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Interruption of platelets and thrombin function as a new approach against liver fibrosis induced experimentally in rats

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ABSTRACT

Aim: Liver fibrosis is a serious health problem which is a critical cause of morbidity and mortality worldwide. It is the main complication of untreated chronic inflammatory liver diseases which can progress to liver cirrhosis, hepatocellular carcinoma, and finally death. Coagulation cascade plays a mechanistic role in the pathogenesis of different chronic inflammatory disease including atherosclerosis, stroke, and tissue fibrosis. The current study was designed to investigate the effect of inhibition of coagulation cascade on carbon tetrachloride (CCl_4)-induced liver fibrosis in rats.

Material and methods: The study was conducted in rats. Rats were treated with CCl_4 subcutaneously for 6 consecutive weeks to determine the onset of coagulation system activation in relation to development of fibrosis. To investigate the effects of coagulation system inhibition in CCl_4-induced liver fibrosis, the anticoagulants drugs dabigatran and clopidogrel were administrated orally concurrently with CCl_4 treatment.

Key findings: The results of our study revealed that during the first week, there were significant elevations of fibrin, tissue factor expressions, and prothrombin time (PT) coupled with neutropenia without significant changes in liver fibrosis markers such as TGF-β, α-SMA and collagen deposition. Starting from the second week, tissue injury markers including the oxidative, inflammatory and fibrosis markers as well as histopathological changes became evident progressively. Intriguingly, dabigatran and clopidogrel significantly normalized the biochemical and pathological changes.

Significance: In conclusion, activation of coagulation cascade is a triggering stimulus in the initiation of CCl_4-induced liver fibrosis and the anticoagulant drugs may exert promising anti-fibrotic effect.

1. Introduction

Liver fibrosis is a serious health problem which represents a critical cause of morbidity and mortality worldwide. It is the main complication of untreated chronic inflammatory liver diseases which can progress to liver cirrhosis, hepatocellular carcinoma and finally death [13]. Previous clinical and experimental studies have demonstrated that prolonged inflammation and oxidative stress may cause uncontrolled pathological wound healing leading to an excessive deposition of extracellular matrix proteins (ECM) and the development of liver fibrosis [30]. The role of coagulation system in CCl_4-induced liver fibrosis is under investigated.

Although former studies considered the coagulation cascade as an acute and transient response to tissue injury responsible only for the formation of fibrin plugs [7,64], recent studies pointed to the potential role of coagulation cascade in the initiation and progression of liver disease [31]. The role of coagulation cascade in liver fibrosis was evolved when thrombotic risk factors were observed in chronic hepatitis patients with risk factor for severe fibrosis [50]. In addition, a direct inhibition of thrombin with synthetic antagonist decreased lung collagen accumulation in experimental pulmonary fibrosis [23]. Furthermore, in hepatic ischemia reperfusion, inhibition of platelets

Abbreviations: CCl_4, carbon tetra chloride; TGF-β, transforming growth factor; α-SMA, alpha smooth muscle actin; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1β; HYP, hydroxyproline; ALT, alanine transaminase; AST, aspartate transaminase; MDA, malondialdehyde; GSH, glutathione; NOx, nitrate/nitrite production; PT, prothrombin time; W, week

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adhesion exerted a protective effects against sinusoidal endothelial cell apoptosis [25]. Pant et al. [49] also reported that coagulation factor X and thrombin exert pro-fibrogenic effects leading to liver fibrosis. Although some studies reported that coagulation system activation shows a detrimental effect on liver fibrosis, other studies show the opposite [37]. Joshi et al. demonstrated that a deficiency of PAR 4 and expression of a mutant form of fibrinogen resulted in more extensive fibrosis compared with wild-type animals in a model of cholestatic liver disease [29]. Under certain experimental conditions, thrombocytopenia and fibrinogen deficiency accelerate liver injury [58]. In addition, fibrin induced release of platelets serotonin which act as an important mediator of liver regeneration in various models of liver injury. Furthermore, fibrin deposition may help in repair of damaged liver tissue either directly or through attraction of platelets [27,48]. On the other hand, in different experimental models, the opposite was seen. Luyendyk et al. [39] also reported that TF contributes to hepatotoxicity.

The potential use of anti-coagulant drugs in the prevention of progression of disease in humans may be dependent on the severity of disease at onset of therapy, the underlying disease and the type and dosing regimen of the drug. Therefore anticoagulation could be beneficial strategy against liver fibrosis [37].

Dabigatran etexilate, a new orally active direct thrombin inhibitor, is a prodrug of the active compound dabigatran, which binds reversibly to thrombin with high affinity and specificity [21]. Dabigatran has a rapid onset of action, a predictable and reproducible pharmacodynamic effect. The pharmacokinetic characteristics of dabigatran permit once-daily dosing [15].

Clopidogrel is an inactive thienopyridine prodrug that requires in vivo conversion in the liver to an active metabolite that exerts its antiplatelet effect [70]. It inhibits the aggregation of the platelets and thus decreases the prevalence of coronary artery stent thrombosis. Therefore, clopidogrel is approved for reduction of stroke, myocardial infarction, and vascular death in patients having atherosclerotic vascular disease [34].

The aim of the present study was to correlate the initiation of activation of coagulation cascade and the onset of CCl_4 induced liver fibrosis and to evaluate the potential usefulness of dabigatran and/or clopidogril as anti-fibrotic therapy the prevention of CCl_4-induced liver fibrosis.

2. Material and methods

2.1. Animals

Adult male albino rats, weighing 200–250 g, were used in the current study. Rats were exposed to 12-h/12-h light/dark cycles at a room temperature of 22 ± 2 °C and a relative humidity of 55–60%. Animals were kept in a pathogen-controlled animal room in the animal facility of the Faculty of Pharmacy, Nahda University for two weeks before being used in our experiments. Animals were fed a standard diet pellet (El-Nasr Company, Abou-Zaabal, Cairo, Egypt) and allowed free access to water ad libitum. All the procedures of animal handling and drug administration were performed according to the recommendations of Animal Care and Use Committee, Faculty of Pharmacy, Beni-Suef University (REC-A-PhBSU-18004)

2.2. Chemicals and reagents

Dabigatran etexilate, clopidogrel, CCl_4 and hydroxyproline ELISA kit for were obtained from Sigma-Aldrich (MO, U.S.A.). Dako solution citrate buffer and Polyclonal rabbit anti-human fibrinogen antibody were purchased from Dako Company (CA, USA.). ELISA kits for TNF-α and IL-1β were obtained from the Glory Science Company (TX, USA). Serum kinetic kits for ALT, AST, albumin and bilirubin were purchased from the Bio Diagnostic Company (Giza, Egypt). The Prothrombin time (PT) kit was obtained from LABiTec GmbH (Ahrensburg, Germany) and from Vitro (Monterrey, Mexico). Universal Quick kit for α-SMA was obtained from Linaris Company (Dossenheim, Germany). The AEC (3-amino-9-ethylcarbazole) substrate kit was purchased from BioGenex (CA, USA). Mouse monoclonal anti-TF antibody was obtained from Thermo Scientific Pierce (IL, USA). Rabbit polyclonal TGF-β1R antibody was obtained Santa Cruz Biotechnology (TX, USA). Goat anti rabbit CY3 conjugated secondary antibody was obtained from Invitrogen (TX, USA). All other chemicals and reagents were of analytical grade.

2.3. Experimental design

The present study consisted of two phases. At the first phase, a time-course study was performed to explore whether there is correlation between the coagulation markers with liver injury and fibrosis markers. The second phase was conducted to estimate the possible protective effects of the selected anticoagulant drugs dabigatran etexilate and clopidogrel against CCl_4-induced liver fibrosis.

