Evaluation of Nicorandil in Treatment of induced pulmonary arterial hypertension in male Rats

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Abstract :

Pulmonary arterial hypertension (PAH) is a chronic, rare, and non-treatable disease, resulting in elevated mean arterial pressure (≥25mmHg) during rest and (≥30mmHg) during exercise. Pulmonary arteries remodeling including endothelial apoptosis, smooth muscle hyperplasia, and endothelial dysfunction are distinct features of PAH. This study aims to evaluate effect of nicorandil as an alternative treatment for PAH in comparison to tadalafil by evaluating its anti-inflammatory effect and histopathological changes. A total of 60 male wistar rats were divided to 6 groups, a control healthy group, and another 5 groups injected with monocrotaline to induce PAH. The induction group was left untreated while the other 4 groups were treated with either nicorandil or tadalafil, with or without treatment blockers (N-Nitroarginine methyl ester and glimepiride), after 21 days they were sacrificed for histopathology and measurement of inflammatory markers. Nicorandil reduced the levels of osteopontin, and cardiac marker brain natriuretic peptide (BNP) significantly ($P \leq 0.05$), also it showed an improved histopathological picture of PAH by reducing smooth muscle proliferation, necrosis, and inflammation in pulmonary arteries. In conclusion, nicorandil in this study showed promising results in reducing inflammation and improving endothelial function.

Key words: Nicorandil, tadalafil, Brain natriuretic peptide, Pulmonary arterial hypertension.

تقييم عقار النيكوراندیل في علاج مرض ارتفاع ضغط دم الشريان الرئوي المستحث في ذكور الجرذان

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الخلاصة:

ارتفاع ضغط الدم الشرياني الرئوي عبارة عن مرض مزمن ونادر وغير قابل للشفاء، مما يؤدي إلى ارتفاع متوسط الضغط الشرياني (≤25 ملم زئبقي) أثناء الراحة و (≤30 ملم زئبقي) أثناء الجهد. يعتبر إعادة تشكل الشرايين الرئوية **(remodeling)**، بما في ذلك موت الخلايا المبرمج البطانة الاوعية**(apoptosis)** ، وتضخم العضلات الملساء، والخلل في عمل بطانة الاوعية**(endothelial dysfunction)** ، من السمات المميزة للمرض. تهدف هذه الدراسة إلى تقييم تأثير نيكوراندیل كعلاج بديل لارتفاع ضغط الدم الشرياني الرئوي بالمقارنة مع تادالافيل من خلال تقييم تأثيره المضاد للالتهابات والتغيرات النسيجية المرضية. تم تقسيم 60 من جرذان ويستار الذكور إلى 6 مجموعات، مجموعة صحية ضابطة، و5 مجموعات أخرى تم حقنها بمادة المونوكروتالين لتحفيز المرض. تُركت المجموعة التحريضية دون علاج بينما عولجت المجموعات الأربع الأخرى إما بالنيكوراندیل أو التادالافيل، مع أو بدون حاصرات العلاج (**N-Nitroarginine methyl ester** و**glimepride**)، وبعد 21 يومًا تمت التضحية بهم من أجل التشريح النسيجي وقياس علامات الالتهاب. قلل النيكوراندیل من الأوستيوبونتين، وببتيد مدر الصوديوم الدماغي (**BNP**) بشكل ملحوظ (***p*<0.05**)، كما أظهر صورة نسيجية مرضية محسنة للمرض عن طريق تقليل تكاثر العضلات الملساء، موت خلايا البطانة الوعائية والالتهاب في

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الشرايين الرئوية. نستنتج ان النيكوراندیل في هذه الدراسة أظهر نتائج واعدة في تقليل الالتهاب وتحسين وظيفة بطانة الأوعية الدموية الرئوية.

الكلمات المفتاحية: نيكوراندیل, تادالافيل, ببتيد مدر الصوديوم الدماغي, ارتفاع ضغط الشريان الرئوي.

