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Exogenous Administration of Nitric Oxide Ameliorate CCl_4 Induced Liver Injury through Inhibition of Hepatic Stellate Cells

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ABSTRACT

Liver fibrosis is characterized by scar tissue due to the accumulation of extracellular matrix proteins generated by activated Hepatic Stellate Cells (aHSCs). The mechanism of selective apoptosis of aHSCs by Nitric Oxide (NO) can provide a breakthrough in liver therapeutics. The aim of the study was to examine the effect of NO released by Sodium Nitroprusside (SNP) on aHSCs and reduction of liver fibrosis induced by Carbon Tetrachloride (CCl_4). Isolated HSCS in both quiescent (qHSCs) and aHSCs were treated with 250 µM SNP. Cell viability assay, RT-PCR and Annexin V staining were performed to access the apoptotic effect of SNP. Mouse liver fibrosis model was prepared by injecting CCl_4.

1 µl/g body weight for four weeks. After fibrosis induction, mice were treated with 50 mM/kg or 100 mM/kg body weight SNP for four weeks along with CCl_4 injections. Blood and liver tissues were analysed for liver functions and fibrosis. *In vitro* results indicated increased apoptotic response in aHSCs after treatment with 250 µM SNP compared to qHSCs. CCl_4-injured mice treated with 100mM SNP showed down regulation of fibrotic markers; αSMA, TIMP, NFkB, iNOS, collagen Iαl. Whereas, the expression of hepatic markers; albumin, CK18, eNOS were upregulated in 100mM SNP treated group. Sirius red staining demonstrated the reduction of liver fibrosis. Decreased serum concentration of bilirubin and alkaline phosphatase confirmed improved liver functions. Above findings not only improve our understanding of the anti-fibrotic role of SNP but also provide foundation for the development of new anti-fibrotic treatment.

Keywords: Liver fibrosis; Quiescent Hepatic Stellate Cells (qHSCs); Activated Hepatic Stellate Cells (aHSCs); Nitric Oxide (NO); Sodium Nitroprusside (SNP)

INTRODUCTION

Liver fibrosis is a process in which wound curing myofibroblasts replace the damaged hepatic tissue with scar [1,2]. aHSCs produce Extra Cellular Matrix (ECM) proteins leading to scar formation [3]. The imperative approach for end stage liver disease is liver transplantation. But due to the deficiency of accessible donors, immune rejection and cost of the transplant, there is a need to develop some other promising therapies [4].

HSCs are positioned within the perisinusoidal space or space of Disse in liver sinusoids and comprising around 15% of the total cells in liver. During the progression of liver injury caused by CCl_4, aHSCs produce large amount of collagens and ECM proteins. In aHSCs, the gene expression of ECM proteins and inhibitors of matrix degrading enzymes are up regulated resulting in the accumulation of ECM proteins at the site of injury [5-8]. Pro-fibrotic cytokines and growth factors such as TGF-β family and from connective tissue are responsible for initiation and maintenance of expression of type I collagen. Conditions of sustained injury results in deposition of type I collagen fibrils. These fibrils are unaffected to proteolytic degradation and results in alteration of liver architecture [9,10].

Spontaneous recovery from liver fibrosis in experimental models is reported through loss of aHSCs due to apoptosis [11]. In CCl_4-induced liver fibrosis models, advanced liver fibrosis and even end stage liver cirrhosis after 4 and 12 weeks of CCl_4 induced injury respectively is reversible and seems to be associated with apoptosis induction in aHSCs [12,13]. This has highlighted the importance of selected induction of apoptosis in aHSCs. In past, certain promising mediators have been reported for the induction of apoptosis in HSCs both *In vitro* and *in vivo* models [13,14].

Nitric Oxide (NO) is a free radical normally produced by nitric oxide synthase and catalytic reaction of L-Arginine. Synthetic compounds such as furoxan, nitrate, diazeniumdiolate and sodium nitroprusside (SNP) are few other sources for the production of NO. NO negatively regulates the HSCs migration, contraction, proliferation and limits the mass of HSCs by promoting their apoptosis. Induction of apoptosis in HSCs by NO involves the changes in mitochondrial membrane potential and is independent of caspase activation [14]. SNP is a NO donor and some of its clinical application are; antihypertensive, vasodilator, antidote and in pulmonary hypertension in pediatric patients etc. Therefore, due to its clinical utility it is useful to evaluate its effectiveness to attenuate CCl_4-induced liver fibrosis in experimental models. The current study was therefore, planned to determine the inhibitory action of NO produced by SNP on cultured HSCs and its effectiveness to reduce CCl_4-induced liver fibrosis in mice model. Our data indicates that SNP may have therapeutic potential through inhibition of aHSCs for the better management of CCl_4-induced liver fibrosis.

