CHAPTER 6

DISCUSSION

6.1 Standardization and validation of rat model of Monocrotaline induced pulmonary hypertension

The present study was undertaken to standardize and validate the model of MCT induced PH in SD rats. In this study two doses of MCT were used and the rats were kept for durations for the development of PH which was confirmed by measuring RVP (a hemodynamic marker of PH) and RVH (a complication associated with PH).

On the basis of previous reports [1-5] experiments were started with two doses of MCT (60 and 80 mg/kg) for 21 days, 28 days and 35 days respectively. There was 100 percent mortality at the dose of 80 mg/kg MCT before fortnight. Therefore, studies with MCT 80 mg/kg were discontinued. Further studies were carried out with the dose of 60 mg/kg only for the period of 21, 28 and 35 days.

The severity of the disease developed by MCT administration is indicated by the significant decrease in the percent survival and the body weight of the MCT treated rats over the period of time. There was increase in the body weight and no mortality in the control rats but, on the other hand, a decrease was observed in the survival and body weight depending on the time period i.e. the longer the time period (21, 28 or 35 days) after MCT administration, the more was the decrease in the survival and body weight in MCT treated rats. These observation regarding survival and body weight after MCT treatment are in line with other studies as well [6-10].

There was a significant increase in RVP, a characteristic feature of PH, following MCT treatment in a time dependent manner. The rise in RVP after 21 days (24.16 ± 0.80) mmHg and 28 days (29.84 ± 1.84
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mmHg) was significant but nearly 2.5 fold increase in the RVP (44.25 ± 1.76 mmHg) was observed after 35 days in MCT treated rats as compared to the control rats. The elevation in RVP in MCT induced PH is due to a rise in the pulmonary arterial pressure and pulmonary vascular resistance [4, 7].

Increase in RVP was also accompanied by RVH following MCT treatment and was evident by an increase in RV/LV+S, RV/BW and RV/HW. RVH was not observed after 21 days of MCT treatment however, there was a mild RVH after 28 days following MCT treatment. Maximum RVH was reached after 35 days following MCT treatment. These observations are in sync with other studies also [5, 11].

In MCT treated rats, there was a time dependent increase (with a maximum increase after 35 days) in LW/BW ratio, another marker of adverse structural changes and proliferation of cells taking place in lungs [3, 12]. MCT treated rats in all the groups had no effect on the systemic hemodynamic parameters (systemic blood pressure and heart rate) as compared to the control rats.

As there was maximum increase in the RVP, RVH and pulmonary vascular remodelling (LW/BW) after 35 days following single administration of MCT (60 mg/kg, s.c), therefore, further studies were carried out with this experimental protocol (single dose of 60 mg/kg of MCT for 35 days) only. This experimental protocol is well accepted and used to study PH and associated RVH complications [2, 13, 14].

Later, to validate the MCT (60 mg/kg) induced model of PH in rats, Bosentan (BOS), a nonspecific competitive endothelin receptor antagonist and a clinically approved drug for the treatment of PH since 2001, was used. Administration of BOS (100 mg/kg) improved the percent survival and attenuated the loss in body weight in MCT treated rats. BOS (50
mg/kg) was found to be ineffective in improving survival and weight gain in MCT treated rats. Treatment with BOS (100 mg/kg) also inhibited the increase in RVP and also checked the compensatory RVH in MCT treated rats as evident by a decrease in ratio of RV/LV+S, RV/BW and RV/HW and histological studies. However, BOS at 50 mg/kg did not prove to be beneficial in reducing RVP and RVH. BOS treatment (100 mg/kg) also ameliorated the pulmonary vascular remodelling of distal pulmonary arteries (confirmed by histological studies) and also decreased the LW/BW ratio in MCT-induced PH which underlines the beneficial effect of BOS on structural remodelling occurring in the lungs. Above results were in agreement with studies done by Chen et al. [15] which showed the protective effect of BOS in hypoxic PH in rats. In all the groups, BOS had no effect on the systemic blood pressure and heart rate.

Therefore, it can be concluded that single administration of the dose of 60 mg/kg MCT was able to increase RVP, RVH, pulmonary vascular remodelling after 35 days and this was reversed by BOS (100 mg/kg), and thereby validating the rat model of MCT induced PH.
6.2 A study on the involvement of poly (ADP-ribose) polymerase-1 (PARP-1) in pulmonary hypertension (PH)

The aim of this part of the study was to explore the involvement of PARP-1 in pathophysiology of PH by using a PARP-1 inhibitor, 1,5-Isoquinolinediol (ISO), as an experimental pharmacological tool.

In the present study, it was observed that PARP-1 inhibition improved severe abnormalities in cardiopulmonary functions and structure resulting in a significantly better survival of MCT-challenged rats as compared to untreated rats after 35 days in both preventive and curative models of PH. Development of PH was also accompanied with weight loss in the MCT treated animals which reflected the severity of the disease [7] but both preventive and curative PARP-1 inhibition by ISO prevented weight loss.

The findings revealed that an increase in the RVP (a characteristic feature of PH) in MCT treated rats which may be due to increased afterload in RV as a result of vasoconstriction and vascular remodeling in the pulmonary vasculature [4, 7, 16]. PARP-1 inhibition significantly reduced the increase in RVP following MCT treatment. As PH is a chronic disease that is not always diagnosed early, so, the effect of PARP-1 inhibition after 3 weeks of MCT treatment, when PH is established or is rapidly progressing, was also studied [3, 17]. Treatment of MCT exposed rats with ISO from days 21 to 35 reversed the increased RVP indicating that inhibition of PARP-1 is effective in curative model of PH also.

