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ORIGINAL ARTICLE

Preventive Effect of MG-132 on Monocrotaline Induced Pulmonary Hypertension in Rat Pulmonary Artery Hypertension Model

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ABSTRACT

Aim of Study: To Prevent the Monocrotaline induced extracellular matrix remodeling of pulmonary artery by inhibiting Ubiquitin Proteasome System in rat pulmonary artery hypertension model.

Study Design: Experimental Study

Place and Duration of Study: Xi'an Jiaotong University, Animal experiment center Xi'an China, from January 2016 to July 2018.

Materials and Methods: Thirty-three male Sprague-Dawley rats were divided in to three groups namely control group, Monocrotaline (M.C.T) induced group and M.G-132 (a proteasome inhibitor) treated group. The pulmonary artery hypertension (P.A.H) model was established by giving intraperitoneal injection of Monocrotaline (M.C.T) furthermore the M.G-132 was given to hold back proteasome function. The right ventricular systolic pressure (R.V.S.P) and the right ventricular hypertrophy index (R.V.H.I) were used to assess the progress developing P.A.H. Vascular remodeling was determined by H&E staining and the level of ubiquitinated-PTEN protein was measured by Immunoblotting.

Results: The final results revealed that the M.C.T increased R.V.S.P and R.V.H.I in rats, whereas these alterations were concealed in P.A.H induced rats which were treated by M.G-132. Also the Zymograghy results in control vs. experimental group revealed that the activity of Matrix metalloproteinase (MMP2/9) in the PAH model group was considerably elevated at ($P<0.05$), while treatment of M.G-132 in M.C.T-induced P.A.H-rats decreased the activity of MMP2/9 at ($p<0.05$).

Conclusions: However, further in-depth investigations are need of a time to explore the transformations of ubiquitin proteasome & MMP activity in chronic MCT induced-pulmonary artery hypertension model.

Keywords: P.A.H, Ubiquitin, Monocrotaline, Extra-cellular remodeling, M.M.P (Matrix metalloproteinases)

INTRODUCTION

Elevated levels of blood pressure in pulmonary arteries, veins and capillaries is called pulmonary hypertension which leads to cause symptoms such as fainting, dizziness, swollen legs and shortness of breath (1, 2). According to W.H.O the phenomenon of developing pulmonary arterial hypertension involves the narrowing of blood vessels which are concerned with the blood supply and drainage of lungs. Over the time, these blood vessels become stiffer & thicker, this phenomenon is recognized as fibrosis. This leads to further deterioration of the condition thus increases the blood pressure and impairs the flow within the lungs (3).The precise mechanism of developing pulmonary arterial hypertension is still unclear, but somehow; it is said that the endothelial dysfunction of a blood vessel resulted in decreased production of endothelium derived vasodilators i.e. nitric oxide & prostacyclin. Furthermore, this initiates the production of vasoconstrictors i.e. vascular endothelial growth factor (VEGF) and thromboxane (4-6).

Ubiquitin is a kind of small size protein only present in eukaryotes (7). Recently the ubiquitin-proteasome-pathway for proteolysis, has gain an importance as a novel "molecular mechanism" which controls many fundamental functions of the nervous system such as development of synaptic plasticity and synaptic connections (8). In Alzheimer's and Parkinson's disease, the role of ubiquitin-proteasome has been established (9). Ubiquitin regulates the protein turnover by modifying the degradation of particular type of proteins. It attaches and tagged them for demolition. These tagged-proteins are exposed to proteasome. These protesomes are a type of cell organelle which are involved in degrading and recycling of old and unneeded proteins (10). During degradation process the proteins are broken down into amino acids and these amino acids again used for synthesis of new ones (11).

There are three critical enzymes namely; (E1, E2, and E3) which plays a major role in ubiquitin-proteasome pathway (12). The E1-enzyme is known as "Ubiquitin-activating enzyme". It amends ubiquitin into its reactive form. The E2-enzyme is known as "ubiquitin-conjugating enzyme", which catalyzes the attachment site of ubiquitin for its substrate proteins. The E3-enzyme is called "ubiquitin-ligase". The E3 & E2 enzyme functions mutually so they are considered in recognizing the substrate protein (13, 14).

