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Growth Factor Receptors and Liver Injury

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Authors' contributions

This work was carried out in collaboration between all authors. Authors EGEE, GMAA and AMIM designed the study, wrote the protocol and supervised the work. Authors EGEE and AMIM carried out the animal modeling. Authors EGEE and AIEDA carried out laboratory work, performed the statistical analysis, wrote the first draft of the manuscript, managed the literature searches and edited the manuscript. Author SAR carried out the histopathological assessment. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study is to investigate the expression pattern of transforming growth factor β receptor I(TGFβRI) and fibroblast growth factor receptor 3 (FGFR3) at the different stages of liver injury including acute injury, fibrosis and cirrhosis.
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: Four Sprague-Dawley rats groups were used for the experiment. **Control group:** 9 rats received corn oil; **Acute toxicity group:** 10 rats were injected 50% CCl_4 in corn oil (4

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ml/kg/IP/single dose); **6 weeks group:** 12 rats were injected with 50% CCl_4 in corn oil (4 ml/kg/IP/twice weekly/6 weeks); **11 weeks group:** 10 rats were injected with 50% CCl_4 in corn oil (4 ml/kg/IP/twice weekly/6 weeks) followed by 5 weeks of CCl_4 treatment with the half previous dose.
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; the first was processed for standard histology and immunofluorescence techniques and the other was homogenized for oxidative status assessment.
Results: TGFβRI and FGFR3 were shown to upregulate in chronic liver injury including stages of fibrosis and cirrhosis. While TGFβRI was shown to be located mainly in the cell membrane, the cytoplasm was shown to be the main site for FGFR3 localization.
Conclusion: TGFβRI and FGFR3 were suggested to be of critical importance in pathogenesis of chronic liver injury so they may be used as a target for chronic liver injury therapy and/or candidate marker for diagnosis and/or prognosis of chronic liver injury.

Keywords: TGFβRI; FGFR3; fibrosis; cirrhosis.

1. INTRODUCTION

Liver plays a limitless 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].

Carbon tetra chloride (CCl_4) is one of the most communal models for inducing hepatotoxicity. It is transformed into a toxic CCl_3^- radical by hepatic cytochrome P4502E1 (CYP2E1). Thus; it brings an acute Centro-lobular necrosis which starts a wound healing response (fibrosis) [3]. 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 [4].

Transforming growth factor β receptors (TGF-βRs) are of 2 types TGF-βRI and TGF-βRII. They mediate hepatic stellate cell (HSC) activation [5] after binding to their ligand cytokine TGFβ, the profibrogenic cytokine that traditionally have been considered the key fibrogenic stimulus to HSC [6].

Fibroblast growth factor receptor 3 (FGFR3) is involved in cell growth control, cell differentiation, and migration [7]. There are many reports that indicated the involvement of FGF/ FGF receptor (FGFR) signaling in the progression liver diseases including fibrosis [8,9] and hepatocellular carcinoma [10-13].

The aim of this study was to investigate the expression pattern of TGFβRI and FGFR3 at different stages of liver injury. This aspect of study was verified by investigation of the localization of these proteins in different liver injury stages.

2. METHODOLOGY

2.1 Animal Model

Adult male Sprague-Dawley rats weighing 250–300 g and aging 70 days were used for 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±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, Egypt), and allowed to food and water *ad libitum*. Animals were randomly divided into four groups: **control group:** 9 rats: receiving corn oil (2 ml/kg/twice weekly/IP); **acute toxicity group:** 10 rats were injected 50 % CCl_4 in corn oil (4 ml/kg/IP/single dose) [14]; **6 weeks group:** 12 rats were injected with 50% CCl_4 in corn oil (4 ml/kg/IP/twice weekly/6weeks) and finally **11 weeks group:** 10 rats were injected with 50% CCl_4 in corn oil (4 ml/kg/IP/twice weekly/6 weeks) followed by 5 weeks of CCl_4 treatment with the half previous dose. At the end of experimental period; rats were anesthetized with diethyl ether and blood samples were withdrawn from ocular vein for

measurement of blood chemistry. The animals then were euthanized, liver tissue samples were harvested and divided into 2 parts; the first 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 [15].