Introduction

Pulmonary arterial hypertension (PAH) is distinguished by pulmonary vasculopathy and pulmonary arterial pressure elevation, the mean pulmonary arterial pressure ≥ 25mm Hg during resting and ≥ 30mm Hg during exercise. It is a progressive, chronic, and deadly disease of the pulmonary arteries characterized by extensive pulmonary arteries constriction accompanied by vascular endothelial and smooth muscle changes, and an increase in mean pulmonary arterial pressure above 25mm Hg, however; PAH is considered a rare disease.[1,2] The tunica intima, tunica media, and tunica adventitia of the pulmonary arteries thicken as a result of remodelling. The development of concentric and plexiform lesions, fibrosis of intima, arterial lumen constriction and ultimately blockage are all indicators of disease progression.[3] The pathophysiological pathways involved in the development of PAH are: nitric oxide (NO), prostacyclin (PGI2), thromboxane A2 (TXA2), and endothelin-1 (ET-1). The production of PGI2 is decreased due to an imbalance in cyclooxygenase-2, eNOS dysfunction, and the vasoconstrictive and mitogenic effects of an increased endothelin-1 signaling pathway. Inflammatory marker osteopontin was shown to be elevated in PAH. Osteopontin (OPN) has been demonstrated to play a significant role in the growth of pulmonary arterial smooth muscle cells and pulmonary adventitial fibroblasts in hypoxic PAH. Nicorandil acts by two mechanisms, firstly as a NO donator, and the second as K^+-ATP channel opener, resulting in vascular dilation, also it shows superiority in preventing cardiac cells apoptosis through K-ATP channel opening.[4,5] In a recent study, it was found that the activation of K^+-ATP channels in monocrotaline-induced PAH in rats resulted in the improvement of PAH treatment as a preventive and curative option.[6] Tadalafil a standard treatment in PAH act as a phosphodiesterase 5 inhibitor preventing the conversion of cAMP to AMP, thus potentiating the effect of nitric oxide, which result in dilation of the vessels and reduction of mPAP. N-Nitroarginine methyl ester (L-NAME) act as nitric oxide synthase inhibitor and shown to elevate mPAP significantly by increasing vascular resistance, while glimepiride act as potassium channel inhibitor by acting at SUR1 subunit of the potassium channel preventing the opening of the channel. [6]
This study aim to assess the activity of nicorandil in reversing monocrotaline-induced PAH and remodeling.

Method

Monocrotaline was used to induce PAH in rats, and then treated with either nicorandil or tadalafil, with or without treatment blockers. N-Nitroarginine methyl ester (L-NAME) from Baoji Guokang Bio-technology – China. Monocrotaline (MCT) from Baoji Guokang Bio-technology– China. Nicorandil from Baoji Guokang Bio-technology – China. Tadalafil from Baoji Guokang Bio-technology – China. Xylazine from Alfasan- Netherland. For western blot technique, detection process done using western blot detection kit-Elabscience (E-IR-R304A), Rat Brain Natriuretic Peptide (BNP) ELISA Kit Enzyme-Linked Immunosorbent Assay (MBS2021774) My BioSource - USA, Recombinant Osteopontin Monoclonal Antibody Western Blot Analysis E AB-81496 Elabscience-USA.

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Animal grouping

A total of 60 male wistar rats were divided into 6 groups with 10 rats in each. A healthy control group I, and the other 5 groups received a monocrotaline injection subcutaneously in the ventral thorax at a dose of (60 mg/kg) as a single dose.[7] Induction group II didn't receive any treatment, nicorandil group III received (10mg/kg/day) of nicorandil orally, [8] tadalafil group IV received (10 mg/kg/day) orally,[9] nicorandil blocker group V received nicorandil (10 mg/kg/day) plus L-NAME (1mg/mL) in drinking water[10] and glimepiride (5mg/kg/day) orally[11], and tadalafil blocker group VI received tadalafil (10 mg/kg/day) plus L-NAME (1mg/mL) in drinking water and glimepiride (5mg/kg/day) orally. Treatment continued for 21 days, according to the animal ethics committee in Mustansiriyah University, file number (12).

Sample collection

After 21 days from the induction, 5 cc of blood were drawn from each animal for the serum collection using cardiac acupuncture in all of the six different groups. To produce the serum, the blood was then put in a clot-activating tube & centrifuged at 3000x for 15 minutes. The serum was stored at a deep freezing (-80°C) for BNP analysis. Rats were slaughtered for the purpose of lung harvest under ketamine/xylazine (80/8 mg/kg) anesthesia, and the lungs were swiftly removed and meticulously cleansed with distilled water. The lungs were then separated into two sections, with one section buffered in neutral formalin 10% for the histopathological assay, and the second section immediately incubated in a deep freezer (-80) for western blot analysis.

Enzyme-linked immunosorbent assay

Biotinylated antibody of BNP ELISA Kit (MBS2021774) was purchased from (My BioSource-USA) (was added and incubated for 60 minutes at (37C), then the wheels washed 3 times. After washing, enzyme conjugate was then added to the wheels and kept for incubation for 30 minutes, then washing for 5 times. Then 100µL of colour reagent added, and colour reaction stopped when the highest intensity of coloration detected. Colour reagent C added and read at (450nm) optical density.[12]