The actual motive of this research is to conclude whether inhibiting the function of Ubiquitin Proteasome System (UPS) ameliorates, the "remodeling of the extracellular matrix" in pulmonary artery along with the development of pulmonary arterial hypertension in P.A.H-rat model. Furthermore, to examine whether MG-132 through inhibition of proteasome functions thus inhibits the activity of MMP, consequently reverses the remodeling of the extra-cellular matrix of pulmonary artery.

MATERIALS AND METHODS

Materials: Tissue extraction buffers were purchased from (Beijing, China). The polyacryamide and sodium dodecyl sulfate (SDS) was purchased from Sigma Aldrich. All others chemicals were of analytical grades. These materials were arranged and bought from local markets for commercial use.

Animals and grouping: This experimental work was performed in "Xi'an Jiaotong University Animal Experiment Centre" under Animal lab guidelines. The animal experimental code of behavior was approved by the "Institutional Animal Care and Use Committee of Xi'an Jiaotong University Animal Experiment Centre" after detailed discussion and review. A total of thirty three (33) Sprague-Dawley male-rats were used in this study i.e. (170-206 gm in weight). All these rats were divided into three groups on random basis i.e. 11 rats per group; control group, received 0.1mg / kg of body weight saline gavage only, a total of 4 weeks; MCT-induced pulmonary artery hypertension group, according to the P.A.H model construction method of giving single intraperitoneal injection of 60 mg / kg of body weight M.C.T, 0.1mg / kg of body

weight saline gavage, a total of 4 weeks; M.G132 treatment group was given monocrotaline (60mg / kg of body weight single intraperitoneal injection), and then given 0.1mg / kg –MG132 treated by gavage, a total of 4 weeks.

Construction of "pulmonary hypertension rat" model: To construct the "pulmonary hypertension rat model'', the rat abdominal skin was disinfected by 75% alcohol disinfectant and the 5ml syringe was used of in experimental rats in left lower quadrant to given Monocrotaline (60mg/kg of body weight) single intraperitoneal injection. After 4 weeks, clear detection of pulmonary artery hypertension rat's model construction was succeeded hemodynamically.

Determination of "right ventricular pressure": After the intraperitoneal anesthesia with 10 % chloral hydrate, right ventricular pressure (RVSP); was then calculated by using pressure transducer and polygraph (Shenzhen Mindray Bio-Medical Electronics Co., Ltd.), until the pressure waveform is stable.

Histological examination of lung and heart tissue: All animals were scarified after RVSP determination. After that the lung and heart tissue were dissociated. The both atria were isolated from heart. Right and left ventricles including interventricular septum were isolated and washed for removing blood. Then the remaining moisture was removed by using the standard filter paper. Weight of tissue then measured by using electric weighing machine. By applying the formula RV / (LV + S), the right ventricular hypertrophy index was calculated.

Lung tissue preparation: By passing normal saline solution via artery, the residual blood was cleansed off from the lung. To expand the lung membrane, 10% neutral formalin was then gradually injected through the trachea under 20cm of (H_2O) pressure into the lung. After five minutes, right lung was dissected out and lower lobe was fixed in 10% neutral formalin. It remained in it for next 48 hrs. Then it was embedded in paraffin after fixation. Tissue was sliced into 5mm small sections and stained with H&E and "Van Gieson".

Zymograghy examination: The standard protocols of Caseinolytic and MMP gelatinolytic activities of control and experimental group were scrutinized, the mini-slab gels containing 10% SDS–polyacrylamide copolymerized with 0·1% gelatin, 12% polyacrylamide gel copolymerized with 0·1% casein. Soluble proteins were extracted by homogenizing the tissue in 2% SDS solution. After centrifugation at 10000g, the supernatants were removed. The protein concentrations were assayed with BCA protein assay reagent. Samples of 10mg of protein were loaded on gels with prestained molecular weight standards. SDS–polyacrylamide electrophoresis was performed at 48C, 125V for 90mins. Gels were soaked in 2·5% Triton X-100 for 30mins at room temperature thus to remove SDS and then incubated for a reaction at 378C overnight to allow proteinase digestion of substrate. These gels were then stained with 0·25% Coomassie brilliant blue in 40% methanol and 10% acetic acid for 2 hours. These were destained with 40% methanol and 10% acetic acid.

