VOLUME 39, NUMBER 4
April 2022

ISSN 0189 - 160X

WAJM

WEST AFRICAN JOURNAL OF MEDICINE

ORIGINALITY AND EXCELLENCE IN MEDICINE AND SURGERY

OFFICIAL PUBLICATION OF
THE WEST AFRICAN COLLEGE OF PHYSICIANS *AND*
WEST AFRICAN COLLEGE OF SURGEONS

www.wajmed.org

WEST AFRICAN JOURNAL OF MEDICINE

WEST AFRICAN JOURNAL OF MEDICINE

ORIGINAL ARTICLE

Targeting Apoptosis Signal-Regulating Kinase-1 (ASK-1) As a Biomarker of Monocrotaline-Induced Pulmonary Hypertension following Administration of Antiretroviral Medications in Rat Model

Cibler la Kinase-1 Régulatrice du Signal d'Apoptose (ASK-1) en Tant Que Biomarqueur de l'Hypertension Pulmonaire Induite par la Monocrotaline Après l'Administration de Médicaments Antirétroviraux Dans Un Modèle de Rat

[1,2]*A. O. Adeoti, [1]A. Nadar, [1]M. L. Channa

ABSTRACT

BACKGROUND: Apoptosis resistance is a recognized pathogenetic mechanism in pulmonary hypertension. However, the link between apoptosis signal-regulating kinase-1 (ASK-1) and pulmonary hypertension (PH) is unclear. This study was conducted to elucidate ASK-1 as a potential biomarker in PH. The study aimed to identify the role of ASK-1 in the mechanism of monocrotaline-induced PH in rats.

METHODS: Forty adult male Sprague-Dawley rats (body weight: 200–250 g) were randomly divided into five groups (n=8 per group). The four treatment groups received a single intraperitoneal injection of monocrotaline (MCT) at a dose of 60 mg. kg^{-1} while the control group received an equivalent volume of intraperitoneal saline injection. Zidovudine (100mg. kg^{-1}), ritonavir (30mg. kg^{-1}), or combination of both drugs (zidovudine 100mg. kg^{-1} and ritonavir 30mg. kg^{-1}) were administrated daily for the study period of 28 days to the rats in three of the four treatment groups with MCT for 28 days. On the twenty-eighth day of the study, rats were sacrificed, and organ harvested with the heart analyzed using RT-PCR for ASK-1. Antioxidant enzyme activities were determined using the colorimetric method.

RESULTS: Animal survival rate was one hundred percent in the treated and control groups while the untreated group recorded 62% survival rate. There was significantly lower mRNA gene expression of ASK-1 in the heart tissues of the treated rats with zidovudine (2.67 ± 0.09, $p < 0.0001$), ritonavir (2.57 ± 0.11, $p < 0.0001$) and a combination of both (2.75 ± 0.06, $p < 0.0001$) when compared to rats in the untreated group. An overexpressed mRNA gene of ASK-1 in the untreated rats was observed (12.0 ± 0.90, $p < 0.0001$) when compared to the controls.

CONCLUSION: ASK-1 is a veritable biomarker for anti-apoptotic characteristics of PH. Our findings will spur new investigations on the role of ASK-1 in PH and the potential therapeutic benefits of antiretroviral medications in the prevention of PH. **WAJM 2022; 39(4): 394–398.**

Keywords: Pulmonary hypertension, apoptosis signal-regulating kinase 1 (ASK-1), zidovudine, ritonavir, HIV/AIDS.

RÉSUMÉ

CONTEXTE: La résistance à l'apoptose est une pathogénétique reconnue mécanisme dans l'hypertension pulmonaire. Cependant, le lien entrekinase-1 régulatrice du signal d'apoptose (ASK-1) et pulmonaire l'hypertension (HTP) n'est pas claire. La présente étude a été menée pour :élucider ASK-1 comme biomarqueur potentiel de l'HTP. L'étude visait à :identifier le rôle de l'ASK-1 dans le mécanisme induit par la monocrotalinePH chez le rat.

