacetamide, halothane and carbon tetrachloride (Shaker et al., 2011).It also protects hepatocytes from injury caused by ischemia, radiation, iron overload and viral hepatitis. Silymarin treatment alleviated the radiation-induced lung injury possibly by decreasing inflammation and fibrosis, which might be related to the improved survival rate (Son et al., 2015). It might be a useful agent for lung cancer patients as a non-toxic complementary approach to alleviate the side effects by thorax irradiation. Silymarin effects against fibrogenic action of bleomycin on lung is reported to be by reduction of collagen deposition and inflammation.

This experiment was designed to study the potential anti-fibrotic, anti-inflammatory and antioxidant effects of naltrexone and silymarin in protection or treatment of amiodarone-induced pulmonary fibrosis in albino rats.

2. Materials and Methods

2.1. Drugs and Chemicals:

Amiodarone HCL:A product of Global Pharmaceutical Industries, Egypt; that was prepared as a suspension in aqueous solution of carboxy methyl cellulose (CMC) 0.5% with a final concentration of 8 mg/ml.Naltrexone HCL: White powder from Sigma Aldrich Company purchased from Egyptian International Centre for Import; that was prepared as a suspension in aqueous solution of carboxy methyl cellulose (CMC) 0.5% with a final concentration of 3 mg/ml. Silymarin:A product of Global Pharmaceutical Industries, Egypt; that was prepared as a suspension in aqueous solution of carboxy methyl cellulose (CMC) 0.5% with a final concentration of15 mg/ml. Carboxymethyl cellulose (CMC) 0.5%:A product of Eastrin Fine Chemicals Company, purchased from El Gomhoria Company, Tanta, Egypt. Aqueous solution of CMC 0.5% was prepared by dissolving 0.5 gm CMC powder in 100 ml distilled water. Formaline fixative solution (10%): A product of Adwic Pharmaceutical Company, purchased from El Gomhoria Company, Tanta, Egypt.Saline (0.9%): A product of Pharmaceutical Solution Laboratories, Cairo, Egypt.

2.2. Groups design and treatment protocols:

This study was carried out in Pharmacology Department, Faculty of Medicine,Tanta University, Egypt. It was conducted on 48 adult Sprague Dawley male albino rats weighing 150-200 g. Rats were housed in wire mesh cages and allowed free access to standard chew and water, and they were divided into 6 equal groups (8 rats for each) as follows: Group I (Normal control group): Rats were received vehicle of CMC (0.5%) by oral gavage once daily for 4 weeks. Group II (untreated amiodarone-diseased group): Rats with amiodarone induced-pulmonary fibrosis for 4 weeks (Agelaki et al., 2007&Sharaf El-Din and Abd Allah, 2016). Group III (naltrexone-protected group): Rats with amiodarone induced-pulmonary fibrosis were administered concomitantly (1 hour after amiodarone) naltrexone in a dose of 20 mg/kg daily by oral gavage for 4 weeks (Ebrahimkhani et al., 2006).Group IV (silymarin-protected group): Rats

with amiodarone induced-pulmonary fibrosis were administered concomitantly (1 hour after amiodarone) silymarin in a dose of 140 mg/kg daily by oral gavage for 4 weeks (Vargas-Mendoza et al., 2014).Group V (naltrexone-treated group): Rats with amiodarone induced-pulmonary fibrosis were administered after 2 weeks naltrexone in a dose of 20 mg/kg by oral gavage and continued concomitantly for 4 weeks. Group VI (silymarin-treated group): Rats with amiodarone induced-pulmonary fibrosis were administered after 2 weeks silymarin in a dose of 100 mg/kg by oral gavage and continued concomitantly for 4 weeks.

2.3. Induction of pulmonary fibrosis:

Pulmonary fibrosis was induced in the rats using amiodarone in a dose of 40 mg/kg daily by oral gavage for 4 weeks (based on a pilot study) in groups II, III, IV and for 6 weeks in groups V and VI.

2.4. Tissue sampling and processing:

At the end of the experiment (at the end of 4th week post-induction for groups I, II, III, IV and at the end of 6th week for groups V, VI) all rats were sacrificed under light general anaesthesia by ether inhalation, the ribs were dissected and thorax was opened longitudinally to expose lungs. Lungs were rinsed 3 times with ice cold saline to remove blood debris.

2.5. Biochemical analysis

3.5.1. **Tissue homogenization**

Right lung was weighted and homogenized with tissue homogenizer for preparations of tissue homogenate in the following ratio (1 lung tissue:10 phosphate buffered saline (PBS) 75mM (pH 7.4). Tissue homogenate was centrifuged at 30,000 rpm for 20 minutes at 4◦C and the resultant supernatant was divided in aliquots and assayed for determination of Transforming growth factor β_1 (TGF- β_1) levels by ELISA, Hydroxyproline levels by ELISA, Tumor necrosis factor-α (TNF- α) levels by ELISA, Spectrophotometric assay of Malondialdehyde (MDA) levels and Spectrophotometric assay of Superoxide dismutase (SOD) activity.

3.5.2. **- Enzyme-linked immunosorbent assay (ELISA) of tissue transforming growth factor β (TGF-β) levels**

Tissue Transforming growth factor β level (pg/gm tissue) was measured by kits obtained from Biodiagnostic Company.(Catalogue No. 201-11-0779).

3.5.3. Enzyme-linked immunosorbent assay (ELISA) of tissue hydroxyproline (Hyp) levels

Tissue Hydroxyproline level (ng/gm tissue)was measured by kits obtained from Biodiagnostic Company, Catalogue No. 201-11-0512 (Reddly and Enwemeka 1996).

3.5.4. Enzyme-linked immunosorbent assay (ELISA) of tissue tumour necrosis factor-alpha (TNF-α) levels

Tissue tumor necrosis factor level (pg/gm tissue) was measured in tissue homogenate by kits obtained from Biodiagnostic Company. Catalogue: ELR-TNF-α).

3.5.5.Determination of lipid peroxidation

Lipid peroxidation was assayed by measuring the levels of malondialdehyde (MDA) (nmol/gm tissue) in lung tissue. MDA level was determined by measuring thiobarbituric reactive species using a kit supplied by Biodiagnostic (Cat. No MD 2529), where the thiobarbituric acid reactive substances react with thiobarbituric acid to produce a pink coloredcomplex.

3.5.6. Determination of tissue superoxide dismutase activity (SOD) activity

Tissue superoxide dismutase activity (SOD) activity (U/gm tissue)was measured by kits obtained from Biodiagnostic Company.This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate - mediated reduction of nitrobluetetra-zoliumdye.