2.2 Antibodies and Chemicals

Rabbit polyclonal TGFβRI and FGFR3 antibodies were 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 indol (DAPI). CCl_4 was purchased from Sigma-Aldrich (MO, USA).

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) [16,17]. Alkaline phosphatase (ALP) activity was assayed according to the method of IFCC [18-20] and serum albumin concentration according to the method described by Gendler [21]. 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 [22]. The activity of SOD was determined using the method described by Marklund [23].

2.4 Immunofluorescence Analysis

Slides containing liver tissue sections were handled according to the method described by Abdel-Bakky and his associates [24]. The primary TGFβRI and FGFR3 antibodies were 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 ± standard error of mean (M±SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post 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.

Table 1. Liver functions and oxidative status 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 single I.P. dose	294±11.9[(a)(b)]	419±11.0[(a)]	395±11.1 [(b)]	3.8±0.06[(b)]	23.7±2.35[(a)(b)]	308±8.05[(a)(b)]
CCl_4 6 weeks I.P.	349±13.4[(a)]	436±15.4[(a)]	570±24.5[(a)]	3.39±0.13	31.9±1.44[(a)]	223±14[(a)]
CCl_4 11 weeks I.P.	298±8.48[(a)]	368±13.4[(a)(b)]	790±24.9[(a)(b)]	2.69±0.07[(a)]	38.1±1.74[(a)]	171±6.57[(a)]

Data are expressed as mean ± SEM
(a) Significantly different from control group
(b) Significantly different from 6 weeks CCl_4 group using one-way ANOVA followed by Tukey-Kramer test for multiple comparison test at P≤0.05

Administration of I.P. CCl_4 significantly elevated serum transaminases activities compared to control group whatever the duration of administration. On the other hand; ALP showed significant elevation only in chronic administration of CCl_4 with minimal change in single dose CCl_4 treatment. Supporting previous results; albumin showed significant decrease only with 11 weeks CCl_4 treatment. On the other hand; tissue MDA content was significantly increased on CCl_4 treated groups compared to control group while SOD was shown to significantly decrease in CCl_4 treated groups compared to control group.

3.2 Expression and Localization of TGFβRI

Fig. 1A shows that TGFβR1protein showed minimal expression liver tissues of control group animals and also animals treated with single CCl_4 dose as a model of acute toxicity. The expression was increased on conversion of toxicity from acute to chronic with maximal expression in 6 and 11 weeks groups. As indicated in Fig. 1B; it was noted that the expression increased by about 146% in 6 weeks CCl_4 treatment group and by 55 % in 11 weeks CCl_4 treatment group. Regarding localization; it was observed that the expression is located in the cell membrane of epithelial hepatic tissue. It was also noted that the expression occurred in areas that shows maximal lesions as necrosis and steatosis. On the other hand; it appears as intense area filling the cytoplasm extending along the fibrous septa and surrounding the cirrhotic nodule in areas that show high density of fibroblastic cells as shown in 11 weeks group. On the other hand there was also minimal expression inside the cirrhotic nodule.

