Effect of Nicorandil on Endothelial Markers and Tissue Remodeling in Pulmonary Arterial Hypertension Model of Male Rats

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Abstract

Background: Chronic pulmonary arterial hypertension (PAH) is a rare, long-lasting illness that makes pulmonary artery endothelial cells (PAEC) not work properly and leads to heart failure and death. Objective: To evaluate the effect of nicorandil in the treatment of PAH compared to tadalafil in a rat model of monocrotaline-induced PAH. Methods: Monocrotaline injection (60 mg/kg) was used for the induction of PAH in male rats; healthy control and induction groups were not treated. The other 4 groups were treated with either nicorandil or tadalafil with or without treatment blockers (glimepiride and N-Nitroarginine methyl ester (L-NAME)) for 21 days orally. Serum was obtained for assessment of endothelin-1 (ET-1) and tissue harvested for nuclear factor kappa B (NFκB) by ELISA, western blot analysis of endothelial nitric oxide synthase (eNOS), and an apoptosis assay to examine the endothelial function. Results: Nicorandil showed a significant reduction in ET-1 and significant elevation in eNOS compared to the induction group, with comparable efficacy to tadalafil; blocker groups showed significantly elevated levels of ET-1 and reduced levels of eNOS compared to healthy control; NFκB was significantly inhibited in nicorandil and tadalafil groups and significantly elevated in blocker and induction groups; while in the TUNEL apoptosis assay, nicorandil showed the highest level of inhibition to apoptosis with apparently normal endothelium lining. Conclusions: Nicorandil shows anti-inflammatory, antiapoptotic, and enhanced endothelial morphology and function compared to the induction model.

Keywords: Anti-apoptotic effect, Endothelin-1, Nicorandil, NFkB, Nitric oxide, Pulmonary arterial hypertension.

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INTRODUCTION

Pulmonary arterial hypertension (PAH) is group 1 of pulmonary hypertension (PH), according to the World Health Organization (WHO). It was first described by the German physician Ernst von Romberg in 1891, and the complete pathophysiology is still unknown [1]. It is a progressive, chronic, and deadly disease of the pulmonary arteries characterized by extensive pulmonary artery constriction accompanied by vascular endothelial and vascular smooth muscle changes and an increase in mean pulmonary arterial pressure above 25mm Hg; however, PAH is considered a rare disease [2,3]. The prevalence and incidence of PAH according to the Registry to Evaluate Early and Long-term PAH (REVEAL) in the US, which uses catheterization as the standard of diagnosis, were 15% and 2.4 per year, respectively [4]. In this registry, the 5-year survival rate was high compared with a previous study [5]. In an already-diagnosed patient, it was above 90%, 76%, and 65% for 1, 3, and 5 years, respectively, while with recently-diagnosed patients, it was 86% for 1 year, 68% for 3 years, and 61% for 5 years [6]. These advancing pulmonary vascular disorders are triggered by the disruption of the three critical signaling pathways, nitric oxide (NO), prostacyclin (PGI2), and thromboxane A2 (TXA2), as well as the increase of endothelial and smooth muscle endothelin A receptors and reduced endothelin B receptors, together with the elevation of endothelin-1 (ET-1). Endothelial nitric oxide synthase (eNOS) dysfunction, reduced PGI2 production (cyclooxygenase-2 imbalance), and the vasoconstrictive and mitogenic consequences of a raised endothelin-1 signaling pathway all contribute to PAH [7,8]. Nicorandil is a drug used for angina pectoris. It acts by two mechanisms: firstly, as a NO donor, and secondly, as a K+-ATP channel opener, resulting in vasodilation due to smooth muscle relaxation [9,10]. Although potassium channels play an important role in PAH development, there is no current treatment for these channelopathies. Monocrotaline is a pyrrolizidine alkaloid derived from the Crotalaria spectabilis plant [11] and was historically utilized for inducing PAH in rats with only a single subcutaneous injection. N-nitroarginine methyl ester (L-NAME) is used as an endothelial nitric oxide synthase inhibitor and glimepiride as an ATP-dependent potassium channel blocker. The point of this study is to find out how well nicorandil works as a medicine to treat PAH in rats that have been given monocrotaline.