Western blot analysis

Samples were prepared for SDS-PAGE, the protein samples diluted in 5xSDS solution and heated at (90C) for 10 minutes, the heated sample were centrifuged at (4C) for 2 min at 12000 rpm, and the upper layer equally loaded into the wheels of 8% gel together with the prestained protein, and run at 120V until the pre-stained protein dye reached the bottom. Transfer of the protein content from the gel to the membrane was done using semi-dry method in 10 minutes. Detection was done using ChemiDoc imaging system from Bio-Rad® through ImageLab software, the pictures for the PVDF membrane were taken. Then processing of the images by the same software to make the bands clearer. By using ImageJ software, the band intensities (concentration of proteins) were measured in comparison with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) polyclonal antibody as a standard housekeeping protein.[13]

Histopathology of lung tissue

After processing the tissue for hematoxylin and eosin staining the slides were examined for the pathological changes like necrosis, edema, inflammatory cells, congestion, thrombosis, and emphysema all were examined and recorded by a professional pathologist. The degree of severity was expressed as (0, 1, 2, 3) corresponding to (absent, mild, moderate, and severe) depending on the mean rank value. [14]

Statistical analysis

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The histopathologic scoring system was calculated using IBM SPSS-20, by applying the non-parametric, Kruskal-Wallis one-way ANOVA k-samples (all pairwise) test. Significant differences were determined utilizing the Kruskal-Wallis test since histopathological changes are not normally distributed and not follow the ordinal level of measurement then non-parametric test makes a useful tool.

Results

Effect of treatment on serum brain natriuretic peptide (BNP)

All treatment groups showed a significant decrease (*P*-value ≤0.05) in BNP level compared to induction group (770.75±28.9 ng/ml). However, nicorandil and tadalafil BNP levels (381.9±15.89& 285.89±15.85 ng/ml, respectively) were significantly lower than that of blocker groups (449.36±15.85& 467.67±13.75 ng/ml, respectively) (*p*-value ≤ 0.05), as shown in (Figure 1).

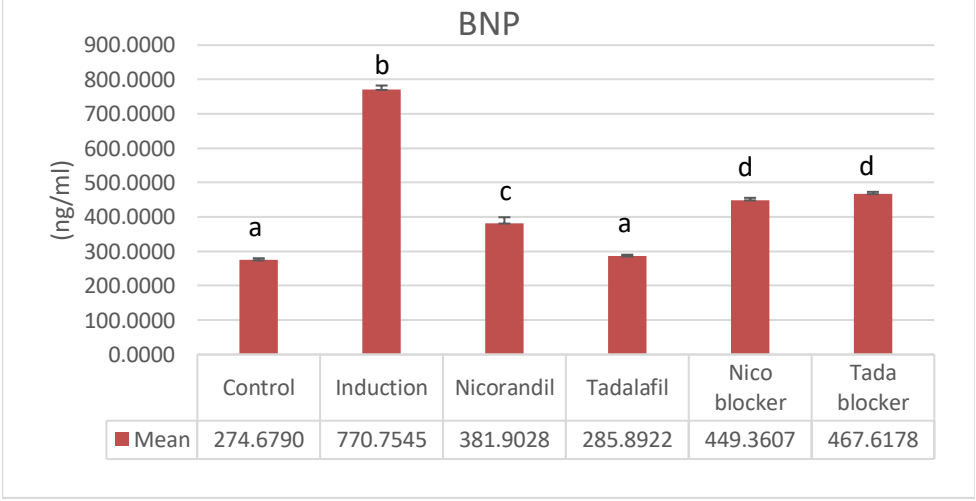

Figure (1): Brain natriuretic peptide among the studied groups. Data are mentioned as means ± S.D. Different lowercase letters indicate significant differences among groups. *p*-value ≤0.05 mean significant difference.

Effect of treatment on osteopontin levels in lung tissue

Osteopontin expression was also compared to GAPDH as shown in (figure 2A), figure (2B) shows that the induction group increased by 43.9 times than the control group, while treatment with 10mg/kg/day of nicorandil decreased total osteopontin level to 8.12 times only. However, the lowest level of 5.45 was observed with 10mg/kg/day tadalafil. The addition of blockers to nicorandil and tadalafil increased the expression to (11.7 and 17.81, respectively) compared to the control group.