3.5.7.Histological examination of Mallory's trichrome stained sections

The left lung was preserved in formalin 10% embedded in paraffin, and 5 µm sections were stained with Masson trichrome and processed for examination of histopathological changes by light microscope.

3.5.8. Statistical analysis

The pulmonary fibrosis score was assessed using non-parametric statistics, Kruskal–Wallis test statistics. Other data were analyzed for normality of distributions and subjected to One-Way ANOVA followed by Post-Hoc Tukey's multiple comparison test. All analyses were performed using SPSS software (version 23) for windows. The values were expressed as mean ± standard error of mean (SEM). The significance was considered when $P < 0.05$.

4. Results

4.1. Results of lung tissue TGF-β1 levels

The untreated induced-PF (group II) exhibited a significant increase in lung tissue TGF-β1 level in comparison to the normal control group (group I). Prophylaxis by naltrexone (group III) or silymarin (group IV) exhibited significant decrease in lung tissue TGF-β1 level in comparison to untreated induced-PF (group II). There was a non-significant difference in lung tissue TGF-β1 level between prophylaxis of induced-PF by either naltrexone (group III) or silymarin (group IV). Treatment of amiodarone induced-PF by either naltrexone (group V) or silymarin (group VI) exhibited significant decrease in lung tissue TGF-β1 level in comparison to untreated induced-PF (group II). There was a non-significant difference in lung tissue TGF-β1 level between the treatment of induced-PF by either naltrexone (group V) or silymarin (group VI), (table 1&figure 1).

4.2.Results of lung tissue Hydroxyproline levels

In the untreated induced-PF (group II); there was a significant increase in lung tissue hydroxyproline in comparison to the normal control group (group I). Prophylaxis of amiodarone induced-PF by either naltrexone (group III) or silymarin (group IV) exhibited significant decrease in lung tissue hydroxyproline in comparison to untreated induced-PF (group II). There was a non-significant difference in lung tissue hydroxyproline between prophylaxis of induced-PF by either naltrexone (group III) or silymarin (group IV).Treatment by naltrexone (group V) or silymarin (group VI) exhibited significant decrease in lung tissue hydroxyproline in comparison to untreated induced-PF (group II). There was a non-significant difference in lung tissue hydroxyproline between the treatment of induced-PF by either naltrexone (group V) or silymarin (group VI), (table 2&figure 2).

Table (1): Comparative statistics of Lung tissue TGF-β_1 levels (pg/gm tissue) among studied groups.

Groups / Parameter	Group I(Normal control)(n=8)	Group II(Untreated)(n=8)	Group III(Naltrexone protected)(n=8)	Group IV (Silymarin protected)(n=8)	Group V(Naltrexone treated)(n=8)	Group VI(Silymarin treated)(n=8)	One-way ANOVA(P value)
Lung tissue TGF-$\beta_1$level (pg/gm tissue) (mean ± SEM)	66.02± 1.681	114.2± 1.186 **P_1<0.001**	68.59± 2.309 **P_2<0.001**	62.19± 1.700 **P_2<0.001** **P_3>0.05**	78.19± 1.006 **P_2< 0.001**	81.95± 1.299 **P_2< 0.001** **P_4>0.05**	(p<0.001)

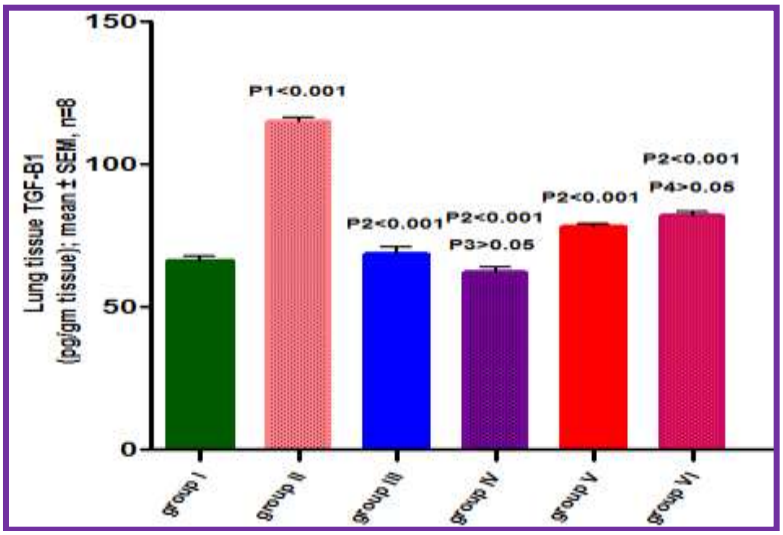

Fig. 1.Lung tissue TGF-β_1 levels (pg/gm tissue) in different studied groups.

Values expressed as mean ± SEM, n=number, Significant at P < 0.05.

TukeytestP$_1$: Untreated induced-PF (group II) versus normal control (group I).

P$_2$: Naltrexone protected (group III), silymarin protected (group IV), naltrexone-treated (group V) andsilymarin treated (group VI), versus untreated induced-PF (group II).

P$_3$: Naltrexone protected (group III) versus silymarin protected (group IV).

P$_4$: Naltrexone treated (group V) versus silymarin treated (group VI).

Table (2): Comparative statistics of Lung tissue hydroxyproline levels (ng/gm tissue) among studied groups.

Groups / Parameter	Group I(Normal control)(n=8)	Group II(Untreated)(n=8)	Group III(Naltrexone protected)(n=8)	Group IV (Silymarin protected)(n=8)	Group V(Naltrexone treated)(n=8)	Group VI(Silymarin treated)(n=8)	One-way ANOVA(P value)
Lung tissue hydroxyproline (ng/gm tissue) (mean ± SEM)	41.94± 0.453	158.5± 2.462 **P_1<0.001**	69.86± 0.832 **P_2<0.001**	71.23± 1.385 **P_2<0.001** **P_3>0.05**	75.96± 0.607 **P_2< 0.001**	73.03± 1.013 **P_2< 0.001** **P_4>0.05**	(P<0.001)

4.3.Results of lung tissue TNF-α levels

The untreated induced-PF (group II) exhibited a significant increase in lung tissue TNF-α level in comparison to the normal control group (group I). Prophylaxis by naltrexone (group III) or silymarin (group IV) exhibited significant decrease in TNF-α level lung tissue in comparison to untreated induced-PF (group II). There was a non-significant difference in lung tissue TNF-α level between prophylaxis of induced-PF by either naltrexone (group III) or silymarin (group IV).Treatment of amiodarone induced-PF by either naltrexone (group V) or silymarin (group VI) exhibited significant decrease in lung tissue TNF-α level in comparison to untreated induced-PF (group II).There was non-significant difference in lung tissue TNF-α level between treatment of induced-PF by either naltrexone (group V) or silymarin (group VI), (table 3&figure 3).

