

Original Research Article

Sulphasalazine Prevents Fibrosis; Relevance of TGFβRI

ABSTRACT

Aims: The aim of this study is to investigate the protective effect of sulphasalazine on liver fibrosis. Besides; investigation of the expression pattern of transforming growth factor β receptor I (TGFβRI) in liver fibrosis and the possible modulatory effect of sulphasalazine on it.

Study design: Controlled experiment

Place and Duration of Study: Department of Biochemistry and Department of Pharmacology and Toxicology, Faculty of pharmacy (boys) Al-Azhar University, between February 2015 and June 2015.

Methodology: Five Sprague-Dawley rat groups were used for the experiment. Control group: receiving corn oil; DMSO group: injected with; 6weeks group: injected with 50% CCL₄ in corn oil; sulphasalazine group: injected with sulphasalazine and CCL₄ and finally mesalazine group: were given mesalazine and CCL₄. On the day after the last dose, rats were anesthetized with diethyl ether and blood samples were collected for measurement of blood chemistry. The animals then were euthanized, and tissue samples from the livers were harvested and divided into 2 parts; one part was processed for standard histology and immunofluorescence techniques and the other was homogenized for oxidative status assessment.

Results: TGFβRI was shown to be upregulated in chronic liver injury; fibrosis stage; with expression occurring mainly in cell membrane of necrotic area. This expression was decreased significantly with sulphasalazine treatment. Sulphasalazine was shown to have ability to diminish fibrous tissue precipitation in chronic liver injury. This decrease in fibrosis observed with sulphasalazine has gone in parallel with decrease in TGFβRI expression. Besides; this action of sulphasalazine was suggested to be due to the whole molecule not to its moiety; mesalazine.

Conclusion: TGFβRI may be used as a candidate marker for diagnosis and prognosis of chronic liver diseases. Besides; it may be used as a target therapy for chronic liver diseases. Moreover; sulphasalazine might be a promising adjuvant therapy for chronic liver diseases.

Keywords: TGFβRI, fibrosis, sulphasalazine, mesalazine

1. INTRODUCTION

The liver plays an unlimited role in the preservation and body homeostasis regulation. It is included in most biochemical pathways to growth, protection against disease, nutrient fund, energy facility and reproduction (1).Hepatotoxicity can be generated by certain common causes including therapeutic agents, natural chemicals, laboratory and manufacturing agents and herbal therapies (2).

Transforming growth factor β receptors (TGFβRs) are of 2 types TGF-βRI and TGF-βRII. They mediate HSC activation (3) after binding to their ligand cytokine TGFβ, the profibrogenic cytokine that traditionally have been considered the key fibrogenic stimulus to HSC (4).

Carbon tetra chloride (CCL₄) is one of the most communal models for inducing hepatotoxicity. It is transformed into a toxic CCl₃· radical by hepatic cytochrome P 450 2E1 (CYP2E1).Thus; it brings an acute Centro-lobular necrosis which starts a wound healing response (fibrosis) (5). However, Contact to these chemicals in humans is rare and generally occurs in the manufacturing during fabrication and in places where these chemicals is usually used (6).

Hepatic stellate cells are the most important player in hepatic injury. Quiescent HSC are characterized by significant desmin expression and vitamin A storage. Following liver injury, HSCs lose their vitamin A content, rise the expression of α-smooth muscle actin (α-SMA) and gain a myofibroblast- like phenotype (7).These events are primarily triggered by Mediators released by activated kupffer cell and injured hepatocytes (8).

Sulfasalazine is a synthetic drug obtained from the grouping of sulfapyridine and 5-aminosalicylic acid, an antibiotic and an anti-inflammatory agent, respectively. This drug is usually used in the inflammatory diseases of the large intestine and rheumatoid arthritis (9)

Despite great knowledge about liver fibrosis, there are little drugs approved for management of it. So the aim of this work is to investigate the possible protective effect of sulphasalazine on liver fibrosis; an aspect that has gone side by side with investigation of expression pattern of TGF β RI in liver fibrosis and investigation of the possible modulatory effect of sulphasalazine on it.