In PH, oxidative injury in lungs precedes vascular remodeling and vasoconstriction [18-21]. In MCT induced PH, there was increased oxidative and nitrosative stress as depicted by enhanced levels of ROS, nitrite, MDA and decreased levels of endogenous antioxidant, GSH. Increased oxidative and nitrosative stress causes severe damage to
biomolecules, especially the DNA which results in single and double-stranded DNA breaks (DSBs). With this, there is phosphorylation of histone H2A variant g-H2AX, a marker for DNA damage [22]. There was, in this study, increased expression of g-H2AX in lung homogenate indicating DNA damage following MCT treatment. Increased DNA damage results in activation of PARP-1, a fact that has also been observed in the present study by increased PARP-1 activity, mRNA and protein expression. There was reduced oxidative stress and consequently g-H2AX expression by PARP-1 inhibition using ISO. This finding is supported by work done by Martinez-Romero et al. 2008 [23] who showed that PARP-1 inhibition or genetic deletion lowered oxidative stress and reduced the expression of g-H2AX. As a result of PARP-1 overactivation, there was enhanced PAR (product of the reaction catalyzed by PARP-1) formation. Pharmacological inhibition of PARP-1 led to a decreased PARP-1 mRNA and protein expression along with low PARP-1 activity and consequently reduced expression of PAR which might be due to the effect of PARP-1 inhibition on amelioration of oxidative and nitrosative stress [23, 24].

PARP-1 activation causes conversion of NAD into nicotinamide and PAR in a reaction consuming ATP thereby lowering the levels of NAD as well as ATP [25]. Similarly, in MCT treated (pulmonary hypertensive) rats there was lower NAD and ATP levels. Inhibition of PARP-1 by ISO checked the consumption of NAD and ATP resulting in their improved levels.

Overactivation of PARP-1 also contributes in oxidative and nitrosative stress by increasing redox imbalance due to lower levels of NAD and ATP [26]. Apart from improving NAD levels, PARP-1 inhibition decreases oxidative and nitrosative stress by having direct effect on decreasing inflammation [27] because it is well known that a oxidative and nitrosative stress induce systemic inflammation [28]. Interestingly,
some PARP-1 inhibitors including ISO are known to possess free radical scavenging potential which may be another reason to attenuate oxidative stress [29]. Also, chronic administration of pharmaceuticals or nutraceuticals with PARP inhibiting activity appears to be beneficial in conditions of chronic oxidative stress [30].

Injury to the pulmonary vascular endothelium (mediated by oxidative stress) precedes pulmonary artery smooth muscle cells (PASMC) proliferation and medial hypertrophy in the distal pulmonary vascular bed of MCT-treated rats [19]. There was endothelial dysfunction in the pulmonary arteries of MCT challenged rats as evident from reduction in acetylcholine (ACh) induced relaxation in phenylepherine precontracted pulmonary arteries. PARP-1 inhibition significantly improved pulmonary arterial endothelial function in rats by improving ACh induced vasorelaxation in isolated pulmonary vessels. This finding is supported by a number of studies where PARP-1 inhibition improved endothelial dysfunction in various diseases like circulatory shock [31] myocardial ischemia reperfusion injury [32], heart failure [32] hypertension [33], diabetes [34, 35] and cardiovascular aging [36]. This improvement in endothelial functions may be due to antioxidative and anti-inflammatory effects following PARP-1 inhibition. This is supported by studies where antioxidants like resveratrol and anti-inflammatory agents like aspirin and hydrocortisone have shown improvement in endothelial function [19]. Apart from this, PARP-1 inhibitor ISO, used in this study possesses antioxidant and free radical scavenging effects which may further add to endothelial improving effect.

Abnormalities in eNOS signaling underlie endothelial dysfunction, a condition common to hypertension, diabetes, aging, and atherosclerosis [37]. Disturbed eNOS signaling has also been witnessed in MCT challenged rats [38, 39] and PH patients [40]. There was also down
regulation of eNOS expression in lungs of pulmonary hypertensive rats, which could be another reason behind endothelial dysfunction in PH. eNOS plays a key role in the function of the vascular endothelium as it catalyses the formation of NO which mediates vasodilation. Alterations in expression of eNOS, eNOS enzymatic activation (e.g., signaling-induced eNOS phosphorylation) and decreased NO bioavailability [41, 42] are responsible for endothelial dysfunction. PARP-1 inhibition improved endothelial function, as discussed above, and eNOS expression. This observation is supported by numerous cell culture and ex-vivo studies which showed that pharmacological inhibition of PARP-1 confers protection against oxidative stress-associated endothelial dysfunction, primarily by increasing the eNOS levels [43, 44].

The results were in agreement with previous reports where there was enhanced medial hypertrophy in pulmonary vasculature of MCT challenged rats [45-47]. Pulmonary vascular remodelling and medial hypertrophy is also observed in patients with primary PH [48]. Pulmonary vascular remodelling as evident from increase in the thickness of the medial layer of pulmonary vessels of MCT challenged rats. This increase in thickness is due proliferation of PAEC and PASMC in MCT-challenged rats [5]. Proliferation of PAEC and PASMC is reflected by a marked increase in the expression of PCNA (a marker of cell proliferation). Inhibition of PARP-1 reduced the medial hypertrophy and vascular remodelling in MCT challenged rats as evident from histological studies and reduced expression of PCNA. This can be because mild activation of PARP-1 promotes cell survival and its inhibition causes delay/no DNA repair and consequently cell death. Also, it has been shown in cancer studies that PARP-1 activation plays a role in increasing cell survival and angiogenesis [49]. Thus, inhibiting PARP-1 proved beneficial in PH.
which decreases the cell survival and proliferation in pulmonary vasculature. A number of recent experimental studies and clinical reports indicate that control of pulmonary arterial pressure, without the reversal of structural changes in pulmonary vasculature is not satisfactory. Therefore, the present findings show that targeting PARP-1 can not only prevent increase in pulmonary arterial pressure but can also reverse pulmonary vascular remodelling.

