

Ormeloxifene, a selective estrogen receptor modulator, protects against pulmonary hypertension

Adam Olaitan Abdulkareem^{a,b,e}, Priya Tiwari^{a,b}, Zahid Rasool Lone^a, Hina Iqbal^c, Satish Gupta^{b,d}, Rajesh Kumar Jha^{b,d}, Debabrata Chanda^c, Kumaravelu Jagavelu^{a,b}, Kashif Hanif^{a,b,*}

^a Division of Pharmacology, CSIR-Central Drug Research Institute, Lucknow, 226031, India

^b Academy of Scientific and Innovative Research, Ghaziabad, Uttar Pradesh, 201002, India

^c Department of Molecular Bio-Prospction, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India

^d Division of Endocrinology, CSIR-Central Drug Research Institute, Lucknow, 226031, India

^e Animal Physiology Unit, Department of Zoology, University of Ilorin, Ilorin, Nigeria

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ABSTRACT

Purpose: Protective effect of 17β-estradiol is well-known in pulmonary hypertension. However, estrogen-based therapy may potentially increase the risk of breast cancer, necessitating a search for novel drugs. This study, therefore, investigated the ameliorative effects of a selective estrogen receptor modulator, ormeloxifene, in pulmonary hypertension.

Methods: Cardiomyocytes (H9C2) and human pulmonary arterial smooth muscle cells (HPASMCs) were exposed to hypoxia (1% O₂) for 42 and 96 h, respectively, with or without ormeloxifene pre-treatment (1 μM). Also, female (ovary-intact or ovariectomized) and male Sprague–Dawley rats received monocrotaline (60 mg/kg, once, subcutaneously), with or without ormeloxifene treatment (2.5 mg/kg, orally) for four weeks.

Results: Hypoxia dysregulated 17β-hydroxysteroid dehydrogenase (17βHSD) 1 & 2 expressions, reducing 17β-estradiol production and estrogen receptors α and β in HPASMC but increasing estrone, proliferation, inflammation, oxidative stress, and mitochondrial dysfunction. Similarly, monocrotaline decreased plasma 17β-estradiol and uterine weight in ovary-intact rats. Further, monocrotaline altered 17βHSD1 & 2 expressions and reduced estrogen receptors α and β, increasing right ventricular pressure, proliferation, inflammation, oxidative stress, endothelial dysfunction, mitochondrial dysfunction, and vascular remodeling in female and male rats, with worsened conditions in ovariectomized rats. Ormeloxifene was less uterotrophic; however, it attenuated both hypoxia and monocrotaline effects by improving pulmonary 17β-estradiol synthesis. Furthermore, ormeloxifene decreased cardiac hypertrophy and right ventricular remodeling induced by hypoxia and monocrotaline.

Conclusion: This study demonstrates that ormeloxifene promoted pulmonary 17β-estradiol synthesis, alleviated inflammation, improved the NOX4/HO1/Nrf/PPARγ/PGC-1α axis, and attenuated pulmonary hypertension. It is evidently safe at tested concentrations and may be effectively repurposed for pulmonary hypertension treatment.

1. Introduction

Pulmonary hypertension (PH) is a fatal disease that affects more females than males but with a better prognosis and survival in females (Sun et al., 2021). Its pathogenesis involves cycles of inflammatory molecules infiltration and oxidative stress, resulting in endothelial

dysfunction, hyperproliferation of pulmonary vascular cells, vascular remodeling, increased pulmonary arterial pressure (>20 mmHg), right ventricular hypertrophy, and eventually death (Simonneau et al., 2019). Currently, there is no ideal treatment option for PH. The available drugs mainly delay disease progression without improving long-term survival (Chen et al., 2017).

The anti-inflammatory activity of estrogens has been previously

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* Corresponding author. Division of Pharmacology, CSIR-Central Drug Research Institute, Sector-10, Jankipuram Extension, Sitapur Rd, Lucknow, 226031, Uttar Pradesh, India.

E-mail address: k_hanif@cdri.res.in (K. Hanif).

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Abbreviations

PH	Pulmonary hypertension
RV	Right ventricle
PASMC	Pulmonary Arterial Smooth Muscle Cells
HPASMC	Human PASMC
LV	Left ventricle
RVP	RV pressure
BW	Body weight
ANP	A-type natriuretic peptide
PSR	Picrosirius red staining
ROS	Reactive oxygen species
mROS	Mitochondrial ROS

reported, mitigating injury-induced vascular remodeling and maintaining proper cardiovascular function through activation of the estrogen receptors α and β (Xing et al., 2009; Murphy, 2011; Streicher et al., 2017). Similarly, studies have linked 17 β -estradiol with better survival in female PH patients (Yuan et al., 2013; Jacobs et al., 2014; Frump et al., 2015; Kozu et al., 2018; Philip et al., 2020). However, considering 17 β -estradiol as a therapeutic strategy for PH treatment in women may be challenging. Firstly, studies have also reported that increased 17 β -estradiol level predisposes women to PH (Mair et al., 2014; Chen et al., 2017). Secondly, estrogen therapy may activate estrogen receptors in the reproductive system, increasing the risk of breast and endometrial cancer (Alencar et al., 2018). Therefore, selective modulation of 17 β -oestradiol metabolism and action is necessary for a better treatment option.

Selective estrogen receptor modulators (SERMs) are nonsteroidal compounds that, unlike estrogens, selectively act as estrogen receptors' antagonists or agonists in a tissue-specific fashion (Patel and Bihani, 2018). They are majorly for treating breast cancer and osteoporosis and maintaining lipid profiles in postmenopausal women (Martinkovich et al., 2014). Their roles in PH have not been well explored; only a few studies reported the protective effects of raloxifene and tamoxifen in monocrotaline and BMPR2-related PH, respectively (Nishida et al., 2009; Chen et al., 2017). However, these studies did not show the efficacy of both compounds on the established PH pathophysiology which includes inflammation, oxidative stress, mitochondrial dysfunction, and endothelial dysfunction, while their detailed effects on estrogen metabolism were lacking. Besides, the risks of thromboembolism and endometrial and uterine cancers in raloxifene and tamoxifen, respectively, may limit their choice (Patel and Bihani, 2018). Thus, searching for safe and effective SERMs with known molecular mechanisms is required for improved PH treatment.

This study, therefore, aimed to investigate the therapeutic potential of ormeloxifene (centchroman) in PH. Ormeloxifene is a potent first nonsteroidal oral contraceptive SERM developed by the CSIR-Central Drug Research Institute, Lucknow, India. Reports have shown its anticancer and anti-osteoporosis effects via downregulations of inflammatory and proliferative markers and improved bone mineral density, respectively (Arshad et al., 2004; Khan et al., 2015; Hafeez et al., 2017). It is relatively non-toxic and well-tolerated, with good bioavailability (Gara et al., 2013). Hence, ormeloxifene may be better repurposed for PH treatment. The present study reveals that ormeloxifene is less uterotrophic; it increases pulmonary arterial estradiol level, inhibits PH development, and attenuates right ventricular remodeling.

2. Materials and methods

2.1. Chemicals and reagents

Antibiotic/antimycotic solution (15240062), trypsin (25200056),

4',6-diamidino-2-phenylindole (DAPI) (D1306), 5,5',6,6' -tetrachloro-1,1',3,3' -tetraethyl benzimidazole carbocyanine Iodide (JC-1; 420,200), mitoSox red (M36008) and Phalloidin (A12381) were purchased from Invitrogen (Carlsbad, CA, USA). Gibco Fetal Bovine Serum (10270-106) was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). 2'-7'-Dichlorodihydrofluorescein diacetate (DCFDA, 35845) was purchased from Sigma, USA. RNAiso Plus reagent (#9108/9109) was obtained from Takara Bio Inc., Shiga, Japan. Primary antibodies to nuclear factor kappa B (NF- κ B) (ab16502), proliferating cell nuclear antigen (PCNA) (sc-25280), caspase 3 (sc-7272), Bcl-2-associated X (Bax) (sc-7480), peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1 α (PGC-1 α) (sc-518025), Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4) (sc-21860), heme oxygenase 1 (HO-1) (sc-7695) and vascular endothelial growth factor (VEGF) (sc-7269) were obtained from Santa Cruz (USA); natriuretic Peptide A (NPPA) (DF6497), B-cell leukemia/lymphoma 2 (Bcl-2) (AF6139), hypoxia-inducible factor-1 α (HIF1A) (AF1009), Estrogen receptor α (ER α) (AF6058), Estrogen receptor β (ER β) (AF6469), 17 β -hydroxysteroid dehydrogenase 1 (HSD17B1) (DF7790), 17 β -hydroxysteroid dehydrogenase 2 (HSD17B2) (DF6734), phosphorylated endothelial nitric oxide synthase (p-eNOS) (AF3247) and β -actin (T0022-HRP) were obtained from Affinity Biosciences (USA); MitoSox (M36008) and secondary HRP-conjugated antibodies were purchased from Santa Cruz Biotechnology (USA). Enzyme link immunosorbent Assay (ELISA) kits for Estradiol (E2) (Cat. no: EU0390) and estrone (Cat no: ELK7837) were procured from Fine Test and ELK biotechnology, respectively, Wuhan, China.

2.2. Cell culture

2.2.1. Cardiomyocytes culture and toxicity study

Cardiomyocytes (H9C2, NCCS, Pune, India) were cultured in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells at confluence were starved with serum-free DMEM, seeded into a 96-well plate (approximately 5000 cells/well), and treated with varying concentrations of ormeloxifene (0, 5, 10, 15, and 20 μ M). The cells were then incubated in a normoxic condition (20% O₂, 5% CO₂, 37 °C) for 48 h (Kanazawa et al., 2017). After 48 h exposure, the medium was discarded, and cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

2.3. Standardization of ormeloxifene concentration

H9C2 cells at confluence were starved with serum-free DMEM and incubated in a normoxic condition (20% O₂, 5% CO₂, 37 °C) or a hypoxic condition (1% O₂, 5% CO₂, 37 °C) with or without ormeloxifene treatment at varying concentrations (0, 1, 2.5, 5 and 10 μ M) for 48 h. The intensity of ANP expression, as well as the cells' surface area, was evaluated by immunocytochemistry. The effect of ormeloxifene per se on ANP was also investigated in normoxic H9C2 by immunocytochemistry.

2.4. Human pulmonary arterial smooth cells (HPASMCs)

HPASMCs (CC-2581, Lonza) were maintained in their prescribed medium, smooth muscle growth medium-2 BulletKit (CC-3181, Lonza) under an atmosphere of 5% CO₂ at 37 °C. Cells at passages 2 to 8 were exposed to hypoxia for 72 h, with or without ormeloxifene treatment (1 μ M), in a plex-glass modular incubator chamber (Billups Rothberg, San Diego, CA) as previously reported (Shafiq et al., 2021).

2.5. MTT assay

Cells were treated with 5 mg/mL MTT and incubated for 3h at 37 °C. After that, cells were solubilized by DMSO and incubated at 37 °C for

another 30 min. The absorbance of the media was measured at 570 nm by a standard microplate reader (Cytation 3; BioTek).

2.6. Cell counting

To assess hypoxia-induced cell proliferation by counting, 5×10^3 HPASMCs were seeded in 96-well plates in duplicate and subjected to normoxic or hypoxic conditions (1% O₂), with or without ormeloxifene treatment (1 μ M), for 72 h. At the end of the exposure, cells were trypsinized and counted by a hemocytometer.