MÉTHODES: Quarante rats Sprague-Dawley mâles adultes (poids corporel:200 à 250 g) ont été divisés au hasard en cinq groupes (n = 8 par groupe).Les quatre groupes de traitement ont reçu une seule injection intrapéritonéalede monocrotaline (TCM) à une dose de 60 mg. kg^{-1} pendant que le témoina reçu un volume équivalent d'injection intrapéritonéale de solution saline.Zidovudine (100 mg kg^{-1}), ritonavir (30 mg kg^{-1}) ou combinaison deles deux médicaments (zidovudine 100 mg. Kg^{-1} et ritonavir 30 mg. kg^{-1}) étaient administré quotidiennement pendant la période d'étude de 28 jours aux rats dans trois des quatre groupes de traitement avec MCT pendant 28 jours. Sur levingt-huitième jour de l'étude, des rats ont été sacrifiés et des organesrécolté avec le cœur analysé à l'aide de rt-PCR pour ASK-1. Les activités enzymatiques antioxydantes ont été déterminées à l'aide de la colorimétrieméthode.

RÉSULTATS: Le taux de survie des animaux était de cent pour cent dans les groupes traités et témoins tandis que le groupe non traité a enregistré 62 %taux de survie. L'expression des gènes de l'ARNm était significativement plus faible d'ASK-1 dans les tissus cardiaques des rats traités par la zidovudine (2.67 ± 0.09, $p < 0.0001$), ritonavir (2.57 ± 0.11, $p < 0.0001$) et acombinaison des deux (2.75 ± 0.06, $p < 0.0001$) par rapport aux rats dans le groupe non traité. Un gène d'ARNm surexprimé d'ASK-1 dans les rats non traités ont été observés (12.0 ± 0.90, $p < 0.0001$) lorsque par rapport aux contrôles.

CONCLUSION: ASK-1 est un véritable biomarqueur anti-apoptotique caractéristiques du pH. Nos conclusions donneront lieu à de nouvelles enquêtes sur le rôle de l'ASK-1 dans l'HTP et les avantages thérapeutiques potentiels demédicaments antirétroviraux dans la prévention de l'HTP. **WAJM 2022; 39(4): 394–398.**

Mots-clés: Hypertension pulmonaire, régulation du signal d'apoptosekinase 1 (ASK-1), zidovudine, ritonavir, VIH/SIDA.

[1]**Department of Human Physiology, College of Health Sciences, School of Laboratory Medicine and Medical Sciences, University of KwaZulu- Natal, Durban, South Africa.**

[2]**Department of Medicine, College of Medicine, Ado-Ekiti, Ekiti State, Nigeria.**

******Correspondence:* **Dr. Adekunle Adeoti,** Department of Human Physiology, College of Health Sciences, School of Laboratory Medicine and Medical Sciences, University of KwaZulu- Natal, Durban, South Africa. Email: kadeoti2002@gmail.com

Abbreviations: ASK-1, Apoptosis Signal-regulating Kinase-1; **MCT,** Monocrotaline.

INTRODUCTION

Pulmonary hypertension (PH) is a fatal progressive disease involving an increase in pulmonary vascular resistance and pulmonary arterial pressure (PAP) which leads to right ventricular (RV) dysfunction, right heart failure and, ultimately, death.[1, 2] It involves vascular endothelial dysfunction, chronic inflammation, smooth muscle cell proliferation, pulmonary arteriolar occlusion, apoptosis resistance, and the hallmark feature of pulmonary vascular remodeling.[3, 4] During this process, the pulmonary vascular pathology evolves from an inert state to a pro-proliferative one and apoptosis-resistant vascular cells develop.[5]

Apoptosis signal-regulating kinase-1 (ASK-1) is an upstream kinase in the p38mitogen-activated protein kinases (MAPKs) signaling pathway that responds to stimuli by activating cellular proliferation, differentiation, survival, apoptosis, and inflammation.[6,7] Its inhibition has been associated with decline in vascular remodeling in MCT-induced pulmonary hypertension experimental rats; however, the contrary is observed when activated.[6,8] The activation of apoptotic pathway by the continuous induction of ASK-1 induces mitochondria-dependent caspases, triggering the intrinsic apoptosis pathway which regulates programmed cell death.[7] ASK-1 is activated in response to several stimuli, most especially oxidative stress, and it is widely expressed in diverse tissues like heart and lungs.[7,9]