2.3.1. Time course study

In the first phase of the study, a time course experiment was conducted using fifty-six weight-matched rats. The rats were randomly allocated into seven groups, eight rats each, namely N, W1, W2, W3, W4, W5 and W6. In group N, rats were kept as a normal control group, receiving the vehicle alone twice weekly for 6 consecutive weeks. For groups W1, W2, W3, W4, W5 and W6, rats received CCl_4 in the indicated schedule twice weekly for 1, 2, 3, 4, 5 and 6 weeks, respectively. Forty-eight hours after the last CCl_4 dose, blood and tissue samples were collected.

2.3.2. Effect of dabigatran etexilate and clopidogrel against CCl4-induced liver fibrosis

In the second phase of this study, thirty-two weight-matched rats were randomly allocated into the following four groups (eight rats each): normal control, fibrosis control, dabigatran-treated, and clopidogrel-treated group. Normal control rats received vehicles only, while fibrosis control rats received CCl_4 alone in the indicated schedule. Rats in dabigatran-treated or clopidogrel-treated group received dabigatran etexilate (20 mg/kg/day, p.o.; [68]) or clopidogrel (20 mg/kg/day, p.o.; [2]) on a daily basis starting 3 days before the first CCl_4 dose until the end of the experiment (6 weeks).

2.4. Methodology

2.4.1. Induction of liver fibrosis

Liver fibrosis was induced in experimental rats as previously described [65]. Rats received CCl_4 (50% solution, v/v, in olive oil) via the subcutaneous route twice weekly for 6 weeks. In the first 2 weeks, CCl_4 solution was given in a dose of 5 ml/kg. In the remaining 4 weeks, the dose was reduced to 3 ml/kg. Forty-eight hours after the last dose, animals were euthanized and samples from blood and liver tissues were collected.

2.4.2. Sample preparation

2.4.2.1. Blood and serum preparation. Blood was withdrawn from the medial epicanthus of the animal's eyes using non-heparinized capillary tube and collected in a glass test-tube. Blood was then centrifuged at 1000 ×*g* for 30 min in a cooling centrifuge (Model 3-30k, Sigma, USA) and the obtained serum was withdrawn and collected in Eppendorf tubes and stored in a deep freezer at −20 °C for spectrophotometrical analyses of serum levels of ALT, AST, bilirubin and albumin.

A portion of the blood was withdrawn directly into sodium citrate tubes and kept for estimation of prothrombin time (Pt) and neutrophil count.

2.4.2.2. Liver tissue preparation. A specimen of the liver tissue was used

to prepare a 10% liver homogenate in normal saline by the aid of a homogenizer (IKA homogenizer, Model T 25 digital ULTRA-TURRAX, Germany), followed by centrifugation at 1000 ×*g* for 15 min at 4 °C in a cooling centrifuge. The obtained supernatant was used for the assessment of tissue MDA, GSH, NOx, TNF-α, IL-1β and hydroxyproline.

Another specimen of the liver tissue was kept in 10% formalin solution in normal saline for routine histopathological examination, immunofluorescence and immunohistochemical assays of tissue factor, fibrin-, TGF-β1 expression and α-SMA deposition.

2.4.3. Estimation of blood and serum biomarkers

Neutrophil count was performed using a hemocytometer. The prothrombin time (Pt) was calculated using Pt kits according to manufacturer's instructions based on the principle described earlier [19,59]. The serum biomarkers ALT, AST, albumin and bilirubin were evaluated using commercially available kits according to manufacturer's instructions based on the method described earlier ([41,52,73].

2.4.4. Estimation of tissue biomarkers

Liver contents of MDA, GSH and NOx were estimated colorimetrically using the standard assay methods [45,56,62]. Liver tissue TNF-α, IL-1β and hydroxyproline were estimated using ELISA kits according to manufacturer's instructions based on the sandwich technique described earlier [6,51,63].

2.4.5. Histopathological study

Histopathological examination of liver sections was performed according to the method described by Banchroft and Steven [4]. Briefly, liver tissue specimen were washed by distilled water followed by serial dilutions of alcohol for dehydration. Specimens were then cleared in xylene and embedded in paraffin at 56 °C in hot air oven for 24 h for preparation of tissue blocks and sectioning at 4 μm thickness by slide microtome. Tissue sections were collected on glass slides, deparaffinized with alcohol, then stained with hematoxylin and eosin (H & E) stain for routine examination, or Masson trichrome (special stain for tissue fibrosis). The stained tissue sections were examined using a light microscope attached to a digital camera.

2.4.6. Immunofluorescence assay

Immunofluorescence staining of liver sections was performed for evaluation of tissue factor, fibrin, and TGF-β1 expression, as described earlier by Abdel-Bakky et al. [1]. Briefly, slides were deparaffinized by using xylene and were gradually hydrated by gradient ethanol series. Antigen retrieval was done by incubation of the sections with citrate buffer (pH 6) in a microwave at 500 watt for 20 min, then allowed to cool down to room temperature. The slides were then washed with 0.05% tween 20 solution in phosphate-buffered saline (TPBS) at pH = 7.4 and fixed with absolute methanol. After washing, sections were blocked by blocking buffer (10% horse serum in 1% BSA-in-PBS for 1 h at room temperature). The slides were incubated overnight at 4 °C, with the appropriate primary antibodies for rabbit anti-goat tissue factor (TF), anti-rabbit fibrin and anti-rabbit TGFβ1. The slides were washed and incubated for 30 min with goat anti rabbit CY3 for (fibrin and TGF β1) and Alexa 488 for TF conjugated secondary antibody for 30 min. After washing, slides were counterstained with DAPI (4′,6-diamidino-2-phenylindole) and mounted for examination and imaging (Leica DM5500B).

2.4.6.1. Immunohistochemical assay. Paraffin sections were incubated for 30 min in xylene for deparafinization, rehydrated through a graded ethanol dilutions and washed in PBS pH 7.4. Antigen retrieval was achieved by incubating the tissue sections for 20 min in 0.01 M sodium citrate buffer, pH 6.0, in a microwave oven (500 W). Tissue sections were incubated with 3% H_2O_2 in methanol for 30 min at room temperature. After blocking the sections with the blocking buffer (10% horse serum, 1% BSA in PBS) for 1 h. The sections were incubated with an Avidin/Biotin Blocking Kit (Linaris, Wertheim, Germany) following the manufacturer protocol. Sections were incubated overnight at 4 °C with rabbit polyclonal α-SMA primary antibody. After washing the tissues, the Universal Quick Kit (Linaris, Wertheim, Germany) was used to stain the kidney sections. As a substrate, the AEC Substrate Kit from Biozol was used to detect the immune complexes. Nucleus was counterstained by hematoxylin (Roth, Karlsruhe, Germany). The sections were inspected with a digital microscope (Leica DM 5500B, Leica Microsystems, Wetzlar, Germany). A minimum of 5 fields of each rat were analyzed using Image-J software (National Institute of Health "NIH", USA).

2.4.7. Statistical analysis

Results were expressed as means of 5–8 values ± standard error of the mean (SEM). All statistical analyses were performed using one-way analysis of variance (ANOVA) test followed by Tukey-Kramer post hoc test using Graph Pad prism version 6 (CA, USA). The value of $P < 0.05$ was set as significance level.

3. Results

3.1. Time course study of CCl_4-induced liver injury

3.1.1. Hematological biomarkers

Our data demonstrated that the mean PT value were significantly increased to about 150%, starting from 1st week after CCl_4 administration, as compared to normal control values. Our data also demonstrated that the elevation of PT values in CCl_4-treated rats is time-dependent. Thus, PT was increased to 208% and 400% of normal control value at the 4th and 6th week post CCl_4 treatment, respectively (Table 1).

Regarding the neutrophil count, our data revealed that neutrophil count exhibited a biphasic changes after CCl_4 treatment. While at the 1st week post treatment neutrophil counts were reduced by 50% compared to the control rats, the neutrophil counts starts to increase at 2nd week and continue to increase progressively until the 6th week posttreatment.