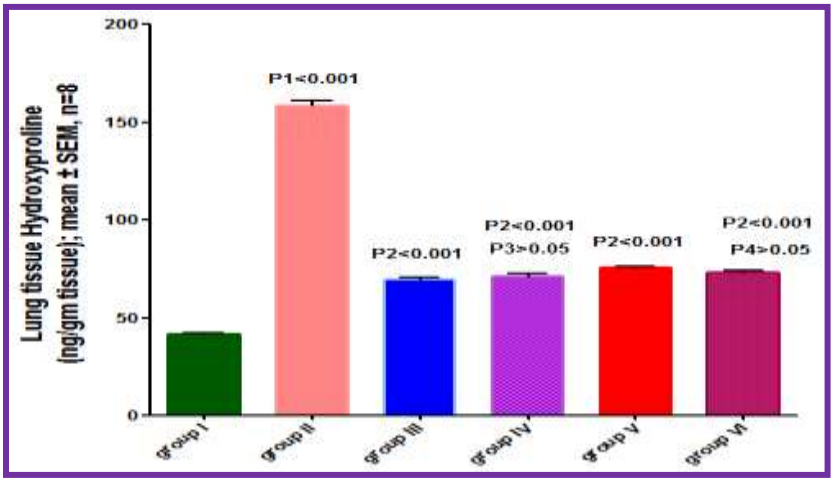

Fig. 2. Lung tissue Hydroxyproline (ng/gm tissue)levels in different studied groups.

Values expressed as mean ± SEM, n=number, Significant at P < 0.05.

Tukey test.P_1: Untreated induced-PF (group II) versus normal control (group I).

P_2: Naltrexone protected (group III), silymarin protected (group IV), naltrexone-treated (group V) andsilymarin treated (group VI), versus untreated induced-PF (group II).

P_3: Naltrexone protected (group III) versus silymarin protected (group IV).

P_4: Naltrexone treated (group V) versus silymarin treated (group VI).

Table (3): Comparative statistics of Lung tissue TNF-α levels (pg/gm tissue) among studied groups.

Groups / Parameter	Group I(Normal control)(n=8)	Group II(Untreated)(n=8)	Group III(Naltrexone protected)(n=8)	Group IV (Silymarin protected)(n=8)	Group V(Naltrexone treated)(n=8)	Group VI(Silymarin treated)(n=8)	One-way ANOVA(P value)
Lung tissueTNF-α level(pg/gm tissue) (mean ± SEM)	145.7 ±1.361	435.2 ± 1.014 **P_1<0.001**	172.8± 0.844 **P_2<0.001**	174.9± 0.547 **P_2<0.001 P_3>0.05**	201.1 ± 0.788 **P_2< 0.001**	196.8 ± 0.801 **P_2< 0.001 P_4>0.05**	(p<0.001)

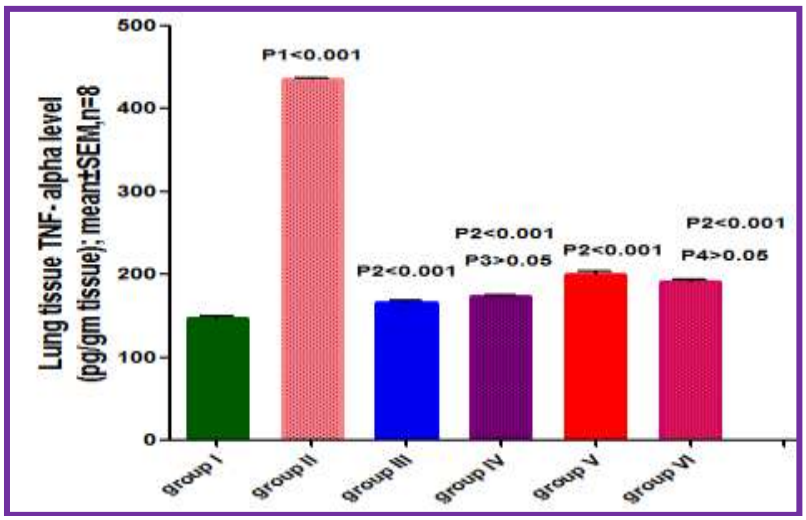

Fig.3. Lung tissue TNF-α levels (pg/gm tissue)in different studied groups.

Values expressed as mean ± SEM, n=number, Significant at P < 0.05.

Tukey test.P_1: Untreated induced-PF (group II) versus normal control (group I).

P_2: Naltrexone protected (group III), silymarin protected (group IV), naltrexone-treated (group V) andsilymarin treated (group VI), versus untreated induced-PF (group II).

P_3: Naltrexone protected (group III) versus silymarin protected (group IV).

P_4: Naltrexone treated (group V) versus silymarin treated (group VI).

4.4.Results of lung tissue MDA levels

The untreated induced-PF (group II) exhibited a significant increase in lung tissue MDA level in comparison to the normal control group (group I). Prophylaxis by naltrexone (group III) or silymarin (group IV) showed a significant decrease in lung tissue MDA activity in comparison to untreated induced-PF (group II).There was a non-significant difference in lung tissue MDA level between prophylaxis of induced-PF by either naltrexone (group III) or silymarin (group IV). Treatment of amiodarone induced-PF by either naltrexone (group V) or silymarin (group VI) exhibited significant decrease in lung tissue MDA levelin comparison to untreated induced-PF (group II).There was a non-significant difference in lung tissue MDA level between the treatment of induced-PF by either naltrexone (group V) or silymarin (group VI) (table 4&figure 4).

Table (4): Comparative statistics of Lung tissue MDA levels (nmol/gm tissue) among studied groups.

Groups / Parameter	Group I(Normal control)(n=8)	Group II(Untreated)(n=8)	Group III(Naltrexone protected)(n=8)	Group IV (Silymarin protected)(n=8)	Group V(Naltrexone treated)(n=8)	Group VI(Silymarin treated)(n=8)	One-way ANOVA(P value)
Lung tissue MDA level(nmol/gm tissue) (mean ± SEM)	15.59 ±1.557	48.58 ± 8.251 **P_1<0.001**	23.75± 3.421 **P_2<0.001**	19.68± 3.320 **P_2<0.001 P_3>0.05**	19.66 ± 2.173 **P_2< 0.001**	19.17 ± 5.137 **P_2< 0.001 P_4>0.05**	(p<0.001)