Human immunodeficiency virus infection is a known risk for the development of severe PH; however, the prevalence of HIV-associated PH (HIV-PH) has remained stable from the pre-anti-retroviral therapy era.[10] The impact of antiretroviral medications on the development of PH is controversial; however, nucleoside reverse transcriptase inhibitors (NRTI) and protease inhibitors like zidovudine and ritonavir, respectively, have been reported to improve outcome and survival of HIV-PH.[11,12] Animal models of pulmonary hypertension developed following the administration of monocrotaline has a good representation of the expectations in humans.[13] Using this validated modality to explore the effects of anti-retroviral therapy on the progression of PH with the aim of identifying biomarkers for early detection and monitoring would give clarity to the pathogenesis of PH.

Despite the current therapeutic advances such as endothelin-1 receptor blockers, type 5 phosphodiesterase inhibitors or platelet derived growth factors (PDGF) receptor blockers in the management of PH, no available literature has explored the role of antiretroviral medications in the development of PH using ASK-1 as a biomarker.[9]

The study aimed at investigating the effect of antiretroviral medication on ASK-1 in monocrotaline-induced pulmonary hypertension in rats.

MATERIALS AND METHODS

Animals

Forty adult male Sprague-Dawley rats (weighing 200–250 g) were supplied by the Biomedical Resource Unit of the University of KwaZulu-Natal, South Africa, and allowed to acclimatize for one week before commencement of the experiments. All the rats were housed in standard cages under standard laboratory conditions of 18–22° C room temperature, 50–70% humidity and 12 hours light/dark cycle with *ad libitum* access to dry feed and water. Animal experiment was approved by the Animal Research Ethics Committee of the institution (AREC/066/018M) and the use of rats complied with the 3R guidelines (3R: Replace, Reduce, and Refine) for the use of experimental animals.[14]

Experimental Design

The 40 rats were randomly assigned into five groups (n=8). Four of the five groups were the treatment groups that received a single intraperitoneal injection of monocrotaline (MCT) at the dose of 60 mg. kg^{-1} while the fifth (control) group received an equivalent volume of intraperitoneal saline injection. MCT (Replamed, South Africa) was dissolved in 1N HCl and buffered to pH 7.0 with 1N NaOH before injection. Subsequently, a daily dose of zidovudine (100mg. kg^{-1}), ritonavir (30 mg. kg^{-1}) or combination of zidovudine/ritonavir was administered to rats in three of the four treatment groups for 28 days.

RNA Extraction and Measurement by RT-Quantitative PCR

Total RNA was extracted from lung tissues using Trizol(Zymo research) agent. Quantitative real-time RT-PCR (qPCR) was performed to assess mRNA expression of the following genes. The primers for the genes were as follows: ASK-1 FW, 5-TGAATCTGAGCCA ACACTACAG-3 and RV, 5-CATCAGCAAGCACGTGCCAAA-3 and purchased from INQABA (South Africa). This primer for gene expression was designed using published sequence information from a previous related study.[15] PCR Detection System (BIORAD Co., CA, USA) was employed to execute Real-Time Polymerase Chain Reaction (PCR) with 0.1 mL tube containing 2 µL cDNA, 2 µL dNTP (1.25 mM each nucleotide), 1 µL reverse and forward prime, 1 µL dH20 and 5 µLSYBRGreen . The PCR conditions were as follows: 10 min at 95°C, followed by 40 cycles of 60 s at 95°C, 30 s at annealing temperature, and 60 s at 72°C. We verified the specificity of PCR by measuring the melting curve of the PCR products at the end of the reaction. Fluorescence data were specified for collection during primer extension. The relative expression of mRNA to GAPDH mRNA was calculated from the cycle threshold (Cq) value by a ΔΔCq method and presented as a relative to control.[16]