3.1.2. Liver toxicity markers

Biochemical analysis of CCl_4-treated rats showed an increase in ALT and AST as compared to the control rats. However, this increase was only significant starting from the 3rd week posttreatment as compared with normal control levels (Table 1).

Serum albumin did not show any significant difference during the first four weeks compared with normal control values. The drop in albumin level was significant in week 5 (about 87%) and continues to decline in week 6 (about 80%) (Table 1).

Regarding serum bilirubin, our result showed that serum bilirubin level started to significantly increase from the 3rd week posttreatment to about 132% compared with normal control value. Further increase in serum bilirubin was evident at weeks 5 and 6, reaching about 242% and 308%, respectively (Table 1).

3.1.3. Oxidative and inflammatory biomarkers

Similarly, hepatic MDA content started to increase significantly at the 3rd week (about 169%). Further significant elevations in subsequent weeks until the 6th week about (about 648%) compared with normal control level (Table 1). Hepatic GSH depletion caused by CCl_4 administration became significant starting mildly from week 2 (78%) compared with normal control level. Depletion in GSH content continued through weeks 3 (67%), 4 (61%), 5 (49%) and 6 (34%; Table 1).

Nitrate/nitrite production started to significantly increase starting from the 4th week through weeks 5 and 6, reaching about 179%, 241% and 340%, respectively, as compared with normal control level (Table 1).

The inflammatory markers TNF-α and IL-1β showed significant

Table 1
Time course study of the effect of carbon tetrachloride administration on serum alanine transaminase and aspartate transaminase levels, serum albumin and bilirubin levels, liver tissue malondialdehyde, glutathione, nitrate/nitrite production, prothrombin time and neutrophil count. Group N: normal control group; Groups W1 through W6 are groups receiving CCl_4 for 1, 2, 3, 4, 5 and 6 weeks, respectively; CCl_4: carbon tetrachloride; ALT: alanine transaminase; AST: aspartate transaminase MDA: malondialdehyde; GSH: glutathione; NOx: nitrate/nitrite production; Pt: prothrombin time.

Group	ALT (U/L)	AST (U/L)	Albumin (g/dl)	Billirubin (g/dl)	Hepatic MDA (nmol/g)	Hepatic GSH (μmol/g)	Hepatic NOx (nmol/g)	Pt (s)	Neutrophil count (10^3/mm^3)
N	34.87 ± 2.20	155.3 ± 9.979	3.42 ± 0.04	0.215 ± 0.007	75.50 ± 1.82	248.4 ± 5.52	62.83 ± 2.30	9.92 ± 0.32	9.17 ± 0.94
W1	104.1 ± 3.6	249.2 ± 7.458	3.39 ± 0.09	0.248 ± 0.006	92.33 ± 4.87	232.5 ± 2.14	71.83 ± 1.97	14.92 ± 0.33[a]	4.50 ± 0.42[a]
W2	124.7 ± 3.198	295.0 ± 6.583	3.35 ± 0.08	0.252 ± 0.006	98.00 ± 1.248	194.9 ± 4.23[a]	79.83 ± 3.52	15.67 ± 0.66[a]	8.667 ± 0.49[b]
W3	149.5 ± 6.013[a]	343.3 ± 11.45	3.23 ± 0.08	0.283 ± 0.004[a]	127.30 ± 1.74[a,b,c]	166.2 ± 3.72[a,b,c]	80.33 ± 3.77	18.67 ± 0.66[a]	12.00 ± 0.93[b,c]
W4	155.8 ± 5.069[a]	404.0 ± 26.13	3.17 ± 0.06	0.285 ± 0.007[a]	292.80 ± 8.65[a,b,c,d]	152.7 ± 3.72[a,b,c]	112.70 ± 3.49[a,b,c,d]	20.67 ± 0.88[a,b,c]	15.67 ± 0.71[a,b,c,d]
W5	521.5 ± 31.84[a,b,c,d,e]	1427 ± 49.10[a,b,c,d,e]	2.98 ± 0.13[a]	0.520 ± 0.028[a,b,c,d,e]	431.31 ± 8.20[a,b,c,d,e]	121.2 ± 3.38[a,b,c,d,e]	151.80 ± 7.28[a,b,c,d,e]	25.50 ± 1.41[a,b,c,d,e]	21.67 ± 0.66[a,b,c,d,e]
W6	1237 ± 42.62[a,b,c,d,e,f]	2412 ± 103.0[a,b,c,d,e,f]	2.72 ± 0.10[a,b,c,d,e]	0.663 ± 0.022[a,b,c,d,e,f]	489.52 ± 4.94[a,b,c,d,e,f]	83.7 ± 2.44[a,b,c,d,e,f]	213.83 ± 3.65[a,b,c,d,e,f]	39.17 ± 1.49[a,b,c,d,e,f]	26.33 ± 0.88[a,b,c,d,e,f]

[a] Significantly different from group N.
[b] Significantly different from group W1.
[c] Significantly different from group W2.
[d] Significantly different from group W3.
[e] Significantly different from group W4.
[f] Significantly different from group W5 at $P < 0.05$.

elevations in liver tissue starting from the 2nd and the 3rd weeks. The elevations continued chronologically until week 6, reaching about 500% and 600% increases, respectively as compared with normal control level (Fig. 1A, B).

3.1.4. Fibrosis biomarkers

Liver tissue hydroxyproline content showed mild significant elevation starting from the 4th week posttreatment. Abrupt elevation of hydroxyproline level was evident in the 5th weeks (about 300%) and the 6th week (about 400%; Fig. 1C). Alternatively, TGF-β1 expression did not show any significant increase in the 1st week, while it started to show a significant expression at the 3rd week (Fig. 2C). Immunohistochemical analysis of liver α-SMA did not show any significant change in the 1st and the 3rd weeks while massive changes was evident in the 6th week posttreatment as compared with basal level (Fig. 3B). Analysis of liver sections stained with the special Masson trichrome stain revealed a mild significant elevation of collagen deposition at the 3rd week, while collagen massively deposited at the 6th week posttreatment (Fig. 3C, D).

3.1.5. Coagulation markers

Immunofluorescence analysis of tissue factor (TF) (Fig. 2A) and fibrin protein expressions (Fig. 2B) revealed that increased the expression of these 2 proteins in the pericentral areas in CCl_4 treated group started from the 1st week compared to the low basal level in normal control rats.

3.1.6. Histopathological analysis

Normal control sections (H&E stain; 400×) showed normal central vein and blood sinusoids, as well as hepatocytes with acidic cytoplasm. At the 1st and 3rd weeks of CCl_4 administration, liver sections showed congested central vein, dilated sinusoids and fatty degeneration of hepatocytes. At the 6th week, distorted hepatic architecture, cytoplasmic vacuolations and pyknotic nuclei became evident (Fig. 3A).

3.2. Effect of dabigatran and clopidogrel on hematological parameters in CCl_4-treated rats

Treatment of rats with dabigatran or clopidogrel significantly corrected the abnormality of PT induced by CCl_4, reaching about 57% and 43%, respectively, as compared with CCl_4 treatment alone (Table 2). Administration of CCl_4 significantly increased neutrophil count to about 3.7 folds compared with normal control level. Treatment with dabigatran and clopidogrel significantly decreased neutrophil count to 56% and 47%, respectively when compared with CCl_4 control level (Table 2).

3.3. Effect of dabigatran and clopidogril on liver function of CCl_4-treated rats

Pre-treatment with dabigatran or clopidogrel significantly decreased serum ALT (15% and 16%, respectively) and serum AST (24% and 8%, respectively) compared with CCl_4 control levels (Table 2). A significant drop in serum albumin level (60%) coupled with significant elevation of serum total bilirubin level (750%) were evident in CCl_4 control rats compared with normal control levels. Dabigatran and clopidogrel pre-treatments significantly restored serum albumin levels, reaching to 136% and 139%, respectively, when compared with CCl_4 control value. Similarly, dabigatran and clopidogrel significantly decreased CCl_4-induced hyperbilirubinemia when compared with CCl_4 control level (Table 2).