2.7. Immunocytochemistry

The effects of hypoxia on H9C2 cell size as well as 17 β -hydroxy steroid dehydrogenase 1 & 2 (17 β HSD 1 & 2) expressions in HPASMCs were assessed by immunocytochemistry. Briefly, cells were grown in 24-well plates and either incubated in normoxic conditions or exposed to hypoxia (1% O₂), with or without ormeloxifene treatment (1 μ M), for 48 h (H9C2) or 72 h (HPASMCs). At the end of the exposure, cells were fixed with cold 4% paraformaldehyde for 10 min, washed with PBS, and then permeabilized with 0.2% Triton X-100 for 40 min at room temperature. After that, cells were washed and blocked with 3% bovine serum albumin (BSA) in PBS for 2 h. The cells were then washed and incubated with primary antibodies, ANP (NPPA) (1:200), 17 β HSD 1 (1:200), and 17 β HSD (1:200) diluted in 3% BSA at 4 °C overnight. Subsequently, the cells were incubated with phalloidin (1:600) and secondary Alexa Fluor 488 IgG conjugated (Invitrogen, USA) at room temperature for 2 h. Phalloidin was used due to its high selectivity for F-actin in a variety of cell and tissue samples. The cells were counterstained for the nucleus with DAPI, and images were captured with an inverted fluorescence microscope (Leica Microscope, DM 6000, Germany). ImageJ software was used to analyze the intensity of primary antibody staining and cell surface area.

2.8. In vivo studies

Female Sprague–Dawley rats (200–250 g) were procured from the Laboratory Animal Division of CSIR-Central Drug Research Institute (CDRI), Lucknow, India, and housed in polyacrylic cages (22.5 cm $\times$ 37.5 cm). They were randomly divided into the following six groups (n = 6) for each group (Fig. 1): i. sham-operated, saline-treated vehicle (2% DMSO) group (S); ii. sham-operated, monocrotaline-treated vehicle group (S + M); iii. sham-operated, monocrotaline-treated, ormeloxifene (2.5 mg/kg) group (S + M + O); iv. ovariectomized, saline-treated vehicle group (O); v. ovariectomized, monocrotaline-treated vehicle group (O + M); vi. ovariectomized, monocrotaline-treated,

ormeloxifene group (O + M + O).

To validate the efficacy of ormeloxifene independent of gender, twelve male Sprague–Dawley (SD) rats (220–250 g) were procured from the Laboratory Animal Division of Central Drug Research Institute (CSIR-CDRI), Lucknow, and divided into 3 groups of 4 rats per group vis-à-vis the Control, saline-treated vehicle (2% DMSO) group, monocrotaline-treated vehicle group, and monocrotaline-treated ormeloxifene (2.5 mg/kg) group. Both the vehicle and ormeloxifene treatments were via the oral route, commencing on the 7th Day post-monocrotaline treatment and lasting for 4 weeks (35th Day post-monocrotaline treatment) (Tian et al., 2020). Ormeloxifene dose was selected based on the previous report of Blesson et al. (2006), with slight modification.

Throughout the experiment, the rats were maintained on husk bedding under standard laboratory conditions (RT 24–27 °C, humidity 60–62%, and 12 h light and dark cycle), provided with dry pellet food and water *ad libitum*, while PH was induced by a single subcutaneous injection of 60 mg/kg body weight monocrotaline (Singh et al., 2016). Monocrotaline was dissolved in 1 N HCl, and pH was maintained at 7.4 with NaOH. All animal procedures were performed according to the protocols approved by the Institutional Animal Ethics Committee (IAEC), CSIR-CDRI, Lucknow, India (IAEC/2020/123/Renew-1/-Dated-23/12/2021), and the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication 8523, revised 1996).

2.9. Echocardiography, right ventricular pressure, and organs weights

For the non-invasive assessment of right ventricular mass, 2D echocardiography was performed using Vivo 770 High-Resolution Image System (Visual Sonics, Toronto, Canada) under urethane (1.5 gm/kg, i. p.) anesthesia. Echocardiograms, including B-mode, M-mode, and spectral Doppler images, were obtained. Subsequently, the development of PH was assessed by measuring the right ventricular pressure using the trans-diaphragmatic approach (Singh et al., 2016). All animals were allowed to stabilize before the final readings were recorded. After the right ventricular pressure measurement, blood was collected through the orbital sinus into citrate-coated tubes. The rats were then sacrificed while the heart and lungs were isolated and weighed. The uterine weight was also determined as a marker of estrogen signaling (Chen et al., 2017). The collected blood was centrifuged at 1000g for 5 min to obtain plasma. The plasma and lungs were kept at –80 °C for further use.

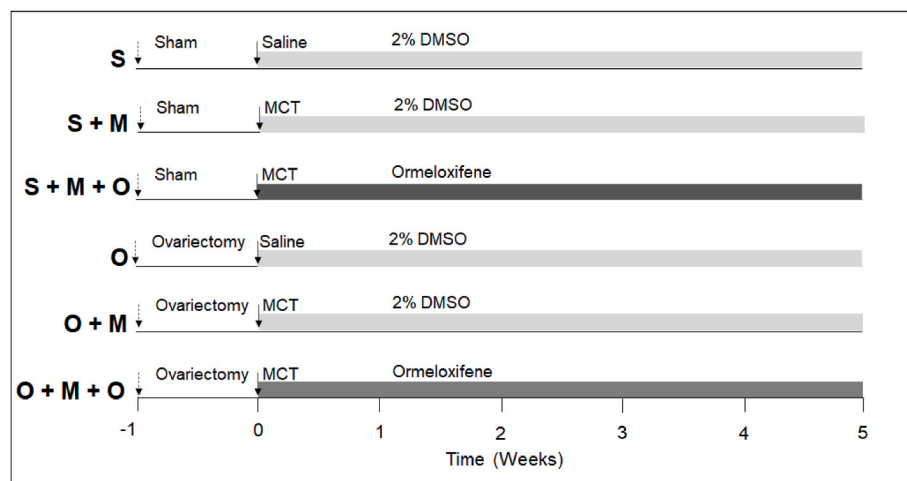


Fig. 1. Experimental protocol for the female rats' studies (n = 6/group). A bilateral ovariectomy or sham operation was performed in female rats at week –1. Rats were injected with 60 mg/kg monocrotaline (MCT) or 0.9% saline at week 0. After Week 1, S + M + O and O + M + O groups received 2.5 mg/kg ormeloxifene, while other groups received the ormeloxifene vehicle (2% DMSO) daily, per oral, for 4 weeks. The duration of each of the experimental settings was represented by the shaded bars.

2.10. Assessment of right ventricular hypertrophy and marker of inflammation

The walls of the right ventricle, left ventricle and ventricular septum were separated and weighed. The Fulton index (ratio of the right ventricle to the summation of the left ventricle and intraventricular septum) and right ventricle (mg)/body weight (g) ratio were calculated for the assessment of the level of right ventricular hypertrophy (Yuan et al., 2013). The lung weight/body weight ratio was also determined as a marker of inflammation (Singh et al., 2016).

2.11. Enzyme-linked immunosorbent assay

The concentrations of 17 β -estradiol were measured in the plasma of monocrotaline-treated female rats and hypoxic HPASMCs by Enzyme-linked immunosorbent assay (ELISA) using the Estradiol kit (Fine Test, China; Cat no: EU0390), while the level of estrone (E1) was measured by the General E1 ELISA kit (ELK Biotechnology, China; Cat no: ELK7837). The manufacturers' instructions were strictly followed in carrying out the assays.

2.12. Histological analysis

For pulmonary vascular remodeling and cardiac hypertrophy, the lungs and hearts were quickly isolated, fixed with 4% paraformaldehyde, and embedded in paraffin. The 10 μ m lung and heart sections were dewaxed and stained with hematoxylin and eosin (H&E) for evaluating pulmonary vascular remodeling and right ventricular hypertrophy, respectively. Similarly, a 10 μ m section of the hearts was stained with picosirius red staining for fibrosis. Images for the lung sections and picosirius red-stained heart sections were examined with Zeiss Axio Imager 2 microscope, BioSciences, Germany, while the images for H&E-stained heart sections were viewed with Nikon SMZ-445 Stereo Zoom microscope, Japan. The wall thickness of the pulmonary artery, right ventricular wall area, and the % picosirius red positive areas were measured using the Fiji ImageJ software, as previously reported (Schindelin et al., 2012; Courtoy et al., 2020).

2.13. Western blotting

Total lysates were obtained from lung tissues or cells by Radio-immunoprecipitation Assay (RIPA) lysis buffer containing 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L Na₃VO₄, 1% Triton X-100, 0.1% Sodium dodecyl sulfate (SDS), 0.5% Sodium deoxycholate, 10 mmol/L Sodium fluoride, and protease inhibitor cocktail (MS-SAFE, Sigma Aldrich, USA). The lysates were centrifuged at 13,000 rpm for 15 min at 4 °C, and supernatants were collected. Total protein concentration was determined using a bicinchoninic acid method, and equal amounts of protein (20–40 μ g) were separated by SDS-PAGE and transferred to the Polyvinylidene difluoride (PVDF) membrane. The immunoblots were subsequently probed with antibodies against HIF-1 α (1:1500), PCNA (1:1000), NF- κ B (1:1000), HO-1 (1:1000), NOX4 (1:1000), VEGF (1:1000), Bax (1:1000), Bcl2 (1:1500), caspase 3 (1:1000), estrogen receptor α & β (1:1500), p-eNOS (1:1500), 17 β HSD1 & 2 (1:1500) and PGC-1 α (1:1000) overnight at 4 °C. After that, the blots were washed with 1 x TBST buffer and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody or β -actin (1:10000) for 2 h at room temperature. Using spot densitometry analysis, the blots were visualized with an enhanced chemiluminescent (ECL) Plus reagent (Sigma, St. Louis, MO, USA). The band intensity was quantified by MYIMAGE analysis software (Thermo Fisher Scientific, Waltham, MA, USA).

2.14. Flow cytometry

Flow cytometry was used to analyze cellular reactive oxygen species,

mitochondrial membrane potential, and mitochondrial superoxide. Briefly, tissue (digested with collagenase II) or cells (HPASMCs) were centrifuged at 5000 rpm and then incubated with 5 μ M 2'-7'-Dichlorodihydrofluorescein diacetate (DCFDA), 2.5 μ M JC1, and 1 μ M mitoSox for 20 min at 37 °C for the estimation of cellular reactive oxygen species, mitochondrial membrane potential and mitochondrial superoxide, respectively, using Fluorescence-activated cell sorting (FACS) Calibur (FACS Calibur, BD Bioscience). The cell population of interest was isolated with gating; data were analyzed and presented as the percentage of DCFDA and mitoSox-positive stained cell populations. Cells were treated with DMSO and 0.3% H₂O₂ as negative and positive controls for oxidative stress, respectively (Pan et al., 2012).

2.15. Real-time PCR

Lung tissues (50 mg) or HPASMC were homogenized, and total RNA was extracted using RNAiso Plus. cDNA was synthesized from 1 μ g total RNA using the High-Capacity cDNA Reverse Transcription Kit (Vilnius, Lithuania; Cat no: 01123700). Genes were amplified using Light Cycler 480 (Roche, USA), and the PowerUp SYBR Green Master Mix was used to determine the mRNA expressions (Vilnius, Lithuania; Cat no: 01059552). Applied Biosystems 7500 Real-time PCR system was used to perform real-time PCR reactions. The comparative CT ($\Delta\Delta C_T$) method was used to calculate the change in mRNA expression, while data were normalized to the GAPDH and expressed as fold change to control (Frump et al., 2015). The primers used are listed as follows: IL-10 (R), 5'-CCTGGTAGAAGTGATGCCCC-3' and 5'-GCAGCTGTATCCAGAGGGTC-3'; IL-1 β (R), 5'-CCTATGTCTTGCCCGTGGAG-3' and 5'-CACACACTAGCAGGTGCTCA-3'; TNF- α (R), 5'-TCAGTAAGTGTCCCAA GCCTC-3' and 5'-TGATCCTGGAAGGGGACAAAG-3'; Nrf1 (R), 5'-TCGCTCTGGATGGTCATTTC-3' and 5'-AGCCCATCTCGTACCATCAC-3'; Nrf2 (R), 5'-TCGCTCTGGATGGTCATTTC-3' and 5'-AGCCCATCTCGTACCATCAC-3'; PPAR γ (R), 5'-GGAGCCGTGACCACTGACA-3' and 5'-TGGTTTGCTGCATGGTTCTG-3'; GAPDH(R), 5'-GGAAGGACATCATGACCACAGT-3' and 5'-GCCATCAGCCACAGTTTC-3'; IL-1 β (H), 5'-GCCAGTGAAATGATGGCTTATT-3' and 5'-AGGAGCACTTCATCTGTTAGG-3'; TNF- α (H), 5'-CTCTTCTGCCTGCTGCACTTTG-3' and 5'-ATGGGCTACAGGCTTGCTCACTC-3'; GAPDH (H) 5'-AGGGCTGCTTTAACTCTGGT-3' and 5'-CCCCACTTGATTTTGAGGGA-3'.