Histomorphometric Analysis

The harvested lung and heart tissues were placed with 10% formalin, embedded at 4°C in paraffin, and sliced to a thickness of 4 µm. Subsequently, the sections were stained with the hematoxylin staining method for 5 min, washed under running water for 1 min, dissimilated 30 s in hydrochloric acid ethanol, and immersed in tap water for 15 min or warm water (about 50!) for 5 min. Sections were stained for 2 min this time with eosin and each section was mounted with DPX and a coverslip. The histological changes were observed under an optical microscope (Leica DM 500, Germany). Ten fields containing terminal arterioles (50–150 mm external diameters) was randomly selected and the dimensions of the external elastic lamina and the internal elastic lamina of each vessel were measured. The wall

thickness (percent) of the arterioles were categorized depending on the degree of vascular muscularization as 1–3, of which: 1=no muscularization, not occluded; 2=partial muscularization, not fully occluded; 3=muscularization, fully occluded.[17]

Drugs

MCT (Sigma-Aldrich, St. Louis, MO, USA) was purchased from REPLAMED, SA. Zidovudine was obtained from Aspen Pharmacare Limited (Aspen, South Africa), while ritonavir was obtained from Abbvie Limited (South Africa). Previous animal studies involving rats were used as template for the dosage of the medications.[12,18]

Data Analysis

Data analysis was performed using one-way ANOVA for statistical differences with Tukey post-hoc tests. Differences for all tests were considered significant when the p value was <0.05 and data recorded as mean ± standard error of mean (SEM). All the analyses were performed using GraphPad Prism, version 6 (GraphPad, San Diego, CA, USA).

RESULTS

Animal Survival Rate

All the rats survived the 1-, 2-, and 3-week and rats in the control and treated (antiretroviral medications following the administration of MCT) groups remained active even till the end of the study. However, three (3) rats in the untreated group died in the 4th week of the study on days 23 and 25 summing up to 38% death rate at the end of the experiment (p = 0.0097). All the rats in the control group (unexposed) survived through the study period. Likewise, those rats in the treatment groups on antiretroviral medication vis-a-vis zidovudine and ritonavir or a combination of both drugs also survived through the study period.

Relative Organ Weight

There was no significant difference (p = 0.3165) in the relative heart weights (RHW) of rats across all groups: control (0.3779 ± 0.023%), MCT (0.3076 ± 0.014%), MCT and zidovudine (0.4805 ± 0.030%), MCT and ritonavir (0.4131 ± 0.022%) and MCT and a combination of zidovudine and ritonavir (0.4302 ± 0.014%).

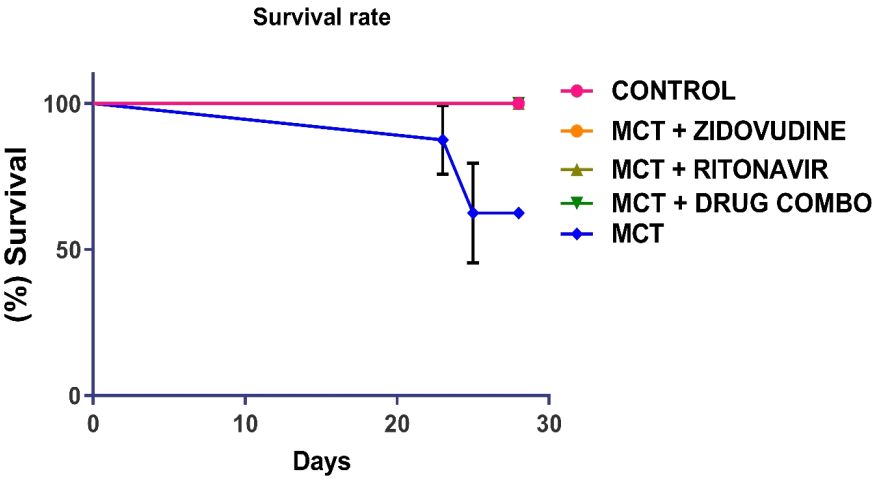

Fig. 1: Survival Rate of the Experimental Animals within 28 Days.
Values are expressed as percent survival.