2. METHODOLOGY

2.1. Animal model.

Adult male Sprague-Dawley (SD) rats weighing 250–300 g were used in the current study. The animals were obtained from the breeding colony maintained at the animal house of the Nile Company for pharmaceuticals, Cairo, Egypt. They were housed in the animal facility of Faculty of Pharmacy, Al-Azhar University in 20 X 18 X 25 cm plastic cages with stainless steel wire lids and mesh floor with 5 animals per cage. They were kept at 23 $\pm$ 1 °C, at 55 % relative humidity, with 12:12-h light: dark cycle, and maintained on a standard rodent chow (El-Nasr Company, Abou-Zaabal, Cairo, Egypt), diets and water ad libitum. Animals were randomly divided into five groups; control group: 9 rats were received corn oil (2mg/kg/twice weekly/IP/for 6 weeks) ; DMSO group: 12 rats were injected with DMSO, the vehicle of sulphasalazine (1ml/kg/day/IP/for 6 weeks) and 50 % CCL₄ in corn oil (4 ml/kg/IP/twice weekly/for 6 weeks); 6weeks group: 12 rats were injected with 50% CCL₄ in corn oil (4 ml/kg/IP/twice weekly/for 6weeks); sulphasalazine group: 12 rats were injected with sulphasalazine (75 mg/kg/day/IP/for 6weeks)(10) and CCL₄ as previously indicated in 6 weeks group and mesalazine group: 12 rats were given mesalazine(100 mg/kg/day/orally by oral gavage/6weeks)(11) and CCL₄ as previously indicated in 6 weeks group . On the day after the last dose, rats were anesthetized with diethyl ether and blood samples were collected for measurement of blood chemistry. The animals then were euthanized, and tissue samples from the livers were harvested and divided into 2 parts; one part was processed by standard histology and immunofluorescence techniques and the other was homogenized in 0.15 M KCl for MDA and SOD assay. All animal procedures were performed in accordance with the international guide for the care and use of laboratory animals (12)

2.2. Antibodies and chemicals.

Rabbit polyclonal TGF β R1 antibody was purchased from Santa Cruz Biotechnology (CA, USA). Cy3-conjugated goat anti-rabbit antibody was purchased from Jackson ImmunoResearch (PA, USA). 4, 6- Diamidino-2- phenyl indole (DAPI). CCL₄ and DMSO were purchased from Sigma-Aldrich (MO, USA). Sulphasalazine was obtained as yellowish powder gift by El-Kahera Pharmaceuticals Company, Cairo, Egypt and mesalazine was obtained as white powder gift by Pharopharm pharmaceuticals company, Alexandria, Egypt

2.3. Biochemical analysis.

Serum enzymatic activities of transaminases (ALT and AST) were estimated by kinetic method according to the method of international federation of clinical chemistry (IFCC) (13;14). Alkaline phosphatase (ALP) activity was assay according to the method of IFCC(15-17) and serum albumin concentration according to the method described by Gendler (18). The liver homogenate was used for the determination of the oxidative stress parameters. The level of thiobarbituric acid-reactive substances (measured as malondialdehyde (MDA) was determined as described by Mihara and Uchiyama (19). The activity of SOD was determined using the method described by Marklund (20).

2.4. Immunofluorescence analysis.

Slides containing liver tissues sections were handled according to method described by Abdel-Bakky and his associates (21). The primary TGF β R1 antibody was diluted in blocking solution in the suitable dilution (1:400) and left overnight in 4 °C. Secondary antibody (cyanine red conjugated)

diluted in the blocking solution was incubated for 30 min and the nuclei were counterstained using DAPI. Finally, all slides were mounted with the fluoromount solution, covered by covering slips, and allowed to stand for detection by immunofluorescence microscope (Leica DM 5500B).

2.5. Statistical analysis.

Data were presented as the mean $\pm$ SE. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post hoc test, according to the number of groups. The 0.05 level of probability was used as the criterion of significance using GraphPad Prism software version 5 (GraphPad Software Inc, CA, USA).

3. RESULTS

3.1. LIVER FUNCTIONS AND OXIDATIVE STATUS ASSESSMENT.

To assess liver functions; we have performed liver function tests including serum transaminases, ALP, and albumin. The oxidative status of liver tissues was determined through MDA and SOD assay as indicated in Table 1.

As indicated in Table 1; Serum ALT and AST activity of control group rats were 46.6 ± 2.85 and 54.1 ± 2.65 respectively. Administration of CCL₄ I.P. for 6 weeks increased ALT and AST to 749% and 806 % respectively if given only. Combination of CCL₄ with DMSO increased serum activities to 712% for ALT and 743% for AST compared to control group. Interestingly concomitant administration of CCL₄ with sulphasalazine reduced serum activities of ALT by 1.2 % and AST by 16 % compared to DMSO group.