2.16. Myograph study (ex vivo experiment)

In a separate experiment, the functioning of pulmonary artery endothelial cells of the experimental rats was assessed using Myograph. The lungs were isolated, while the endothelium-intact intra-pulmonary arteries were dissected and cut into rings of 3–4 mm. The rings were subsequently mounted on a Danish Myo Technology wire myograph (Aarhus, Denmark) for isometric recordings and maintained in Krebs physiological saline solution (119 mM NaCl, 4.7 mM KCl, 1.5 mM CaCl₂, 1.17 mM MgSO₄, 1.18 mM KH₂PO₄, 25 mM NaHCO₃, and 5.5 mM glucose) at 37 °C. The mounted arteries were subjected to a basal tension of 5 mN and left to equilibrate for 1 h. They were intermittently aerated with a mixture of 95% O₂ and 5% CO₂, while the buffer was changed every 15 min.

Prior to every experiment, the endothelium viability of each arterial ring was ensured by the contraction response induced by 80 mM KCl. Finally, the endothelium integrity of the arterial rings was assessed via a concentration-dependent relaxation response of acetylcholine (1 nM–10 μ M) over thromboxane A₂ analog, U46619 (200 nM) pre-constricted arterial rings. Changes in isometric tension were recorded by using PowerLab data collection and Chart 5 software.

2.17. Statistical analysis

Data were analyzed and presented as the mean $\pm$ S.E.M, using PRISM 8.0 (GraphPad Software, USA). A One-way ANOVA, followed by the

Tukey post-hoc test, was used to compare mean values among the *in vitro* and male rat groups. Meanwhile, A Two-way ANOVA, followed by the Tukey post-hoc test, was used to compare mean values among individual groups of ovary-intact and ovariectomized female rats and to determine the differences among the corresponding ovary-intact and ovariectomized rats. Values were considered significant when $p < 0.05$.

3. Results

3.1. Cytotoxicity and ormeloxifene concentration-finding range

For selecting non-toxic, effective ormeloxifene concentrations, cytotoxicity was evaluated in H9C2 with different concentrations (5–20 μM) of ormeloxifene. As presented in Fig. S1, concentrations ≤ 10 μM were less toxic, while 15 and 20 μM significantly reduced viability ($p < 0.05$ and $p < 0.01$, respectively). Next, we treated hypoxic H9C2 with 1–10 μM ormeloxifene. Our data (Fig. S2) show that hypoxia increased the expression of natriuretic Peptide A, ANP (marker of cardiac stress and hypertrophy), and cell size, while 1 and 2.5 μM ormeloxifene but not 5 and 10 μM reduced ANP expression and decreased cell size ($p < 0.001$). Although more effects were found in 2.5 μM , they were not significantly different ($p > 0.05$) from those of 1 μM . Therefore, 1 μM ormeloxifene was used for subsequent *in vitro* studies. We further investigated whether ormeloxifene per se has a dose-dependent effect on ANP intensity in normoxic H9C2. Our findings showed that ormeloxifene per se has no effect on ANP intensity at tested concentrations (1.0, 2.5, 5.0, and 10.0 μM) when compared to the control (Fig. S3).

3.2. Ormeloxifene reverses proliferation in hypoxic HPASMCs

Hypoxia is an established stimulus for the proliferation of pulmonary arterial smooth muscle cells (PASMCs), leading to vascular remodeling, vasoconstriction, and PH. Hence, we investigated the anti-proliferative potential of ormeloxifene in hypoxic HPASMCs. As presented in Fig. S4 and Fig. 2 a-c, hypoxia increased hypoxia-inducible factor-1 alpha (HIF-1 α) protein expression, cell viability, cell number, and protein expression of proliferating cell nuclear antigen (PCNA), a proliferating marker. More so, there was a reduced expression of caspase 3 in hypoxic HPASMC (Fig. 2d), indicating apoptosis resistance. Pre-treatment of hypoxic HPASMC with ormeloxifene reduced HIF-1 α expression, reversed hypoxia-induced proliferation, and increased caspase 3 expression.

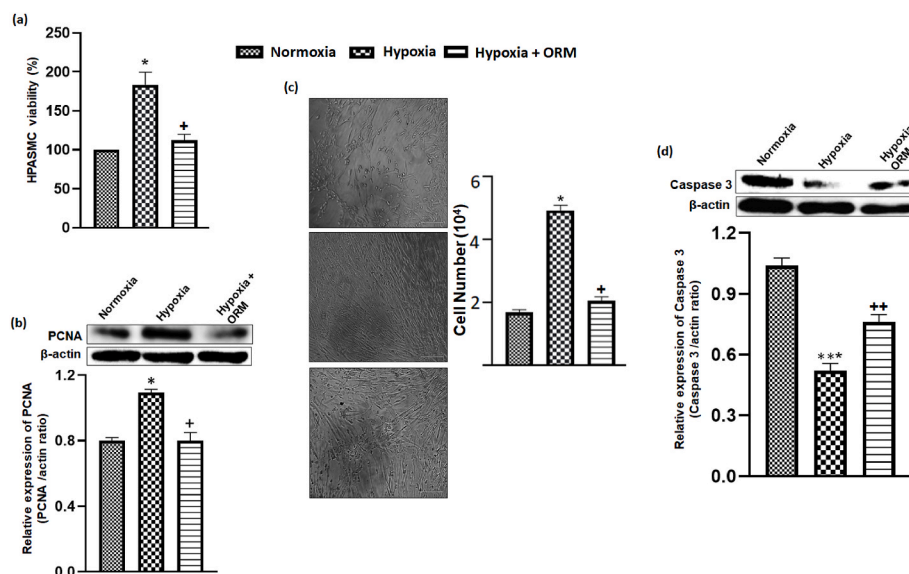


Fig. 2. Effect of ormeloxifene (ORM) on hypoxia-induced proliferation and apoptosis resistance in HPASMC. (a) viability (MTT) assay, (b) Western blot analysis of PCNA expression, (c) representative HPASMC confluence per group and cell number; Scale bar, 200 μm (d) Western blot analysis of caspase 3 expression. Hypoxia increased cell proliferation and hindered apoptosis. In contrast, ormeloxifene treatment reduced proliferation and promoted apoptosis. * $p < 0.05$ vs Normoxia; *** $p < 0.001$ vs Normoxia; + $p < 0.05$ vs Hypoxia; ++ $p < 0.01$ vs Hypoxia ($n = 3$).

3.3. Hypoxia-induced inflammation in HPASMCs is attenuated by ormeloxifene

Infiltration of inflammatory cytokines is a key disease mediator in PH. Therefore, the effect of ormeloxifene on hypoxia-induced inflammation was evaluated in HPASMCs. Our study revealed increased protein expression of nuclear factor kappa B (NF- κB) p65 (Fig. 3a) and elevated mRNA expressions of Interleukin 1 beta (IL-1 β) and tumor necrosis factor-alpha (TNF- α) in hypoxic HPASMCs (Fig. 3 b and c), while ormeloxifene reduced the expressions of NF- κB and the inflammatory cytokines, IL-1 β , and TNF- α (Fig. 3 a - c).

3.4. Ormeloxifene reduced NOX4 expression and reversed mitochondrial membrane hyperpolarization in hypoxic HPASMCs

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4) is a major source of reactive oxygen species accumulation that perturbs mitochondrial function and promotes hyperproliferation in pulmonary arterial smooth cells (PASMCs) (Bernard et al., 2017; Wang et al., 2022). Our data showed that exposure of HPASMCs to 72 h hypoxia increased NOX4 protein expression, reduced protein expression of peroxisome proliferator-activated receptor-gamma coactivator -1 alpha (PGC-1 α), and increased mitochondrial membrane potential (Fig. 4 a and b). Meanwhile, ormeloxifene pre-treatment reduced NOX4 expression, increased PGC-1 α expression, and attenuated hypoxia-induced mitochondrial membrane hyperpolarization.

3.5. Ormeloxifene promotes 17 β -estradiol synthesis in hypoxic HPASMCs

The 17 β -hydroxysteroid dehydrogenases 1 & 2 (17 β HSD 1 & 2) control the biological actions of estrogen by regulating the interconversion of 17 β -estradiol and estrone (Tofovic and Jackson, 2020). While estrogenic 17 β HSD 1 promotes estradiol synthesis by converting estrone to estradiol, the anti-estrogenic 17 β HSD 2 decreases estradiol level via the conversion of estradiol to estrone. In this study, the protein expression of 17 β HSD1 and 17 β -estradiol level were significantly reduced in HPASMCs following hypoxia exposure (Fig. 5a and c), while the 17 β HSD2 protein expression and estrone level increased (Fig. 5b and d). Conversely, hypoxia downregulated estrogen receptors α and β protein expressions (Fig. 5 e). However, pre-treatment with ormeloxifene elevated 17 β HSD1, and reduced 17 β HSD2 expression, promoting the conversion of less estrogenic estrone to a more estrogenic 17 β -estradiol and, thus, upregulating ER α and ER β expressions.

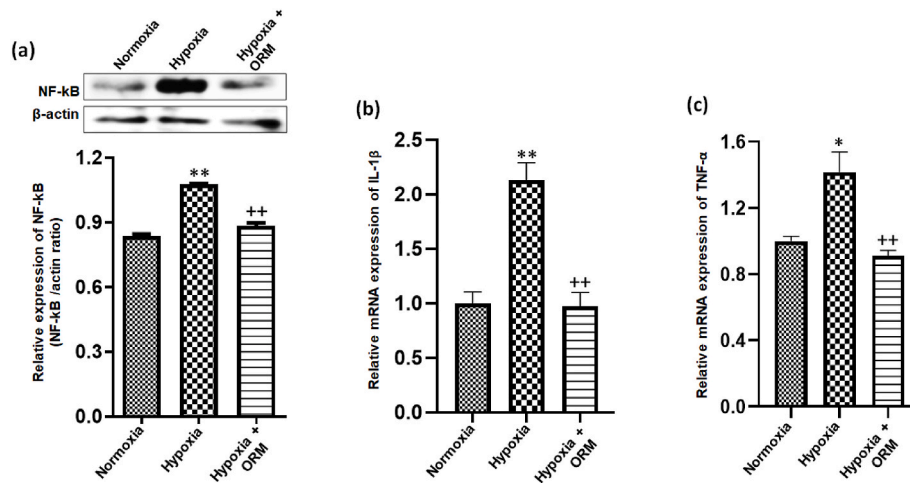


Fig. 3. Ormeloxifene (ORM) effect on hypoxia-induced inflammation in HPASMCs. (a) Western blot analysis of NF-κB (b) mRNA expression of IL-1β (c) mRNA expression of TNF-α. Hypoxia promoted inflammation in HPASMCs after 72 h exposure, while ormeloxifene reduced the expressions of the inflammatory cytokines. * $p < 0.05$ vs Normoxia; ** $p < 0.01$ vs Normoxia; ++ $p < 0.01$ vs Hypoxia ($n = 3$).