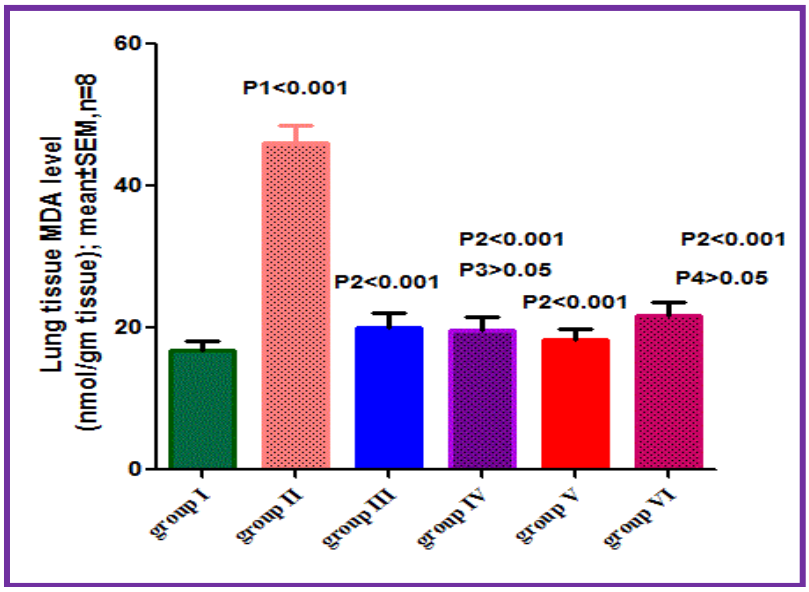

Fig.4. Lung tissue MDA levels (nmol/gm tissue) in different studied groups.

Values expressed as mean ± SEM, n=number, Significant at P < 0.05.

Tukeytest.P_1: Untreated induced-PF (group II) versus normal control (group I).

P_2: Naltrexone protected (group III), silymarin protected (group IV), naltrexone-treated (group V) andsilymarin treated (group VI), versus untreated induced-PF (group II).

P_3: Naltrexone protected (group III) versus silymarin protected (group IV).

P_4: Naltrexone treated (group V) versus silymarin treated (group VI).

4.5.Results of lung tissue SOD activity

In the untreated induced-PF (group II); there was a significant decrease in lung tissue SOD activity in comparison to the normal control group (group I). Prophylaxis by naltrexone (group III) or silymarin (group IV) exhibited significant increase in lung tissue SOD activity in comparison to untreated induced-PF (group II).There was a non-significant difference in lung tissue SOD activity between prophylaxis of induced-PF by either naltrexone (group III) or silymarin (group IV).Treatment of amiodarone induced-PF by either naltrexone (group V) or silymarin (group VI) exhibited significant increase in lung tissue SOD activity in comparison to untreated induced-PF (group II).There was a significant difference in lung tissue SOD activity between the treatment of induced-PF by either naltrexone (group V) or silymarin (group VI), (table 5&figure 5).

Table (5): Comparative statistics of Lung tissue SOD activity (U/gm tissue) among studied groups.

Groups / Parameter	Group I(Normal control)(n=8)	Group II(Untreated)(n=8)	Group III(Naltrexone protected)(n=8)	Group IV(Silymarin protected)(n=8)	Group V(Naltrexone treated)(n=8)	Group VI(Silymarin treated)(n=8)	One-way ANOVA(P value)
Lung tissueSOD activity(U/gm tissue) (mean ± SEM)	601.9 ±0.936	269.4 ± 1.636 **P_1<0.001**	313.2± 1.682 **P_2<0.01**	304.1± 1.348 **P_2<0.01** **P_3>0.05**	361.7±4.604 **P_2< 0.001**	452.7 ± 7.582 **P_2< 0.001** **P_4<0.01**	(p<0.001)

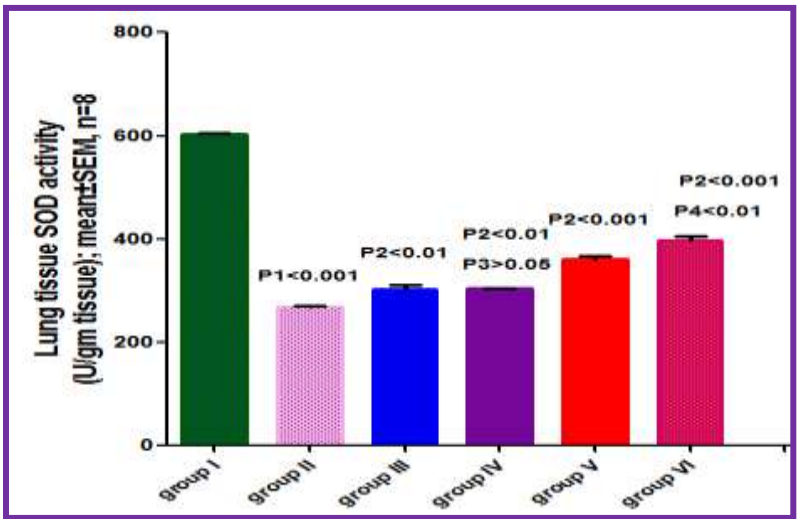

Fig.5. Lung tissue SOD activity (U/gm tissue) in different studied groups.

Values expressed as mean ± SEM, n=number, Significant at P < 0.05.

Tukeytest.P_1: Untreated induced-PF (group II) versus normal control (group I).

P_2: Naltrexone protected (group III), silymarin protected (group IV), naltrexone-treated (group V) andsilymarin treated (group VI), versus untreated induced-PF (group II).

P_3: Naltrexone protected (group III) versus silymarin protected (group IV).

P_4: Naltrexone treated (group V) versus silymarin treated (group VI).

4.6. Histopathological scoring of pulmonary fibrosis

In the untreated induced-PF (group II); there was a significant increase in fibrosis score compared to the normal control group.Prophylaxis of amiodarone induced-PF by either naltrexone (group III), silymarin (group IV) exhibited a significant decrease in fibrosis score compared to untreated induced-PF (group II).Treatment of amiodarone induced-PF by either naltrexone (group V) or silymarin (group VI) exhibited a non-significant decrease in fibrosis score compared to untreated induced-PF (group II).There was a non-significant difference in fibrosis score between prophylaxis and treatment of induced-PF by either naltrexone (group III&IV) or silymarin (group V&VI), (table 6&figure 6).

Table (6): Lung fibrosis score in different studied groups.