In MCT treated rats, there was an increase in LW/BW ratio, another marker of adverse structural changes and proliferation of cells and inflammation (infiltration of various proinflammatory cytokines and immune cells) taking place in lungs which was in agreement with previous reports [3, 12, 50]. Following the treatment with ISO in both preventive and curative studies, there was a decrease in the LW/BW ratio. This again confirms the beneficial effect that PARP-1 inhibition exerts on pulmonary vascular structural remodeling and inflammation occurring in PH.

There exists solid evidence suggesting that pulmonary inflammatory conditions are prevailing in PH [51]. In the present study also, the level of pro-inflammatory cytokine TNF-α and expression of p65 subunit of NF-κB (a central transcriptional mediator of inflammatory response) and a decrease in anti-inflammatory cytokine IL-10 was observed in the lungs of MCT treated rats. A number of previous studies support this observation that an intense perivascular inflammation, pulmonary arterial medial hypertrophy and vascular remodelling follows initial inflammatory phase, after MCT administration [51-53]. PARP-1 inhibition proved anti-inflammatory by increasing anti-inflammatory cytokine IL-10, decreasing pro-inflammatory cytokine TNF-α levels and expression of NF-κB. This can be because PARP-1 contributes to inflammatory processes through the regulation of several transcription factors, cytokines, adhesion factors and inflammatory mediators. PARP-1
is known to be a co-activator of NFκB and also PARylates NFκB [28, 54, 55]. PARP-1 deletion abrogates NFκB activity [28]. PARP-1 inhibition also served to protect cells in oxidative and nitrosative stress induced systemic inflammatory response [56]. Therefore, it can be stated that inhibition of PARP-1 served to attenuate the inflammatory response by abrogating NFκB signalling and TNF levels and increasing the levels of IL-10.

Structural remodelling of pulmonary vessels is an important feature of PH, which reflects distal artery muscularization and matrix remodelling. The matrix metalloproteinases (MMPs) play an important role in matrix remodelling as they are involved in extra-cellular matrix turnover and hence, in SMC and EC migration and proliferation. Pharmacological inhibition of MMPs has beneficial effects in a number of pathological conditions [57-60]. Upregulation of MMP-2 and MMP-9 have been found in experimental PH which degrade basement membrane and promote cell proliferation and migration [7, 61]. In this study there was an amplified expression of MMP-2 and MMP-9 and reduced expression of their endogenous inhibitor, TIMP2, in MCT treated rats as compared to control rats. However, in ISO-treated groups there was restoration of deregulated TIMP2/MMPs balance (by downregulating the MMP-2 and MMP-9 and enhancing TIMP2 expression) that would potentiate the reversal of remodelling in PH. PARP-1 deficiency by genetic deletion or by pharmacological inhibition of PARP-1 using its inhibitor have shown to have direct effect on TIMP2 expression thereby having inhibitory effect on MMPs [62]. It is possible that part of the observed in vivo effect of PARP inhibitors in PH may involve the attenuation of intracellular TIMP/MMPs imbalance which might have led to preservation of ECM integrity.
In recent years, novel cancer-like concept for PH has emerged due to structural and functional changes in the pulmonary vasculature implicating exuberant proliferation, migration and survival and apoptosis resistance of pulmonary vascular cells within the pulmonary arterial wall (i.e. SMC, myofibroblasts and EC). These structural changes suggest a switch from a quiescent state to a pro-proliferative and apoptosis-resistant cellular phenotype [4, 63-65]. Apoptosis resistance was observed as evident by lower number of TUNEL positive cells and decreased caspase-3 activity along with no signs of PARP-1 cleavage (a marker of apoptosis) in lungs of MCT treated rats. PARP-1 inhibition induced apoptosis as shown by more TUNEL positive cells and increased activity of caspase-3 in MCT exposed lungs. Also, immunoblotting experiments revealed increased PARP-1 cleavage by inhibiting PARP-1 in MCT challenged rats which may be due to increased caspase-3 activity. Therapies, which induced apoptosis were found to be beneficial in PH as they reversed the structural changes in the pulmonary arteries e.g. Dichloroacetate, PDGF inhibitors, etc [64]. PARP-1 inhibition promotes apoptosis in lungs by inhibiting the expression and activity of several cell survival proteins including HIF-1α [66].

GSK3β participates in a wide spectrum of cellular processes, including glycogen metabolism, transcription, translation, cytoskeleton regulation, intracellular vesicular transport, cell cycle progression and apoptosis [67]. Sklepiewicz et al (2011) [67] have observed increased levels of inactivated or phosphorylated GSK3β in lung homogenates and PASMC after 35 days of MCT administration. In the present study also PARP-1 inhibition led to a decreased expression of phosphorylated GSK3β. The decrease in the inactivated form of GSK3β might be beneficial in PH by promoting apoptosis and inhibiting cell proliferation as studies have shown that by increasing active GSK3β levels by active
gene transfer of GSK3β, and decreasing the phosphorylation or inactivation of GSK3β caused a significant inhibition of smooth muscle proliferation, sustained apoptosis along with reduction in neointima formation in the restenosis model of balloon injury in rat carotid arteries [68, 69].