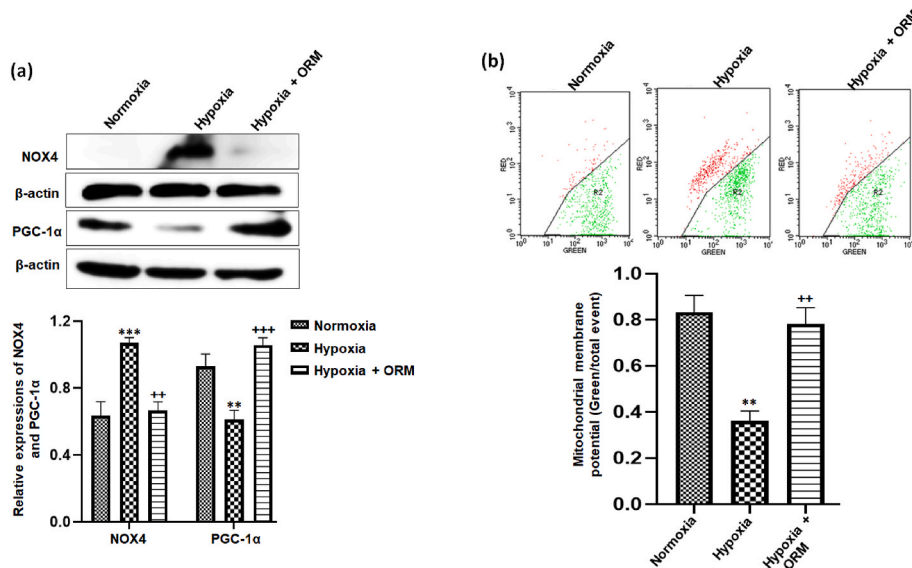


Fig. 4. Effect of ormeloxifene (ORM) on hypoxia-induced mitochondrial stress in HPASMCs exposed to 72 h hypoxia (1% O₂). (a) Analysis of NOX4 and PGC-1α protein expressions by Western blot, and (b) mitochondrial membrane potential. Hypoxia increased expression of NOX4, reduced PGC-1α expression, and hyperpolarized the mitochondrial membrane, while ormeloxifene reversed the hypoxia effects ** $p < 0.01$ vs Normoxia; *** $p < 0.001$ vs Normoxia; ++ $p < 0.01$ vs Hypoxia; +++ $p < 0.001$ vs Hypoxia ($n = 3$).

3.6. Ormeloxifene attenuates right ventricular pressure and right ventricular hypertrophy, reduces right ventricular fibrosis, and improves the survival rate in monocrotaline-treated female rats

Right ventricular dysfunction is a key determinant of disease prognosis and survival in PH. Thus, we assessed the effects of ormeloxifene on hypoxia-induced hypertrophy in H9C2 as a pilot study. Our immunocytochemistry experiment in Fig. 6 a & b showed that hypoxia increased natriuretic peptide A (ANP) intensity, enlarged cells size, and downregulated estrogen receptors α and β expressions in H9C2 cells ($p < 0.001$), while ormeloxifene decreased ANP intensity, reduced the size of the cells, and increased expressions of estrogen receptors α and β ($p < 0.001$). Further, the *in vitro* ormeloxifene effects were validated in female rats. As presented in Fig. 6 c - f and Fig. S5, assessments of pulmonary hemodynamics and echocardiographic analyses showed that the monocrotaline-treated groups developed severe PH, with increased right ventricular pressure, Fulton index, right ventricular to body weight ratio, heart weight to body weight ratio, and right ventricular mass when compared with the sham-operated or ovariectomized control

groups. Remarkably, ovariectomized, monocrotaline-treated rats displayed higher right ventricular pressure ($p < 0.01$) and right ventricular hypertrophy { $p < 0.05$ (right ventricular mass) and $P < 0.01$ (right ventricular wall area)} compared with sham-operated, monocrotaline-treated rats (Fig. 6 c - f). Also, analysis of picrosirius red staining showed increased fibrosis (collagen deposition) in monocrotaline-treated rats, with more effect in ovariectomized rats ($p < 0.05$) (Fig. 6 g). Administration of ormeloxifene significantly lowered right ventricular pressure, right ventricular hypertrophy, and fibrosis in female rats, with or without ovariectomy.

There were no differences in final body weight and survival rate among all the groups of ovary-intact rats (Fig. S6). However, ovariectomy increased final body weight compared to the vehicle-treated sham control group, while monocrotaline administration inhibited ovariectomy-induced body weight increase, regardless of ormeloxifene treatment. Also, monocrotaline reduced the survival rate in ovariectomized rats compared, while ormeloxifene improved the survival rate (Fig. S6).

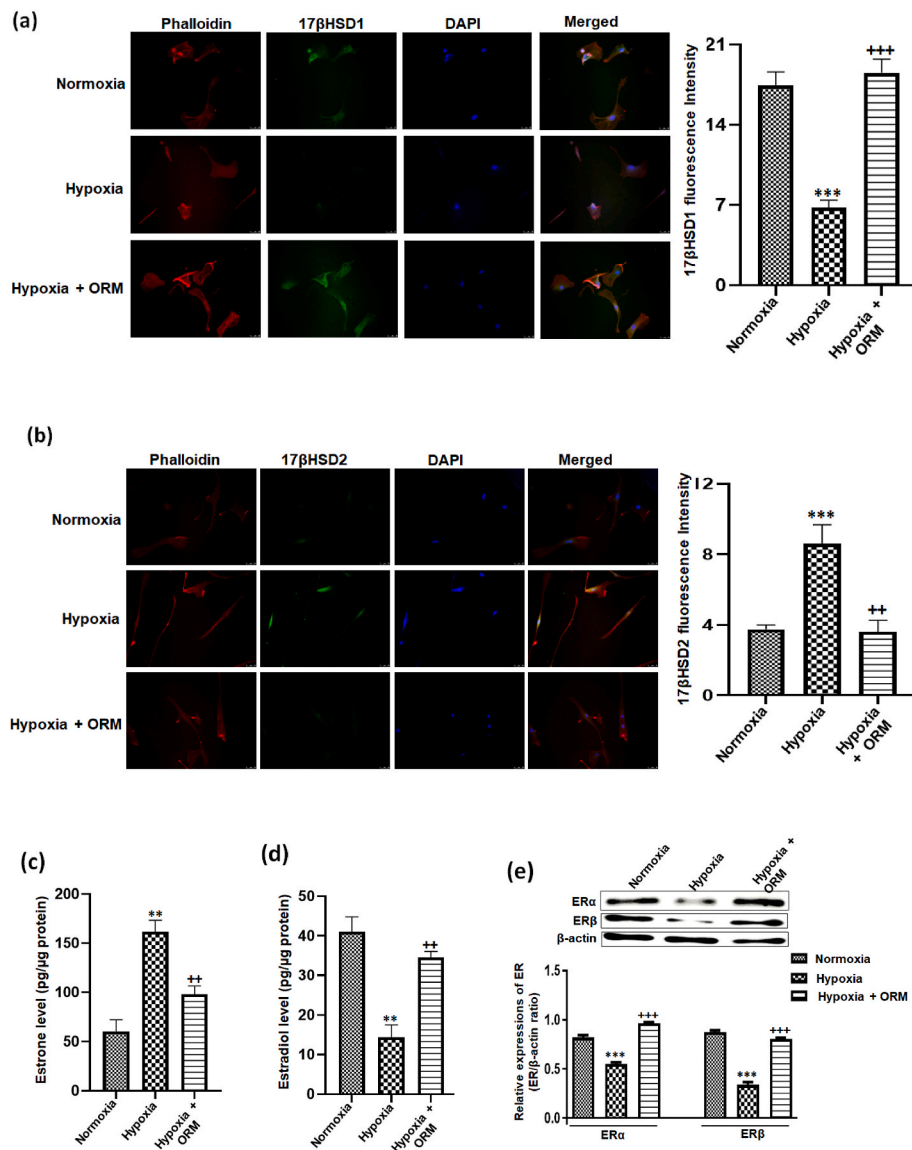


Fig. 5. Ormeloxifene (ORM) increases 17β-estradiol synthesis and upregulated estrogen receptors α and β (ERα and ERβ) in HPASMCs exposed to 72 h hypoxia. (a) Immunocytochemistry analyses of 17β-hydroxysteroid dehydrogenase 1 intensity (17βHSD 1), and (b) 17β-hydroxysteroid dehydrogenase 2 (17βHSD 2); Scale bar, 50 μm, (c) 17β-estradiol level (d) Estrone level (e) Western blot analyses of estrogen receptors α and β. Hypoxia significantly decreased 17βHSD 1 expression and increased expression of 17βHSD 2, promoting conversion of 17β-estradiol to estrone and decreasing estrogen receptors α and β expressions, while ormeloxifene attenuated the hypoxia effect on estrogen metabolizing enzymes and upregulated estrogen receptors α and β expressions. ** $p < 0.01$ vs Normoxia of the corresponding group; *** $p < 0.001$ vs Normoxia of the corresponding group; ++ $p < 0.01$ vs Hypoxia of the corresponding group; +++ $p < 0.001$ vs Hypoxia of the corresponding group ($n = 3$).

3.7. Ormeloxifene reverses pulmonary apoptosis-resistance, proliferation, and vascular remodeling in monocrotaline-induced PH in female rats

There was a decrease in pulmonary Bcl-2-associated X (Bax) expression, while pulmonary B-cell leukemia/lymphoma 2 (Bcl-2), proliferating cell nuclear antigen (PCNA), and vascular endothelial growth factor (VEGF) expressions increased in monocrotaline-treated rats (Fig. 7 a - e), indicating proliferation of pulmonary vascular cells. In addition, pulmonary arterial medial wall thickness was significantly increased in response to monocrotaline as compared to the control rats, with more thickness in the ovariectomized rats ($p < 0.001$) (Fig. 7 f). Meanwhile, ormeloxifene treatment significantly reduced protein expressions of PCNA, VEGF, and Bcl2 expressions, increased Bax expression, and reversed arterial medial thickness to normal levels, attenuating vascular remodeling (Fig. 7 a - f).

3.8. Ormeloxifene mitigates pulmonary inflammation in monocrotaline-induced PH in female rats

Monocrotaline elevated the ratio of lung weight to body weight (Fig. 8 a), which indicates inflammation (Singh et al., 2016). Further, monocrotaline promoted pulmonary protein expressions of NF-kB p65

and pulmonary mRNA expressions of IL-1β and TNF-α but decreased mRNA expressions of IL-10 (Fig. 8 b-f) in both sham-operated and ovariectomized rats. In contrast, ormeloxifene significantly decreased the ratio of lung weight to body weight as well as the pulmonary expressions of NF-kB, IL-1β, and TNF-α, while raising the IL-10 mRNA expressions (Fig. 8 a -f).

3.9. Ormeloxifene attenuates monocrotaline-induced pulmonary oxidative stress in female rats

Flow cytometry and Western blot analyses revealed the increased generation of pulmonary cellular reactive oxygen species and elevated NOX4 protein in monocrotaline-treated rats (Fig. 9 a & b). Further, monocrotaline reduced the expressions of pulmonary heme oxygenase 1 (HO-1) protein as well as mRNA expressions of nuclear respiratory factor (Nrf) 1 & 2 (Fig. 9 b - d). More decrease in mRNA expression of Nrf1 ($p < 0.01$) was found in monocrotaline-treated ovariectomized rats compared with monocrotaline-treated ovary-intact rats, suggesting more stress in ovariectomized rats. Meanwhile, administration of ormeloxifene significantly lowered pulmonary reactive oxygen species production, reduced NOX4 expression, and elevated mRNA expressions of Nrf 1 & 2.