Groups / Parameter	Group I(Normal control)(n=8)	Group II(Diseased-Untreated)(n=8)	Group III(Naltrexone protected)(n=8)	Group IV(Silymarin protected)(n=8)	Group V(Naltrexone treated)(n=8)	Group VI(Silymarin treated)(n=8)	#Kruskal-Wallis Test X^2 value(P value)
Pulmonary fibrosis scoring (median) (mean ± SEM)	0	7 **P_1<0.001**	2.5 **P_2<0.01**	2 **P_2<0.01** **P_3>0.05**	4.5 **P_2<0.01**	4 **P_2<0.01** **P_4>0.05**	42.16 **P<0.001**

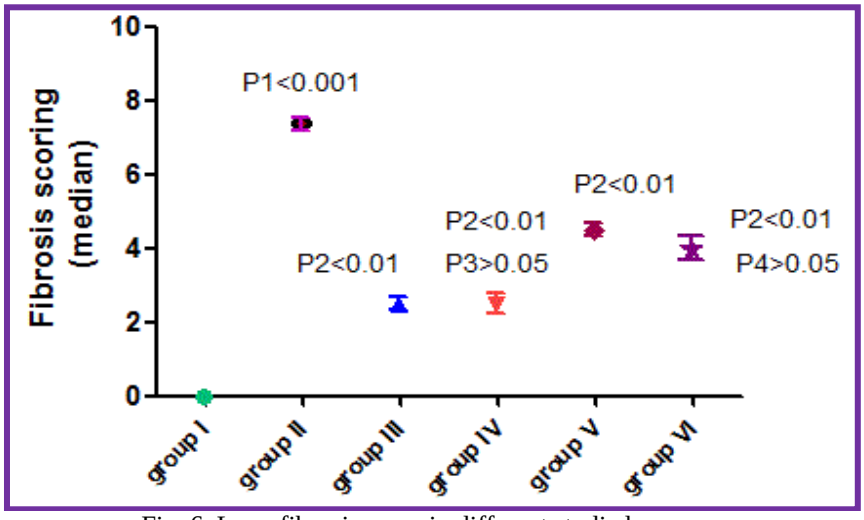

Fig. 6. Lung fibrosis score in different studied groups.

Values expressed as median, n=number, Significant at P < 0.05.

Non-parametric statistics, Kruskal–Wallis test

P_1: Untreated induced-PF (group II) versus normal control (group I).

P_2: Naltrexone protected (group III), silymarin protected (group IV), naltrexone-treated (group V) andsilymarin treated (group VI), versus untreated induced-PF (group II).

P_3: Naltrexone protected (group III) versus silymarin protected (group IV).

P_4: Naltrexone treated (group V) versus silymarin treated (group VI).

4.7.Histopathological examination of stained sections (Mallory's trichrome stain) of the lung tissue

Lung sections from all animals of control group showed normal lung architecture regarding alveolar walls, normal interalveolar spaces (regarding blood capillaries and connective tissue), normal bronchi and bronchioles and peri-bronchial tissue (figure 7). Histopathological examination of lung sections from untreated induced-PF group showed severe dense interstitial inflammatory cellular infiltration with mononuclear cells and widened interalveolar shape associated with vascular congestion, perivascular edema, and hyalinization with inflammatory and peribronchiolar inflammatory infiltration (figure 8,9). Lung sections of naltrexone and silymarin received groups showed a good response in the form of mild to moderate interstitial and interalveolar cellular infiltration with reserved alveolar shapes and architecture (figures 10,12). Lung sections of silymarin received groups showed a good response in the form of mild to moderate collagen deposition interstitial and interalveolar cellular infiltration with reserved alveolar shapes and architecture (figures 11,13).

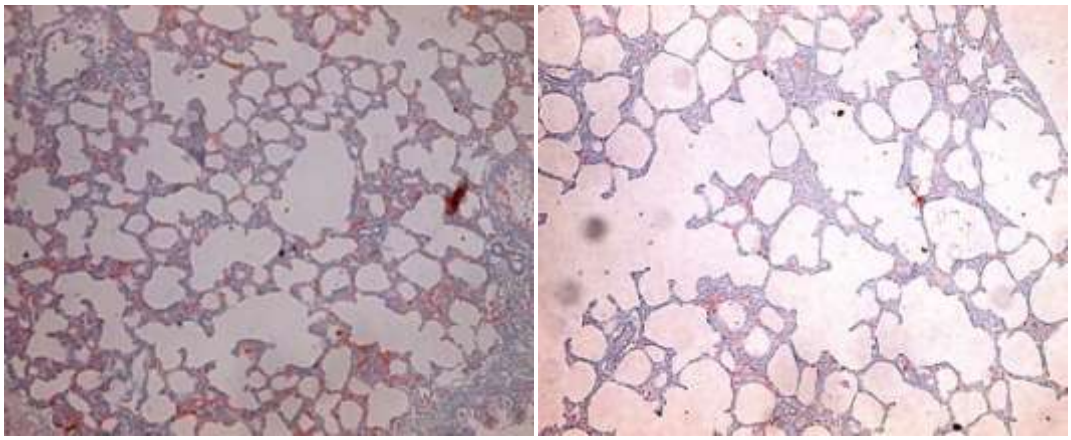

Fig.7: Photomicrograph of section of control group (group I) showing normal lung architecture with thin interalveolar septa,notice few apparent minimal collagen fibers (blue color) in the interstitium. Mallory's Trichrome stain x 400.

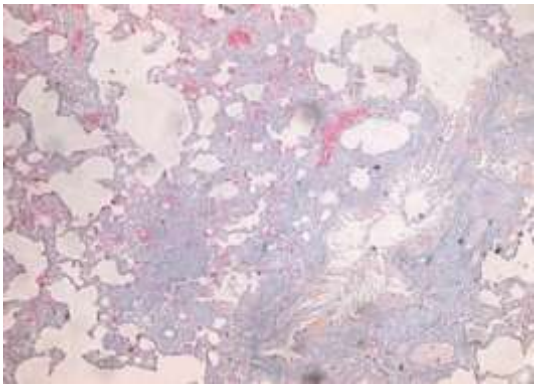

Fig.8: Photomicrograph of section of diseased untreated group (group II) showing increased deposition of collagen fibers (blue color) around a large bronchiole with thickened interalveolar septum (increased deposition of collagen). Mallory's Trichrome stain x 400.

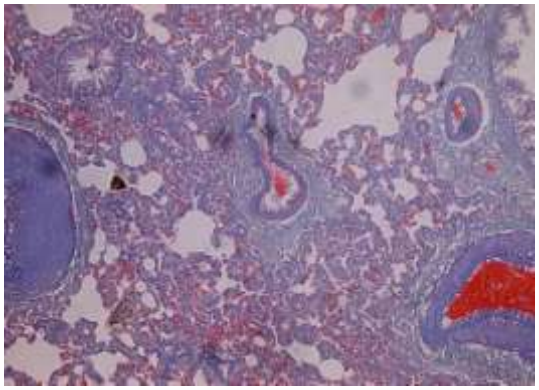

Fig.9: Photomicrograph of section of diseased untreated group (group II) showing massive increased deposition of collagen fibers (blue color) around alveoli, bronchioles and blood vessels of the lung. Mallory's Trichrome stain x 400.