As shown in Table 1 ALP activity of control group rats was 312 ± 21.9 . Administration of CCL₄ I.P. for 6 weeks increased ALP significantly by 83 % if given only and by 86 % in combination with DMSO compared to control group. Concomitant administration of sulphasalazine with CCL₄ decreased ALP activity by 10 % compared to DMSO group.

Serum albumin of control group was 3.81 ± 0.08 g/dl. A quite similar result was obtained upon administration of CCL₄ with mesalazine for 6 weeks where albumin concentration was 3.18 ± 0.16 g/dl as indicated in Table 1

As shown in Table 1; the tissue MDA content of control group was 4.73 ± 0.318 nmol/g tissue. This content was significantly increased on CCL₄ treated groups to be 31.9 ± 1.44 , 31.7 ± 1.82 and 25.7 ± 1.5 (nmol/g tissue) for 6 weeks, DMSO and sulphasalazine groups respectively. On the other hand; combination of CCL₄ with mesalazine showed MDA content of 9.86 ± 0.339 respectively; results that showed significant decrease compared to 6 weeks CCL₄ treatment although they appeared non-significantly changed from control group. Finally sulphasalazine showed a significant decrease in MDA content compared to DMSO group.

Table 1 shows the M $\pm$ SEM for SOD of studied groups. The control group showed tissue SOD activity of 526 ± 25.2 U/mg tissue. This activity was significantly decreased in other groups to become 242 ± 15.4 , 223 ± 14 , 281 ± 16 and 225 ± 9.65 for DMSO, 6 weeks, sulphasalazine, and mesalazine groups.

Table 1: Liver functions and oxidative status assessment tests.

	ALT(IU/L)	AST(IU/L)	ALP(IU/L)	Albumin(g/dl)	MDA(nmol/g tissue)	SOD(U/mg tissue)
Control	46.6 ± 2.85	54.1 ± 2.65	312 ± 21.9	3.81 ± 0.08	4.73 ± 0.318	526 ± 25.2
CCL ₄ 6 weeks I.P.	$349 \pm 13.4^{(a)}$	$436 \pm 15.4^{(a)}$	$570 \pm 24.5^{(a)}$	3.39 ± 0.13	$31.9 \pm 1.44^{(a)}$	$223 \pm 14^{(a)}$
CCL ₄ 6 weeks I.P. +DMSO I.P.	$332 \pm 12.8^{(a)}$	$402 \pm 8.91^{(a)}$	$580 \pm 21.1^{(a)}$	3.35 ± 0.12	$31.7 \pm 1.82^{(a)}$	$242 \pm 15.4^{(a)}$
CCL ₄ 6 weeks I.P. + sulphasalazine	$328 \pm 19^{(a)}$	$338 \pm 14.5^{(a)}$ (b)(c)	$523 \pm 19.8^{(a)}$	3.34 ± 0.12	$25.7 \pm 1.5^{(a)}$	$281 \pm 16^{(a)}$

I.P.

CCL ₄ 6 weeks I.P. + oral mesalazine	315±19 ^(a)	486±24.2 ^(a)	701±44 ^{(a)(b)}	3.18±0.16 ^(a)	9.86 ±0.339 ^(b)	225±9.65 ^(a)
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Data are expressed as mean ± SEM

(a) Significantly different from control group

(b) Significantly different from 6 weeks CCL₄ group

(c) Significantly different from 6 weeks CCL₄ + DMSO (for sulphasalazine) using one-way ANOVA followed by Tukey-Kramer test for multiple comparison test at P≤0.05

3.2. EXPRESSION AND LOCALIZATION OF TGFβR1.

Figure 1 and Graph 1 show that TGFβR1 protein showed minimal expression in normal liver tissues. The tissue expression was shown to be maximal in 6 weeks CCL₄ groups. It was noted that the expression increased by about 146% in 6 weeks CCL₄ treatment group. It was also observed that the expression is located in the cell membrane of epithelial hepatic tissue in areas that shows lesions.

It was also noted that concomitant administration of DMSO and CCL₄ for 6 weeks produced non-significant change from CCL₄ only. On the other hand; Concomitant administration of sulphasalazine and CCL₄ decreased the expression by about 46% compared to DMSO group while concomitant administration of mesalazine with CCL₄ reduced the expression by about 36% compared to 6 weeks CCL₄ group.