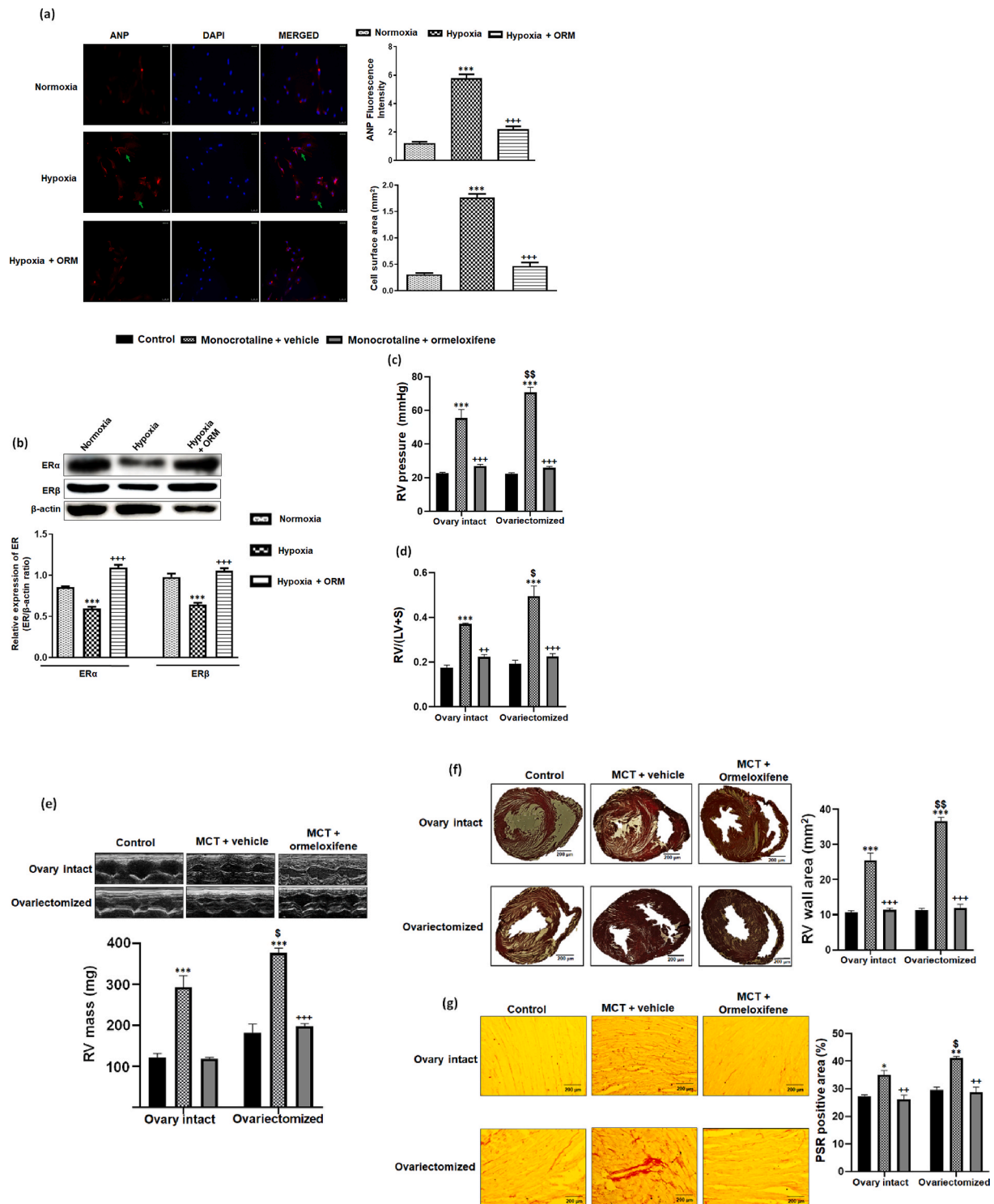


Fig. 6. Effect of ormeloxifene (ORM) on right ventricular (RV) pressure, RV mass and RV fibrosis in monocrotaline-treated ovary intact and ovariectomized Sprague-Dawley rats and hypoxic cardiomyocytes. (a) Immunocytochemistry analysis of ANP expression (Scale bar, 50 μ m) and H9C2 cell surface area, (b) protein expressions of estrogen receptors (ER α and β) in hypoxic H9C2 (c) right ventricular (RV) pressure, (d) ratio of right the ventricular weight to the weight of left ventricle + intraventricular septum (RV/LV + S), the Fulton Index (e) 2-D echocardiography (f) H & E staining of the heart sections (Scale bar, 200 μ m) (g) Picrosirius red (PSR) staining of the heart sections (Scale bar, 200 μ m). Monocrotaline increased right ventricular pressure and induced hypertrophy, with more effects in ovariectomized rats, while ormeloxifene significantly reduced the right ventricular pressure and attenuated the hypertrophy. * $p < 0.05$ vs corresponding control; ** $p < 0.01$ vs corresponding control; *** $p < 0.001$ vs corresponding control; + $p < 0.05$ vs corresponding MCT; ++ $p < 0.01$ vs corresponding MCT; +++ $p < 0.001$ vs corresponding MCT; $^{\$}$ $p < 0.01$ vs corresponding ovary intact; $^{\$ \$}$ $p < 0.01$ vs corresponding ovary intact (n = 6).

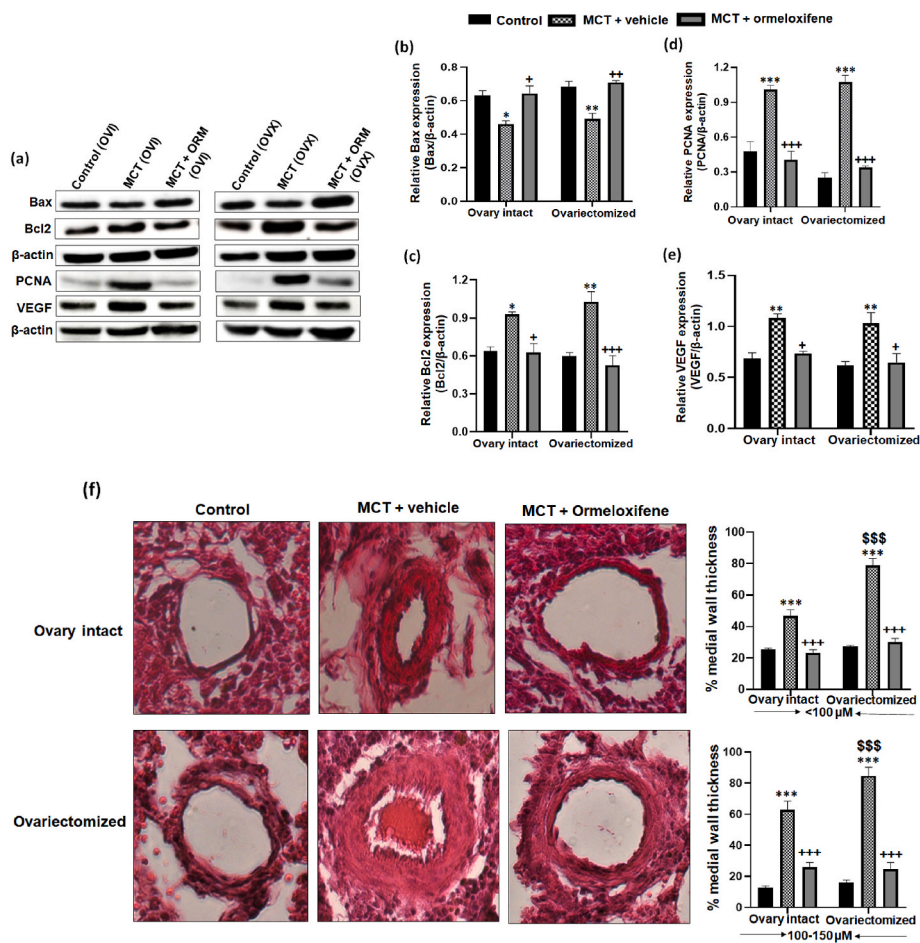


Fig. 7. Effect of ormeloxifene (ORM) on apoptosis resistance, proliferation, and vascular remodeling in lungs of monocrotaline (MCT)-treated ovary intact (OVI) and ovariectomized (OVX) Sprague-Dawley rats. (a) Representative blots of Bax, Bcl2, PCNA, and VEGF (b) Relative expression of Bax (c) Relative expression of Bcl2 (d) Relative expression of PCNA (e) Relative expression of VEGF (f) H & E staining of the pulmonary artery (Scale bar, 200 μm) and % medial wall thickness. Monocrotaline induced hyperproliferation of vascular cells and increased medial wall thickness (with more effects in OVX), while ormeloxifene significantly attenuated proliferation and reduced vascular remodeling. * $p < 0.05$ vs corresponding control; ** $p < 0.01$ vs corresponding control; *** $p < 0.001$ vs corresponding control; + $p < 0.05$ vs corresponding monocrotaline + vehicle; ++ $p < 0.01$ vs corresponding monocrotaline + vehicle; +++ $p < 0.001$ vs corresponding monocrotaline + vehicle; \$\$\$ $p < 0.001$ vs corresponding ovary intact (n = 4).

3.10. Ormeloxifene improves monocrotaline-induced endothelial dysfunction in female rats

Vasoreactivity experiments showed that acetylcholine-induced vasorelaxation was significantly reduced in U46619 precontracted pulmonary artery rings of monocrotaline-treated female rats (Fig. 10 a). The impaired vasorelaxation recorded in monocrotaline-treated ovariectomized rats was more compared to monocrotaline-treated ovary-intact rats, which signifies an enhanced endothelial dysfunction. Meanwhile, ormeloxifene improved vasorelaxation in pulmonary artery rings of both ovary-intact and ovariectomized groups. Also, monocrotaline reduced pulmonary protein expressions of phosphorylated endothelial nitric oxide synthase (p-eNOS) in both ovary-intact and ovariectomized rats, while ormeloxifene increased p-eNOS protein expressions (Fig. 10 b).

3.11. Improvement of pulmonary vascular mitochondrial function in monocrotaline-induced PH in female rats by ormeloxifene

There was an increase in pulmonary mitochondrial membrane potential (evident by a decrease in JC-1 green fluorescence) in monocrotaline-treated rats compared to the control rats (Fig. 11 a). Also, monocrotaline reduced pulmonary mitochondrial superoxide (evident by the decreased number of mitoSox-positive cells) in ovariectomized and ovary-intact rats (Fig. 11 b). Contrarily, mRNA and protein expressions of peroxisome proliferator-activated receptor-γ (PPARγ) and PGC-1α, respectively, were decreased in monocrotaline-treated ovary-intact and ovariectomized rats (Fig. 11 c & d). Ormeloxifene treatment reduced mitochondrial membrane potential and improved the expressions of PPARγ and PGC-1α in both ovary-intact and ovariectomized rats, while it increased mitochondrial superoxide in ovariectomized rats.

3.12. Ormeloxifene regulates pulmonary vascular 17β-hydroxysteroid dehydrogenases protein expressions and upregulates pulmonary protein expressions of estrogen receptors in monocrotaline-induced PH in female rats

Regardless of ormeloxifene treatment, analyses of uterine weight and plasma 17β-estradiol concentration in Fig. 12 a & b showed a significant decrease in uterine weights and reduced plasma 17β-estradiol levels in all ovariectomized groups, which is an indication of a successful ovariectomy and a less uterotrophic activity by ormeloxifene (Schermuly et al., 2011). Similarly, there was a significant reduction ($p < 0.01$) in uterine weight as well as a decreased level ($p < 0.001$) of plasma 17β-estradiol in monocrotaline-treated ovary-intact rats, as compared to the vehicle-treated control rats (Fig. 12 a & b). While administration of ormeloxifene had no significant effect on uterine weights in ovary-intact rats, it elevated plasma 17β-estradiol level ($p < 0.05$) but to a lower extent when compared with the vehicle-treated control rats (Fig. 12 a & b). Also, monocrotaline reduced the pulmonary protein expressions of 17βHSD 1, increased protein expressions of 17βHSD 2, and decreased protein expressions of estrogen receptors α and β (Fig. 12 c & d). In contrast, ormeloxifene administration significantly decreased 17βHSD 2 expressions and upregulated expressions of 17βHSD 1 and estrogen receptors α and β.