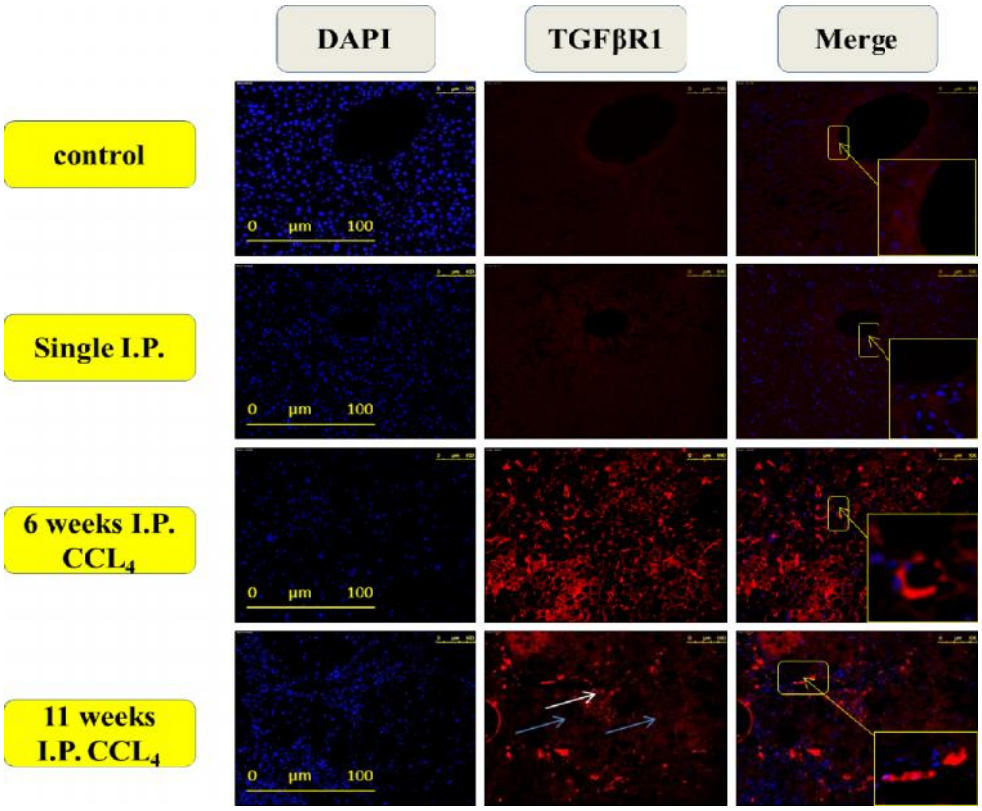

Fig. 1A. Effect of CCl4 administration on hepatic expression of TGFβRI protein

Immunofluorescence staining of liver sections of CCl_4 treated rats showing minimal TGFβR1 expression in control group and also in single I.P. treated group. Six weeks CCl_4 treated group showed maximal expression located mainly in cell membrane (yellow rectangle).Eleven weeks treatment showed constitutive expression (yellow rectangle) occurred mainly at area of fibrous septa or nodule border (white arrows) with minimal expression in cirrhotic nodule (blue arrows)

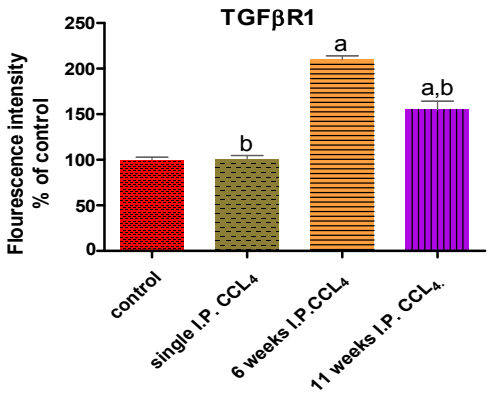

Fig. 1B. Effect of CCl4 administration on hepatic expression of TGFβRl protein

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

Data are expressed as mean ± SEM

a) Significantly different from control group

b) Significantly different from 6 weeks CCl_4 group one-way ANOVA followed by Tukey-Kramer test for multiple comparison test at P≤0.05

3.3 Expression and Localization of FGFR3

Fig. 2A and Fig. 2B show that FGFR3 protein shows minimal expression in liver tissues of control group animals and also animals treated with single CCl_4 dose as a model of acute toxicity. The expression was increased on conversion of toxicity from acute to chronic with maximal expression in 6 and 11 weeks groups. It was observed that FGFR3 expression occurs in the cytoplasm of the cells that found in the affected areas as areas of steatosis, underlying fibrosis and fibrous septa. It was noted that the expression increased by about 146% in 6 weeks CCl_4 treatment group and. Intraperitoneal administration of CCl_4 for 11 weeks resulted in maximal expression that increased by about 222% compared to control.