METHODS

Study design

In this experimental animal study, sixty Wistar albino male rats were used, obtained from the Animal House of the Iraqi Center for Cancer and Medical Genetic Research (ICCMGR) at Mustansiriya University. They were handled according to the Ethics Committee in the College of Pharmacy, Al-Mustansiriya University. Rats weighing 200–250 g were maintained in an animal house at 22°C under a 12-hour dark-light cycle and were allocated into 6 groups based on age, and weight-matched animals were selected. Sixty Wistar albino male rats were distributed in 6 groups, 10 in each, as follows: (I) negative control group injected 0.5 ml/kg normal saline subcutaneously (S.C) single dose, then 5% carboxyl methyl cellulose (CMC) orally for 21 days, (II) induction group induced pulmonary arteries remodeling by S.C injection of monocrotaline (MCT) (60 mg/kg/day) at day 1 as a single dose, then 5% CMC solution orally for 21 days, (III) nicorandil group induced pulmonary arteries remodeling by S.C injection monocrotaline (MCT) (60 mg/kg/day) at day 1 as a single dose, then nicorandil (10 mg/kg) once daily by oral gavages for 21 days, (IV) standard group induced pulmonary arteries remodeling by S.C injection monocrotaline (MCT) (60 mg/kg/day) at day 1 as single dose, then tadalafil (10 mg/kg) once daily by oral gavages for 21 days, (V) nicorandil with blocker group induced pulmonary arteries remodeling by S.C injection monocrotaline MCT (60 mg/kg/day) at day 1 as a single dose, then nicorandil (10 mg/kg) once daily by oral gavages for 21 days, pretreated with NOS inhibitor (L-NAME) 1mg/ml in drinking water, and ATP-dependent K+-channel blocker (glimepiride) at a dose of 5mg/kg/day orally 1 hour before nicorandil, (VI) tadalafil with blocker group induced pulmonary arteries remodeling by S.C injection monocrotaline (MCT) (60 mg/kg/day) at day 1 as a single dose, then tadalafil (10 mg/kg) once daily by oral gavages for 21 days, pretreated with NOS inhibitor (L-NAME) 1mg/ml in drinking water, and ATP-dependent K+-channel blocker (glimepiride) at a dose of 5mg/kg/day orally 1 hour before tadalafil.

Preparation and drug doses

Monocrotaline was prepared by dissolving monocrotaline in 3 ml of 1 N HCl and then 10 N NaOH to prepare a solution of 7.4 pH. The volume was then completed by distilled water to prepare a solution of monocrotaline containing 20 mg/ml. [12] Groups II to VI were injected S.C. in the ventral thorax. Nicorandil was prepared by dissolving nicorandil in a 5% solution of CMC to prepare a final concentration containing 10 mg/mL of nicorandil. It was given by oral gavage at a dose of 10 mg/kg/day [13] for 21 days to groups III and V. Tadalafil solution was prepared by dissolving 10 mg in 1 ml of 5% CMC solution. It was given orally at a dose of 10 mg/kg/day [14] to groups IV and VI. N-Nitroarginine methyl ester (L-NAME) is fed to animals in drinking water at a dose of 1 mg/ml to groups V and VI [15]. Glimepiride solution was prepared by dissolving 10mg in 1 ml of 5% CMC solution. It was given orally at a dose of 5 mg/kg/day to groups V and VI [16].

Sample collection

For serum collection, 5 ml of blood was taken from each animal by heart acupuncture in each of the six groups 21 days after the induction to measure ET-1 levels. The blood was then placed in a clot-activating tube and centrifuged at 3000x for 15 minutes to prepare the serum. For ELISA analysis, the serum was kept at a temperature of -80 °C. For lung harvest, rats were sacrificed under ketamine/xylazine (at a dose of 80/8 mg/kg) anesthesia,
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and the lungs were immediately removed and thoroughly cleaned with distilled water. Then the lungs were divided into 2 parts, one buffered in neutral formalin at 10% for the TUNEL apoptosis assay, and the second part incubated immediately in a deep freeze (-80) for western blot analysis (eNOS) and ELISA (NFκB).