3.13. Ormeloxifene ameliorates right ventricular pressure, right ventricular hypertrophy, and pulmonary PCNA expression in monocrotaline-treated male rats

To substantiate our findings in female rats, we validated the efficacy of ormeloxifene in monocrotaline-treated male rats. Similar to female

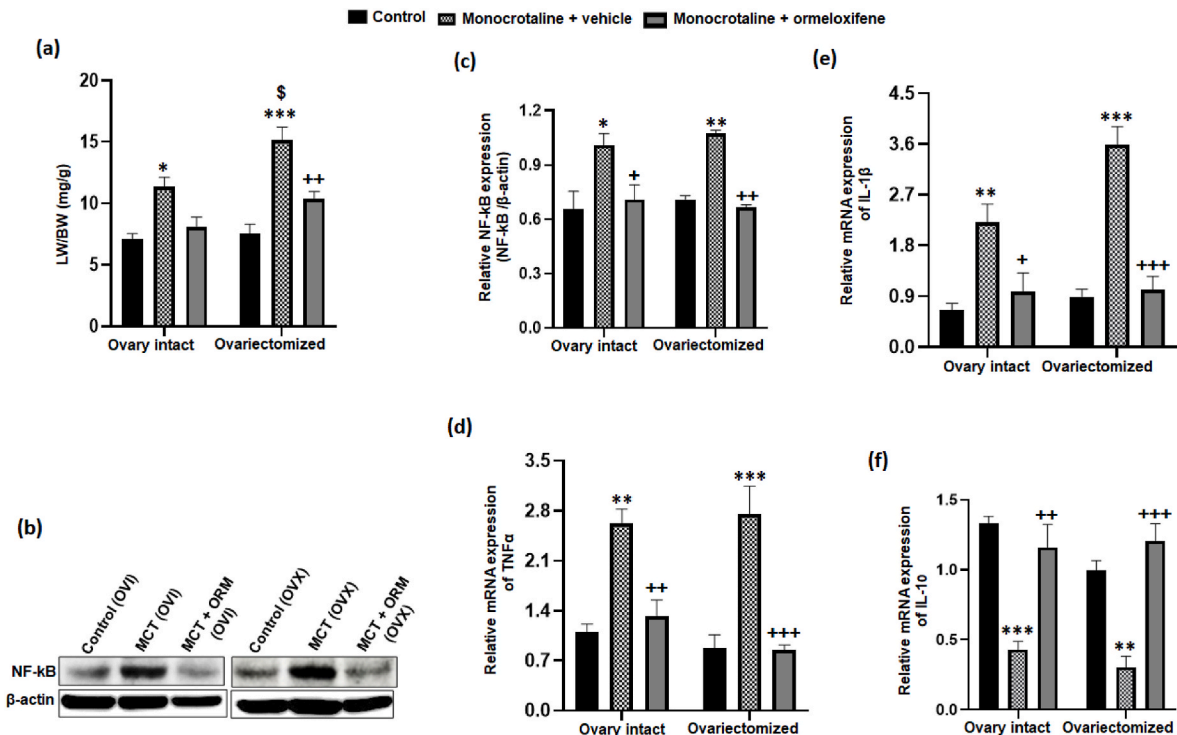


Fig. 8. Effect of ormeloxifene (ORM) on markers of inflammation in monocrotaline-treated ovary intact and ovariectomized Sprague-Dawley rats. (a) Lung/body weight ratio (LW/BW) (b) & (c) Western blot analysis of NF-κB expression, (d) TNF-α mRNA expression, (e) IL-1β mRNA expression, and (f) IL-10 mRNA expression. Monocrotaline (MCT) increased lung-to-body weight ratio and elevated expressions of NF-κB, TNF-α, and IL-1β in both ovary-intact and ovariectomized rats but lowered IL-10 expression. The effects of monocrotaline on markers of inflammation were attenuated by ormeloxifene treatment. * $p < 0.05$ vs corresponding control; ** $p < 0.01$ vs corresponding control; *** $p < 0.001$ vs corresponding control; + $p < 0.05$ vs corresponding monocrotaline + vehicle; ++ $p < 0.01$ vs corresponding monocrotaline + vehicle; +++ $p < 0.001$ vs corresponding monocrotaline + vehicle; \$ $p < 0.05$ vs corresponding ovary intact (n = 4).

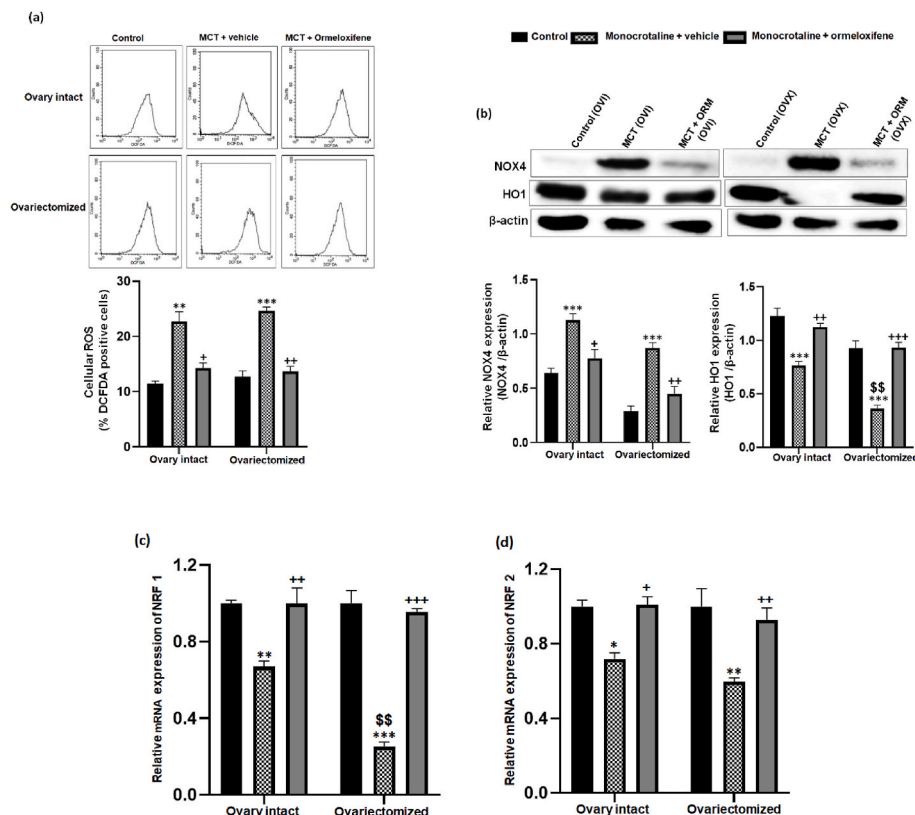


Fig. 9. Ormeloxifene (ORM) attenuates oxidative stress in monocrotaline-treated ovary intact (OVI) and ovariectomized (OVX) Sprague-Dawley rats. (a) DCFDA positive cells (b) Western blot analyses of NOX4 and HO1 expressions (c) Relative mRNA expressions of Nrf1 (d) Relative mRNA expressions of Nrf2. Monocrotaline (MCT) increased NOX4 expression, decreased HO1/Nrf axis, and induced pulmonary production of reactive oxygen species, while ormeloxifene elevated HO1/Nrf axis, attenuated NOX4 expression, and lowered reactive oxygen species generation. * $p < 0.05$ vs corresponding control; ** $p < 0.01$ vs corresponding control; *** $p < 0.001$ vs corresponding control; + $p < 0.05$ vs corresponding monocrotaline + vehicle; ++ $p < 0.01$ vs corresponding monocrotaline + vehicle; +++ $p < 0.001$ vs corresponding monocrotaline + vehicle; \$\$ $p < 0.01$ vs corresponding ovary intact (n = 4).

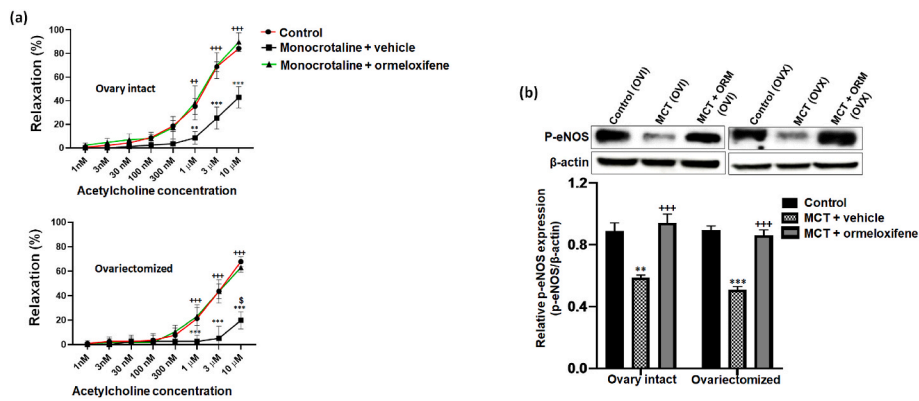


Fig. 10. Ormeloxifene (ORM) improves endothelial function in monocrotaline-treated ovary intact (OVI) and ovariectomized (OVX) Sprague-Dawley rats. (a) acetylcholine-induced vasorelaxation in U46619 pre-contracted rings from pulmonary arteries of ovary intact and ovariectomized rats (b) Western blot analysis of P-eNOS protein. $^{**}p < 0.01$ vs corresponding control; $^{***}p < 0.001$ vs corresponding control; $^{++}p < 0.01$ vs corresponding monocrotaline + vehicle; $^{+++}p < 0.001$ vs corresponding monocrotaline + vehicle; $^{\$}p < 0.05$ vs corresponding ovary intact (n = 5).

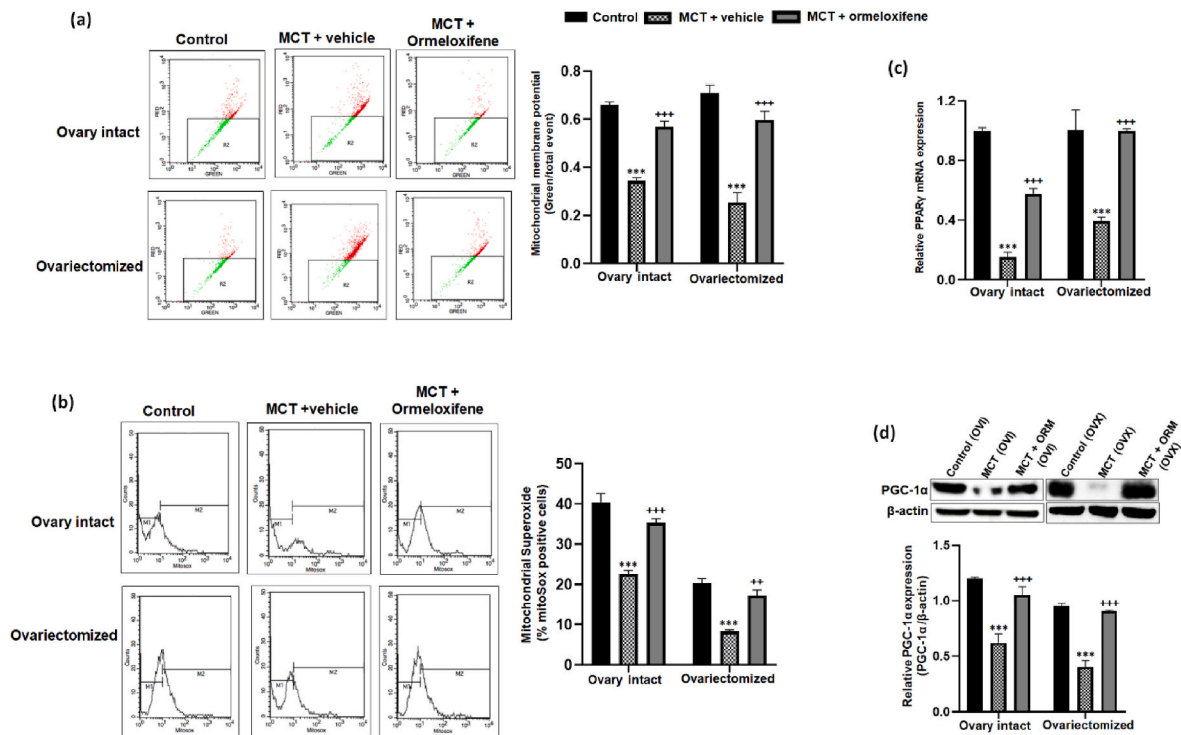


Fig. 11. Ormeloxifene improves mitochondrial health in monocrotaline (MCT)-treated ovary intact (OVI) and ovariectomized (OVX) Sprague-Dawley rats. (a) Mitochondrial membrane potential (JC-1 dye) (b) Mitochondrial superoxide (mitoSox dye) (c) Pulmonary mRNA expression of PPARγ (d) Western blot analysis of pulmonary PGC-1α protein. Monocrotaline resulted in mitochondrial dysfunction, while ormeloxifene treatment significantly improved mitochondrial function. $^{***}p < 0.001$ vs corresponding control; $^{++}p < 0.01$ vs corresponding monocrotaline + vehicle; $^{+++}p < 0.001$ vs corresponding MCT (n = 4).

rats, ormeloxifene significantly reduced right ventricular pressure, Fulton index, and pulmonary PCNA expression in monocrotaline-treated male rats (Fig. 13 a-c).