In PH, hypoxic condition occurs in lungs, in response to which Hypoxia-inducible factor-1α (HIF-1α), (one of the pivotal mediators in the lungs) gets upregulated and thereby participates in the PH pathogenesis. In fact, induction of HIF-1α by cobalt chloride, inhibitor of prolyl hydroxylase dehydrogenase, promotes PH in rats [70]. There was enhanced HIF-1α expression in lungs of MCT challenged rats which was lowered by PARP-1 inhibition. This observation is in agreement with studies where partial deficiency of HIF-1α markedly attenuated the increase in pulmonary arterial pressure and RVH in mice [71,72]. Moreover, inhibition of HIF-1α by Digoxin and Acriflavin also prevented the development of PH [73]. PARP-1 gene deletion or inhibition attenuates HIF-1α mediated responses by decreasing HIF-1α stabilization and accumulation [23]. Also, PARP-1 forms a complex with HIF-1α and activates it. PARP-1 inhibition causes HIF-1α downregulation which proves beneficial in PH because it regulates a number of genes involved in vascular remodelling like VEGF, proliferative and vasoactive factors such as endothelin-1, platelet-derived growth factor (PDGF) and angiotensin-converting enzyme (ACE) which have an important role in pathophysiology of PH [74]. There was increased expression of VEGF in MCT treated rats which indicated vascular remodelling taking place in lungs. Also, PARP-1 inhibition led to attenuation VEGF expression. This might be an indirect effect of PARP-1 via HIF-1α downregulation as discussed above.
This part of the study can be concluded as-- PARP-1 plays an important role in PH because inhibition of PARP-1 by its pharmacological inhibitor (ISO) decreased RVP along with improvement in survival and body weight. The increase in RVP takes place due to pulmonary vascular remodelling which further results in RVH. Pulmonary vascular remodelling and RVH are hallmarks of PH and both of these MCT induced effects were ameliorated by PARP-1 inhibition. Oxidative stress in MCT treated rats was evidenced by increased levels of MDA, nitrite and ROS and decreased level of GSH; all these parameters were modulated in a beneficial way by PARP-1 inhibition. Increased oxidative stress induced DNA damage and subsequent PARP-1 activation was curbed by ISO treatment. In this study, ACh induced relaxation in pulmonary vessels isolated from MCT challenged rats was impaired. Treatment with PARP-1 inhibitor led to attenuated endothelial dysfunction in MCT treated rats. Pulmonary vascular remodelling and matrix degradation was also prevented by PARP-1 treatment. PARP-1 inhibition led to increase in the IL-10 levels, decrease in the TNF-α and LW/BW ratio in MCT treated rats, thereby, attenuating inflammation associated contributions in PH pathogenesis. PARP-1 inhibition also modulated the expression of a number of genes (HIF-1α, VEGF, GSK3β, NFκB) participating in the pathogenesis of PH. Thus, it can be concluded that PARP-1 inhibition may be a useful approach to treat PH.
6.3 Effect of PARP-1 inhibition on right ventricle hypertrophy in MCT induced pulmonary hypertension in rats

PH is a deadly disease in which vasoconstriction and vascular remodelling both lead to a progressive increase in pulmonary vascular resistance and RVP. An important adaptation of the RV to the high pressure and increased resistance in pulmonary arteries is to increase wall thickness by accumulating muscle mass [75]. Similarly, the RVH was evident by an increase in RV/LV+S, RV/BW and RV/HW and histological studies, following MCT treatment, an observation in sync with other studies also [73]. Both preventive and curative treatment with ISO checked the compensatory RVH in MCT treated rats as evident by a decrease in ratio of RV/LV+S, RV/BW and RV/HW and decrease in the RV wall area and RV/LV+S (wall area %). The reduction in RVH upon ISO administration can be attributed to its ability to inhibit PARP-1 overactivation. As, in a number of studies genetic deletion and pharmacological inhibition of PARP-1 has been found to improve contractile function, reduced myocyte death, hypertrophy, collagen formation and, overall mortality in heart diseases [76- 83].