Statistical analysis: All these experiments were executed into triplicate. Student's t test was performed to check the impact. The marker of significance level was set at $P < 0.05$. Mean and ±SD were used to present data.

RESULTS

In control group, food intake is stable, there is skin luster, normal breathing, heartbeat and activity; weight increased significantly and even we did not find a case of death. In PAH model group, reduced food intake, reduced activity, rough fur, slow weight gain, total 3 rats died in 4 weeks, with the mortality rate 37.5%. However, MG-132 treatment group, the food intake was increased as in comparison with the model group, the hairs were slightly smooth, increased activity, weight also increased in correlation to the Control Group, 1 rat died in a total of 4 weeks with the mortality rate 11% as shown in FIG-I.

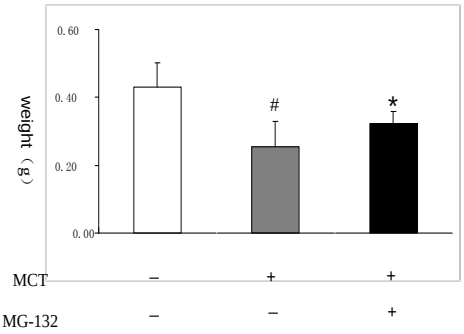


Fig I: The changes in weight between the control groups, PAH model group, and treatment group. All these groups were considerably different from the Control, at the level of ($p < 0.05$); data was presented; mean of averages and ± SD (bar).

Determination of Right ventricular pressure: For control group, the RVSP was in the range of (24.69 + 4.55) mmHg, for PAH model group, the RVSP was in the range of (42.51 + 6.34) mmHg, which showed that the RVSP was considerably increased in comparison with the control group; for MG-132 treatment group, the RVSP was in the range of (33.28+5.17) mmHg, as compared with "Model group" it was considerably decreased, but the pressure is still elevated than "Control group" (FIG-II).

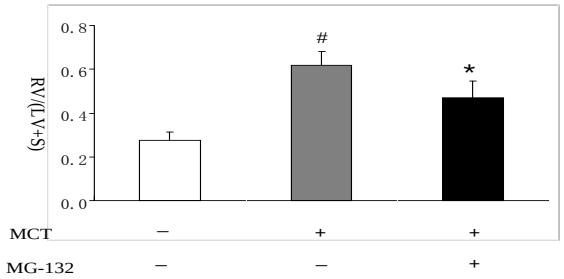


Fig 2: The determination of right ventricular pressure among different groups. All these groups were considerably dissimilar from the Control Group at ($p < 0.05$); the data was presented; mean of average ± SD (bar).

Right ventricular hypertrophy index

In Control group RV / (LV+S) was lying in range i.e. (0.28 + 0.04); in PAH model group, RV / (LV+S) was considerably increased (0.62 + 0.04) as compared with control group, suggesting that the right ventricular hypertrophy of PAH model group. MG132 treatment group RV / (LV+S) was lying in the range of (0.47 + 0.07), as in contrast with PAH model group, the degree of right ventricular hypertrophy was significantly decreased (FIG-III; A and B).

Also to elevate the accumulation of Extra Cellular Matrix (ECM) component in pulmonary vessels in pulmonary artery hypertension (PAH) collagen deposition was determined by Masson staining as shown in FIG-IV: in which mature collagen fiber were stained blue, collagen deposition was considerably raised in lungs of M.C.T-treated rat as compared to control group however after administration of MG132 deposition of collagen in pulmonary vessel in MCT treated rats was dramatically reduced.