3.4. Effect of dabigatran and clopidogril on the redox status of liver tissue homogenate in CCl_4-treated rats

Pre-treatment with dabigatran or clopidogrel significantly

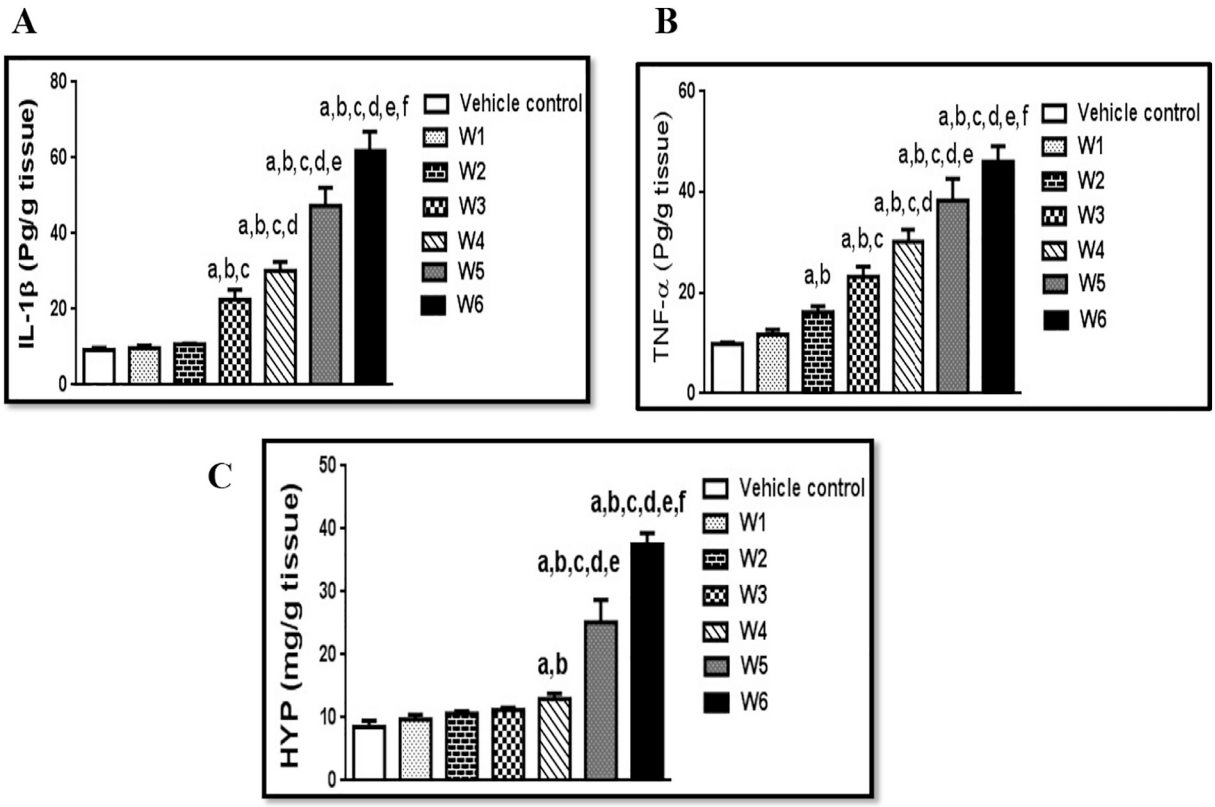

Fig. 1. Time course study of the effect of carbon tetrachloride administration on A) liver tissue tumor necrosis factor-alpha, B) interleukin-1β and C) liver tissue hydoxyproline level. CCl_4: carbon tetrachloride; TNF-α: tumor necrosis factor-alpha; IL-1β: interleukin-1β and HYP: hydroxyproline. [a]Significantly different from the normal control group. [b]Significantly different from week 1 value. [c]Significantly different from week 2 value. [d]Significantly different from week 3 value. [e]Significantly different from week 4 value. [f]Significantly different from week 5 value at $P < 0.05$.

decreased MDA (52% and 46%), and NOx (51% and 48%) and NOx (51% and 48%), while increased GSH content (148% and 153%), respectively, when compared with CCl_4 control values (Table 2).

About 6-fold increases in liver tissue TNF-α and IL-1β levels were recorded in rats receiving CCl_4 alone. Pre-treatments with dabigatran and clopidogrel significantly corrected these elevations. The effect of clopidogrel was better than dabigatran regarding both markers (Fig. 4A and B).

3.5. Effect of dabigatran or clopidogril on liver fibrogenesis in CCl_4-treated rats

Rats receiving CCl_4 alone showed about 6-fold increase in liver tissue hydroxyproline expression compared with normal control value. Dabigatran and clopidogrel significantly decreased this value, with the effect of clopidogrel being more potent (Fig. 4C).

TGF-β1 is one of the most important inducers of collagen I and other matrix components; therefore its inhibition has a major role in the progression of liver fibrosis [24]. TGF-β1 expression was significantly increased in liver sections obtained from the CCl_4-treated group comparing with normal control. Protein expression of TGF-β1 in rats pretreated with dabigatran or clopidogril was significantly lower than that in rats pretreated with CCl_4 alone (Fig. 5C).

As a marker of HSCs activation, α-SMA is one of the sensitive indicators of the rate of fibrogenesis. Our results showed that there was no tissue expression of α-SMA in normal control rats. By contrast, considerable expression of α-SMA in the pericentral sinusoidal spaces where HSCs is normally located was detected in sections obtained from the CCl_4 group. Expression of α-SMA in the CCl_4 plus dabigatran or clopidogril-treated groups showed a remarkable reduction of tissue expression of α-SMA in the pericentral area, compared with the CCl_4 control group (Fig. 6B).

With the special Masson trichrome stain, liver sections obtained from CCl_4-intoxicated rats showed extensively increased collagen deposition compared with normal rats. Significant drop of deposition was evident with dabigatran and clopidogrel pretreatments (Fig. 6C, D).

3.6. Effect of dabigatran and clopidogril on coagulation markers in CCl_4-treated rats

As a marker for coagulation activation, immunofluorescence analysis for TF and fibrin was carried out. Vehicle-treated group showed no or basal expression of TF and fibrin proteins in the liver tissues. On the other hand, treatment with CCl_4 significantly increased pericentral expression of TF and fibrin in the sinusoidal area around the central veins. On contrast, pretreatment with dabigatran or clopidogrel decreased TF and fibrin protein expressions as compared to CCl_4 treatment alone (Fig. 5A, B).

3.7. Histopathological study

Normal control sections (H&E; 400×) showed normal hepatic architectures and normal hepatocytes. CCl_4 control sections showed massively distorted architecture with extensive fibrosis and fatty vacuoles. Dilated congested central vein and pyknotic nuclei were also evident. Dabigatran or clopidogrel pre-treatments showed significant improvements of hepatic architecture to almost normal pattern (Fig. 6A).