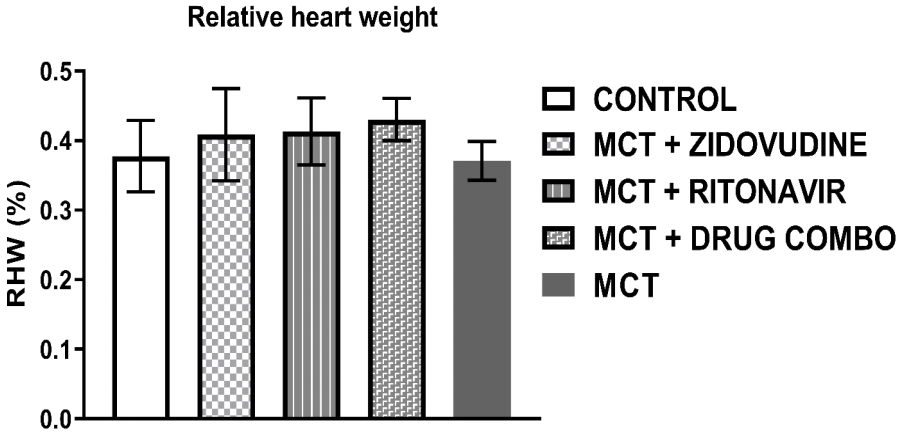

Fig. 2: Relative Heart Weight (RHW) of the Experimental Animals.
Values are expressed as mean ± standard error of mean (SEM).

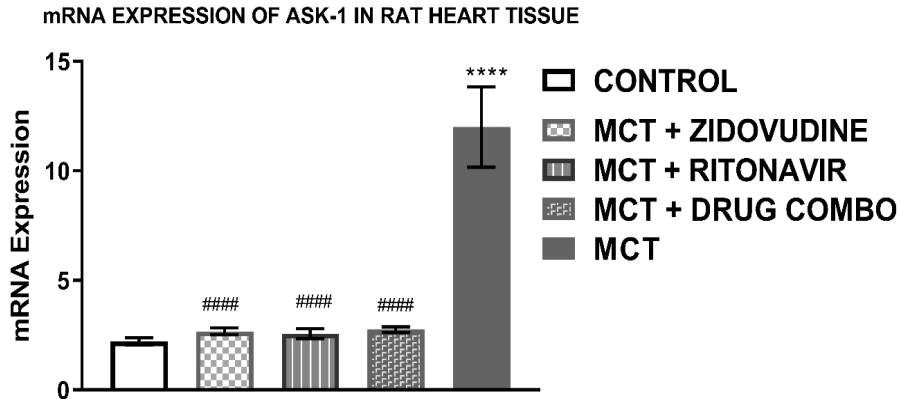

Fig. 3: mRNA Expression of ASK-1 in the Heart Tissue of Experimental Rats.
Values are expressed as mean ± standard error of mean (SEM). ****P < 0.0001 when compared with Control; ####P < 0.0001 when compared with MCT

Assessment on the mRNA Expression of ASK-1 in Rat Heart

Figure 3 shows an overexpression of ASK-1 in the heart tissue of untreated rats (12.0 ± 0.90, $p < 0.0001$) when compared to the rats in the control group. The mRNA expressions in the hearts of rats with zidovudine (2.67 ± 0.09, $p < 0.0001$), ritonavir (2.57 ±0.11, $p < 0.0001$) and a combination of both (2.75 ± 0.06, $p < 0.0001$) were lower and statistically significant when compared to rats in the untreated group. Likewise, the mRNA expression of ASK-1 was similar to the control rats (2.22 ± 0.08) but not statistically significant.

Histopathological Indices

The light microscope examination of the slides with representative photo-microscopic features showed marked thickening in the media walls of the muscular pulmonary arteries in the lungs of the untreated rats following MCT administration. The rats with anti-retroviral drugs (zidovudine, ritonavir and both drugs combined) show significant improvement in their media wall thickness when compared to the untreated MCTgroup.

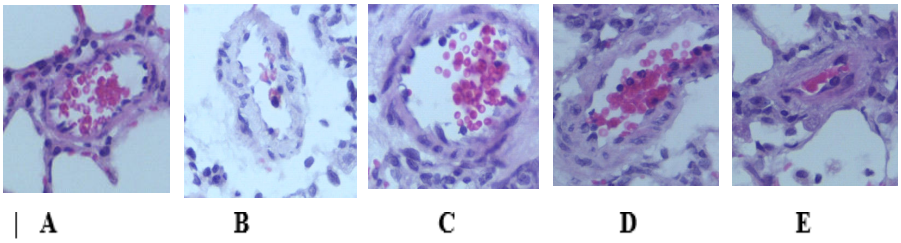

Fig. 4a: Representative Images of Lung Stained with H and E for the different Groups (Image A- Control, Image B- MCT+Drug A, Image C- MCT+Drug B, Image D- MCT+Drugs combo and Image E- MCT)