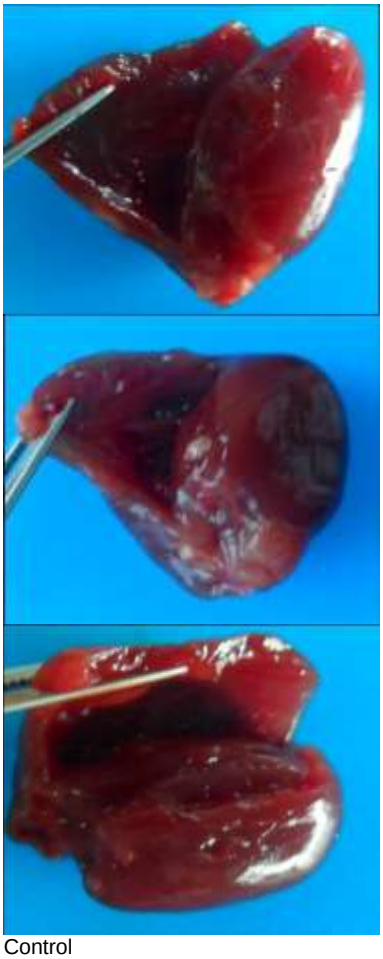
Our results were consistent with the result reported by (15)

Zymology Assay: Due to the activities of MMP2_MMP9 dictate vascular structural collagen remodeling, we investigated the activities of MMP2_MMP9 in the lung. The activity of MMP2_MMP9 were assessed by Zymograghy, as shown in FIG-V the activities of MMP2 was considerably increased in M.C.T-Treated group as in contrast to Control group, which was declined in MG132-treated rats at; $p<0.05$ Vs M.C.T group. It demonstrated

the activities of MMP9 reached a fold over increase against control in M.C.T-treated rats (P<0.05), which was reduced unto a fold over control group in MG132, in accordance to a significant up regulation of “MMP-2” & “MMP-9”. Thus there is an increase in gelatinolytic activity of isolated pulmonary arteries from M.C.T-treated rats was finally established.

Our results were consistent or even better than the results reported in (16-19).

(A)



Control
MCT
MG132

(B)

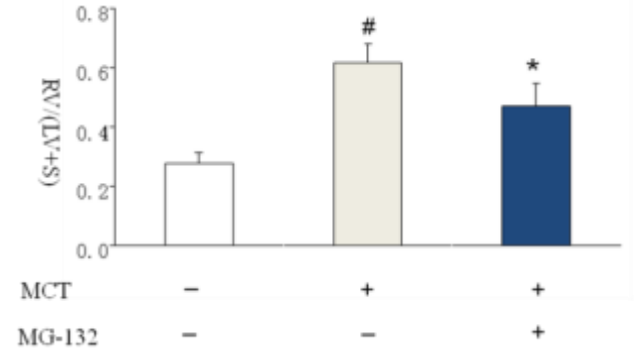
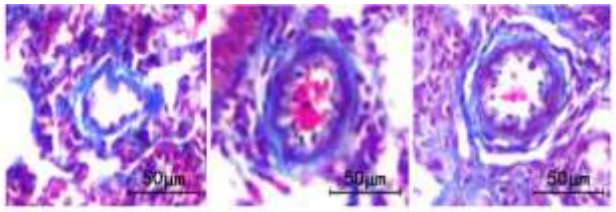


Fig 3: (A); Image of specimen after surgery (B), the determination of right ventricular hypertrophy index among different groups. All groups were considerably dissimilar from the Control group at ($p < 0.05$) and the data was expressed as the mean of average ± SD (bar).



Control (×400) MCT (×400) MG132 (×400)

Fig 4: MG132 prevented MCT induced collagen deposition; collagen in small pulmonary arteries has been investigated by Masson staining.