4. Discussion

This study examined the influence of 17β-estradiol deficiency in monocrotaline-induced PH in female rats. Further, it evaluated the ormeloxifene effects in hypoxic H9C2, HPASMCs, and monocrotaline-treated rats for its possible repurpose in PH treatment. The novelties of our findings are that: 1) hypoxia reduced 17β-estradiol concentration in HPASMCs and increased estrone; 2) monocrotaline and hypoxia dysregulated 17β-HSD 1 & 2 expressions; 3) ormeloxifene modulated pulmonary 17β-HSD 1 & 2 expressions and upregulated estrogen receptors α and β expressions in monocrotaline-treated female rats and hypoxic HPASMCs; 4) ormeloxifene selectively modulated 17β-estradiol

synthesis in a tissue-specific manner, prevented PH progression and attenuated right ventricular hypertrophy in both male and female rats.

Hyperproliferation of pulmonary artery smooth muscle cells (PASMCs) is the critical feature of PH that results in vascular remodeling as well as right ventricular hypertrophy and failure (Farkas and Goncharova, 2022). Conforming with the previously reported anticancer effect of ormeloxifene (Maher et al., 2015; Khan et al., 2015; Hafeez et al., 2017), our study demonstrated that ormeloxifene mitigated hypoxia and monocrotaline-induced proliferation of pulmonary vascular cells and reversed vascular remodeling. In PH, inflammatory cells infiltrate the remodeled pulmonary vessels and contribute largely to survival (KueblerWolfgang et al., 2018; Wang et al., 2022). Our study showed evidence of inflammatory cells' infiltration following hypoxia and monocrotaline treatments through the increased expressions of inflammatory molecules (NF-κB protein, and IL-1 β and TNF-α mRNA) and low mRNA expression of the anti-inflammatory cytokine, IL-10 (*in vivo*).

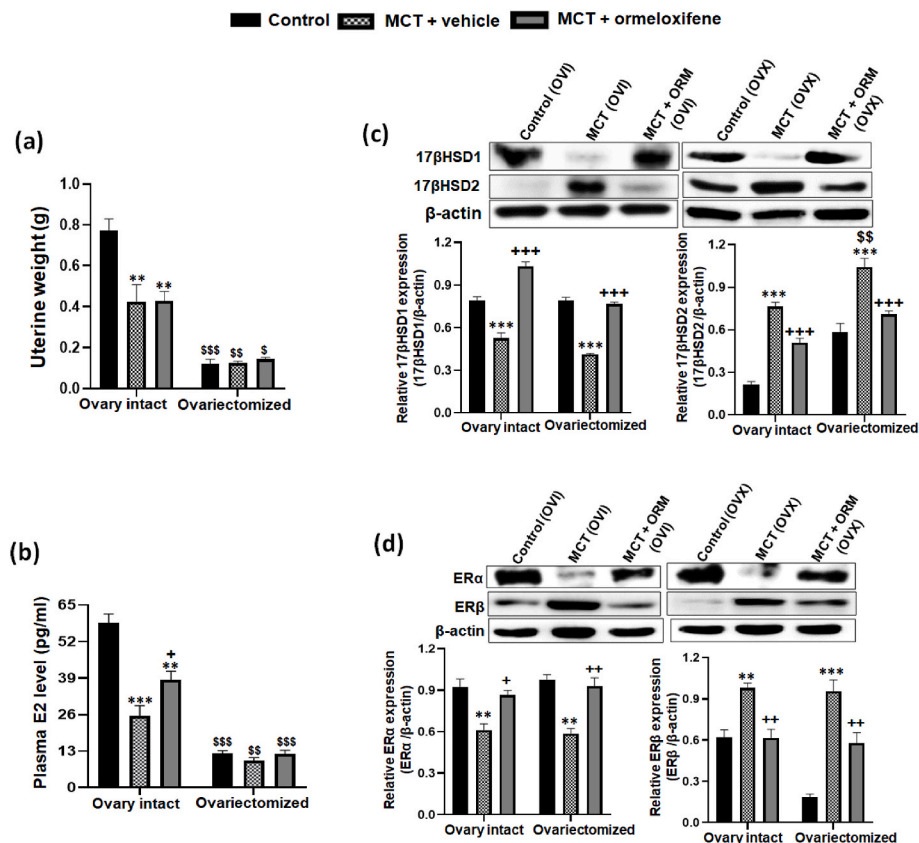


Fig. 12. Effect of ormeloxifene (ORM) on plasma 17β-estradiol synthesis as well as lung expressions of estrogen metabolizing enzymes and estrogen receptors α and β (ER α and β) in monocrotaline-treated ovary intact (OVI) and ovariectomized (OVX) Sprague-Dawley rats. (a) uterine weight (b) 17β-estradiol (E2) (c) Western blot analyses of 17β-hydroxy steroid dehydrogenase (17βHSD) 1 & 2 (d) Western blot analyses of estrogen receptors α and β. Ovariectomy, as well as monocrotaline (MCT), significantly decreased uterine weight and plasma 17β-estradiol level. Also, monocrotaline reduced the 17βHSD1 expression, upregulated 17βHSD 2 expressions, and downregulated expressions of estrogen receptors α and β. In contrast, ormeloxifene improved plasma 17β-estradiol levels, reversed the effect of monocrotaline on lung 17βHSD1 & 2 expressions, and upregulated estrogen receptors α and β expressions without affecting the uterine weight. ** $p < 0.01$ vs corresponding control; *** $p < 0.001$ vs corresponding control; + $p < 0.05$ vs corresponding monocrotaline + vehicle; +++ $p < 0.001$ vs corresponding monocrotaline + vehicle; \$ $p < 0.05$ vs corresponding ovary intact; \$\$\$ $p < 0.001$ vs corresponding ovary intact ($n = 4$).

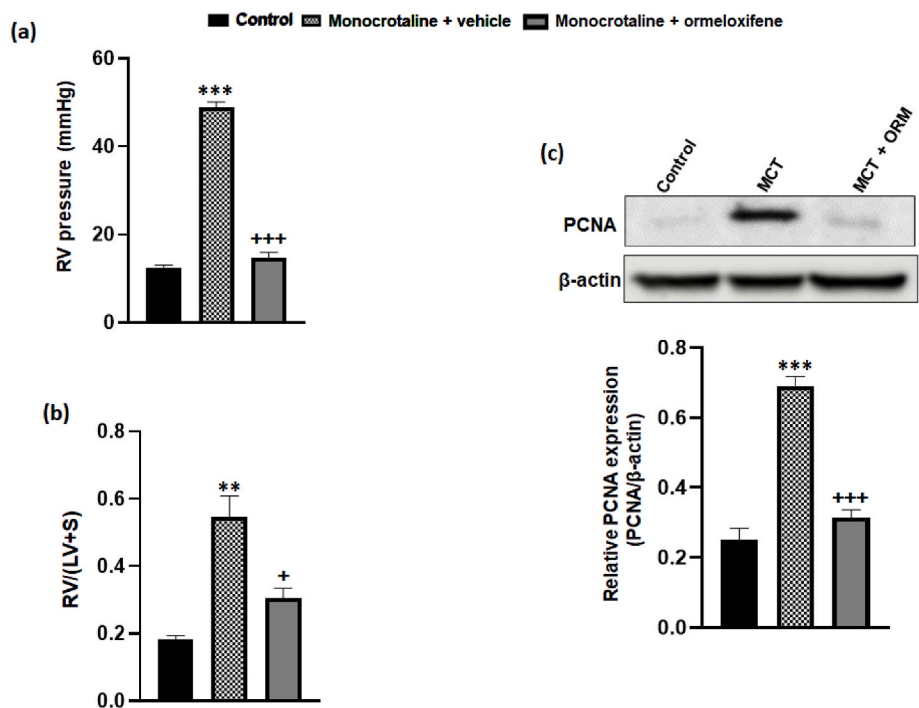


Fig. 13. Effect of ormeloxifene (ORM) on (a) right ventricular pressure (RVP), (b) The Fulton index, ratio of right the ventricular weight to the weight of left ventricle + intraventricular septum (RV/LV + S), and (c) pulmonary PCNA expression in monocrotaline (MCT)-treated male Sprague-Dawley rats. Ormeloxifene significantly lowered the monocrotaline-induced increase in right ventricular pressure, Fulton index, and pulmonary PCNA expression. ** $p < 0.01$ vs control; *** $p < 0.001$ vs control; + $p < 0.05$ vs monocrotaline; +++ $p < 0.001$ vs monocrotaline ($n = 4$).

The Ormeloxifene administration alleviated the induced inflammation, suggesting that it possesses anti-inflammatory properties which may be beneficial for PH treatment.

Inflammation modulates oxidative stress via upregulation of the NOX complex (Anrather et al., 2006; Yu et al., 2017). Besides, increased

expression of NOX4 reportedly plays a vital role in promoting the hyperproliferation of PSMCs, PH development, and cardiac hypertrophy (Wedgwood et al., 2013; Barman et al., 2014; Guo et al., 2018; Wang et al., 2022). Similar to those studies, the observed inflammation in hypoxic and monocrotaline-treated conditions of our study was

associated with increased NOX4 protein expression. Further, our *in vivo* findings showed increased oxidative stress in the lung of monocrotaline-treated female rats, while the HO-1/Nrf axis was down-regulated. Administration of ormeloxifene decreased NOX4 expression, attenuated oxidative stress, and upregulated HO-1/Nrf. Since the HO-1/Nrf axis plays an important role in controlling oxidative stress and improving lung and cardiac functions (Wu et al., 2018; Cui et al., 2021), the increased expressions of HO-1, Nrf1, and Nrf2 by ormeloxifene suggest its antioxidant potency which may be due to its pulmonary estrogenic property (Castracani et al., 2020).