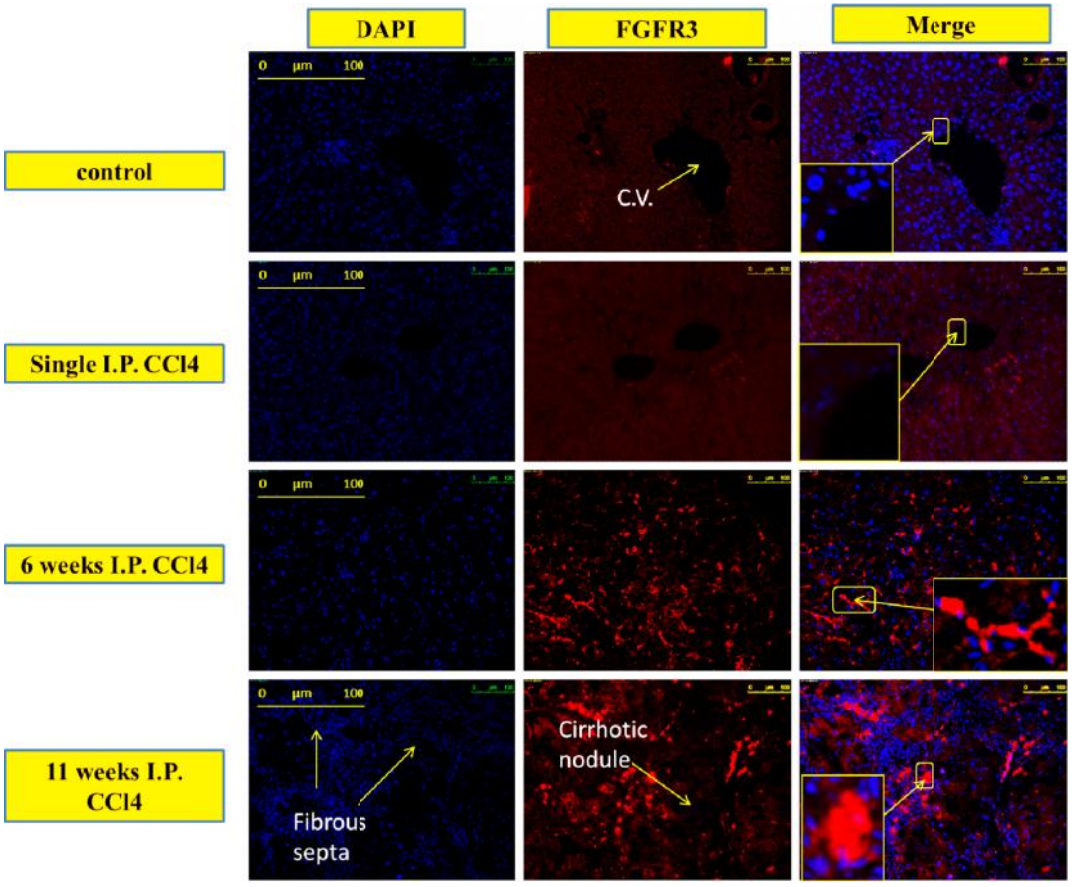

Fig. 2A. Effect of CCl_4 administration on hepatic expression and localization of FGFR3 in CCl4 treated rats

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

Data are expressed as mean ± SEM

(a) Significantly different from control group
(b) Significantly different from 6 weeks CCl_4 group one-way ANOVA followed by Tukey-Kramer test for multiple comparison test at P≤0.05

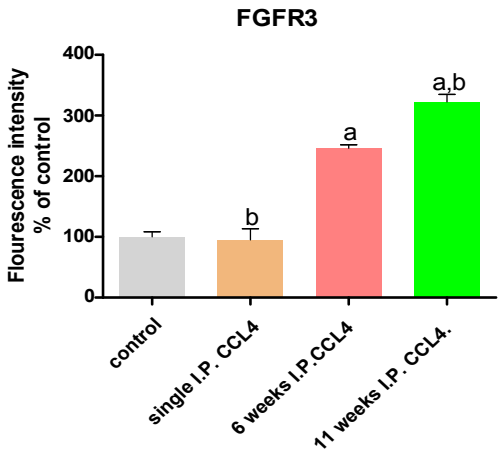

Fig. 1B. Effect of CCl4 administration on hepatic expression and localization of FGFR3 protein

Data are analysed using one-way ANOVA followed by Tukey-Kramer test for multiple comparison at P≤0.05.

a) Significantly different from control.
b) Significantly different from 6 weeks CCl_4 group.