Oxidative and nitrosative stress have been intertwined with a number of cardiovascular disorders including cardiac hypertrophy and heart failure [84]. Increased oxidative stress plays a pivotal role in RV dysfunction in MCT induced PH and antioxidant treatment have ameliorated the development of PH and improved RV dysfunction [85]. Similarly, there was a increase in the oxidative and nitrosative stress as evident from increased MDA, ROS and nitrite levels and decreased GSH levels in RV of MCT treated rats and pharmacological inhibition of PARP-1 by ISO reduced the oxidative stress which is in consistent with previous reports where genetic and pharmacological PARP-1 inhibition have reduced oxidative stress by lowering ROS [24, 86]. The increased oxidative and nitrosative stress damage DNA by inducing single and
double-stranded DNA breaks. DNA damage initiates DNA repair mechanisms which involve a number of DNA repair enzymes chiefly PARP-1 [25]. These facts support the observations as there was enhanced expression of g-H2AX (a marker for DNA damage) in RV of MCT treated rats. Enhanced expression of g-H2AX following DNA damage has been reported in a number of studies [87, 88]. To rescue the DNA from damage, DNA repairing enzyme PARP-1 gets activated as and there was a significant increase in PARP-1 activity, mRNA and protein expression in RV of MCT challenged rats. Increase in PARP-1 activity is further evidenced by augmented level of PAR formation, a product of PARP-1 activation. Overactivation of PARP-1 results in PAR formation at the expense of NAD which diminishes NAD levels [83] and similar findings were observed in the present study. Reduced NAD levels create a redox imbalance contributing to an overall increase in oxidative stress [89, 90]. Thus, overactivation of PARP-1 may be another reason for increase in the oxidative stress in the RV of MCT treated rats. Pharmacological inhibition of PARP-1 by ISO protected DNA from further damage as shown by decreased g-H2AX expression which is in accordance with a study where PARP-1 deficient cells showed reduced DNA damage as evident by comet assay and lower g-H2AX expression [24]. There was also reduced PARP-1 activity and expression in the RV of pulmonary hypertensive rats thereby suppressing the oxidative and nitrosative stress (by restoring NAD levels). Also, some PARP-1 inhibitors including ISO have potent antioxidant and free radical scavenging properties which might be an added advantage in combating oxidative and nitrosative stress [29]. Increased PARP-1 activity also augments inflammation which further propels oxidative stress by increasing ROS production and thereby causing more PARP-1 activation as a consequence [28] which becomes a vicious cycle. Therefore, inhibition of PARP-1 lowered oxidative and nitrosative stress (by augmenting NAD levels and restoring redox balance) and by reducing inflammation in RV of MCT treated PH.
A number of experimental evidences suggest that inflammation plays a critical role in adverse cardiac remodelling and heart failure [91, 92]. MCT administration activates NFκB in the RV and its genetic inhibition ameliorates MCT induced RV dysfunction in PH [93]. In agreement to this, there was augmented inflammation in the right heart of MCT treated rats as evident from increased TNF-α levels and expression of p65 subunit of NFκB. However, PARP-1 inhibition caused a relief in inflammation by lowering TNF-α levels and NFκB (p65 subunit) expression. This might be because PARP-1 regulates the expression of various proteins responsible for inflammation at transcriptional level including NFκB (which is one of the most important transcription factor with which PARP-1 interacts) and acts as a co-activator in the NF-κB-mediated transcription [25]. Also, it has been recently demonstrated that PARP-1 activation is an important upstream event of NFκB activation [94]. This explains the diminished NFκB expression in response to PARP-1 inhibition. Also, PARP inhibition or the PARP-1 knockout genotype has resulted in the suppressed levels of the inflammatory cytokines like TNF-α and IL-1β in various animal models of inflammation [95, 96]. Following ISO treatment (as compared to MCT treated rats), there occurred a significant increase in the levels of anti-inflammatory cytokine IL-10 in RV which might be due to inhibition of NFκB [97].

Matrix Metalloproteinases (MMPs) play a key role as the most potent stimulator of cardiac hypertrophy and their activation results in widespread matrix degradation [62]. Similarly, enhanced MMP-2 and MMP-9 expression in the RV following MCT treatment was observed in rats. Inhibition of MMPs prevent cardiac hypertrophy in rats [98] and their endogenous inhibition is achieved by TIMPs [99]. During cardiac hypertrophy, the activity of MMPs increases while that of TIMPs decreases. Decrease in expression of TIMP-2 was observed in RV of rats suffering from PH induced by MCT. The beneficial effect of inhibition of
PARP-1 by ISO on RVH is further strengthened by a decrease in the expression of MMPs (MMP-2 and MMP-9) and increase in expression of TIMP-2. Present work also finds echo in the work of Hans et al. (2011) [62] who demonstrated the protective effects of PARP-1 gene deletion against cardiac hypertrophy is mediated by increasing TIMP-2 expression and decreasing MMP activity. Thus, PARP-1 inhibition may have exhibited cardioprotection by maintaining the optimum TIMP-2/MMPs balance and preventing ECM degradation.

Several studies report mitochondrial dysfunction in cardiac hypertrophy as evident from metabolic dysfunction and [100, 101] and impaired ATP generation. In this study, mitochondrial dysfunction as apparent from loss of mitochondrial membrane potential, reduced levels of NAD and ATP in MCT challenged rats. This mitochondrial dysfunction was reversed by PARP-1 inhibition by ISO. It is in sync with a previous report where absence of PARP-1 exerted a marked protection against oxidant induced mitochondrial dysfunction in cardiac myocytes by restoring the mitochondrial membrane potential [102]. PARP-1 inhibition also preserved the levels of NAD and ATP, a finding supported by work of Pillai et al (2006) [83]. Thus PARP-1 inhibition kept the mitochondrial and metabolic integrity in RV of MCT treated rats.

Cardiac hypertrophy and cardiac failure are associated with death of cardiomyocytes due to apoptosis. Similarly, there is apoptotic death of cardiomyocytes in hypertrophied RV associated with PH [103]. The results were also in similar lines as there was extensive apoptosis in RV as apparent from increased number of TUNEL positive cells. Inhibition of PARP-1 proved cardioprotective by attenuating apoptosis in cardiomyocytes of RV which was obvious by a decrease in the number of TUNEL positive cells. This observation finds support from various studies in which PARP-1 inhibition prevented apoptosis [25].
Increased apoptosis witnessed in RV of pulmonary hypertensive rats might be because of number of reasons. Excessive PARP-1 activation causes formation of PAR which is transported out of the nucleus to the mitochondria and directly affects the mitochondrial membrane by causing its depolarization and decreasing the mitochondrial membrane potential [43]. This results in opening of mitochondrial transition pore and release of cell death factors like apoptosis inducing factor (AIF) and cytochrome c (cyt c) to nucleus and cytosol respectively [104, 105]. In this study also, there was decrease in mitochondrial expression of cyt c and AIF and increase in expression of cytosolic cyt c and nuclear AIF. PARP-1 inhibition checked the release of cyt c and AIF from mitochondria. This may be because inhibition of PARP-1 reduced PAR formation and its journey to the mitochondria thereby maintaining the mitochondrial membrane potential and stopping the release of cell death factors. Reduced PARP-1 activation also maintains NAD levels which further prevents AIF release because replenishment of NAD levels suppress the translocation of mitochondrial AIF to the nucleus [104].