4. Discussion

Liver fibrosis is a serious chronic disease characterized by excessive

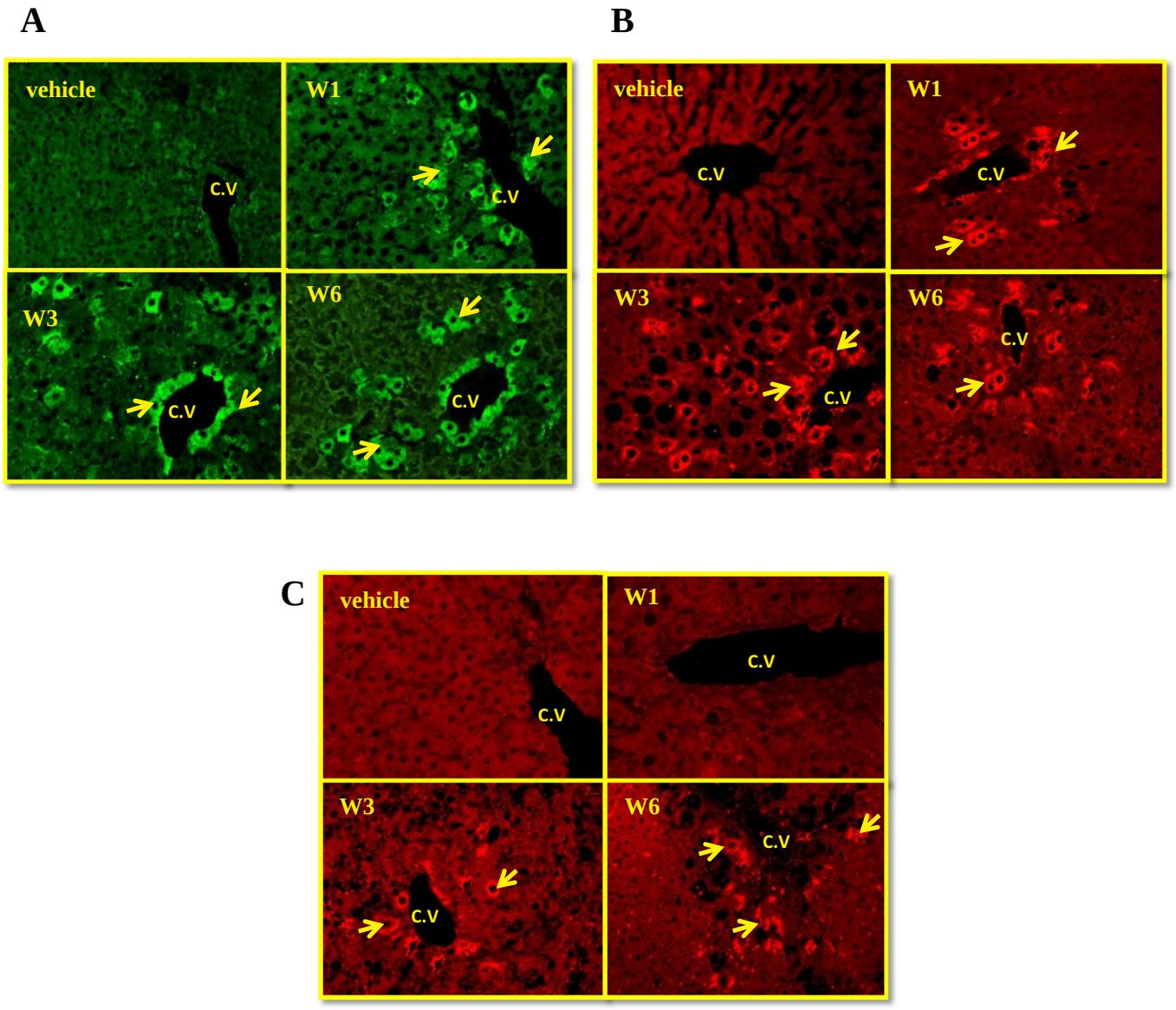

Fig. 2. A) Photomicrographs of immunofluorescence-stained liver sections showing time course effect of carbon tetrachloride (CCl_4) on a) tissue factor expression B) fibrin expression- and C) transforming growth factor (TGF-β1) expression. Groups W1, W3 and W6 are groups receiving carbon tetrachloride (CCl_4) for 1, 3 and 6 weeks, respectively.

deposition of extracellular matrix (ECM) in the liver parenchyma. It usually progresses to hepatocellular carcinoma (HCC), since > 80% of HCCs develop in patients with liver fibrosis [38]. Coagulation cascade has indispensable role in the progression of tissue injury, including fibrosis [44].

Pro-thrombotic state is reported to promote fibrosis in the liver [32], kidney and lung [16]. These findings prompted us to investigate the role of coagulation cascade, as well as anticoagulant agents, in the prevention of CCl_4- induced liver fibrosis.

Data of the present investigation revealed that CCl_4 administration to rats resulted in progressive increase in coagulation, oxidative, inflammatory and fibrotic markers in a time dependent manner. Interestingly, PT time and neutrophil count increased significantly from the first week, coupled with significant increases in liver tissue expressions of tissue factor and fibrin even before any significant change in other live injury biomarkers. These findings might be attributed to notion that coagulation plays a causative role in liver fibrosis and its activation starts prior to tissue injury. This plausible explanation is substantiated by the finding of Tripodi et al. who reported that the peripheral consumption of plasma coagulating factors and the intrahepatic thrombosis associated with liver fibrosis is an evidence for activation of coagulation cascade [60]. Accordingly, it has been shown that there is lower level of balance between pro and antithrombotic factors in patients with chronic liver disease. Therefore, these patients might not be really anticoagulated in stable condition [57].

Similarly, neutropenia became evident in the first week after CCl_4 administration due to tissue migration. It is worth mentioning that the drop in neutrophil count in the first week might be compensated in the subsequent weeks through mobilization from bone marrow and commencement of inflammatory neutrophil proliferation as described by [40,54].

Several previous studies have established a correlation between coagulation system and HSCs activation. The in vitro study of Anstee et al. [3] demonstrate that using selective PAR-1 and PAR-4 agonists are able to induce stellate cell activation. Dhar et al. [11] also reported that FXa promotes stellate cell contractility and activation. Hepatic stellate cells (HSCs) are the principal cells involved in liver fibrosis and their activation is characterized by transformation from the quiescent form