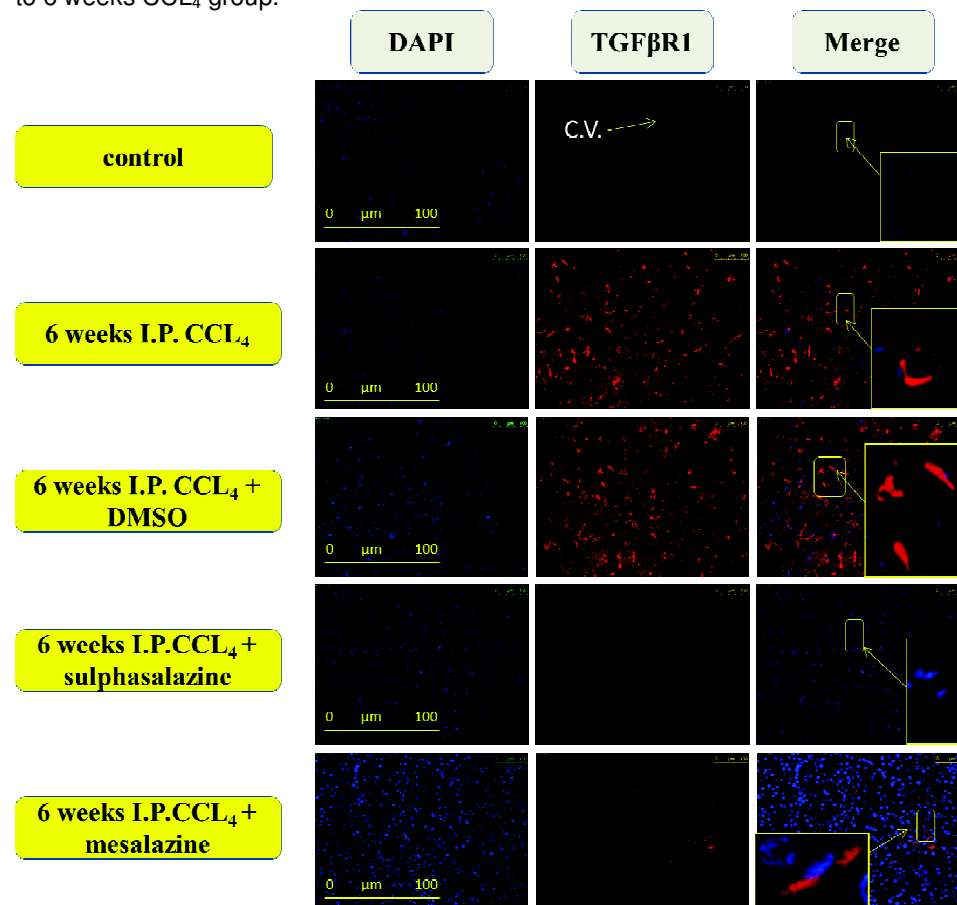
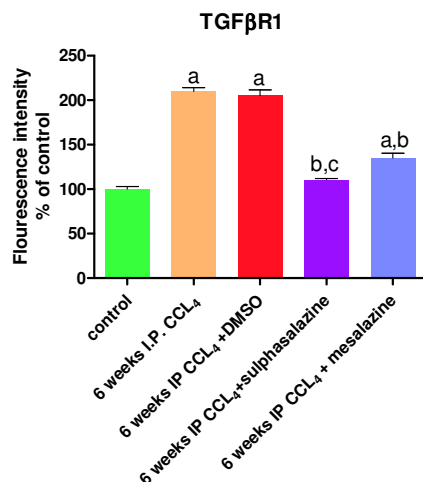


Figure 1: Immunofluorescence staining of liver sections of CCL₄ treated rats showing minimal TGFβR1 expression in control group. Six weeks CCL₄ treated group showed maximal expression located mainly in cell membrane (yellow rectangle). It was noted also that there is non-significant expression change between 6 weeks CCL₄ treatment alone or combined with DMSO. Combination of CCL₄ with sulphasalazine or mesalazine showed decrease in the expression compared to 6 weeks CCL₄ treatment with maximal decrease occurred with sulphasalazine.



Graph 1: Fluorescence intensity was obtained from 5 fields of each section (minimally 2 rats of each group) using ImageJ software.