**Enzyme-linked immunosorbent assay**

After the preparation of serum and tissue samples, they were kept at -80 °C until the time of analysis. The biotinylated antibodies of endothelin-1 (ET-1 ELISA kit MBS263783) and nuclear factor kappa B (NFkB ELISA kit MBS453975) were purchased from My BioSource, USA, and the procedure was followed by the manufacturer’s guidelines [17].

**Western blot analysis**

The Western blot technique was used to measure the concentration of eNOS in lung tissues. Samples were prepared for SDS-PAGE, the protein samples were diluted in a 5x dodecyl sulfate polyacrylamide (SDS) solution and heated at 90 °C for 10 minutes. The heated samples were centrifuged at 4 °C for 2 min at 12000 rpm, and the upper layer was equally loaded into the wheels of 8% gel together with the pre-stained protein [19].

(endothelial nitric oxide synthase (eNOS) monoclonal antibody E-AB-22088) and BCA kit E-BC-K318-M) purchased from Elabscience, the procedure was followed according to the manufacturer’s guidelines. By using ImageJ software, the band intensities (concentration of proteins) were measured, and by multiplying the intensity of the targeted protein over the housekeeping protein producing relative intensity to each group, this relative intensity was then multiplied by the relative intensity of the control to calculate the number of folds in comparison to the control (Figure 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) polyclonal antibody was used as a standard housekeeping protein [18].

**Figure 1**: Endothelial nitric oxide synthase (NOS-3) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression on PVDF membrane of the studied groups.

**One-step TUNEL apoptosis assay**

Following the fixation, samples were labeled and placed in blocks of paraffin wax according to manufacturer protocol (one-step TUNEL in situ Apoptosis Kit (Green, FITC) fluorescence E-CK-A320, Elabscince, USA). Apoptotic cells were detected utilizing a fluorescent microscope with a FITC filter from Zeiss-Germany and counted computationally by ImageJ macro software; the green fluorescent color indicates the presence of apoptotic fragments [19].

**Statistical analysis**

Statistical analysis was done utilizing SPSS-20® and Microsoft Excel 2022. Mean and standard deviations were calculated. Analysis of variance (ANOVA) and post hoc Tukey test were used for comparing differences among groups. Statistical significance was considered at p<0.05.

**RESULTS**

The levels of endothelin-1 were significantly lower (p<0.05) in the nicorandil and tadalafil groups (50.06±5.88 and 42.1±1.21 ng/ml, respectively) compared to the induction group (199.08±11.49). The nicorandil blocker and tadalafil blocker groups were about the same in terms of ET-1 levels reduction compared to nicorandil or tadalafil alone (p<0.05), as shown in (Figure 2). The number of folds for each sample showed how the treatment changed the levels of endothelial nitric oxide synthase (eNOS) in lung tissue.

**Figure 2**: Serum levels of Endothelin-1 among studied groups compared to the induction group. Data are presented as means±SD. Statistically significant at p<0.05 (ANOVA followed Tukey test).

Figure 3 shows that the level of eNOS expression was greatly decreased by 59% in the induction group (0.41-fold) compared to the control (1-fold), while treatment with 10 mg/kg/day of nicorandil elevated eNOS to 84% of the control group. Tadalafil at a dose of 10mg/kg/day only showed 61% of that for the control group, nicorandil with blocker group and tadalafil with blocker group
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showed lower levels of eNOS expression (24 and 16%), respectively.

Figure 3: Number of folds expressed as percentages for endothelial nitric oxide synthase (eNOS) expression in studied groups. Statistically significant at p<0.05 (ANOVA followed Tukey test).