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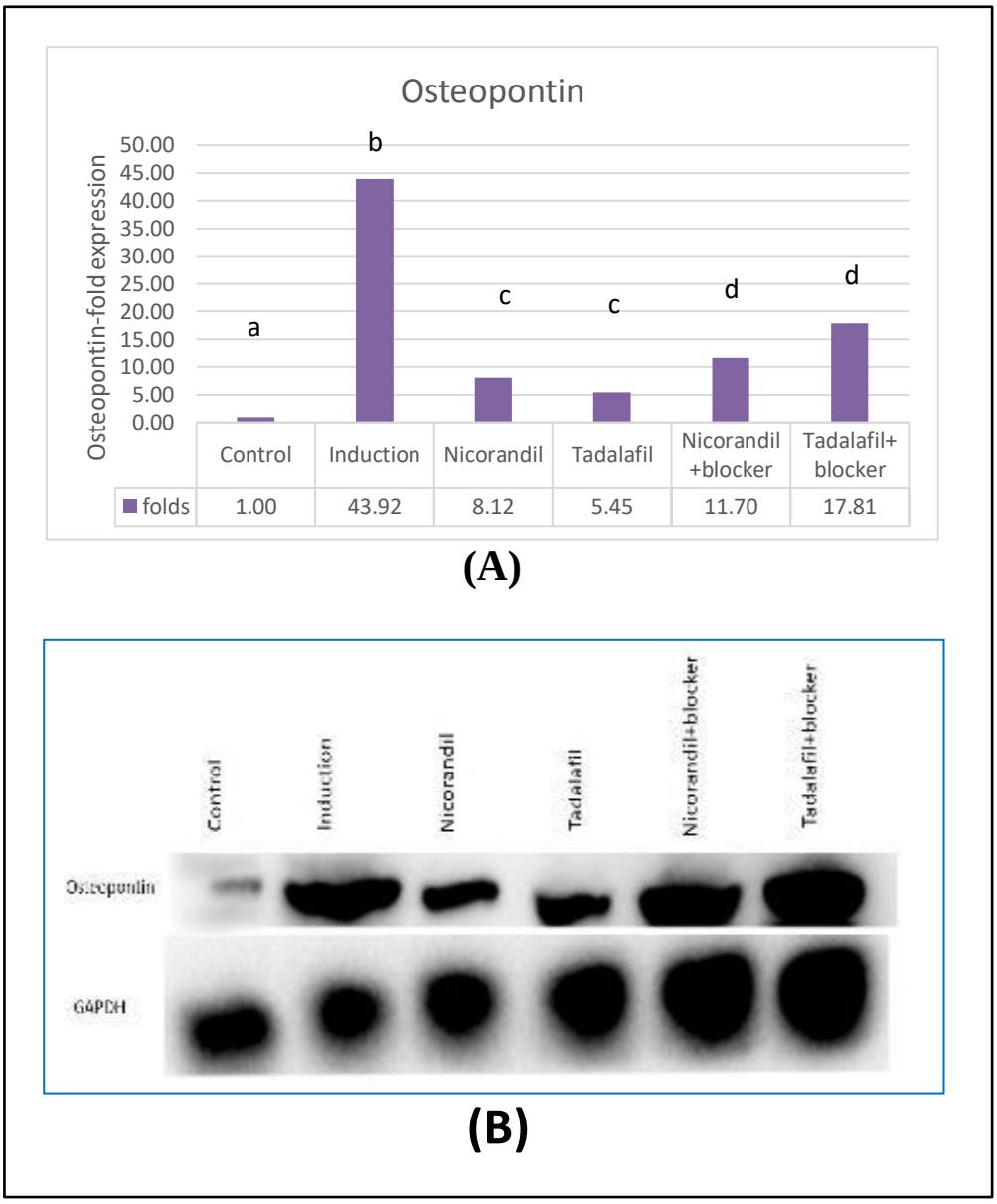

Figure (2): Western blot analysis. A: Number of folds for osteopontin expression among the studied groups. B: Protein expression detected by western blot for osteopontin and Glyceraldehyde-3-phosphate dehydrogenase. Different lowercase letters indicate significant differences among groups. *p*-value ≤0.05 mean significant difference.

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Effect of treatment on histopathological changes

The control group histopathological section of lung tissue was illustrated in Figure (3) which revealed apparently normal lung tissue morphology. While, the induction group cross-section of the lung revealed marked interstitial pneumonia that is characterized by the thickening of interstitial tissue associated with massive infiltration of mononuclear leukocytes involved lymphocytes and macrophages and collapsed alveoli, and pulmonary edema with peripheral lobular emphysema. The pulmonary vessels revealed marked medial degeneration and thrombosis (figure 4). Slides of the lung tissue within the nicorandil group showed mild interstitial thickening and apparently normal segments of bronchi, with almost normal pulmonary artery and alveolus (figure 5). Sections of the lung tissue within the tadalafil group showed moderate interstitial pneumonia associated with the thickening of the interstitium by infiltration of mononuclear leukocytes without exudation (figure 6).

Slides of the lung tissue of the nicorandil blocker group were similar to those of the induction group which revealed severe interstitial bronchopneumonia and emphysema (figure 7). While, Cross-section of the lung tissue within the tadalafil blocker group showed severe parenchymal consolidation associated with severe interstitial pneumonia and severe pulmonary emphysema, marked pulmonary arteritis with moderate medial thickening, and per vascular cuffing (figure 8).