MATERIALS AND METHODS

Animals

C57 BL/6 mice (n = 10 each group) were treated according to procedures approved by the Institutional Review Board (IRB) and ethical committee of the XXX.

Hepatic stellate cells isolation & culturing

Hepatic stellate cells from C57 BL/6 mice were isolated and purified as reported by Blomhoff and Berg, [15]. Isolated HSCs were washed in 10 ml Dulbecco's Modified Eagles Medium

(DMEM; Gibco, UK) twice. The final pellet was re-suspended in 10 ml DMEM containing 20% FBS and 0.1% penicillin/streptomycin and seeded in a 6 well plate. Medium of the cells was changed 2 hours after culture. HSCs cultured in 6-well plates, showed attachment after two hours of culturing. At 2^{nd} day (qHSCs) and 8^{th} day (aHSCs) of culturing, both qHSCs and aHSCs were characterized by RT-PCR.

SNP treatment

2.5×10^3 HSCs per well were used for apoptosis analysis in response to SNP as cells were more resistant to apoptotic stimuli in more confluent conditions [16]. The cells at 8^{th} day (aHSCs) of culturing were washed and serum free fresh medium was added in four experimental groups; control group, 250 µM SNP group, 250 µM SNP + 1mM L-NG-Nitroarginine Methyl Ester (L-NAME) group and 1 mM L-NAME group. L-NAME is the NOS inhibitor.

Assay for nitric oxide release

NO produced in the culture medium after 6, 12 and 18 hours of SNP treatment in all groups was evaluated from the total amount of nitrite concentration present in the medium. Briefly, medium was collected and centrifuged to pellet down the suspended cells. 100 µl

of medium was reacted with equal volume of Griess reagents 1 and (Oxford Biomedical Research, Germany) and placed on a shaker at room temperature for 10 minutes. The color product was measured at a wavelength of 538 nm by spectrophotometer. The concentration of nitrite was determined using a standard graph curve made from $NaNO_2$ concentration.

Cell viability assay

After 18 hours of SNP treatment, cell viability was evaluated by trypan blue assay in all experimental groups. Briefly, trypan blue solution was added to cells after removal of medium. The cells were incubated at 37°C for 10 minutes in CO_2 incubator then washed with PBS and examined under phase contrast microscope. Six high power fields were selected and trypan blue positive cells were counted to estimate the viability of cells.

Apoptosis analysis

Apoptosis of HSCs was assessed after staining with Annexin V primary antibody followed by immunostaining with Flourescein Isothiocyanate (FITC) conjugated secondary antibody. Stained slides were further analyzed for fluorescence under BX61 Olympus microscope equipped with DP70 camera (Olympus, Japan). Six high power fields were selected and annexin V positive cells were counted. The percentage of annexin V positive cells was calculated in all treatment groups.

CCl_4-induced liver fibrosis model and SNP treatment

6-8 weeks old C57 BL/6 mice weighing 15-20 grams were used in experiments. All animals were kept in standard conditions of temperature (25 ± 2°C), relative humidity (50% ± 10%), and light illumination (2 h/day). Mice were divided into four groups; vehicle control group 1 received Olive oil only, CCl_4 control group, 50 mM SNP group and 100 mM SNP group. All groups except vehicle control group received CCl_4, dissolved in olive oil (1:1) in a concentration of 1 ul/g of body weight, twice a week for 4 weeks [17]. After 4 weeks of CCl_4 injury, 50 mM and 100 mM SNP was given to SNP groups twice a week for four weeks [18]. During SNP treatment, animals were continuously received CCl_4 injections twice a week. The animals were sacrificed at the end of 8th week of experiment. The liver tissues and blood samples were collected.