A previous study reported that NOX4-mediated oxidative stress promotes the accumulation of superoxide and degradation of nitric oxide, resulting in endothelial dysfunction that characterizes PH (Novelli et al., 2019). Accordingly, our findings showed that monocrotaline impaired the pulmonary arterioles' vascular tone and decreased pulmonary p-eNOS (Ser1177) expression in lung homogenates, which were improved by ormeloxifene treatment. Superoxide from NOX signaling is a well-known stimulator of protein phosphatase 2A (PP2A), which reduces p-eNOS (Ser1177) expression, and lowers NO content, leading to endothelial dysfunction (Han et al., 2010; Ding et al., 2020). It is, therefore, possible that the improved endothelial function in ormeloxifene-administered rats is via the downregulation of PP2A. Apart from endothelial dysfunction, a report has also shown that NOX4 affects mitochondrial function via the Nrf2 pathway (Bernard et al., 2017). In agreement with that, we presented evidence that elevated NOX4 expression was accompanied by repressed mitochondrial fitness in this study, both *in vitro* and *in vivo*. Contrarily, ormeloxifene improved PPAR γ /Nrf/PGC-1 α path and reversed mitochondrial membrane hyperpolarization, suggesting improved mitochondrial metabolism in ormeloxifene-treated groups.

Our findings revealed a decrease in plasma 17 β -estradiol levels by monocrotaline. Similarly, *in vitro* study showed that hypoxia reduced 17 β -estradiol level in HPASMC and elevated estrone, which presumably indicates the conversion of 17 β -estradiol to estrone by a change in estrogen metabolizing enzymes, thus, resulting in a less protective role of 17 β -estradiol in pulmonary arteries. Interestingly, a previous study has reported alterations in pulmonary expressions of CYP-19, CYP-1A1, and CYP-1B1 in monocrotaline-induced PH (Yuan et al., 2013); however, the role of 17 β -HSDs has not been previously evaluated in PH. Although aromatase is the major metabolizing enzyme for 17 β -estradiol synthesis, the 17 β -HSD 1 & 2 control the biological properties of estrogens and regulate the interconversion of 17 β -estradiol and estrone (Tofovic and Jackson, 2020). Thus, we assessed the effects of hypoxia and monocrotaline on 17 β -HSD 1 & 2 expressions in HPASMC and lungs of monocrotaline-treated female rats, respectively. Our findings revealed that both hypoxia and monocrotaline reduced the expressions of 17 β -HSD 1 and elevated 17 β -HSD 2 expressions. The significance of these findings is that 17 β -HSD likely regulates 17 β -estradiol production and action in PSMCs and therefore plays crucial roles in the pathogenesis/pathophysiology of PH.

Administration of ormeloxifene increased the concentration of 17 β -estradiol in HPASMC and plasma of the ovary-intact rats. It, however, did not stimulate 17 β -estradiol synthesis in ovariectomized rats and had no effect on uterine weight. This may mean that ormeloxifene is less estrogenic in reproductive tissues (Chen et al., 2017), having a tissue-specific effect on 17 β -estradiol biosynthesis. The finding agrees with a previous study where ormeloxifene showed the least uterotrophic activity in rats, as compared to 17 β -estradiol and tamoxifen (Blesson et al., 2006). The improved plasma 17 β -estradiol level observed in ormeloxifene-treated ovary-intact rats might be due to the suppressed PH progression, especially when the increase is significantly less than that of the control rats. Also, a possible explanation for the increased 17 β -estradiol in HPASMC following ormeloxifene treatment is that ormeloxifene increased 17 β -HSD 1 expression and inversely regulated 17 β -HSD 2 expressions, promoting 17 β -estradiol production from estrone.

Our data that demonstrated increased 17 β -HSD1 and decreased 17 β -HSD 2 expressions in lungs of monocrotaline-treated rats and hypoxic HPASMC concur with the previous study that reported the growth-inhibitory effect of retinoic acid (an inducer of 17 β HSD1) in serotonin-induced proliferated PSMCs (Preston et al., 2005). However, the findings are contrary to the study of Dwivedi et al. (2005), who showed increased activity of 17 β HSD but decreased 17 β -estradiol and estrogen receptor levels in the uterine tissue of ormeloxifene-treated rats. This further indicates that ormeloxifene selectively regulates 17 β HSD activity/expression and 17 β -estradiol synthesis in a tissue-specific manner.

The actions of 17 β -estradiol are mainly via estrogen receptors α and β , both of which are present in the lungs and heart (Yuan et al., 2013). Previous studies have shown an estrogen receptor β -mediated protective effect of estrogen in hypoxia- and monocrotaline-induced PH (Umar et al., 2011; Lahm et al., 2012; Yuan et al., 2013). However, the present study demonstrated that both estrogen receptors α and β are down-regulated by hypoxia and monocrotaline. Upregulation of estrogen receptors by ormeloxifene administration suggests the possible involvement of estrogen receptor signaling in ormeloxifene action, which will be thoroughly investigated in our future studies. Anti-proliferation of 17 β -estradiol has been earlier reported (Prieto et al., 2008), while evidence exists that the estradiol-receptor binding complex in vasculature cells alters gene expressions, acts as an antioxidant, reduces inflammation, and induces vasodilation (Aiping et al., 2017; Nita et al., 2021). More so, previous studies have demonstrated that estradiol increased Nrf2 and PGC-1 α expressions and improved mitochondrial fitness (Castracani et al., 2020; He et al., 2020; Kalkhoran and Kararigas, 2022). Hence, the activity of ormeloxifene in promoting local pulmonary 17 β -estradiol synthesis, with consequent upregulation of estrogen receptors α and β , may be responsible for its anti-proliferative, anti-inflammatory, and antioxidant properties as well as its improved effects on endothelial and mitochondrial functions, thereby preventing vascular remodeling, and attenuating PH development.

Cardiac failure is the main cause of death in PH patients, correlating with the degree of right ventricular hypertrophy (Alencar et al., 2018). Thus, the response of the right ventricle to therapy determines survival (Rodriguez-Arias and García-Álvarez, 2021). This study demonstrated that hypoxia increased ANP intensity and promoted hypertrophy in H9C2 cardiomyocytes, which were inhibited by ormeloxifene pretreatment. Hence, we report for this time, the potentiality of ormeloxifene in maintaining cardiac morphology. Validating our *in vitro* study, we showed that ormeloxifene attenuated monocrotaline-induced high right ventricular pressure, hypertrophy, and fibrosis in female rats, implying that it reversed right ventricular remodeling, enhanced right ventricular structure, and seemingly improved right ventricular function in the treated rats. The cardioprotective effect of ormeloxifene may be a consequence of its enhancing effect on 17 β -estradiol in non-reproductive tissues, which probably led to estrogen receptor upregulation, as we demonstrated increased estrogen receptors α and β in hypoxic ormeloxifene-pretreated H9C2. The severe right ventricular pressure and hypertrophy observed in ovariectomized rats agree with the previous studies (Nishida et al., 2009; Yuan et al., 2013), confirming the 17 β -estradiol cardioprotective effect.

Similar to the female studies, ormeloxifene alleviated right ventricular pressure and hypertrophy and reduced pulmonary PCNA expression in male rats, validating its therapeutic potential in PH regardless of gender. Our data on the effect of ormeloxifene per se on ANP intensity in normoxic H9C2, which conform to a previous report (Gara et al., 2013), suggest that ormeloxifene can be well-tolerated at recommended doses.

5. Conclusion

The present study presents evidence that ormeloxifene is less uterotrophic but promotes pulmonary 17 β -estradiol synthesis, attenuating inflammation, improving the NOX4/HO1/Nrf/PPAR γ /PGC-1 α axis and

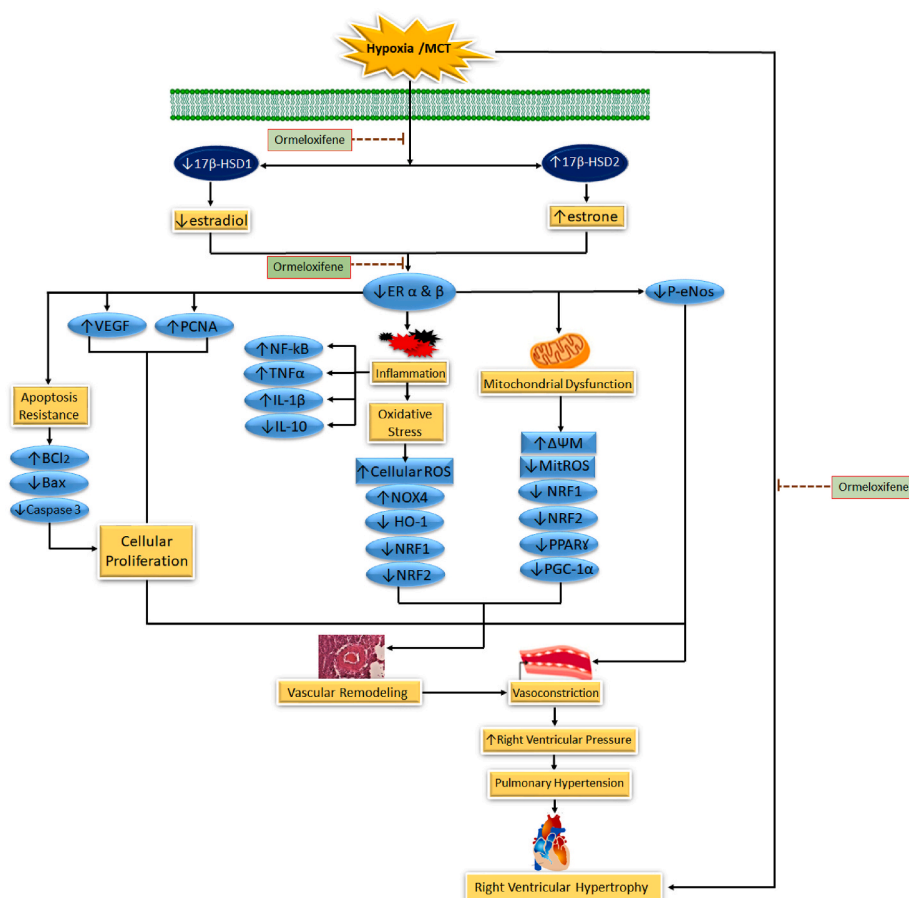


Fig. 14. The Proposed pathway for the mechanism of ormeloxifene action.

inhibiting the progression of PH (Fig. 14). Our findings have some limitations. We could not definitively correlate 17 β -HSD 1 & 2 expressions with the progression of PH. More so, we could not evaluate whether the ormeloxifene effect is estrogen receptor-dependent or not. Nevertheless, our findings are noteworthy and have clinical relevance in PH patients. The study clearly shows, for the first time to our knowledge, the possible deficiency of local pulmonary 17 β -estradiol in PH, mediated by a change in 17 β -HSDs protein expressions. Also, for the first time, our study reports the efficacy of ormeloxifene in inhibiting PH development and associated right ventricular hypertrophy, which may warrant its repurpose for better PH treatment. Our future studies will concentrate on the potential downstream mechanism of ormeloxifene action via the estrogen receptors signaling pathway by knocking down the receptors. Hypoxia and monocrotaline are widely used classical experimental models of pulmonary hypertension. They have good reproducibility and are suitable for studying PH pathophysiology and screening novel pharmacological compounds; however, they are not without limitations. For instance, hypoxia only represents a mild disease as the pulmonary endothelial architecture is largely unaltered and the developed RV hypertrophy does not translate to RV failure. On the other hand, monocrotaline has systemic toxicity, causing hepatic venous-occlusive disease and renal insufficiency (Dignam et al., 2022). Therefore, the evaluation of ormeloxifene efficacy in other models of PH and clinical findings will be another focus of future studies.

Ethics statement

The approval for animal use was given by Institutional Animal Ethics Committee (IAEC), CSIR-CDRI, Lucknow, India (IAEC/2020/123/Renew-1/Dated-23/12/2021).

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CRediT authorship contribution statement

Adam Olaitan Abdulkareem: Methodology, Data curation, Investigation, Writing – original draft, Writing – review & editing. **Priya Tiwari:** Methodology. **Zahid Rasool Lone:** Methodology. **Hina Iqbal:** Methodology. **Satish Gupta:** Methodology. **Rajesh Kumar Jha:** Resources. **Debabrata Chanda:** Resources. **Kumaravelu Jagavelu:** Resources. **Kashif Hanif:** Conceptualization, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no competing interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejphar.2023.175558>.

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