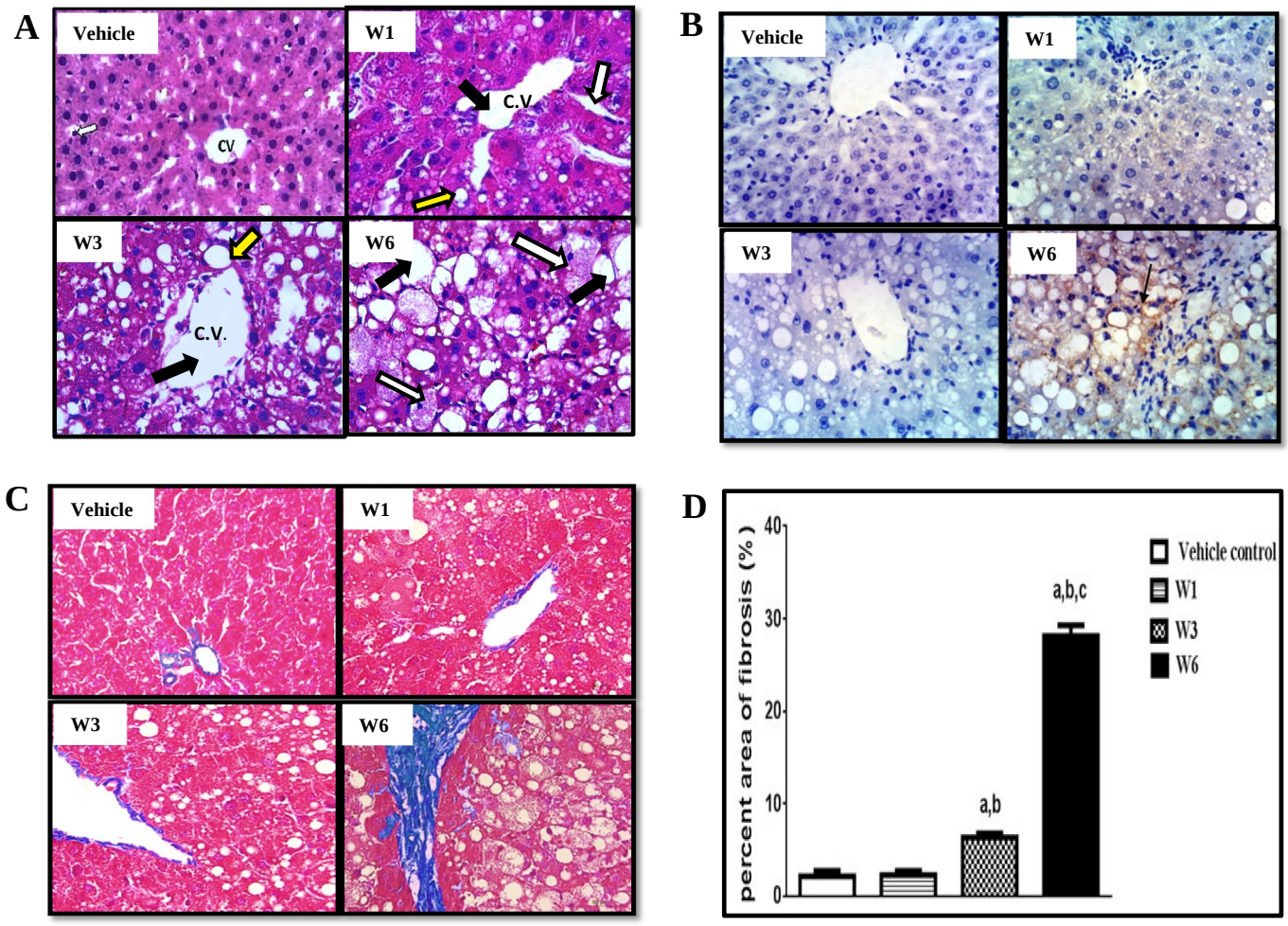

Fig. 3. A) Photomicrographs of liver sections (H&E; 400×) showing time course effect of carbon tetrachloride administration. Groups W1, W3 and W6 are groups receiving carbon tetrachloride (CCl_4) for 1, 3 and 6 weeks, respectively. Normal (vehicle) control section shows normal central vein (C.V.) and blood sinusoids (black arrows). Hepatocytes are normal with acidophilic cytoplasm and vesicular nuclei (white arrows). The W1 panel shows mildly congested central vein (black arrow), hepatocytes with fatty degeneration (yellow arrow) and dilated blood sinusoids (white arrow). The W3 panel shows dilated central vein (black arrow) and hepatocytes with fatty degeneration (signet ring appearance; yellow arrow). The W6 panel shows distortion of normal hepatic architecture, cytoplasmic vacuolations (black arrow) and pyknotic nuclei (white arrow). B) Immunohistochemical time course study of the effect of carbon tetrachloride administration on b) liver tissue alpha smooth muscle actin (α-SMA) level. C) Photomicrographs of liver sections (Masson trichrome stain; 400×) showing time course effect of carbon tetrachloride administration on tissue fibrosis. Groups W1, W3 and W6 are groups receiving carbon tetrachloride (CCl_4) for 1, 3 and 6 weeks, respectively. Proliferation of fibrous connective tissues and fiber extension within portal areas were significantly detected in W6 panel. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

into proliferative, fibrogenic, and contractile myofibroblasts [24]. The expression levels of α-SMA and TGF-β1 are sensitive indicators of HSC activation into fibrogenic myofibroblasts [10,53]. This explanation supports our findings that α-SMA is expressed by activated HSCs. In addition, hydroxyproline is the main component of collagen and its level is associated with indicative of liver fibrosis [66,72], which also

Table 2
Effect of dabigatran and clopidogrel administration on carbon tetrachloride-induced injury regarding serum alanine transaminase and aspartate transaminase levels, serum albumin and bilirubin levels, tissue malondialdehyde, glutathione and nitrate/nitrite production, prothrombin time and neutrophil count. CCl_4: carbon tetrachloride; ALT: alanine transaminase; AST: aspartate transaminase MDA: malondialdehyde; GSH: glutathione; NOx: nitrate/nitrite production; Pt: prothrombin time.

Groups	ALT (U/L)	AST (U/L)	Albumin (g/dl)	Billirubin (g/dl)	Hepatic MDA (nmol/g)	Hepatic GSH (μmol/g)	Hepatic NOx (nmol/g)	Pt (s)	Neutrophil count (10^3/mm^3)
Normal control	47.6 ± 1.6	118.5 ± 3.3	4.7 ± 0.07	0.28 ± 0.011	34.3 ± 2.71	512.6 ± 6.11	82.1 ± 5.8	20.5 ± 0.9	9.8 ± 0.6
CCl_4 control	701.7 ± 51.1[a]	1320.0 ± 82.1	2.8 ± 0.11[a]	2.10 ± 0.12[a]	419.4 ± 27.48[a]	228.9 ± 5.94[a]	425.2 ± 18.4[a]	49.9 ± 1.7[a]	36.0 ± 1.3[a]
Dabigatran/CCl_4	112.9 ± 1.9[b]	317.2 ± 12.9[a,b]	3.8 ± 0.04[a,b]	0.35 ± 0.01[b]	218.7 ± 4.40[a,b]	338.6 ± 7.46[a,b]	215.2 ± 3.5[a,b]	28.3 ± 1.4[a,b]	20.0 ± 0.8[b]
Clopidogrel/CCl_4	105.1 ± 4.8[b]	124.7 ± 1.667[b,c]	3.9 ± 0.06[a,b]	0.31 ± 0.01[b]	194.1 ± 7.468[a,b]	350.1 ± 4.66[a,b]	203.0 ± 3.5[a,b]	21.4 ± 0.6[b]	16.8 ± 0.6[a,b]

[a] Significantly different from the normal control group.
[b] Significantly different from the CCl_4 control group at $P < 0.05$.
[c] Significantly different from the dabigatran group at $P < 0.05$.

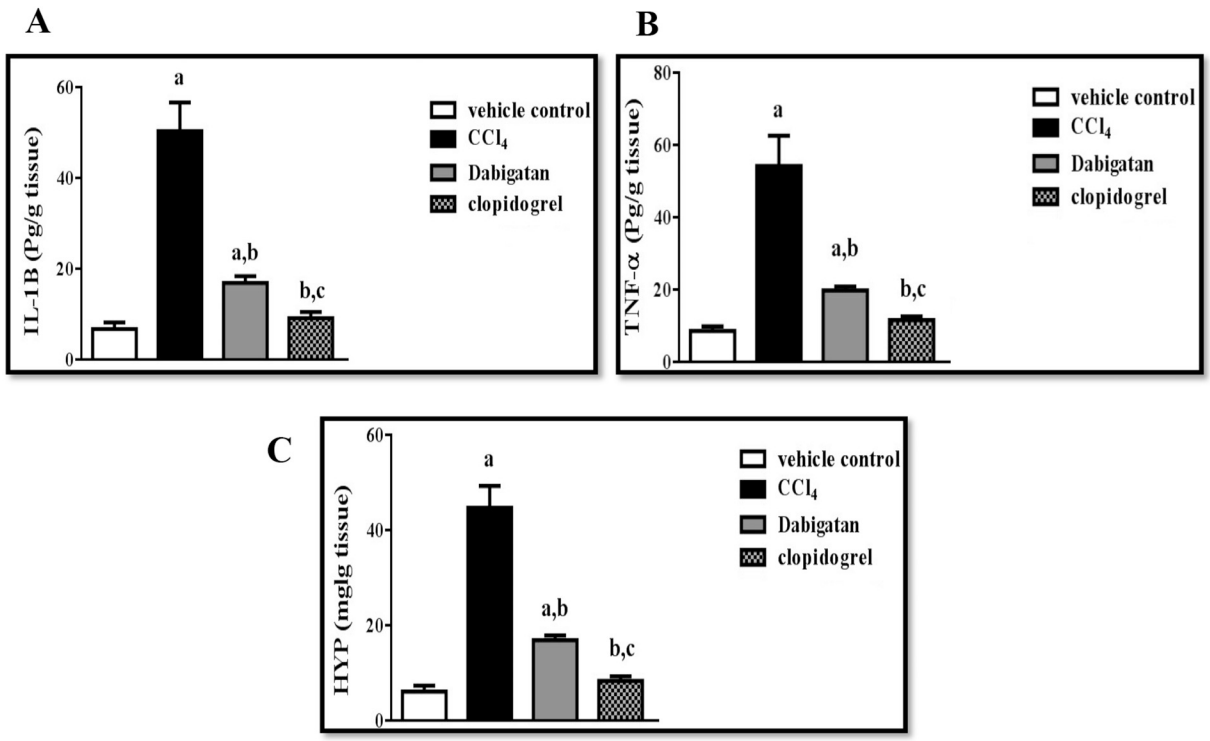

Fig. 4. Effect of dabigatran and clopidogrel administration on carbon tetrachloride-induced injury regarding A) liver tissue tumor necrosis factor-alpha B) interleukin-1β and C) liver tissue hydroxyproline level. CCl_4: carbon tetrachloride; TNF-α: tumor necrosis factor-alpha; IL-1β: interleukin-1β and HYP: hydroxyproline. [a]Significantly different from the normal control group; [b]Significantly different from the CCl_4 control group; [c]Significantly different from the dabigateran/CCl_4 group at $P < 0.05$.

supports our findings. The increased level of HYP in fibrotic liver was further confirmed by histopathological examination with Masson trichrome stain showed late fibrogenesis.