Gene expression analysis

Total RNA was extracted from four in vitro HSCs groups and liver tissues of all experimental groups by using TRIZOL reagent according to manufacturer's protocol (Invitrogen, CA). cDNA was synthesized using 2 µg total RNA by M-MLV reverse transcriptase (Invitrogen, CA). The cDNA was amplified by PCR using the primers listed in table I and the thermal conditions at 94°C for 4 minutes, followed by 31-35 cycles of 94°C for 45 seconds, 56-58°C for 45 seconds, and 72°C for 45 seconds and a final extension of 10 minutes at 72°C. PCR products were analysed after electrophoresis in 2% (w/v) agarose gels containing ethidium bromide.

Immunostaining

4% Paraformaldehyde (PFA) was used to fix the liver tissue sections. After washing with PBS, 10% donkey serum was used for blocking followed by labelling with primary antibodies specific to anti mouse αSMA (1:400; Sigma, USA), CK18 (1:50; Santa Cruz, USA), iNOS (1:50; abcam, USA) and eNOS (1:50, abcam, USA). Anti-mouse FITC, TRITC and peroxidase conjugated secondary antibodies were used for fluorescence. DP-70 camera loaded Olympus BX-61 microscope was used to capture images (Olympus, Japan).

Sirius red staining

Liver tissue fixed in 4% PFA were dehydrated through increasing ethanol grades and embedded in paraffin. Paraffin-embedded tissues were further sectioned into 5 µm thick slices. Sirius red staining was performed to access the accumulation of Collagen in the hepatic tissues. Morphometric analysis was performed through image J software as reported previously.

Blood biochemistry

Blood was collected from each mouse in all groups and sera were stored at -80°C for further analysis. Concentrations of serum bilirubin and Alkaline Phosphatase (ALP) were tested to evaluate the liver function. Bilirubin and ALP tests were performed according to manufacturer's instructions (DiaSys, Germany).

Statistical analysis

3 slides per animal and 10 animals per experimental group were used to obtain the quantitative data of Sirius red staining and stated as ± SEM. One-way ANOVA was applied for statistical analysis of percent fibrosis area, ALP and bilirubin between treatment groups compared to control. p-value ≤ 0.05 was taken as statistically significant.

RESULTS

Increased nitric oxide concentration induces apoptosis in HSCs

Both qHSCs and aHSCs were characterized through the expression of αSMA, Desmin, MMP13, TIMP and collagen1α1 genes. RT-PCR analysis exhibited significant increase in expression of genes specific for HSCs in aHSCs compared to qHSCs. Fibrotic liver tissue was taken as a control (Figure 1). Nitric Oxide (NO) produced by 250 µM SNP after 6, 12 and 18 hours was 1.01 µM, 3.03 µM and 4.13 µM respectively in SNP group. The level of nitrite concentration was increased after SNP treatment while, L-NAME inhibited the effect of SNP (Figure 2A).

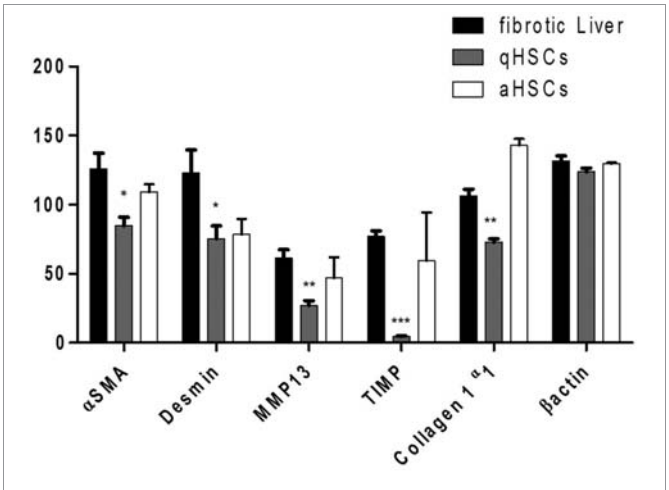

Figure 1: Characterization of cultured HSCs by RT-PCR. Cultured HSCs expressed αSMA, Desmin, MMP 13, TIMP and collagen 1α1. Bactin was kept as a control. qHSCs (quiescent HSCs after 2 days of culture), aHSCs (activated HSCs after 8 days of culture).