The effect of treatment on nuclear factor kappa B (NFκB) expression in lung tissue of rats as measured by ELISA showed a significant reduction (p<0.05) of NFκB tissue levels (Figure 4) in both nicorandil and tadalafil groups (1.8±0.09 and 1.57±0.13 ng/ml, respectively) in comparison to induction (5.93±0.09 ng/ml). The most reduction was seen in the tadalafil group in comparison to all studied groups. The nicorandil with blocker group and the tadalafil with blocker group had significantly (p<0.05) higher levels of NFκB (2.31±0.13 and 2.19±0.12 ng/ml, respectively) compared to the nicorandil or tadalafil alone group and were not statistically different from each other (p=0.727).

Figure 4: Nuclear Factor Kappa B (NFκB) levels treated groups compared to the control group. Data are mentioned as means±SD. Statistically significant at p<0.05 (ANOVA followed Tukey test).

Figure 5: one-step TUNEL apoptosis assay of pulmonary arteries shows apoptotic fragments as green fluorescent. Control group (A), induction group (B), nicorandil group, tadalafil group (D), nicorandil blocker group (E), and tadalafil blocker group (F).

Results illustrated in Figure 6 showed that the apoptotic rate was highest in the pulmonary arteries of the induction group (564.33 cell fragments), while administration of nicorandil or tadalafil suppressed this apoptosis to only 5 and 26 cell fragments, respectively, in pulmonary arteries. The effect of blockers on either nicorandil or tadalafil was obvious in elevating the rate of apoptosis up to 76 and 156, respectively.

Figure 6: Number of apoptotic fragments in pulmonary arteries for the studied animal groups using TUNEL assay. Data are mentioned as means±SD. Statistically significant at p<0.05 (ANOVA followed Tukey test).