Cytochrome c is a regulator of apoptosis as it precedes morphological changes associated with apoptosis [106]. Once cytochrome c is released from mitochondria it binds with proapoptotic proteins to create apoptosome which in turn activates the caspase-3. Accordingly, increased caspase-3 activity and increased presence of cytosolic cyt c (as discussed earlier) in RV of MCT treated rats was found. For apoptosis to proceed further, caspase-3 cleaves PARP-1 to prevent NAD consumption and depletion of ATP pools (which are required in the later events during apoptosis). Like studies by Yu et al. [105], the increased caspase 3 activity was paralleled with increase in cleaved PARP-1 expression [107] in MCT treated rats. Increased activity of caspase-3 was checked by ISO treatment due to likely incarceration of cyt c within mitochondria. The reduction in caspase-3 activity, following ISO treatment, also decreased
the PARP-1 cleavage. Thus, PARP-1 inhibition prevented both caspase independent (due to AIF release) and caspase dependent (due to caspase activation) apoptosis in the RV of MCT treated rats.

There was significant decrease in the expression of eNOS in MCT treated rats and this was increased by PARP-1 inhibition. As it is well known that there is development of cardiac hypertrophy in eNOS deficient mice, also, overexpression of eNOS attenuates cardiac hypertrophy [108, 109]. Thus, ISO by increasing eNOS expression might be beneficial in reducing MCT induced cardiac hypertrophy in rats.

There is a dysfunctional oxygen supply (myocardial ischaemia) to the heart in cardiac hypertrophy as a consequence of increased mechanical load. This is due to vessel occlusion and capillary rarefaction, respectively [87]. The hypoxia-inducible factor (HIF-1α) plays a pivotal role and gets upregulated in the transcriptional response to changes in oxygen availability [110, 111]. Similar results were found in RV hypertrophied heart after MCT treatment where increased expression of HIF-1α was found. PARP-1 regulates the expression of HIF-1α by forming a complex with HIF-1α and directly activating HIF-1α–dependent gene expression [112, 113]. Transcriptional activation of HIF-1α is dependent on PARP-1 enzymatic activity and gets reduced in PARP-1 deficient cells. Upon PARP-1 inhibition by ISO, there was reduction in HIF-1α expression which might contribute in protection against RVH after MCT challenge. This is in sync with a study where down regulation of HIF-1α has prevented cardiac hypertrophy [114].

Studies were done on left ventricle (LV) also (as control tissue) to check left ventricle hypertrophy (LVH) and to see if there was any oxidative stress induced PARP-1 activation and inflammation in it. MCT did not lead to LVH after 35 days of its administration. Also, there was no change in the oxidative stress and inflammation in LV amongst various groups.
In conclusion, this study demonstrated that PARP-1 inhibition attenuated RVP and RVH in preventive as well as curative treatment. Preventive treatment with ISO decreased oxidative stress, inflammation, mitochondrial dysfunction and apoptosis in hypertrophied RV with PH. In a disease where treatment options are limited, this study provides proof of concept that PARP-1 inhibition could be beneficial and suggests that further investigation is warranted.

**Schematic representation of the involvement of PARP-1 in the pathophysiology of pulmonary hypertension**
6.4 Modulation of PARP-1 expression by an herbal, *Withania somnifera*, in monocrotaline induced pulmonary hypertension in rats

The thrust of current therapies against PH is on vasodilatation only and not on the structural changes occurring in pulmonary vessels, hence they are partially effective and give only symptomatic relief [4]. Further, use of existing therapies us fraught with number of undesirable side effects as mentioned in review of literature. Therefore, there is a pressing need to look for agents who can act on vasoconstriction as well as vascular remodelling. Thus, the best option is to explore the alternative medicines. In the present study, WS has been evaluated in MCT model of PH due to its cardioprotective, antioxidant, pro-apoptotic and anti-inflammatory properties. Further, studies were carried out to see if the protective effects of WS in PH are mediated through PARP-1 or not.

For this study, first commercially available root powder of WS was standardized by HPLC methods. HPLC profile showed that root powder of WS contained withaferin A and withanolide A. These bioactive compounds present in WS are the major constituents which are responsible for biological activities [115, 116].

The elevation in RVP is due to structural changes and vasoconstriction occurring in pulmonary vasculature [4, 7]. A preventive treatment with WS inhibited increase in RVP at both the doses of 50 mg/kg and 100 mg/kg. Curative intervention by WS at the dose of 100 mg/kg was protective and led to a significant attenuation of RVP. Development of PH was further associated by weight loss and mortality of the animals because of the severity of the disease [7]. Protection by preventive and curative treatment with WS stemmed weight loss and increased percent survival implying its ability to prevent the severity of MCT induced PH in rats.
PH is associated with increase in RVP and subsequent RVH which was evident by an increase in RV/LV+S, RV/HW and RV/BW following MCT treatment and is in agreement with earlier findings [7, 11]. Interestingly, both preventive and curative WS treatment checked the compensatory RVH by decrease in the ratio of RV/LV+S, RV/BW and RV/HW and histological studies in MCT treated rats. This observation is supported by earlier findings showing WS root extract to be cardioprotective in experimental myocardial infarction [117] and ischemia reperfusion injury [118].