HSCs revealed a significant increase in the number of apoptotic cells (45.5 cell/field) when exposed to 250 µM SNP after 18 hours stained with trypan blue. However, L-NAME reduced the effect of SNP on HSCs and showed lesser number of apoptotic cells (14.40 cells/field) (Figure 2B). Morphology of aHSCs was changed from round and flattened to condensed and smaller after treatment with 250 µM SNP. However, the effect of SNP was reduced in group treated with L-NAME (Figure 2C). Gene expression regulation of αSMA, MMP13, TIMP and collagen 1α1 genes has further showed the inhibitory effect of SNP on aHSCs while L-NAME has inhibited the effect of SNP (Figure 2D). In order to confirm whether apoptosis has been induced by SNP, Annexin V apoptosis detection assay was performed. The number of annexin V positive cells increased to 46.67 ± 2.87 in SNP treated group versus 6.83 ± 1.67 in control group. This increase in cell death was diminished in SNP + L-NAME group to 15.33 ± 1.86 cells/field (Figure 2E1-E2).

Exogenous administration of SNP reduced fibrosis in CCl_4 injured liver

After CCl_4 induced liver fibrosis, mice in two separate groups were treated with 50 mM and 100 mM SNP twice a week for 4 weeks. After 24 hours of last SNP injection, mice were sacrificed and fibrosis