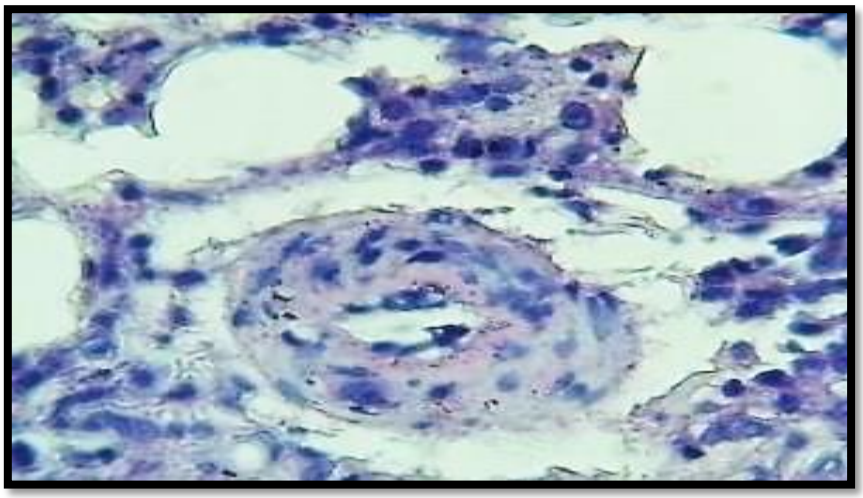

Figure (3): cross-section of the lung tissue for the control group. Section of the lung (control) shows normal appearance of inter alveolar septa, pulmonary artery. H&E stain.400x.

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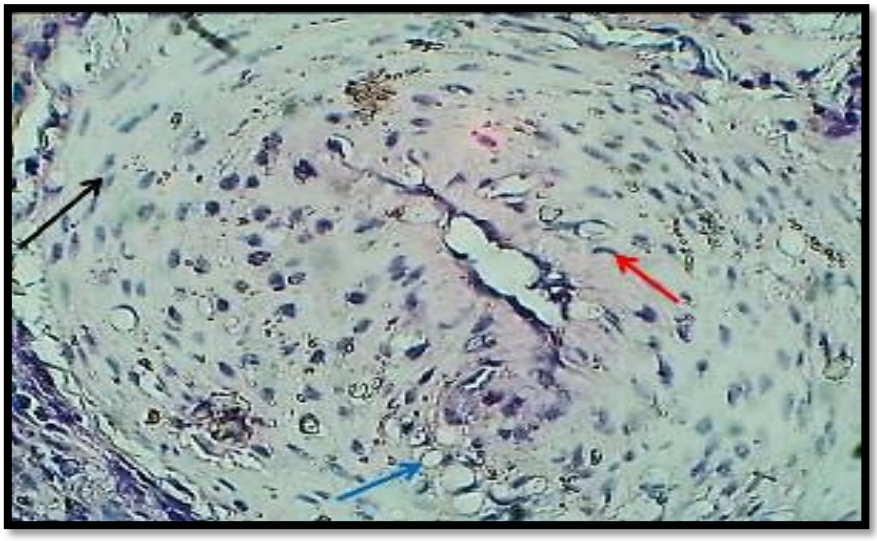

Figure (4): Cross-section of the lung tissue for the induction group. Section of the lung (induction) shows marked medial thickening (black arrow), sub endothelial concentric fibrosis (Red arrow) & sub endothelial myxoid changes (blue arrow) .H&E stain.400x.

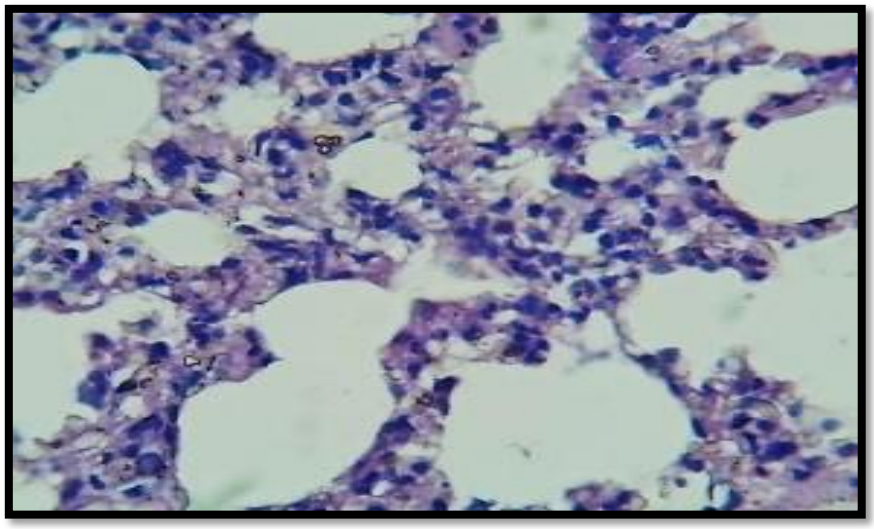

Figure (5): cross-section of the lung tissue for the nicorandil group. Section of the lung (nicorandil) shows mild thickening of the interalveolar septum associated with little infiltration of leukocytes. H&E stain.400x.