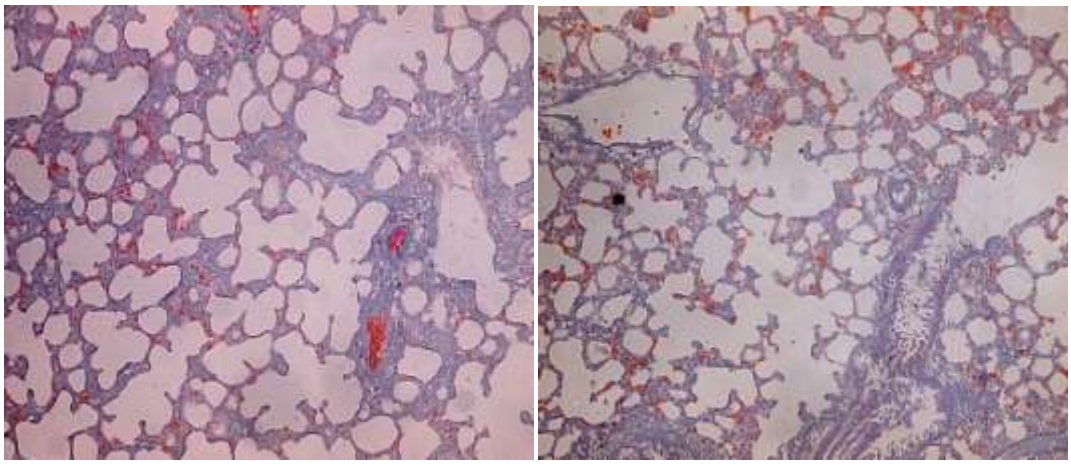

Fig.10: Photomicrograph of section of naltrexone protected group (group III) showing mild deposition of collagen fibers (blue color) with mild interstitial and interalveolar cellular infiltration. Mallory's Trichrome stain x 400.

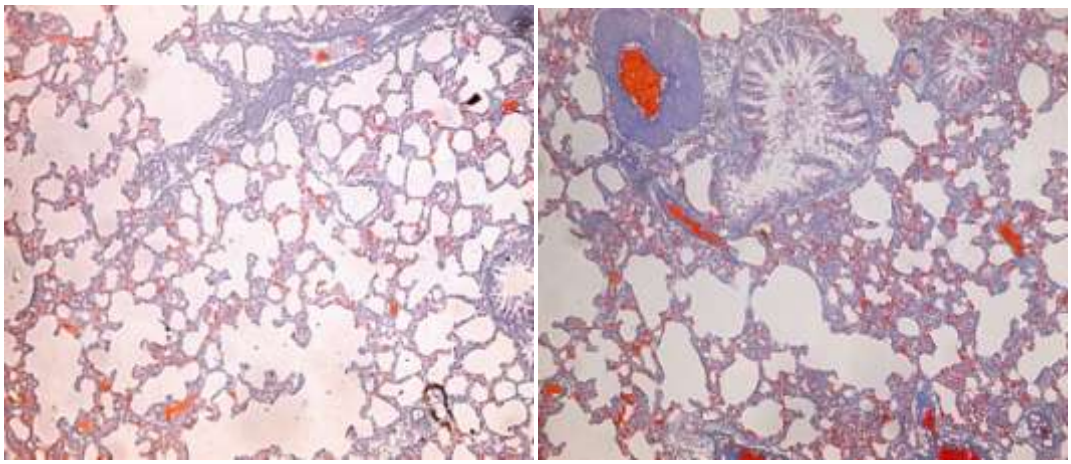

Fig.11: Photomicrograph of section of silymarin protected group (group IV) showing mild deposition of collagen fibers (blue color) with mild interstitial and interalveolar cellular infiltration. Mallory's Trichrome stain x 400.

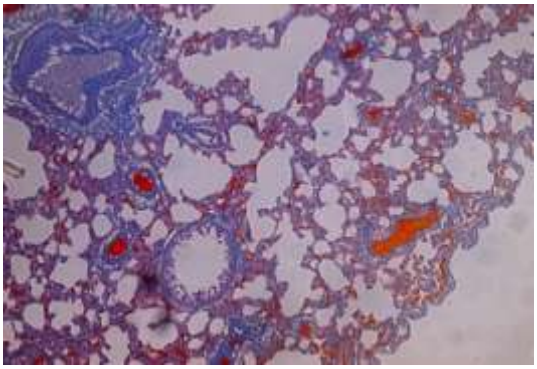

Fig.12: Photomicrograph of section of naltrexone treated group (group V) showing moderate deposition of collagen fibers (blue color) with mild interstitial and interalveolar cellular infiltration. Mallory's Trichrome stain x 400.

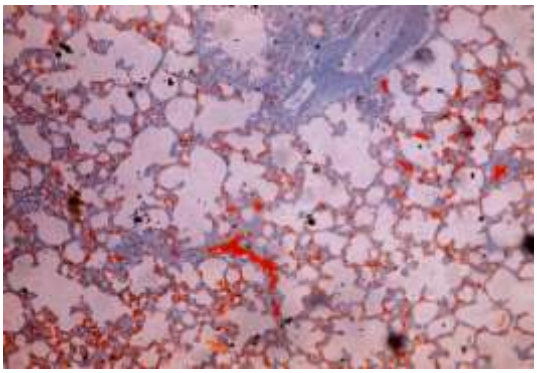

Fig.13: Photomicrograph of section of silymarin treated group (group VI) showing moderate deposition of collagen fibers (blue color) with mild interstitial and inter-alveolar cellular infiltration. Mallory's Trichrome stain x 400.

4. Discussion

This experiment was designed was to evaluate the possible anti-fibrotic, anti-inflammatory and anti-oxidant effects of naltrexone and silymarin in amiodarone induced-PF in albino rats.In the present study, the induction of PF by amiodarone showed a significant increase in transforming growth factor-β1 (TGF- β1) and tumor necrosis factor-α (TNF-α) levels in lung tissue when compared to the normal control group. These results denoting the implication of pro-inflammatory cytokines in the pathogenesis of pulmonary fibrosis. Many other studies reported most of these findings produced by amiodarone induced-PF (Zaeemzadeh et al., 2011;Sharaf El-Din and Abd Allah, 2016;Al-Shammari et al., 2016; Nasri et al., 2016; Cheng et al., 2017).

It is well known that there is a crosstalk between lipid peroxidation products and the induced over expression of fibrogenic cytokines as well as increased transcription and synthesis of collagen (El-Mohandes et al., 2017). Much of the recent mechanistic work regarding oxidative mechanisms in PF has centered around the two-way interplay between TGF-β1 and ROS mediated processes. ROS have been shown to activate latent TGF-β1 and TGF-β1 increases the production of ROS in human lung fibroblasts (Todd et al., 2012).The results obtained in the present work provide the evidence for the contribution of oxidative stress in induction of PF that presented as increased MDA and decreased SOD levels in lung tissue; respectively.