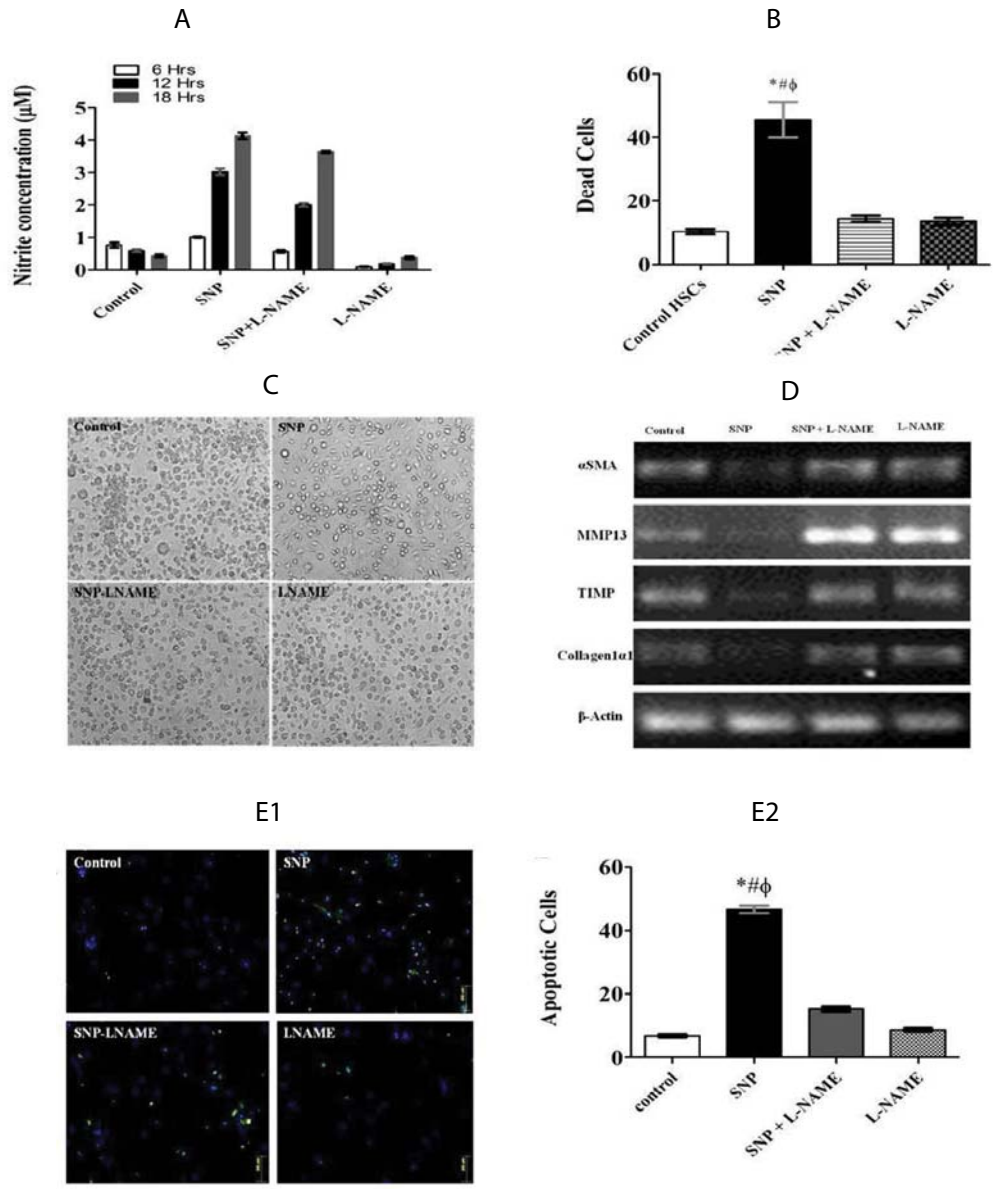

Figure 2: Apoptosis analysis of HSCs by NO. A) Nitrite concentration (µM) in HSCs cultures after 6, 12 and 18 hours. B) Effect of SNP treatment on HSCs viability. C) Morphology of the activated HSCs after treatment with SNP and L-NAME. D) SNP treatment reduced the expression HSCs specific genes. E) Annexin V binding assay for different treatments. DAPI was used to counter stain nuclei. Significantly high number of annexin V positive cells were detected in SNP treated group compared to control and other groups (E1). Quantification of Annexin V positive cells (E2). All values were expressed as mean ± SEM (p^* < 0.05 for SNP vs. control, $p\#$ < 0.05 for SNP vs. SNP + L-NAME, $p\phi$ < 0.05 for SNP vs. L-NAME).

was analysed by gene expression analysis, immunostaining of αSMA, CK18, iNOS and eNOS, Sirius red staining and liver function tests.

Gene expression analysis

To establish whether NO generated through SNP can induce the apoptosis or inhibition of aHSCs in fibrotic liver to resolve the fibrosis; the gene expression of fibrotic (αSMA, Collagen IαI, TIMP and NFκB) and hepatic (CK18) markers was analyzed. The expression of αSMA, Collagen IαI, TIMP and NFκB genes was increased in CCl_4 group (48.815, 69.534, 35.191, 62.624) as compared to vehicle controls while, the expression of CK18 was decreased in CCl_4 group (4.87). αSMA, Collagen IαI, TIMP and NFκB mRNA expression was notably lowered in 100 mM SNP group (5.504, 25.269, 2.79, 9.038) as compared to the 50 mM SNP (27.941, 33.056, 7.625, 38.362) and CCl_4 groups. In contrast, CK18 expression was increased in 100 mM SNP group (24.794) compared to 50 mM SNP (8.106) and CCl_4 groups (Figure 3).

Histological analysis

Immunofluorescence analysis of both fibrosis and liver markers further confirmed the inhibitory and apoptotic effects of SNP induced NO. The expression of αSMA was increased in CCl_4 group compared to vehicle control and lowered in 100 mM SNP group as compared to other treated groups. This showed that the NO produced by SNP induced the inhibition of aHSCs in fibrotic liver. However, the increased expression of CK18 in 100 mM SNP group showed the normalization of liver structure. In contrast, to the increased expression of iNOS, a clear reduction in the expression of eNOS was shown in CCl_4 group. The expression of iNOS was increased in 100 mM SNP group. However, down regulation of eNOS induced by CCl_4 induced was overcome in the SNP treated groups (Figure 4).

Sirius red staining

The results of Sirius red staining of different experimental groups showed only minor stains around the central vein and pericellular regions in normal mice liver compared to numerous sites of collagen deposition detected in CCl_4-injured mice. Furthermore, 100 mM SNP group illustrated suppression in the accumulation of collagens compared to 50 mM SNP and CCl_4 groups (Figure 4). Statistical analysis further confirmed a significant decrease in fibrotic area (%) to 1.45% in 100 mM SNP group compared to 3.35% in 50 mM SNP group and 4.00% in CCl_4 group (Figure 5).

Functional recovery after SNP treatment

The hepato-protective effect of SNP was further evaluated by differential quantification of bilirubin and ALP concentrations in serum of treatment groups. After 4 weeks of SNP treatment, the concentration of serum bilirubin in 100 mM SNP group (0.54 mg/dl) was significantly lesser than CCl_4 (1.3 mg/dl) and 50 mM SNP (0.96 mg/dl) groups (Figure 6A). Similarly, the level of serum ALP in 100 mM SNP group (455.4 units/L) was also significantly lesser than CCl_4 (801.60 units/L) and 50 mM SNP (627.6 units/L) groups (Figure 6B). Together, this data indicate the positive role of SNP to improve liver functions.