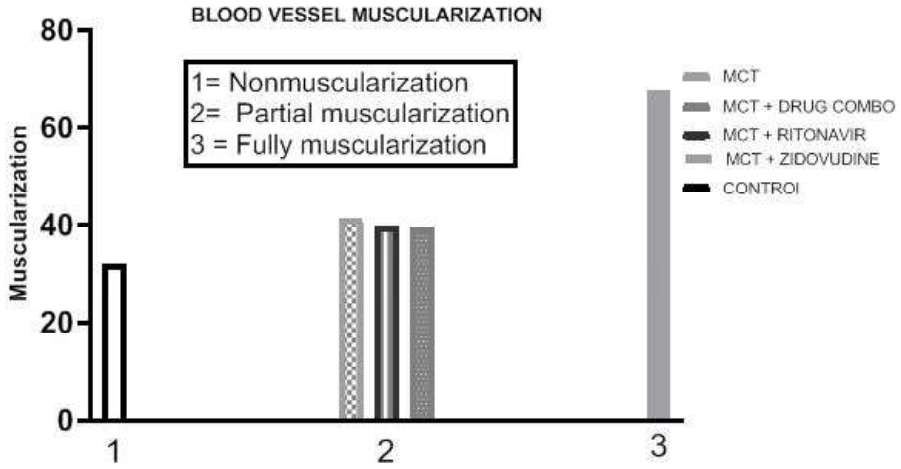

Fig. 4b: shows Degree of Pulmonary Arterioles Muscularization in Lung Tissues of Experimental Rats.

DISCUSSION

In this study, there was a reduction in the animal survival rate from one hundred percent in the treated and control groups to 62% in the untreated group. However, there was no significant difference in the relative heart weight in the experimental groups. This study showed a significantly lower mRNA gene expression of ASK-1 in the heart tissues of the treated rats when compared to rats in the untreated group as well as an overexpressed mRNA gene of ASK-1 in the untreated rats relative to the control. The survival rate of the untreated rats dropped to 87.0% on day 23 which further declined to 62.0% by day 25. This reduction in the animal survival rate of untreated rats after the three weeks was similar to previous studies where survival rates of 70% to 90% were observed by the 21st day following MCT injection.[19,20] As observed in this study and previous studies, rats were reported to have died after the third week of MCT administration in the untreated group. This finding could be as a result of the severity of the PH in the rats due to the pulmonary vascular endothelial damage caused by MCT which has been reported to be maximum after the third week of its administration.[21] Therefore, the therapeutic effects of the antiretroviral medications in the prevention of mortality among the treated rats would suggest the likely beneficial role in mitigating the development of PH in these groups.

In this study, the ASK-1 mRNA gene expression in the treated rats was higher compared to the control rats but lower than in the untreated rats. This agrees with previous studies where ASK-1 stimulation of cellular apoptotic response plays a major role in the signaling pathways and treatment modalities.[15,22] ASK-1 is fundamental to programmed cell death, of which its failure affects apoptosis, and its inhibition could reduce pathological remodeling of the pulmonary vasculature and the right ventricle and this progresses to pulmonary hypertension in animal models.[23]

In a similar study involving monocrotaline-induced pulmonary hypertension in rats, the overexpression of ASK-1 mRNA gene in the untreated rats in relation to the control group could be evidence of the ASK-1 resistance which is anti-apoptotic.[8] Such presentations of apoptosis-resistance could manifest as increased proliferative vascular changes of the endothelial cells (EC), thereby obliterating the vessels due to the anti-apoptotic effects.[24]

The histopathology results of the lungs revealed marked thickening in the media walls of the muscular pulmonary arteries in the untreated rats. However, improvement in this media wall thickness was observed in the rats treated with zidovudine and/or ritonavir following MCT-induced PH. This histologic finding is comparable to those reported prominent medial hypertrophy of the muscular pulmonary arteries in MCT-induced PH in rats.[25] This is an indication that zidovudine, ritonavir and a combination of the drugs were able to mitigate the deteriorative effects of MCT.