Data are expressed as mean $\pm$ SEM

(a) Significantly different from control group

(b) Significantly different from 6 weeks CCL₄ group

(c) Significantly different from 6 weeks CCL₄ + DMSO using one-way ANOVA followed by Tukey-Kramer test for multiple comparison test at $P \leq 0.05$

3.3. HISTOPATHOLOGICAL FINDINGS

Effects of CCL₄ treatment on histopathological findings of liver tissue are represented in

Table 2 and **Error! Reference source not found.**

The control group showed normal hepatic architecture. On contrast, Six weeks CCL₄ treatment caused expanded PT with fibrous septa extending from PT to PT (yellow arrows), marked micro- and macro-vesicular steatosis. The hepatocytes exhibited some aspects of single cell necrosis with dilated central veins and mild interface activity.

It was noted that sulphasalazine had minimal effect on steatosis. Interestingly, despite sulphasalazine group animals showed marked steatosis, they also showed completely diminished underlying fibrosis in their liver tissues.

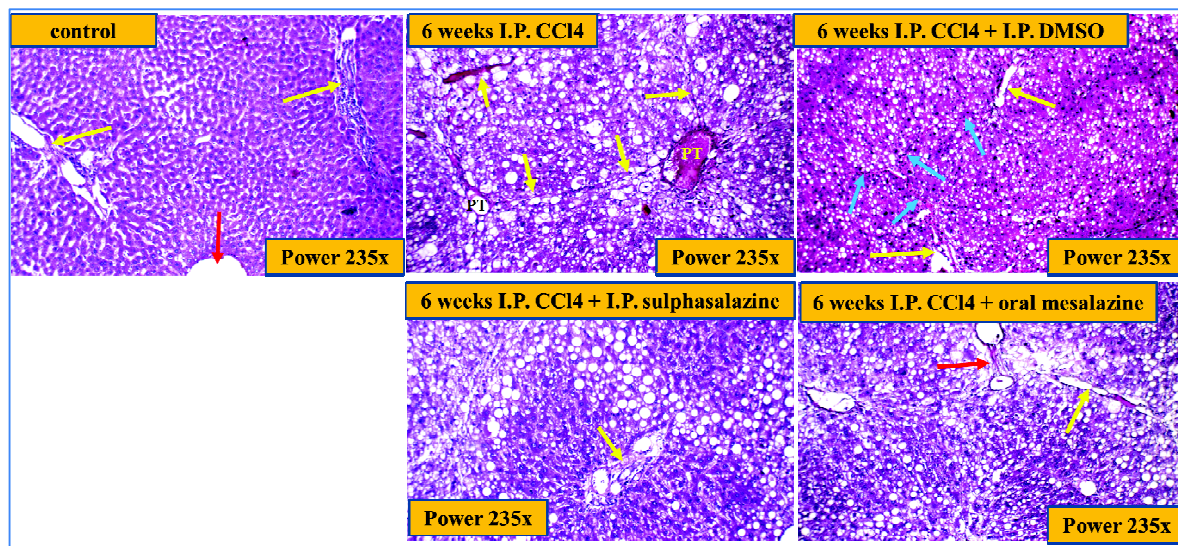
Finally, combination of mesalazine with CCL₄ resulted in some alleviation in injury signs. However, it seemed not to have positive effects regarding steatosis or fibrosis.

Table 2: Histopathological findings of the studied groups

	control	CCL ₄ 6 weeks	CCL ₄ weeks DMSO	6 + CCL ₄ + sulphasalazine	6 weeks CCL ₄ weeks mesalazine	6 +
CV	0	+	0	0	0	
Steatosis	0	+++	++	+++	++	
Hepatocyte	0	+	0	0	0	
Spotty necrosis	0	0	+	0	0	

Interface activity	0	+	0	0	0
PT	0	+	++	0	0
Fibrosis	0	++	++	0	++

Central vein (CV):
0: within normal +: dilated ++: markedly dilated
Steatosis, Spotty necrosis, Interface activity:
0: no +: mild ++: moderate to marked
Hepatocytes:
0: within normal +: single cell necrosis ++: confluent or diffuse necrosis
Portal tract (PT):
0: within normal +: expanded ++: expanded with inflammatory infiltrate
Fibrosis:
0: no fibrosis
+: fibrosis confined to enlarged portal zones
++: fibrosis of peri-portal or portal-portal septa with intact architecture
+++: architectural distortion (septal or bridging fibrosis) without obvious cirrhosis
++++: probable or definite cirrhosis



4. DISCUSSION

Administration of CCL₄ I.P. doses for 6 weeks significantly elevated serum transaminases enzymes activities suggesting hepatocellular damage. These results agreed with previous reports that CCL₄ significantly increases serum transaminases (22) (23;24). This CCL₄ induced hepatic damage was reported to be due to oxidative stress (25;26).