DISCUSSION

Liver comprises of 10-15% of non-parenchymal cells (HSCs) which plays a central role in liver fibrosis [1]. In damaged liver, injured hepatocytes along with their metabolites are known to activate kupffer cells. These triggered Kupffer cells activate HSCs by releasing cytokines such as Transforming Growth Factor-α (TGF-α), Platelet-Derived Growth Factor (PDGF), Tumor Necrosis Factor-α (TNF-α) and Reactive Oxygen Species [19]. aHSCs express αSMA and collagens leading to their deposition. On the other hand, enzymes that inhibit degeneration of ECM and cytokines (IL-6, IL-8, MCP-1 and ICAM-1) that promote fibrosis are also produced by aHSCs [17]. Thus activation of aHSCs leads to an imbalance between the synthesis and degeneration of ECM proteins and results in the development

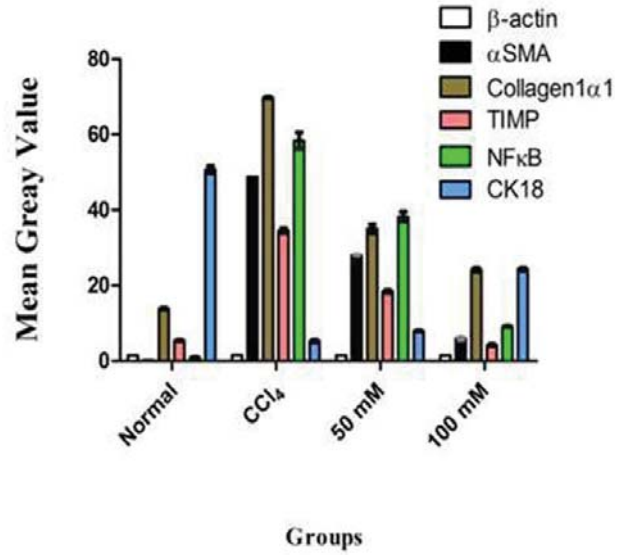

Figure 3: RT-PCR analysis of fibrotic and hepatic markers. A) 100 mM SNP reduced the expression of αSMA, collagen1α1, TIMP and NF-κB genes in fibrotic mouse while the expression of CK18 gene was increased. B) Mean grey value calculated by image J showed that the expression of fibrotic markers was decreased in 100mM SNP group while hepatic marker expression was increased.

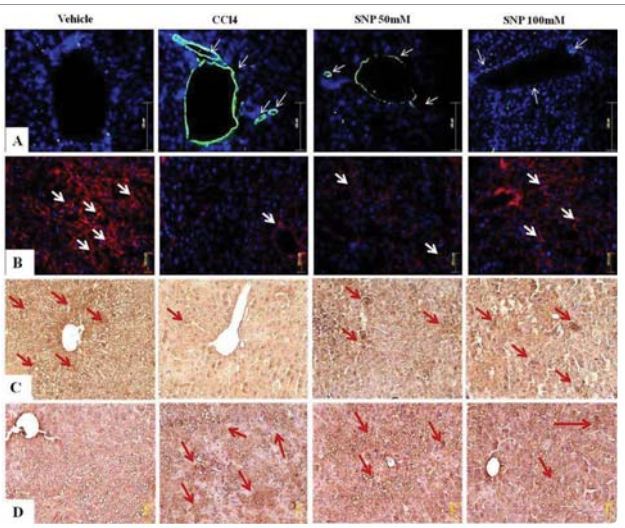

Figure 4: Immunohistochemical expression of treatment groups. A) 100 mM SNP group showed decreased expression of αSMA as compared to 50 mM SNP and CCl_4 control groups. B) 100 mM SNP resulted in the increased expression of CK18 as compared to 50mM SNP and CCl_4 groups. C) 100 mM SNP group showed increased expression of eNOS as compared to 50mM SNP and CCl_4 groups. D) 100 mM SNP group showed decreased expression of iNOS as compared to 50 mM SNP and CCl_4 control groups. (Magnification 200X).

of hepatic fibrosis [20]. Several key steps in the activation of aHSCs have been proved to be potential therapeutic targets for treating liver fibrosis. Apoptosis is known to be induced in several different cell types by the action of NO through peroxynitrite mediated mitochondrial damage [21]. Moreover, NO donors also recognized for their anti-fibro genic action through negative regulation of HSCs migration, contraction and proliferation. Furthermore, it also promotes the apoptosis of HSCs through mitochondrial dysfunction.