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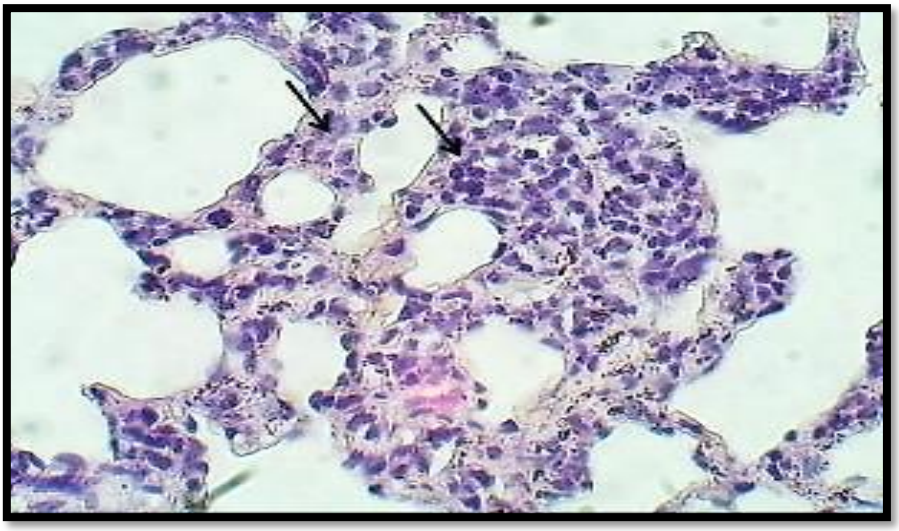

Figure (6): Cross-section of the lung tissue for the tadalafil group. Section of the lung (tadalafil group) mild interstitial thickening associated with mild lymphocyte infiltrate (Arrows).H&E stain.400x.

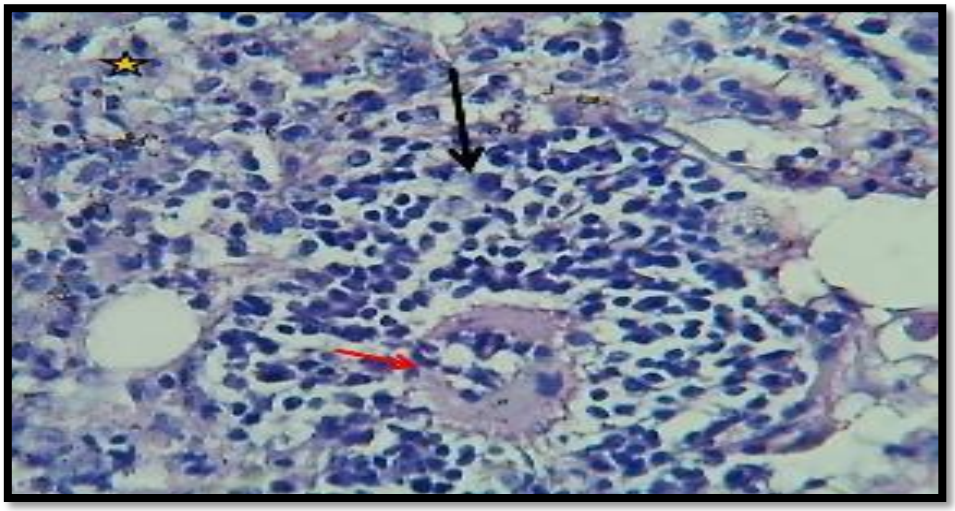

Figure (7): cross-section of the lung tissue for the nicorandil blocker group. Section of lung (nicorandil blocker) shows: marked pulmonary arteritis with marked medial degeneration (Red arrow) & per vascular cuffing (Black arrow). H&E stain.400x