In the present experiment, either protection or treatment by silymarin showed a significant decrease in hydroxyproline, TNF-α, TGF-β and MDA levels and a significant increase in SOD activity in lung tissue when compared to the untreated induced-PF group. These results were in agreement with histopathological examination of lung sections which showed restoration of part of the normal pattern of alveoli, minimal infiltration of inter-alveolar and

interbronchiolar spaces with inflammatory cells and minimal collagen deposition. The established PF induced by amiodarone has been assayed as an increased hydroxyproline levels in lung tissue and further confirmed by the high scoring of histopathological changes in lung sections**.**

Son et al. (2015) showed that silymarin treatment significantly reduced inflammation and fibrosis in lung tissue which detected by reduction of the number of inflammatory cells in the bronchoalveolar lavage fluid and decreasing inflammatory cell infiltration in the respiratory tract. Al-Shammari et al. (2016) showed that SOD activity was significantly decreased in lung tissues of amiodarone-induced PF group and stated that the alteration in SOD activity produced by administration of amiodarone for three weeks is indicative of changes in the handling of reactive oxygen species.

Regarding histopathological findings, the present study confirmed that amiodarone-induced PF group showed marked distorted lung architecture and thickened interalveolar septa and peri-bronchial inflammatory cellular infiltration with the formation of large emphysematous spaces. These findings were in agreement with Zickri et al. (2014) who observed similar findings as thickening of the inter-alveolar septa and dense cellular infiltration and extravasated RBCs. Moreover, Mahavadi et al. (2014) confirmed the thickening of alveolar septa with patchy fibrosis and cellular infiltration. In addition, these findings are matched with Nasri et al., (2016)&Naglaa and Mona, (2013) who mentioned marked inflammatory polymorph cell infiltration mainly lymphocytes and macrophages and interstitial fibrosis with focal areas of obvious congestion of the pulmonary vessels.

5. Conclusion

The current study had attempted to show the potential role of naltrexone and silymarin when given either for protection or treatment in PF induced by amiodarone. Naltrexone and silymarin have a preventive and curative role on the fibrosis process which evidenced by decreasing growth factors, cytokines, and inflammatory cells. Also, they have a role as antioxidant denoted by the increase of SOD activity, in addition to the improvement in the histologic morphology and fibrosis score of the lung.

As a conclusion either naltrexone or silymarin exhibited a promising role in amiodarone-induced pulmonary fibrosis and could be introduced as new therapeutic approaches that have to be investigated for protection or treatment of different types and models of PF. Moreover, the combination of naltrexone and silymarin necessitates to be investigated to evaluate if they could provide more effectiveness.

6. Acknowledgement

Special thanks to Prof. Dr. Karima El-Desoky Professor of Histopathology, Faculty of Medicine, Tanta University, and Prof.Dr. GehanSoliman, Professor of Histology, Faculty of Medicine, Tanta University,for their cooperation in carrying out the histopathological examination and helping us in photo-imaging and in finalizing this work.

References

1. Abdel-Moneim AM, Al-Kahtani MA, El-Kersh MA and Al-Omair MA. (2015): Free Radical Scavenging, Antiinflammatory/Antifibrotic and Hepatoprotective Actions of Taurine and Silymarin against CCl4 Induced Rat Liver Damage. PLoS One.;10(12):e0144509.
2. Agelaki MG, Pantos P, Korantzopoulos DG, Tsalikakis GG, Baltogiannis A, Fotopoulos and Kolettis TM. (2007): Comparative antiarrhythmic efficacy of amiodarone and dronedarone during acute myocardial infarction in rats. Eur. J. Pharmacol.;564: 150-57.
3. Ahn JY, Kim MH, Lim MJ, Park S, Lee SL, Yun YS and Song JY. (2011): The inhibitory effect of ginsan on TGF-beta mediated fibrotic process. J Cell Physiol 226, 1241-1247.
4. Al-Shammari B, Khalifa M, Bakheet SA and Yasser M. (2016): A Mechanistic Study on the Amiodarone Induced Pulmonary Toxicity. Oxid Med Cell Longev.;2016:6265853.
5. Arizmendi N, Puttagunta L, Chung KL, Davidson C, Rey-Parra J and Chao DV. (2014): Rac2 is involved in bleomycin-induced lung inflammation leading to pulmonary fibrosis. Respir Res.;15(71)15-71.
6. Baddini-Martinez J, Baldi BG, Costa CH, Jezler S, Lima MS and Rufino R. (2015): Update on diagnosis and treatment of idiopathic pulmonary fibrosis. J Bras Pneumol.; 41 (5):454-466.
7. Bihari B (2013): Bernard Bihari, MD: low-dose naltrexone for normalizing immune system function. Altern Ther Health Med.;19 (2):56–65.
8. Cheng L, Li R, Zhou M, Li F, Chang Q, Li C and Lu X. (2017): Moxibustion has a positive effect on pulmonary fibrosis: an alternative approach. Afr J Tradit Complement Altern Med.;14(2):125-9.
9. Cohen-Naftaly M and Friedman SL. (2011): Current status of novelantifibrotic therapies in patients with chronic liver disease. Therap Adv Gastroenterol.;4 (6):391-417.
10. De Minicis S, Candelaresi C, Marzioni M, Saccomano S, Roskams T, Casini A, Risaliti A, Salzano R, Cautero N, di Francesco F, Benedetti A and Svegliati-Baroni G. (2008): Role of endogenous opioids in modulating HSC activity in vitro and liver fibrosis in vivo. Gut.;57(3):352-364.
11. Ebrahimkhani MR, Kiani S, Oakley F, Kendall T, Shariftabrizi A, Tavangar SM, Moezi L, Payabvash S, Karoon A, Hoseininik H, Mann DA, Moore KP, Mani AR and Dehpour AR. (2006): Naltrexone, an opioid receptor antagonist, attenuates liver fibrosis in bile duct ligated rats. Gut.;55 (11): 1606-1616.
12. Ebrahimkhani MR, Moezi L, Kiani S, Merat S and Dehpour AR. (2008): Opioid Receptor Blockade Improves Mesenteric Responsiveness in Biliary Cirrhosis.;53 (11):3007-3011.
13. El-Mohandes EM, Moustafa AM, Khalaf HA and Hassan YF. (2017): The role of mast cells and macrophages in