In conclusion, the data from this research indicate that zidovudine and ritonavir attenuate MCT- induced PH in rats by down-regulation of ASK-1 which could serve as a useful biomarker for the anti-apoptotic characteristics of PH.

ACKNOWLEDGEMENTS

This study was supported by the research grant provided by College of Health Sciences (CHS) of the University of KwaZulu-Natal, South Africa.

Competing Interests

The authors declare that they have no competing interests.

REFERENCES

1. Zhang Z, Zhang L, Sun C, Kong F, Wang J, Xin Q, *et al*. Baicalin attenuates monocrotaline-induced pulmonary hypertension through bone morphogenetic protein signaling pathway. *Oncotarget.* 2017; **8:** 63430-41. DOI: 10.18632/oncotarget.18825
2. Varshney R, Ali Q, Wu C, Sun Z. Monocrotaline-Induced Pulmonary Hypertension Involves Downregulation of Antiaging Protein Klotho and eNOS Activity. Hypertension (Dallas, Tex : 1979). 2016; **68:**1255–1263. DOI: 10.1161/hypertensionaha.116.08184.
3. Zhang M, Chang Z, Zhao F, Zhang P, Hao YJ, Yan L, *et al.* Protective Effects of 18beta-Glycyrrhetinic Acid on Monocrotaline-Induced Pulmonary Arterial Hypertension in Rats. Frontiers in pharmacology. 2019; **10:** 13. DOI: 10.3389/fphar.2019.00013
4. Cai Z, Li J, Zhuang Q, Zhang X, Yuan A, Shen L, *et al*. MiR-125a-5p ameliorates monocrotaline-induced pulmonary arterial hypertension by targeting the TGF-beta1 and IL-6/STAT3 signaling pathways. Experimental & molecular medicine. 2018; **50:** 45. DOI: 10.1038/s12276-018-0068-3
5. Gamen E, Seeger W, Pullamsetti SS. The emerging role of epigenetics in pulmonary hypertension. *The European Respiratory Journal.* 2016; **48:** 903–917. DOI: 10.1183/13993003.01714-2015
6. Qin N, Yang W, Feng D, Wang X, Qi M, Du T, *et al.* Total ginsenosides suppress monocrotaline-induced pulmonary hypertension in rats: involvement of nitric oxide and mitogen-activated protein kinase pathways. *Journal of Ginseng Research.* 2016; **40:** 285–291. DOI: 10.1016/j.jgr.2015.09.005
7. Tesch GH, Ma FY, Han Y, Liles JT, Breckenridge DG, Nikolic-Paterson DJ. ASK1 Inhibitor Halts Progression of Diabetic Nephropathy in Nos3-Deficient Mice. *Diabetes*. 2015; **64:** 3903–3913. DOI: 10.2337/db15-0384
8. Budas GR, Boehm M, Kojonazarov B, Viswanathan G, Tian X, Veeroju S, *et al*. ASK1 Inhibition Halts Disease Progression in Preclinical Models of Pulmonary Arterial Hypertension. American journal of respiratory and critical care medicine. 2018; **197:** 373–385. DOI: 10.1164/rccm.201703-0502OC
9. Courboulin A, Tremblay VL, Barrier M, Meloche J, Jacob MH, Chapolard M, *et al.* Kruppel-like factor 5 contributes to pulmonary artery smooth muscle proliferation and resistance to apoptosis in human pulmonary arterial hypertension. Respiratory research. 2011; **12:** 128. DOI: 10.1186/1465-9921-12-128
10. Opravil M, Sereni D. Natural history of HIV-associated pulmonary arterial hypertension: trends in the HAART era. *AIDS.* 2008; **22:** S35–S40. DOI: 10.1097/01.aids.0000327514.60879.47
11. Pugliese A, Isnardi D, Saini A, Scarabelli T, Raddino R, Torre D. Impact of highly active antiretroviral therapy in HIV-positive patients with cardiac involvement. *J Infect.* 2000; **40:** 282–284. DOI: 10.1053/jinf.2000.0672