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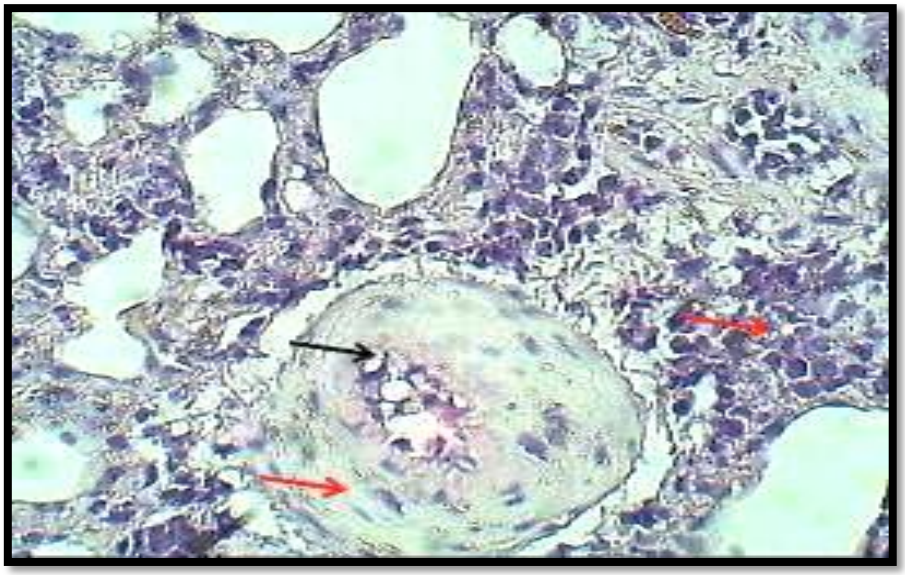

Figure (8): cross-section of the lung tissue for the tadalafil blocker group. Section of the lung (tadalafil blocker) shows mild myxoid degeneration of endothelial cells (Black arrow) and moderate medial thickening (Red arrow) .H&E stain.400x.

Histopathological scoring system

As shown in table (1) histopathological score system , a dose of 10 mg/kg/day of either nicorandil or tadalafil showed significant reduction *(p≤0.05)* in necrosis (13& 11, mean rank respectively) compared to the induction group (mean rank= 30) and was non-significant compared to the control group (nicorandil *p*=0.392& tadalafil *p*=0.669) with an almost absence of necrosis, while nicorandil blocker and tadalafil blocker groups showed moderate changes with a significant increase in necrosis compared to either nicorandil or tadalafil alone *(p≤0.05)*.

Edema in lung tissue showed the same distribution of data as necrosis. On the other hand, nicorandil and tadalafil significantly reduced inflammatory cells (*P≤0.05)* by (10 and 12 mean rank, respectively) compared to the induction group (mean rank=29), and nicorandil blocker group (mean rank=24) was significant (*P*≤0.05) in revealing a moderate increase in inflammatory cells compared to nicorandil and tadalafil alone groups. Also, the tadalafil blocker group showed similar results as the induction group (mean rank=29). Regarding congestion and thrombosis, both were significantly reduced (*p*≤0.05) by nicorandil (mean rank=12) and tadalafil (mean rank=15) compared to induction (mean rank=29), where both nicorandil and tadalafil demonstrated almost the absence of congestion and thrombosis as in the control group.

Table (1): Histopathologic score system of the studied groups.

Control	Mean rank	9 a	9.5 a	7 a	8 a	9 a
	Score	0 a	0a	0 a	0 a	0 a

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	value					
Induction	Mean rank	30 [b]	30 [b]	29 [b]	29 [b]	30 [b]
	Score value	3 [b]	3 [b]	3 [b]	3 [b]	3 [b]
Nicorandil	Mean rank	13 [a]	12 [a]	10 [a]	12 [a]	11 [a]
	Score value	0 [a]	0 [a]	0 [a]	0 [a]	0 [a]
Tadalafil	Mean rank	11 [a]	10 [a]	12 [a]	15 [a]	13 [a]
	Score value	0 [a]	0 [a]	0 [a]	1 [a]	0 [a]
Nicorandil blocker	Mean rank	26 [b]	24 [b]	26 [b]	23 [b]	29 [b]
	Score value	2 [b]	2 [b]	2 [b]	2 [b]	3 [b]
Tadalafil blocker	Mean rank	25 [b]	27 [b]	29 [b]	25 [b]	18 [a]
	Score value	2 [b]	2 [b]	3 [b]	2 [b]	1 [a]

The nicorandil blocker group and tadalafil blocker group demonstrated a moderate increase in thrombosis and congestion (mean rank =23 and 25, respectively) and did not differ statistically compared to the induction group (*p=0.291, p=0.519,* respectively). Emphysema was highest in the induction group (mean rank=30) and nicorandil blocker group (mean rank=29), while nicorandil and tadalafil were as the control in the absence of emphysema (*p=0.714, p=0.463,* respectively). Mild emphysema was observed in the tadalafil blocker group (mean rank=18) and was not differ statistically compared to the control (*p=0.089).*

Discussion

Pulmonary arterial hypertension disease is usually accompanied by right ventricular failure and cardiac remodeling, nicorandil is known to have antiapoptotic effect reducing cardiac remodeling, thus patient with PAH may benefit from the dual function of nicorandil in alleviation of PAH symptoms together with its cardioprotective effect.