amiodarone-induced pulmonary fibrosis and the possible attenuating role of atorvastatin. Biotech Histochem.;92(7):467-80.
14. Fernandez IE and Eickelberg O. (2012): The impact of TGF-beta on lung fibrosis: from targeting to biomarkers. Proc Am Thorac Soc.; 9(3):111-116.
15. Hudzik B and Polonski L. (2012): Amiodarone-induced pulmonary toxicity. CMAJ.; 184(15): E819.
16. Joukar S, Zarisfi Z, Sepehri G and Bashiri A. (2014): Efficacy of Melissa officinalis in suppressing ventricular arrhythmias following ischemia-reperfusion of the heart: a comparison with amiodarone. Med Princ Pract.; 23: 340–345.
17. Kalueff AV. (2016): Commentary: Supplier dependent differences in intermittent voluntary alcohol intake and response to naltrexone in Wistar rats. Front Neurosci.;10:82.
18. Kosmidis C, Newton P, Muldoon EG and Denning DW. (2016): Chronic fibrosing pulmonary aspergillosis: a cause of 'destroyed lung' syndrome. Infect Dis.:1-6.
19. Lafuente-Lafuente C, Alvarez J C, Leenhardt A, Mouly S, Extramiana F, Caulin C, Funck-Brentano C and Bergmann J F. (2009): Amiodarone Concentrations in Plasma and Fat Tissue During Chronic Treatment and Related Toxicity. Br J Clin Pharmacol.; 67:511-519.
20. Li X, Shen Y, Lu Y and Yang J. (2015): Amelioration of Bleomycin induced Pulmonary Fibrosis of Rats by an Aldose Reductase Inhibitor, Epalrestat. Korean J Physiol Pharmacol.; 19 (5):401-411.
21. Mahavadi P, Henneke I, Ruppert C, Knudsen L, Venkatesan S, Liebisch G, Chambers RC, Ochs M, Schmitz G, Vancheri C, Seeger W, Korfei M and Guenther A. (2014): Altered surfactant homeostasis and alveolar epithelial cell stress in amiodarone-induced lung fibrosis. Toxicol Sci.; 142(1):285-297.
22. Mata-Santos HA, Dutra FF, Rocha CC, Lino FG, Xavier FR, Chinalia LA, Hossy BH, Castelo-Branco MT, Teodoro AJ, Paiva CN and dos Santos Pyrrho A. (2014): Silymarin reduces profibrogenic cytokines and reverses hepatic fibrosis in chronic murine schistosomiasis. Antimicrob Agents Chemother.; 58(4): 2076-2083.
23. Molyneaux PL and Maher TM. (2013): The role of infection in the pathogenesis of idiopathic pulmonary fibrosis. Eur Respir Rev.;22 (129):376-381.
24. Naglaa K and Mona A. (2013): Amelioration of amiodarone-induced lung fibrosis in rats by grape seed extract. Journal of Applied Sciences Research.;9(6): 3698-707.
25. Nasri HR, Joukar S, Kheradmand H, Poursalehi HR and Dabiri S. (2015): Coadministration of atorvastatin and amiodarone increases the risk of pulmonary fibrosis in rats. Med Princ Pract.;25(2):150-154.
26. Nicolescu AC, Comeau J L, Hill B C, Bedard L L, Takahashi T, Brien J F, Racz W J and Massey T E. (2007): Aryl Radical Involvement in Amiodarone-Induced Pulmonary Toxicity: Investigation of Protection by Spin-Trapping Nitrones. Toxicol Appl Pharmacol.; 220: 60-71.
27. Nicolescu AC, Ji Y, Comeau J L, Hill B C, Takahashi T, Brien J F, Racz W J and Massey T E. (2008): Direct Mitochondrial Dysfunction Precedes Reactive Oxygen Species Production in Amiodarone-Induced Toxicity in Human Peripheral Lung Epithelial HPL1A Cells. Toxicol Appl Pharmacol.; 227: 370-379.
28. Razavi-Azarkhiavi K, Ali-Omrani M, Solgi R, Bagheri P, Haji-Noormohammadi M, Amani N and Sepand MR. (2014): Silymarin alleviates bleomycin induced pulmonary toxicity and lipid peroxidation in mice. Pharm Biol.;52 (10):1267-1271.
29. Reddy GK and Enwemeka CS. (1996): A simplified method for the analysis of hydroxyproline in biological tissues. Clin Biochem.;29(3):225-29.
30. Shaker ME, Zalata KR, Mehal WZ, Shiha GE and Ibrahim TM. (2011): Comparison of imatinib, nilotinib and silymarin inthe treatment of carbon tetrachloride-induced hepatic oxidative stress, injury and fibrosis. Toxicol Appl Pharmacol.;252(2):165-175.
31. Sharaf El-Din AA and Abd Allah OM. (2016): Impact of olmesartan, medoxomil on amiodarone-induced pulmonary toxicity in rats: Focus on transforming growth factor-β1. Basic ClinPharmacol Toxicol.;119(1):58-67.
32. Son Y, Lee HJ, Rho JK, Chung SY, Lee CG, Yang K, Kim SH, Lee M, Shin IS and Kim JS. (2015): The ameliorative effect of silibinin against radiation-induced lung injury: protection of normal tissue without decreasing therapeutic efficacy in lung cancer. BMC Pulm Med.5;15:68.
33. Sushchyk S, Xi ZX and Wang JB. (2016): Combination of levotetrahydropalmatine and low dose naltrexone: A promising treatment for prevention of cocaine relapse. J Pharmacol Exp Ther.;357(2):248-57.
34. Todd N, Luzina I and Atamas S. (2012): Molecular and cellular mechanisms of pulmonary fibrosis. Fibrogenesis Tissue Repair.;5(1):11.
35. Vargas-Mendoza N, Madrigal-Santillan E, Morales-Gonzalez A, Esquivel-Soto J, Esquivel-Chirino C, Garcia-Luna Y Gonzalez-Rubio M, Gayosso-de-Lucio JA and Morales-Gonzalez JA. (2014): Hepatoprotective effect of silymarin. World J Hepatol.;6(3):144-49.
36. Younger J, Parkitny L and McLain D. (2014): The use of low-dose naltrexone (LDN) as a novel anti-inflammatory treatment for chronic pain. Clin Rheumatol.;33 (4):451-459.
37. Zaeemzadeh N, Hemmati A, Arzi A, Jalali M and Rashidi I. (2011): Protective effect of caffeic acid phenethyl ester (CAPE) on amiodarone-induced pulmonary fibrosis in rat. Iran J Pharm Res.;10(2):321-28.
38. Zickri MB, Fadl SG and Metwally HG. (2014): Comparative study between intravenous and intraperitoneal stem cell therapy in amiodarone induced lung injury in rat. Int J Stem Cells.;7 (1): 1-11.

12/17/2019