Considering the cross-talk between coagulation system and HSC activation, it seems reasonable to hypothesize those anti-coagulants or coagulation-related drugs can affect fibrogenesis and could be considered for prevention of liver fibrosis. Dabigatran is a direct thrombin inhibitor that prevents thrombin-mediated cleavage of fibrinogen into fibrin as well as thrombin-induced platelet aggregation and thus prevents thrombi formation [20]. Results of the current study revealed that dabigatran could ameliorate CCl_4-induced liver injury, where dabigatran pre-treatment normalized all CCl_4-induced oxidative and fibroproliferative outcomes. Earlier studies reported that thrombin enhances fibrin-induced inflammation in obese patients, which was ameliorated by dabigatran [32]. Our data also showed that dabigatran decreased tissue factor and fibrin protein expression in CCl_4-treated rats. Direct thrombin inhibition has been shown to decrease tissue factor production through reducing the adherence of platelets to monocytes and granulocytes in myocardial infarction patients [9]. This may be explained by the earlier findings that dabigatran inhibits thrombin binding to protease-activated receptors (PAR-1) which is expressed on a number of cells, including HSC, thus inhibits the production of extracellular matrix proteins as well as the recruitment of inflammatory cells to the site of injury [42].

Platelets play diverse roles in pathophysiology of liver fibrosis. Studies suggest that platelets can either promote or reduce liver injury and fibrosis. For example, Murata et al. [47] and Maruyama et al. [43] reported that the increase in platelets induced by platelet transfusion can improve the liver function of patients with liver fibrosis in a clinical setting. One reason may be that platelets enhanced the expression of hepatocyte growth factor (HGF) and matrix metalloproteinase 9 (MMP9), thereby stimulating fibrolysis and decreasing pro-fibrotic growth factor TGF-β [67]. MMP-9 may also indirectly contribute to fibrolysis by accelerating HSC apoptosis [22]. In Addition, platelet-derived serotonin interacts with both hepatocytes and HSCs to modulate the phenotypic plasticity of these cells that helps in liver regeneration after injury [8].

However, there are many contrasting data report that platelets have harmful effects on liver fibrosis. Zaldivar et al. [71], concluded that platelet-derived chemokine CXCL4 associated with liver fibrosis in humans with different liver diseases. Additionally, the use of anticoagulant and antiplatelet drugs reduce the severity of liver injury in different animal models [17,26]. Platelets recruit and activate inflammatory cells, including granulocytes, macrophages, and T cells through PDGF-β, CXCL4 or seroton into the liver and thereby perpetuate liver inflammation [25] [46]. Furthermore, platelet-derived mediators, such as PDGF-β, are potent inducers of HSC transformation to pro-fibrotic myofibroblasts [69]. Serotonin released by platelets could mediate vasoconstriction and reduce blood flow within the hepatic sinusoidal microcirculation. Serotonin also activates the contraction of HSCs or liver sinusoidal endothelial cells (LSECs), resulting in hepatic hypoperfusion [5,55].

The paradox of platelets being both deleterious and beneficial to liver function appears to be context dependent. It is determined by the cellular and cytokine microenvironment specific to the stage and type of liver injury [8]. In acute liver injury, up-regulation of 5-hydroxytryptamine (5HT) pro-regenerative effects [35]. In the late stages of liver injury, however, HSCs participate in termination of the regeneration process and actually promote fibrosis. At this time point, platelet-derived serotonin interacts with HSC 5-HT 2β receptors stimulating HSC and TGF-β expression [12,14].

In the present study, the antiplatelet agent clopidogrel was shown to exert anti-fibrotic effect against CCl_4-induced liver fibrosis as evidenced by reduced levels of hepatic α-SMA, TGF-β1 and hydroxyproline as compared with CCl_4 control rats. These effects were also coupled with significant normalization of all other inflammatory markers as

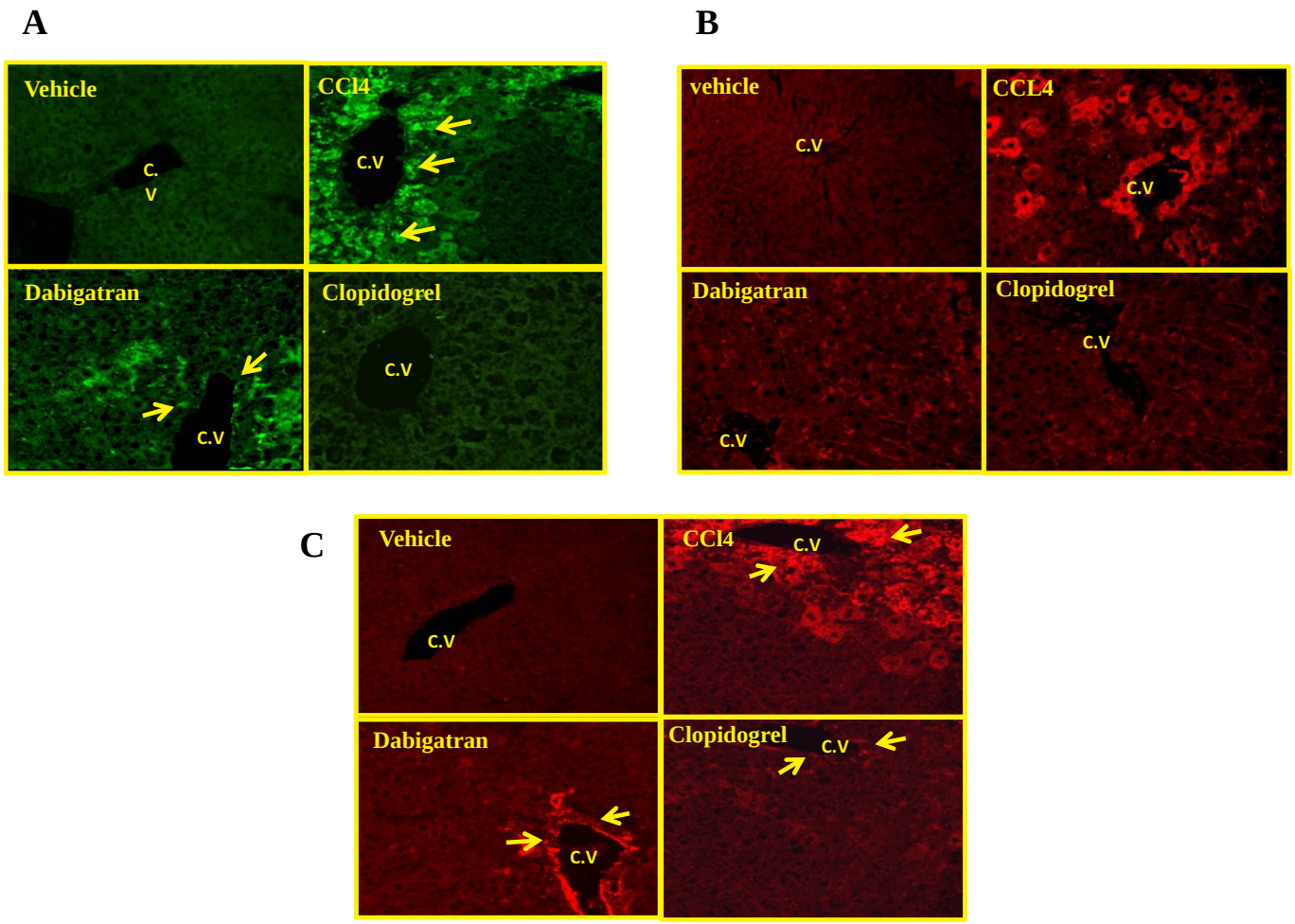

Fig. 5. A) Photomicrographs of immunofluorescence-stained liver sections showing the effect of dabigatran and clopidogrel on carbon-tetrachloride (CCl_4)-induced a) tissue factor expression B) fibrin expression deposition and C) transforming growth factor (TGF-β1) expression.