Brain natriuretic peptide (BNP) is a hormone largely released by the ventricular myocardium in response to wall stress caused by conditions like pressure overload and volume expansion.[15] It has a strong correlation with mortality.[16]

In the current study, BNP levels were elevated in the induction group, which is in line with the previous study in which induction of PAH elevated levels of BNP, reflecting an increase in pulmonary arterial pressure and right ventricular hypertrophy.[17,18] Brain natriuretic peptide was reduced in both nicorandil and tadalafil groups. The reduction of BNP in the nicorandil group is consistent with a previous study which found that nicorandil reduces BNP by decreasing right ventricular remodeling through decreasing right ventricular

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pressure and activation of mitochondrial K^+-ATP channels (mATP-K^+).[19] The reduction of BNP in the tadalafil group goes in line with a previous clinical study that revealed BNP reduction after administration of tadalafil to patients with resistant pulmonary hypertension, indicating an improvement in cardiac status.[20] Blocker groups showed moderate changes due to the effect of L-NAME, that blocks eNOS and leading to increased arterial pressure.[21]

Osteopontin is a multifunctional 44 kDa phosphoprotein that is widely present in many different tissue types, including bone, GI tract epithelial cells, lungs, breasts, salivary glands, inner ear, placenta, and kidneys.[22] Additionally, osteopontin interacts with CD44, a cell-surface receptor that is widely distributed and expressed by pulmonary artery endothelial cells (PAECs) which resemble endothelial-to-mesenchymal transitions in pulmonary arteries that have neointimal hyperplasia or blocked capillaries, including plexiform lesions. Also, in vascular smooth muscle cells(VSMC), it mediated cell proliferation leading to VSMC hyperplasia through activation of the p38 MAPK signaling pathway.[23]

In the current study, monocrotaline elevated the level of osteopontin similar to a previous study, in which osteopontin was elevated due to overexpression of serotonin transporter (SERT), ERK1/2 signaling pathway, which is linked directly to overexpression of OPN.[24] Nicorandil and tadalafil groups both decreased OPN in comparison to the induction group. In previous studies, OPN production was inhibited by selective activation of cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG), which is a downstream signalling pathway from NO and cGMP, it is believed that PKG suppresses the steady state of mRNA encoding for OPN.[25,26] The blocker groups were intermediate in the reduction of OPN with the preference of the nicorandil blocker group over the tadalafil blocker group, this can be explained by the role of mK^+-ATP sensitive channels because nicorandil can indirectly reduce generation of ROS, which is a promotor for OPN synthesis.[27]

Pulmonary arteries in the lungs of those with PAH typically undergo total vessel obliteration, which increases vascular resistance. Complex, multicellular vascular lesions that block and obliterate pulmonary arterioles are a frequent histology finding.[28] The blocked blood vessels greatly reduce the amount of blood that can flow through the pulmonary arteries and significantly elevate the right ventricular (RV) afterload, which helps to cause RV dysfunction and failure. Massive influxes of inflammatory cells in the lungs are a hallmark of PAH.[29] Alveolar edema, alveolar septal cell hyperplasia, and pulmonary vein blockage are all brought on by monocrotaline.[30]

In this study, monocrotaline produced severe inflammation within lung tissue accompanied by leukocyte infiltration, smooth muscle hyperplasia, emphysema, and occlusion of pulmonary arteries, which is consistent with the previous studies that found monocrotaline administration results in marked medial thickening, diffused edema, and leukocyte infiltration.[31,32] Nicorandil attenuated these changes and showed apparently normal arterial wall without medial thickening which is the hallmark of PAH remodelling and minimized inflammation. In a previous study, it was found that the administration of K^+ channel openers prevented PAH rat model inducing tunica media thickening and endothelial induction of apoptosis.[33] Tadalafil produces similar results to that of the nicorandil group by inhibiting smooth muscle proliferation and inflammation. Tadalafil showed a similar result in decreasing vascular remodelling in MCT-induced PAH in rats in which media

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thickening was prevented by tadalafil administration.[34,35] In the blocker groups, both treatments showed moderate changes regarding medial thickening, inflammation, and edema. This is due to the blocking effect of L-NAME, in which blocking of eNOS reduces the activity of NO, which plays a crucial role in preventing vascular remodelling by inhibiting endothelial apoptosis, inducing smooth muscle apoptosis, reduction of ROS, and regulating the inflammatory response.[36]

Conclusion

In the current study, nicorandil showed anti-inflammatory action by reducing osteopontin, improved cardiac status by reducing BNP levels, and attenuated vascular changes induced by monocrotaline.

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