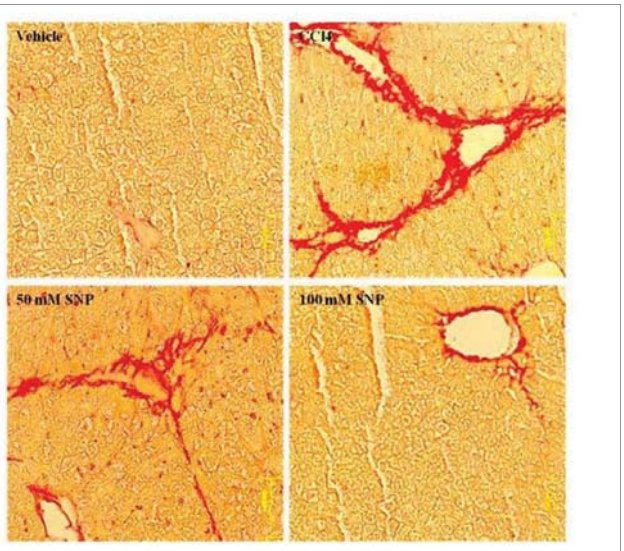

Figure 5: Estimation of collagen levels in different treatment groups. A) Staining with Sirius red demonstrated that collagen levels were greater after CCl_4 injury and were reduced by treatment with 100 mM SNP. B) Graphical representation of %fibrotic area estimated by image J software. All values were expressed as mean ± SEM. (p^* < 0.05 for 100 mM SNP vs CCl_4 control and $p\#$ < 0.05 for 100 mM SNP vs 50 mM SNP).

Figure 6: Serum concentrations of A) bilirubin and B) ALP in different experimental groups. All values were expressed as mean ± SEM (p^* < 0.05 for 100mM SNP vs CCl_4 control and $p\#$ < 0.05 for 100 mM SNP vs 50 mM SNP).

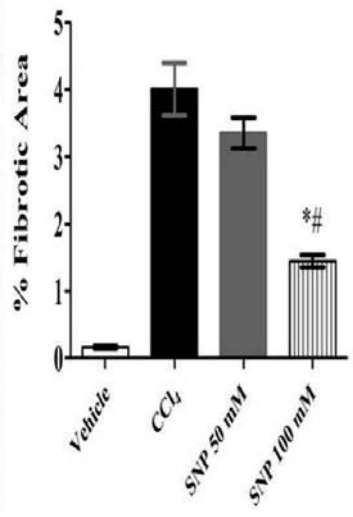

Figure 6: Serum concentrations of A) bilirubin and B) ALP in different experimental groups. All values were expressed as mean ± SEM (p^* < 0.05 for 100mM SNP vs CCl_4 control and $p\#$ < 0.05 for 100 mM SNP vs 50 mM SNP).

Table 1: Primer sequences of various gene.

	Tm °C		Primer Sequence
αSMA	58	Forward,	CTGACAGAGGCACCACTGAA
		Reverse,	AGAGGCATAGAGGGACAGCA
Collagen1α1	57	Forward,	GCCAAGAAGACATCCCTGAA
		Reverse,	GGCAGAAAGCACAGCACTC
TIMP	57	Forward,	CATCTGGCATCCTCTTGTTG
		Reverse,	CTCGTTGATTTCTGGGGAAC
CK18	56	Forward,	TGAGACAGAACTAGCCATGC
		Reverse,	CACTTCCACAGTCAATCCAG
NFκB	58	Forward,	GCACCTGTTCCAAAGAGCAC
		Reverse,	GTGGAGTGAGACATGGACACAC
Desmin	58	Forward,	GTGAAGATGGCCTTGGATGT
		Reverse,	TGTGTAGCCTCGCTGACAAC
MMP13	58	Forward,	ATGGACCTTCTGGTCTTCTGG
		Reverse,	ATGGCTTTTGCCAGTGTAGG
β-Actin	58	Forward,	ACTGCTCTGGCTCCTAGCAC
		Reverse,	ACATCTGCTGGAAGGTGGAC

Contrary to this, NO is a crucial mediator in juvenile hepatocytes proliferation during liver regeneration [22]. Therefore, it was proposed that inhibiting the aHSCs by exogenous NO administration can be a potential therapeutic strategy for treating liver fibrosis [23].