**DISCUSSION**

Pulmonary arterial hypertension (PAH) is a rare, fast-progressing, incapacitating, and potentially fatal pulmonary vascular illness. Pulmonary vasoconstriction, vascular remodeling, endothelial dysfunction, smooth muscle proliferation, and thrombosis are all components of PAH’s pathological mechanisms. These modifications elevate the resistance of the pulmonary arteries, which leads to right heart failure and then death. Potassium channel channelopathies are now recognized as a
pathogenic cause for the development of PAH. Two channeledopathies have been recognized due to genetic alterations in genes encoding for potassium channel subfamily K\(^+\) member 3 (KCNK3) and ATP-binding cassette subfamily C member 8 (ABCC8). [20] In this study, the administration of monocrotaline (MCT) to induce PAH significantly elevated the level of ET-1. This elevation is consistent with a previous study done by Nagendran et al. (2013), who reported an elevation of ET-1 and endothelin-A receptors [21]. This can explain the increased vasoconstriction and endothelial dysfunction in PAH. Nicorandil decreased the level of ET-1, which corresponds to the previous study done by Chen et al. (2015), which found that nicorandil decreased the level of ET-1 and elevated the level of NO in patients with coronary artery disease [22]. This reduction of ET-1 can be attributed to ATP-K\(^+\) and the known effect of nicorandil, which increases the level of NO [23]. Nitric oxide contributes to the regulation and synthesis of ET-1. The administration of nitric oxide donors down-regulates the synthesis of ET-1 by suppressing the gene responsible for its production [22,24]. The Tadalafil group showed the same results because tadalafil also increases the level of NO in the endothelial tissue due to its PDE5-inhibiting activity. In the blocker groups, the results were intermediate between treatment groups (nicorandil and tadalafil) and the induction group; however, there was a significant difference because L-NAME suppressed the production of nitric oxide, which led to overexpression of ET-1. This went along with a previous study done by Takahashi et al. (2005), in which L-NAME increased the level of ET-1 [25]. It has been shown that endothelial NO prevents hyperplasia of vascular smooth muscle cells (VSMCs) and that under hypoxic conditions, the expression of eNOS genes is reduced, suggesting that endothelial dysfunction can be attributed to eNOS reduction [26]. Monocrotaline (MCT) in the induction group of the current study decreased the level of eNOS significantly compared to the control group. In previous studies, it was found that MCT reduced both the expression and activity of eNOS [27,28]. Nicorandil elevated the level of eNOS to approximate that of the control (0.84-fold). Refaie et al. (2020) showed that potassium channel openers elevate eNOS through ATP-sensitive potassium channels [29]. Additionally, protein kinase C reduces the activity of eNOS by phosphorylating threonine 495 of eNOS, while nicorandil blocks this phosphorylation by the NO pathway, preserving eNOS activity [30]. Accordingly, nicorandil can increase both the quantity and activity of eNOS [30]. Tadalafil increased eNOS compared to the induction group, and it was 0.61-fold greater than that of the control group. This result is consistent with the previous study done by Vignozzi et al. (2016), which showed an increase in eNOS after tadalafil administration [31]. However, the exact mechanism is not clear. In this study, it is suggested that the reduction of inflammation through reduction of NFκB (Figure 4) and improvement in endothelial function after administration of tadalafil could be the reason for the elevation of eNOS levels, because tadalafil only potentiates the effect of NO and does not increase the level of eNOS. Blocker groups also showed reduced levels of eNOS in western blotting; L-NAME, together with nicorandil or tadalafil, reduced the level of eNOS, and this suggests that the nitric oxide pathway could indirectly regulate eNOS expression through decreasing ROS and restoring endothelial function [32]. In the pathogenesis of PAH, NFκB plays an important function by increasing ET-1 and chemokine ligand 5 by elevating these mediators’ mRNA [33]. In this study, monocrotaline increased the level of NFκB, which is consistent with a study that found that monocrotaline mediates the initiation of an inflammatory response that activates NFκB signaling through increasing inflammatory mediators [34]. Nicorandil effectively reduced NFκB to reach that of the control group. In line with the previous study, it was found that nicorandil inhibits toll-like receptors (TLR) that are responsible for NFκB activation and its downstream signaling through nucleotide-binding domain leucine-rich-containing family pyrin domain-containing-3 (NLRP3) that lead to activation of caspase 1 and pyroptosis [35]. The Tadalafil group also reduced NFκB, suggesting that NO is the regulatory mediator to control NFκB. Tadalafil increases the level of NO in PAH, and nitric oxide has a negative effect on NFκB, which is in tune with the previous study, which found that NO elevation mediates a suppressor action on the activation of NFκB and synthesis in VSMCs [36]. This also applies to nicorandil. In the blocker groups, there was a significant elevation of NFκB; this can be attributed to eNOS inhibition that led to decreased levels of NO, thus the partial loss of nicorandil and the tadalafil effect [37]. Using the TUNEL technique with FITC-fluorescent dye, the nicorandil group showed apparently normal PAECs compared to the control, with almost the absence of apoptosis fragments, which was even better than the tadalafil group. NFκB is a known factor that controls apoptosis in endothelial cells. Overexpression of NFκB can be mediated by different factors; these include reactive oxygen species (ROS) and hypoxia [38]. Nitric oxide regulates the level of NFκB, exerting negative feedback on the synthesis of NFκB. Upregulation of eNOS by nicorandil and tadalafil resulted in inhibition of NFκB and consequent apoptosis [39]. In the current study, glimepiride did not effectively block ATP-K\(^+\) channels in the pulmonary arteries, whether sacrolemmal or mitochondrial. This may be due to the difference between sulfonylurea, in which glibenclamide impairs ischemic conditions but glimepiride does not [41], and only L-NAME acted on eNOS. However, the nicorandil blocker group still demonstrated a better result than the tadalafil blocker group. This is in tune with a previous study that found that the second pathway of nicorandil, that is, mK\(^{\text{ATP}}\)-ATP channel opening, resulted in decreased production of ROS and prevented ROS activation of NFκB and apoptosis, as well as ROS-mediated apoptosis independent of NFκB [42].

**Conclusion**

Nicorandil manifested effective anti-apoptotic activity in the vascular endothelium, was anti-inflammatory, and improved vascular function, as represented by decreased levels of ET-1 and elevated levels of eNOS, which is comparable to the standard treatment tadalafil.
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Conflict of interests

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Supplementary data can be shared with the corresponding author upon reasonable request.

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