In PH, oxidative injury precedes proliferation of PASMC to cause medial hypertrophy [19] and increase in RV cell mass to cause RVH. In this study, as shown and discussed earlier, MCT caused oxidative stress as evident by increased ROS in both lungs and RV. Treatment with WS lowered ROS levels thus decreasing oxidative stress in lungs as well as RV. WS owes strong antioxidant properties to its active principle constituent i.e. withaferin A which increases endogenous antioxidant superoxide dismutase with a concomitant decrease in lipid peroxidation [119]. In a previous study, WS treatment also gave protection by increasing the level of endogenous antioxidants like glutathione and glutathione peroxidase in isoprenaline induced myocardial infarction in rats [117].

Oxidative stress (whether it is endogenous or exogenous) causes DNA damage, which is always followed by the phosphorylation of the histone, g-H2AX. g-H2AX is a key factor in the repair process of damaged DNA. It is recruited to DNA damage sites, which in turn recruits other proteins for DNA repair (e.g. PARP-1, one of the most important DNA repair enzyme). There was increase in the expression of g-H2AX in the lungs and RV of the MCT-treated rats showing the increased DNA damage, probably due to increased oxidative stress after MCT
administration. This increased expression of g-H2AX was ameliorated by WS treatment at both doses indicating reduced DNA damage. This DNA protective effect following WS treatment may be due to antioxidant potential of WS. Upon DNA damage, PARP-1 is one of the first enzymes which gets activated to repair the DNA and maintain the genomic integrity [120-122]. Treatment of MCT exposed rats with WS also resulted in reduced PARP-1 expression. Reduced DNA damage (as indicated by reduced expression of g-H2AX) in WS treated rats following MCT challenge, might have resulted in reduced PARP-1 expression in lungs and RV of the rats. Also, treatment with antioxidants like curcumin and vitamin E have found to have attenuated PARP-1 expression in a study on lung epithelial cells [27], thus, antioxidants present in WS might be responsible for lowering PARP-1 expression in MCT challenged rats.

Oxidative injury as well as inflammation in lungs has adverse effects on pulmonary vascular endothelium causing its dysfunction [19, 123]. There was impairment in ACh induced relaxation in pulmonary vessels isolated from MCT treated rats and WS improved endothelial dysfunction at both the doses. This improvement in endothelial functions seems due to anti-oxidative properties of WS because antioxidant like resveratrol improved endothelial function (in pulmonary arteries) of pulmonary hypertensive rats [19]. Also, there was reduced expression of eNOS in lungs of MCT challenged rats, which is in sync with the clinical studies [40]. WS increased the expression of eNOS in lungs. This effect of WS may be responsible for protection in PH because NO donors, and overexpression of eNOS attenuate PH [19]. Increased eNOS activity augments NO level which has vasodilatory, anti-proliferative and pro-apoptotic effects [4]. Also, WS attenuated PARP-1 expression which might be another important factor responsible for improving endothelial function. Increased PARP-1 activation is responsible for endothelial
dysfunction in a number of pathophysiological conditions like shock [36, 124], complement-mediated endothelial injury, myocardial infarction and various forms of myocardial reperfusion injury, and heart transplantation, as well as the endothelial dysfunction associated with chronic heart failure, aging, hypertension, and diabetes mellitus [43]. Earlier, the experiment with PARP-1 inhibitor ISO also showed improved endothelial dysfunction in PH, which has been discussed previously in Section 5.2.

Pulmonary vascular remodelling observed in MCT-induced PH resembles the characteristics of human PH in terms of marked medial wall thickening [3, 7, 45]. The results are in agreement with the previous reports as MCT-challenged rats had shown excessive medial hypertrophy in pulmonary arteries. This increase in medial hypertrophy is due to the proliferation of EC and PASMC [3, 4]. Further, marked increase in the expression of PCNA, demonstrating the proliferation of cells was observed in lungs of MCT-challenged rats. WS treatment attenuated the medial hypertrophy at both the doses, along with decreased expression of PCNA in lungs. This effect of WS is supported by the study where WS root extract has led to a significant decrease in the PCNA expression in cancer cells [125]. The down regulation of PARP-1 expression may also contribute in anti-proliferative action of WS because PARP-1 activation is responsible for the exuberant cellular proliferation as it activates factors like AP-2 (responsible for evasion to growth suppressor signals), NFκB (thereby sustained proliferative signalling and inflammation) causing medial hypertrophy in the pulmonary vessels [28]. Also, it has been suggested by previous experiments and discussed in Section 5.2 that inhibition of PARP-1 prevents cellular proliferation.

PH has similarities to cancer as it is marked by a hyperproliferative diathesis and apoptosis-resistance in lung vascular cells [64]. Apoptosis resistance as evident by lower number of TUNEL positive cells and
decreased procaspase-3 expression was observed in the lungs of MCT treated rats. Dichloroacetate, which induced apoptosis in cancer, was found to be useful in PH [126]. In the same way, treatment with WS (50 and 100 mg/kg) induced apoptosis and increased expression of procaspase-3 in lung. Moreover, Withanolides present in WS potentiate apoptosis by suppressing the expression of anti-apoptotic genes [127]. In vivo studies also confirmed anti-proliferative and apoptosis inducing effect of WS when aqueous root extract of WS reduced cancer cell number in mice with Dalton’s ascitic leukemia [128]. Weakening in apoptosis resistance in lungs of MCT challenged rats after WS treatment may be due to its effect on PARP-1 inhibition. Studies by Meloche et al. (2014) [66] and by us showed that pharmacological PARP-1 inhibition increases apoptosis in lungs of pulmonary hypertensive rats. Thus, WS by inhibiting PARP-1 curbed the exuberant cellular survival and proliferation in lungs and increased apoptosis.