12. Gary-Bobo G, Houssaini A, Amsellem V, Rideau D, Pacaud P, Perrin A, *et al.* Effects of HIV protease inhibitors on progression of monocrotaline- and hypoxia-induced pulmonary hypertension in rats. Circulation. 2010; **122:** 1937–1947. DOI: 10.1161/circulationaha.110.973750
13. Nogueira-Ferreira R, Vitorino R, Ferreira R, Henriques-Coelho T. Exploring the monocrotaline animal model for the study of pulmonary arterial hypertension: A network approach. *Pulm Pharmacol Ther.* 2015; **35:** 8–16. DOI: 10.1016/j.pupt.2015.09.007
14. Nam MH, Chun MS, Seong JK, Kim HG. Ensuring reproducibility and ethics in animal experiments reporting in Korea using the ARRIVE guideline. *Lab Anim Res.* 2018; **34:** 11–19. DOI: 10.5625/lar.2018.34.1.11
15. Kherrouche Z, Blais A, Ferreira E, De Launoit Y, Monte D. ASK-1 (apoptosis signal-regulating kinase 1) is a direct E2F target gene. *Biochem J.* 2006; **396:** 547–556. DOI: 10.1042/bj20051981
16. Coates DE, Zafar S, Milne TJ. Quantitative Real-Time Gene Profiling of Human Alveolar Osteoblasts. *Methods Mol Biol.* 2017; **1537:** 447–459. DOI: 10.1007/978-1-4939-6685-1_27
17. Han X, Zhang Y, Zhou Z, Zhang X, Long Y. Hydroxysafflor yellow A improves established monocrotaline-induced pulmonary arterial hypertension in rats. *J Int Med Res.* 2016; **44:** 569–584. DOI: 10.1177/0300060515597931
18. Adebiyi OO, Adebiyi OA, Owira PM. Naringin Reverses Hepatocyte Apoptosis and Oxidative Stress Associated with HIV-1 Nucleotide Reverse Transcriptase Inhibitors-Induced Metabolic Complications. *Nutrients.* 2015; **7:** 10352–10368. DOI: 10.3390/nu7125540
19. Itoh T, Nagaya N, Fujii T, Iwase T, Nakanishi N, Hamada K, *et al*. A combination of oral sildenafil and beraprost ameliorates pulmonary hypertension in rats. *American Journal of Respiratory and Critical Care Medicine.* 2004; **169:** 34–38. DOI: 10.1164/rccm.200303-346OC
20. Nagaya N, Okumura H, Uematsu M, Shimizu W, Ono F, Shirai M, *et al.* Repeated inhalation of adrenomedullin ameliorates pulmonary hypertension and survival in monocrotaline rats. *American Journal of Physiology Heart and Circulatory Physiology.* 2003; **285:** H2125-31. DOI: 10.1152/ajpheart.00548.2002
21. Ruiter G, de Man FS, Schalij I, Sairras S, Grunberg K, Westerhof N, *et al.* Reversibility of the monocrotaline pulmonary hypertension rat model. *The European Respiratory Journal.* 2013; **42:** 553–556. DOI: 10.1183/09031936.00012313
22. Kolliputi N, Waxman AB. IL-6 cytoprotection in hyperoxic acute lung injury occurs via suppressor of cytokine signaling-1-induced apoptosis signal-regulating kinase-1 degradation. American Journal of Respiratory Cell and Molecular Biology. 2009; **40:** 314–324. DOI: 10.1165/rcmb.2007-0287OC
23. Xu D, Li Y, Zhang B, Wang Y, Liu Y, Luo Y, *et al*. Resveratrol alleviate hypoxic pulmonary hypertension via anti-inflammation and anti-oxidant pathways in rats. *International Journal of Medical Sciences.* 2016; **13:** 942–954. DOI: 10.7150/ijms.16810
24. Suzuki YJ, Ibrahim YF, Shults NV. Apoptosis-based therapy to treat pulmonary arterial hypertension. *J Rare Dis Res Treat.* 2016; **1:** 17–24.
25. Lee YS, Byun J, Kim JA, Lee JS, Kim KL, Suh YL, *et al.* Monocrotaline-induced pulmonary hypertension correlates with upregulation of connective tissue growth factor expression in the lung. *Exp Mol Med.* 2005; **37:** 27–35. DOI: 10.1038/emm. 2005.4