Myofibroblast like phenotype of HSCs is a typical manifestation of liver inflammation. The phenotypic changes identified in the culture conditions were similar to the injured liver as the expression of αSMA is the hallmark of HSCs activation [24]. aHSCs are further characterized by loss of vitamin A storing ability and enhanced collagen and TIMP expression [6,8]. In the first phase of this study, HSCs were isolated from CCl_4-injured mouse liver and cultured. HSCs transform from a non-proliferative to a highly proliferative state over a period of eight to ten days and express αSMA and other profibrotic genes [25]. Both qHSCs and aHSCs were characterized by gene expression analysis and our results are in accordance with reports that the expression of aHSCs specific genes: *αSMA, TIMP, MMP13 and Collagen IαI* was increased in aHSCs compared to qHSCs [26].

CCl_4-induced liver fibrosis approach has been adopted in our mice model and the parameters of hepatic functions and histo-pathological consequences have been closely monitored. CCl_4 is a widely used compound in animal models of liver fibrosis causing severe fibrosis [24]. The CCl_4-induced injury has been confirmed by serum concentrations of bilirubin and ALP and liver tissue histology. CCl_4-induced liver fibrosis also resulted in an increase in % fibrotic

area, reduced expression of CK18 and increased expression of fibrotic markers signifying the destruction of liver structure and function. Nitric oxide has both water soluble and lipid soluble properties and can pass through cell membranes. When entered in cells, it induces target molecules and induces apoptosis both by caspase-dependent and caspase-independent pathways in various tissues [27]. Therefore, the exogenous NO administration to CCl_4-induced liver fibrosis model was beneficial in reducing liver fibrosis as demonstrated in the 2nd phase of this study. Considering the in vitro apoptotic effect of SNP treatment on aHSCs, it was applied to CCl_4-induced liver fibrosis mouse model.

Reduced expression of αSMA in CCl_4-injured liver under 100 mM SNP showed that NO restrained the aHSCs. Apoptosis of aHSCs in the present study may provide a possible explanation for the reduction in collagen-IαI and diminution of Sirius red stained collagens. However, exact mechanism which regulates the matrix degradation required further clarification. The expression of interstitial collagens was analysed in parallel with collagenase inhibitor TIMP1 during recovery from liver fibrosis. Results indicate that the extensive remodelling is correlated with a diminution of TIMP1 expression. This highlighted the importance of the removal of the inhibitory influence of the TIMPs on collagenase activity. As some studies reported that TIMP1 and TIMP2 are produced by aHSCs and the reduction of these enzymes is a consequence of the reduction in number of aHSCs [28].

Nitric Oxide is a key factor of hepatic blood supply. In CCl_4-induced liver fibrosis model, expression of eNOS was significantly decreased compared to enhanced expression of iNOS. Therefore, the increased iNOS and decreased eNOS or together, they have contributed their adverse effects in chronic liver injury [29]. 100 mM SNP has produced a high level of NO in the serum as reported earlier. 100 mM SNP showed a reduction in iNOS and increase in the eNOS levels contrary to CCl_4-treated mice (Figure 4). The interaction between NO and apoptosis of HSCs in vivo seems to be too complex to be described as stimulatory/inhibitory categories. *In vitro* analysis showed that NO induced the apoptosis of HSCs. There might be the similar mechanism involved *In vivo* in our study which leads to the selective apoptosis either by inactivating the aHSCs or by inhibiting the activation of HSCs. In brief, we performed a primary selection and evaluation of SNP as a NO donor compound effective in reducing liver fibrosis.

CONCLUSIONS

It is concluded from the present study that NO released by SNP significantly induced apoptosis in the aHSCs and substantially diminished the CCl_4-induced hepatic injury. Consequently, improvement in liver functions and reduction in collagen deposition and fibrosis occurs. Hence, the potential use of HSCs apoptosis induced by NO appears to hold remarkable potential in the therapy of hepatic fibrosis.

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