However, the scenario in right heart of pulmonary hypertensive rats is just the reverse as there is death of cardiac myocytes due to apoptosis in cardiac hypertrophy and failure. In this study, there was augmented apoptosis as evident from increased number of TUNEL positive cells in hypertrophied RV of MCT challenged rats which are in line with studies done by Zuo et al (2012) [105]. WS treatment led to the decrease in the RV myocyte apoptosis and this may be because of its effect on PARP-1 inhibition because PARP-1 overactivation results in cell death by apoptosis. Checking the overactivation of PARP-1 by administration of its pharmacological inhibitor, ISO, have weakened apoptosis and even initiated cell survival by repairing the damaged DNA. This effect of ISO on apoptosis has also been confirmed and discussed in Section 5.3 where ISO treatment attenuated apoptosis in the RV of MCT exposed rats.
In RV of MCT treated rats there was decrease in eNOS expression which was upregulated by WS treatment. eNOS deficiency leads to cardiac hypertrophy also eNOS overexpression is beneficial in cardiac hypertrophy. There are reports which suggest that cardiac hypertrophy develops in eNOS deficient mice and eNOS overexpression prevents the development of cardiac hypertrophy. WS improved eNOS expression via PARP-1 inhibition as it has been found that PARP-1 deficiency leads to preservation of eNOS activity in ApoE−/− mice.

There is modulation of inflammatory cytokines during initiation and progression of PH. A number of pro-inflammatory cytokines like MCP-1, TNF-α, IL-6, IL-1β get up-regulated in the lung vasculature and RV of patients and animal models of PH [4]. Similarly, the results demonstrated an increase in the level of pro-inflammatory cytokine TNF-α and NF-κB (p65 subunit) expression (a key transcription factor regulating the expression of several cytokines, chemokines, adhesion molecules, and inflammatory mediators) and a decrease in anti-inflammatory cytokine IL-10 level in the lungs and RV of MCT treated rats which were reversed with WS treatment showing its anti-inflammatory effect. WS has been used for its anti-inflammatory activity as evident from studies in which WS inhibited inflammation induced by Freund’s complete adjuvant in rats as well as in rheumatological conditions [129]. Withanolides in WS also inhibited TNF-α induced NF-κB activation [127]. Inhibition of the PARP-1 expression may be another factor in anti-inflammatory effect of by WS because PARP-1 directly activates NF-κB.

In MCT treated rats, there was an increase in LW/BW ratio, another marker of adverse structural changes, proliferation of cells and inflammation taking place in lungs [3]. Following the treatment with WS, in both preventive and curative studies, there was a decrease in the LW/BW ratio. This again underlines the favourable effect WS exerts on
structural remodelling occurring in the lungs. PARP-1 inhibitory effect of WS may have reduced the LW/BW as it has been studied that PARP-1 inhibition reduces LPS-induced acute lung inflammation in mice [130].

In response to hypoxia, HIF-1α is one of the pivotal mediators in the lungs and is involved in the pathogenesis of PH and partial deficiency of HIF-1α markedly attenuated the increase in pulmonary arterial pressure and right ventricular hypertrophy in mice [71]. Induction of HIF-1α by cobalt chloride promotes PH in rats [131]. Moreover, inhibition of HIF-1α by Digoxin and Acriflavin prevented the development of PH [11]. In this study, there was a significant increase in the expression of HIF-1α in lungs of rats treated with MCT. One of the reason of protection by WS in PH may be down regulation of HIF-1α because it regulates a number of genes involved in vascular remodelling like vascular endothelial growth factor (VEGF), proliferative and vasoactive factors such as endothelin-1, platelet-derived growth factor (PDGF) and angiotensin-converting enzyme (ACE) which have an important role in pathophysiology of PH [132]. Of note, PARP-1 forms a functional complex with hypoxia inducible factor-1α (HIF-1α), which promotes gene expression to adapt cells to hypoxic conditions, and whose activity requires the transcriptional co-activation by PARP-1 [112]. PARP-1 also plays a role in accumulation of HIF-1α and thus WS treatment might have decreased the HIF-1α by its inhibitory effect on PARP-1. Increased expression of HIF-1α was also found in RV of MCT treated rats, and was reversed by WS treatment which is in agreement with previous studies where decreased expression of HIF-1α has been found to be beneficial in cardiac hypertrophy. WS treatment attenuated the increased expression of HIF-1α.

It can be concluded that WS treatment attenuated RVP and RVH in preventive as well as curative treatment. WS also decreased oxidative stress, inflammation, improved endothelial dysfunction and reversed the
pulmonary vascular remodelling associated with PH. The protective effect of WS is also mediated by its inhibitory effect on PARP-1 whose pharmacological inhibition proved protective in MCT induced PH in rats. Thus, this study has reconfirmed the status of WS as a vaso- and cardioprotective herbal in Indian medicinal system.

Schematic representation of the beneficial effects of Withania somnifera in monocrotaline induced pulmonary hypertension
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DISCUSSION


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