Concomitant administration of sulphasalazine with CCL₄ reduced AST not ALT activity compared to DMSO group suggesting the positive effect toward liver injury. These effects might be due to the anti-inflammatory action of sulphasalazine. This disagrees with what has been reported by Jennings and his coworkers a result that was interpreted by the relationship of hepatic injury, steatosis, fibrosis and cirrhosis to ulcerative colitis not sulphasalazine medication (27).

ALP activity caused by IP CCL₄ administration for 6 weeks and DMSO group shows significant increase compared to control which is less than 3 times as control; a result that might

suggest hepatotoxicity and mild biliary toxicity. This matches with that previously reported by Posen and Doherty (28).

Concomitant administration of mesalazine and CCL₄ for 6 weeks significantly increased ALP activity compared to 6 weeks CCL₄ treatment. This result might suggest the toxic effect of mesalazine with the mentioned regimen on hepatic tissue or synergistic effect of mesalazine on CCL₄ toxicity; a suggestion that might be clear upon ALP and albumin result.

Meanwhile; it was clarified that free radicals production and oxidative stress are main players in liver injury especially CCL₄ induced liver injury (25;26). In our study; administration of CCL₄ significantly elevated tissue content of MDA and reduced serum SOD activity. These results agreed with the previous reports that demonstrated the great role of oxidative stress in CCL₄ induced liver injury. (29-31).

Supporting the measured biochemical data: Our histopathological findings demonstrated that administration of CCL₄ for 6 weeks produced fibrosis of peri-portal or portal-portal septa with intact architecture. This is similar to what has been reported that continued administration of CCL₄ leads to hepatic fibrosis, cirrhosis, and HCC (32)

It was found that sulphasalazine, concomitant administration of sulphasalazine and CCL₄ has a marked clear prevention of fibrous lesions or fibrous septa; major findings seen in CCL₄. However, there is no positive effect on steatosis. This may be an expected result according to reports of Oakley and his team concerning the inhibitory effect of sulphasalazine on the machinery and machinery of fibrous tissue synthesis (33).

As an interesting matter; the decrease in histopathological scores observed in mesalazine group didn't agree with sulphasalazine group although it was documented that sulphasalazine is a prodrug for mesalazine in some pharmacological actions(34). This might suggest that the action of sulphasalazine previously reported by Oakley and his associates may be related to the sulphasalazine molecule at all not related to its moiety; mesalazine or route of administration might be of great impact.

The TGF- β is the principal regulator in chronic liver injury sharing in all stages of disease progression (35). Its action begins by binding to its receptor TGF β R1 and TGF β RII(36). It was observed that TGF β R1 expression occurs intracellularly in the cell membrane. This agrees with Massague and Chen reports(37). It was also shown that TGF β R1 expression was only upregulated in chronic liver toxicity; specifically phases that showed some extent of tissue remodeling prescribed by Devaux and his team (38) suggesting involvement of TGF β R1 in cellular processes involved in chronic not acute liver injury. This was similar to the previous reports in myocardial infarction (MI) by Devaux and his team (38). Besides; they also showed that the maximal expression occurs in lesion areas suggesting the direct relationship between TGF β R1 expression and lesions grade; a result that also is quite similar to Devaux and his team reports (38).

It was also observed that TGF β R1 expression was nearly diminished in sulphasalazine group suggesting the tight linkage between TGF β R1 expression and fibrosis not steatosis. a result that can be interpreted by the apoptosis activation property of sulphasalazine on activated HSC as major site of fibrogenesis (39).

5. CONCLUSION

In conclusion, our findings indicate, for the first time, that TGF β R1 is upregulated in chronic liver injury. TGF β R1 is upregulated in injury combined with underlying fibrosis; a result that may suggest usage of TGF β R1 as a candidate marker for diagnosis and prognosis of chronic liver diseases and a target for liver disease therapy. Moreover, sulphasalazine exhibits a fibroprotective effect in experimental liver fibrosis a result that may usefulness of sulphasalazine as adjuvant therapy in chronic liver diseases.

6. References

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