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

3.4 Histopathological Findings

Effects of CCl_4 treatment on histopathological findings of liver tissue are represented in Table 2 and Fig. 3. The control group showed normal hepatic architecture. On contrast, CCl_4 treated groups showed pathological findings changes differed with the duration of treatment.

Single I.P. dose treated group showed average portal tract without interface activity or underlying fibrosis. On the other hand; it showed spotty necrosis, and mild micro-vesicular steatosis and dilated central vein. Six weeks treatment caused expanded portal tract with fibrous septa extending from a portal tract to another, marked micro- and macro-vesicular steatosis. The hepatocytes exhibited some aspects of single cell necrosis with dilated central veins and mild interface activity.

Eleven weeks treatment showed markedly expanded portal tract with definite inflammatory infiltrate, complete nodular formation (definite cirrhosis) and marked micro- and macro-vesicular steatosis. The hepatocytes exhibited single cell necrosis with mild spotty necrosis. Finally; the central veins were markedly dilated with marked interface activity.

Table 1. Histopathological findings of the studied groups

	Control	Single I.P. CCl_4	CCl_4 6 weeks	CCl_4 11 weeks
CV	0	+	+	++
Steatosis	0	+	+++	+++
Hepatocyte	0	++	+	+
Spotty necrosis	0	++	0	+
Interface activity	0	0	+	++
PT	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

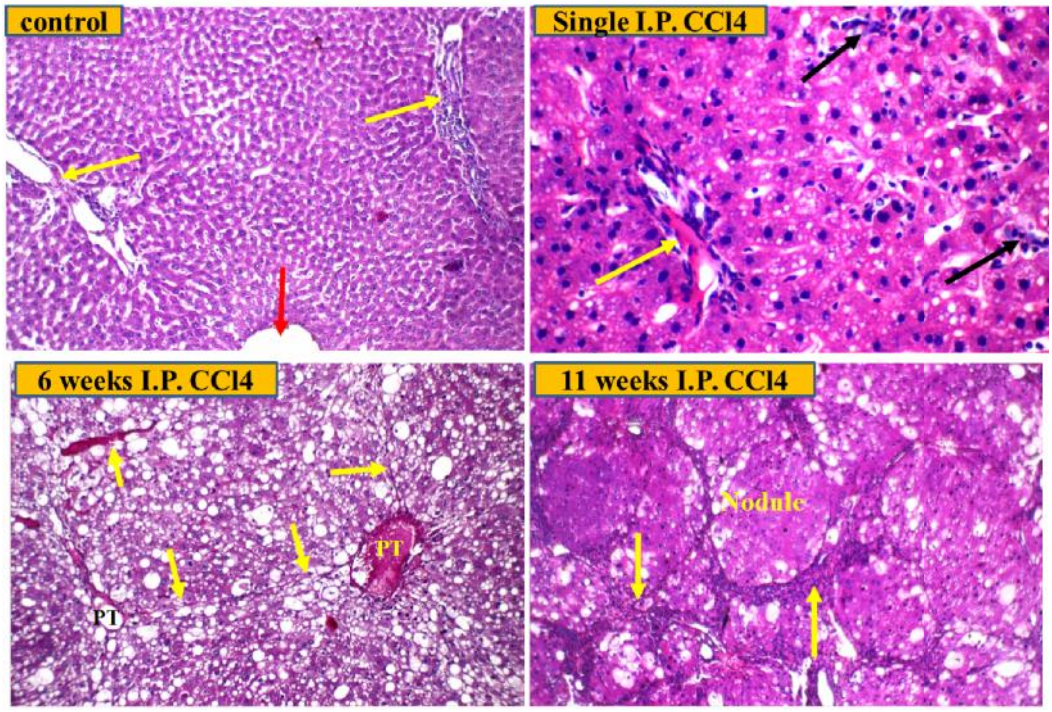

Fig. 3. Histopathological micrograph of liver samples of CCl_4 treated groups ×235 using H and E stain. Control: liver tissue showing average portal tract (yellow arrows), average central vein (red arrow), and average hepatocytes arranged in cords. Single I.P. CCl_4 dose: liver tissue showing average portal tract (yellow arrow), spotty necrosis (black arrow), and mild micro-vesicular steatosis.6 weeks I.P. CCl_4: liver tissue showing expanded portal tract with underlying fibrosis and fibrous septa extending from a portal tract to another (yellow arrows), marked micro- and macro-vesicular steatosis.11 weeks I.P. CCl_4: liver tissue showing markedly expanded portal tract (yellow arrows), complete nodular formation, marked micro- and macro-vesicular steatosis