MMP9
MMP2

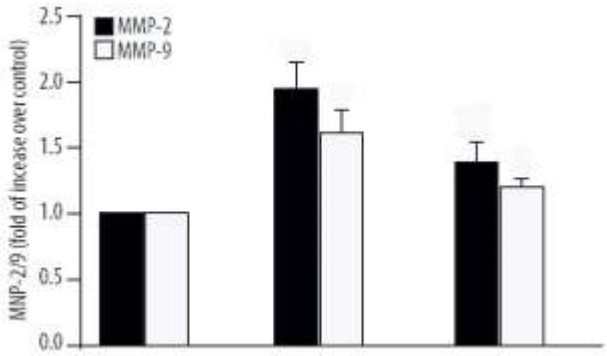


Fig 5: “Effect of MCT and MG132 on the activity of MMP2/MMP9”

DISCUSSION

The Endothelial cell (E.C) is documented as most important regulators of functions in cardiovascular system. The endothelial dysfunction entails complex disproportion between E.C production of vasodilators Vs vasoconstrictors, antithrombotic Vs prothrombotic mediators, inhibitors Vs activators and anti-inflammatory Vs pro-inflammatory signals. Therefore, the present study focuses on the underlying mechanism of heart failure through the pulmonary arterial hypertension (PAH). (20, 21).

The results presented in this study were consistent with the previously reported result of (22). Treatment causes inflammatory response in the lungs, endothelial cell injury and consequent proliferation in the vascular smooth muscle. Thus it has leaded to develop severe degree of P.A.H, associated RVH, and pulmonary vascular lesions. Injured endothelial cells and inflammatory cells secrete more MMPs. Furthermore, the results presented in FIG-II were consistent the previous reported results by (23).

Also, it has been demonstrated that the MMPs in either way which could directly/indirectly effects the proliferation, invasion, migration and programmed cell death of endothelial cells along with smooth muscle cells (24). These conclusions are in correlation and suggested that MMPs plays an important role in developing PAH. In support, MMP inhibitor has been established as a protective role in M.C.T-induced P.A.H in rats (25).

Increased in the levels of deposition of elastin & collagen are recognized as an imperative determinants in development of P.A.H (26). The accumulation of collagen contributes to develop stiffening in pulmonary artery. It is concerned in development of P.H and right ventricular dysfunction via two mechanisms i.e. increased in

distal arterial cyclic strain damage and secondly promotes smooth muscle cell (S.M.C) proliferation & proximal wave reflections. This increased the after load in right ventricle (27, 28).

This current research and in context to previous studies, MMP activity was increased during M.C.T treatment, which is suggestive of its input in development of cardiopulmonary pathological changes in experimental rats having “MCT-Induced Pulmonary Hypertension”.

In our study it was noted that the mean body weight of MCT-Treated rats was remarkably decreased as in comparison with our control group. This revealed signs of developing cachexia in patients with lower ejection fraction in chronic heart failure. It was seen that the “lungs/Body-weight-ratio” was considerably increased in our M.C.T-group in contrast to control group, which directed us towards the occurrence of proliferative pulmonary response in the M.C.T-treated rats.

These results were in correlation with the previously reported studies by (29). R.V/(L.V + Septum) ratio can be measured, as index of R.V.H. This was considerably augmented in M.C.T group since 14 days.

CONCLUSION

In M.C.T-induced rat model of PAH, there were obvious, ECM remodelling in pulmonary artery which was accompanied with the elevated activity of MMP2/9, suggesting that the abnormality of MMP signal pathway was invloved in progression of developing “Pulmonary Artery Hypertension” (P.A.H). Also; inhibition of ubiquitin proteasome system can ameliorate the remodeling of the “Extra-cellular matrix of pulmonary artery” & collagen deposition. Furthermore, M.G-132 inhibits the function of ubiquitin proteasome system, and subsequently inhibits the activity of MMP, leading to suppression of the remodeling of the extra-cellular matrix of pulmonary artery along with collagen deposition and amelioration of P.A.H.

Further in-depth researches are required for further changes regarding ubiquitin proteasome (UPS) & M.M.P activity in “Chronic M.C.T induced Pulmonary artery hypertension model”.

Conflict of interest: There is no conflict of interest in this study.

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