described with dabigatran. Inhibition of platelet aggregation was reported to attenuate liver fibrosis, which was supported by Li et al. [36] who concluded that aspirin could reduce liver fibrosis in rats. Similarly, Jia et al. [28] demonstrated that clopidogrel inhibited the expression of α-SMA and TGF-β and consequently cardiac fibrosis. The effect of clopidogrel on the pro-inflammatory cytokines TNF-α and IL-1β observed in the current study came in agreement with Tu et al. [61] who proved the anti-inflammatory reno-protective effect of clopidogrel in chronic renal Injury. Intriguingly, it was recently reported that platelets represent an important source of TGF-β1required for HSC activation in a mouse model of liver fibrosis, where the authors suggested that targeting platelets may represent an anti-fibrotic strategy [18].

5. Conclusion

In conclusion, coagulation seems to play an important mechanistic role in the pathogenesis of liver fibrosis, where coagulation cascade initiates a series of inflammatory and fibro-proliferative events. Targeting coagulation cascade by the thrombin inhibitor dabigatran or by the antiplatelet agent clopidogrel may represent an attractive prophylactic strategy against liver fibrosis, but further clinical trials are claimed to confirm such effects.

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Declaration of Competing Interests

None.

Authors' contribution

Nesreen, IM performed data collection, carried out the practical experiments and biochemical assay, performed statistical analysis and drafted the manuscript. Messiha, BA participated in the design of the study, supervision of practical work, manuscript editing and overall manuscript revision. Abdel-Bakky MS participated in the design of the study and its co-ordination, shared in the supervision of the practical study and performed and imaging the immunofluorescence technique as well as interpreted immunofluorescence data. Abo-Saif, AA conceived the study, participated in its design and performed overall revision on the study. Ibrahim, GS helped in the imaging in immunofluorescence technique. All authors read and approved the final manuscript.

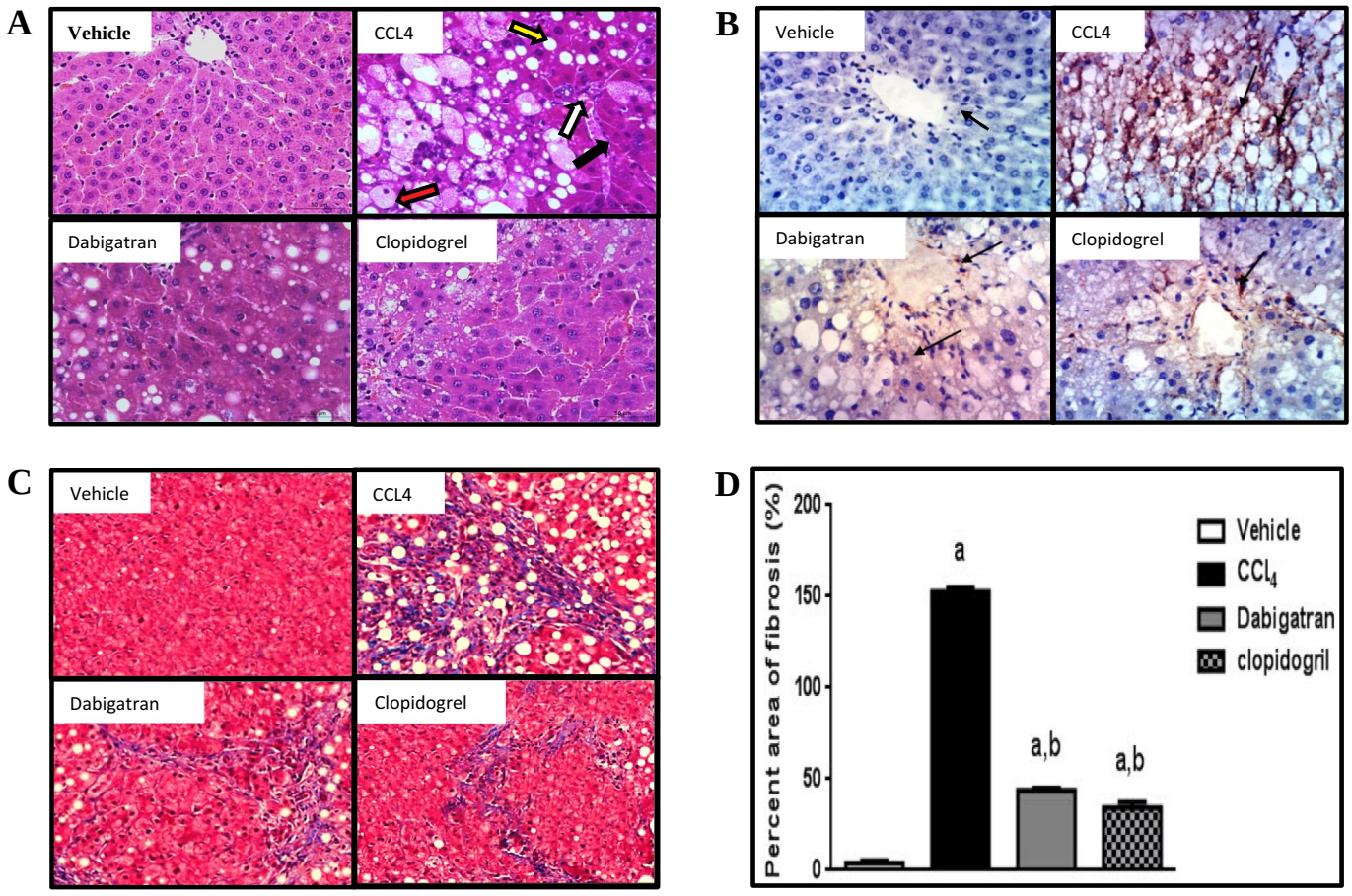

Fig. 6. A) Photomicrographs of liver sections (H&E; 400×) showing the effect of dabigatran and clopidogrel on carbon tetrachloride (CCl_4)-induced liver injury. Normal (vehicle) control section shows normal hepatic architectures with the absence of any pathologic lesions. The CCl_4 control section shows distorted architecture with extensive fibrosis combined with development of massive fatty vacuoles (yellow arrow). Dilated congested central vein (black arrow) and fibrous tissue (white arrow) and appearance of pyknotic nuclei (red arrow) are evident. Liver sections of dabigatran- or clopidogrel-treated rats showed almost normal hepatic architecture. B) Immunohistochemical examination of the effect of dabigatran and clopidogrel on carbon tetrachloride (CCl_4)-induced injury regarding liver tissue alpha smooth muscle actin (α-SMA) level. C) Photomicrographs of liver sections (Masson trichrome stain; 400×) showing the effect of dabigatran and clopidogrel on carbon tetrachloride (CCl_4)-induced liver fibrosis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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