4. DISCUSSION

Administration of CCl_4 either a single I.P. dose or chronic I.P. doses for 6 or 11 weeks significantly elevated serum transaminases enzymes activities suggesting hepatocellular damage. These results agreed with previous reports that indicated that CCl_4 significantly increases serum transaminases [25-27]. This CCl_4 induced hepatic damage was reported to be due to oxidative stress [28,29]. This hepatocellular damage was supported to some extent by serum ALP activity that showed non-significant increase after single I.P. CCl_4 treatment compared to control group suggesting minimal biliary injury detected upon that model.

This result opposes what was reported by Kālu's team [26]. This difference might be due difference in animal strain or route of administration that has not been mentioned. On the other hand; ALP elevation in chronic models including 6 weeks, 11 weeks groups showed 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 [30]. Continuous CCl_4 for 11 weeks showed significant elevation in ALP activity compared to 6 weeks CCl_4 treatment suggesting that chronic hepatocellular intoxication might lead to biliary injury. Supporting previous data; Serum albumin

concentration showed only significant decrease from control at 11 weeks CCl_4 treatment, a group that showed cirrhotic nodule formation suggesting decreased functioning tissue mass as reported by Giannini and his team [31].

Meanwhile; it was clarified that free radicals production and oxidative stress are main players in liver injury especially CCl_4 induced liver injury [28,29,32-34]; reports that agreed with our results that showed that administration of CCl_4 significantly elevated tissue content of MDA and reduced serum SOD activity.

Supporting the measured biochemical data: Our findings demonstrated that single I.P. dose of CCl_4 resulted in marked spotty necrosis and confluent hepatocytes necrosis. On the other hand prolonged administration of CCl_4 to 6 weeks produced fibrosis with intact architecture while prolonged CCl_4 to 11 weeks produced definite cirrhosis with clear cirrhotic nodule. This agreed with what has been reported that histopathological changes occur according to CCl_4 administration period. A single dose of CCl_4 leads to centrizonal necrosis and steatosis [35], while continued administration leads to hepatic fibrosis, cirrhosis, and HCC [36].

The TGF-β is the principal regulator in chronic liver injury sharing in all stages of disease progression [37]. Its action begins by binding to its receptor TGFβRI and TGFβRII [38]. It was observed that TGFβR1expression occurs intracellularly in the cell membrane. This agrees with Massague and Chen reports [39]. It was also shown that TGFβR1 expression was only upregulated in chronic liver toxicity; specifically phases that showed some extent of tissue remodeling prescribed by Devaux and his team [40] suggesting involvement of TGFβR1in 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 [40]. Besides; they also showed that the maximal expression occurs in lesion areas suggesting the direct relationship between TGFβR1expression and lesions grade; a result that also is quite similar to Devaux and his team reports [40].

The FGF/FGFR signaling axis has been reported to be a contributor to fibrosis in the liver [8,9]. On the other hand FGFR3 was reported to be activated by FGF2 [41]; a cytokine that has been believed to be involved in fibrotic disorders [42,43].

Our results showed that FGFR3 expression increased in chronically intoxicated groups suggesting involvement of FGFR3 in chronic liver injury processes. A result that is quite similar to reports of Lee's team [44]. Lee and his team have also reported that expression of all FGFR seem to be cytoplasmic. This is quite similar to our results. Our results showed that the expression of FGFR3 appeared as large spread spot that may indicate that the expression was cytoplasmic.

5. CONCLUSION

In conclusion, our findings indicate, for the first time, that TGFβR1 and FGFR3 are upregulated in chronic not acute liver injury. TGFβR1 is upregulated in injury combined with underlying fibrosis or cirrhosis while FGFR3 is upregulated in injury combined with steatosis, fibrosis and cirrhosis; results that may suggest usage of TGFβR1 and FGFR3 as a candidate markers for diagnosis and prognosis of chronic liver diseases and a target for liver disease therapy.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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