

The preventive effect of low molecular weight heparin on CCL₄-induced necrosis and apoptosis in rat liver

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ABSTRACT

Introduction. Heparin having anti-inflammatory and anti-fibrotic properties may have therapeutic effect on liver injury. The present study investigated the effect of low molecular weight heparin (Enoxaparin) on carbon tetrachloride (CCL₄) induced hepatic necrosis and apoptosis in rats. **Material and methods.** Thirty male rats were divided into 5 groups. Group I: Control; Group II: Olive oil dissolved CCL₄ at dose of 1 mL/kg, ip, twice per week; Group III: CCL₄ and Enoxaparin at dose of 180 IU/kg, sc, daily; Group IV: Enoxaparin; Group V: Olive oil at dose of 1 mL, ip, twice per week. The liver histology at the fourth week was examined by haematoxylin-eosin, Masson's trichrome, Toluidine blue and Periodic acid schiff stains. Proliferative and apoptotic activities were assessed semi-quantitatively by proliferating cell nuclear antigen (PCNA) and caspase-3 immune staining and TUNEL method. Semi-quantitative values formulated by the equation HSCORE = $\sum P_i (i+1)$ including both distribution and intensity of staining. Additionally, nidogen and α -smooth muscle actin were labeled by immunohistochemistry. **Results.** CCL₄ group had marked hepatocellular necrosis around the vena centralis and increased inflammatory cells and mast cells. Hepatocytes showed deposition of lipid droplets, decrease in glycogen, apoptosis, and picnotic or enlarged nuclei. Enoxaparin reduced necrosis, apoptosis, and number of mast cells but had no effect on lipid droplets in hepatocytes. HSCORE's of caspase-3 and PCNA were also significantly decreased by administration. **Conclusion.** Enoxaparin have beneficial effects against necrosis as well as apoptosis at the early stage of CCL₄ induced liver injury.

Key words. Apoptosis. Enoxaparin. CCL₄. Liver. PCNA.

INTRODUCTION

The capacity of regeneration distinguishes the liver from the other essential organs in adults. Incomplete regeneration known as hepatic fibrosis is now regarded as a common response to liver injury and, regardless of its origin (viral infections, alcohol abuse, metal overload), is characterized by excessive accumulation of extracellular matrix (ECM) components.¹⁻⁴ Emerging experimental evidence indicates that oxidative stress could represent a common link

between the different types of chronic liver injuries and hepatic fibrosis. In fact, lipid peroxidation has been associated with liver fibrosis due to iron overload, ethanol, carbon tetrachloride, and virus C hepatitis.⁴⁻⁸ Independent stimulation of ECM deposition seems to occur at a pre-necrotic stage during oxidative stress-associated liver injury, although it is difficult to discriminate between profibrogenic and true fibrogenic effects induced by free radical species. Hepatic stellate cells (HSCs) are a major source of ECM in normal and pathological conditions. During fibrogenesis, HSCs undergo a process of activation, developing a myofibroblast-like phenotype associated with increased proliferation and collagen synthesis.⁹ Activation of stellate cells is regulated by several soluble factors, including cytokines, chemokines, growth factors (hepatocyte growth factor, epidermal growth factor,

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transforming growth factor), and also products of oxidative stress.^{4,10,11}

Mast cells are distributed in all organs including liver which have pro-fibrogenic and anti-fibrotic mediators. They are multi-effector cells with important roles in the tissue destruction and remodeling in liver injury.¹²⁻¹⁵

CCl₄ is known to be hepatotoxic as well as nephrotoxic to humans.^{16,17} CCl₄ induces necrosis and apoptosis of the liver in a variety of experimental animal models.^{5,9} While a single oral, intraperitoneal, subcutaneous dose of CCl₄ will lead to reversible liver injury, a prolonged exposure will result with fibrosis, cirrhosis and hepatic carcinoma.^{2,18,19}

Treatment of liver fibrosis still remains as a therapeutic challenge. There is a need for a therapy that will decrease the extent of fibrosis or enhance the liver regeneration. Few treatments have been proposed to alter the hepatocyte regenerative activity but none has proven clinically effective.^{6,13,19-21}

Low-molecular-weight heparins (LMWH) are fragments of commercial grade heparin produced by either chemical or enzymatic depolymerization. Deaminative cleavage with nitrous acid is used in the manufacture of dalteparin, reviparin and nadroparin. Alkaline beta-eliminative cleavage of the benzyl ester of heparin is used in the manufacturing of Enoxaparin. LMWHs are potentially more advantageous pharmacologically and pharmacokinetically than heparin due to their reduced hemorrhagic to antithrombotic ratio, lesser risk of bleeding, greater bioavailability at low doses, and longer half-life.²² The antifibrotic role of a LMWH was shown as an experimental model of glomerulotoxicity and lung fibrosis.^{17,22,23} In endotoxin-induced liver injury, Dalteparin reduces sinusoidal capillarization and the production of inflammatory cytokine in Kupffer cells.²⁴ Additionally, recent clinical data have suggested a beneficial effect for LMWH as hepatic antifibrotic agent.^{8,25-28}

The present study aimed to examine antifibrotic and regenerative effects of LMWH on CCl₄ induced toxic liver injury by immunohistochemistry with light and electron microscopy.

MATERIAL AND METHODS

Animals

The present study was approved by the laboratory animals ethic committee of university (2005/300-39). Experiments were carried out on 30

Sprague-Dawley male rats of 260–320 g body weight. They were housed at 22–24 °C and were exposed to alternate cycles of 12 h light and darkness. They were given free access to a standard pellet diet and tapwater.

Experimental procedure

The animals were divided into 5 groups with 6 rats in each group. Group I was control rats. Group II was administered CCl₄ (Merck) at a dose of 1 mL/kg of body weight, intraperitoneal, dissolved in olive oil, twice per week for four weeks. Group III had CCl₄ and Enoxaparin (Enoxaparin Sodium, Aventis Pharma) 180 IU/kg of body weight, subcutaneously daily. Group IV was given Enoxaparin 180 IU/kg of body weight subcutaneously daily. Group V was administered olive oil at a dose of 1 mL, ip, twice per week. At the end of four weeks, animals were anesthetized with ketamine/xylazine (90/10 mg/kg) and sacrificed by decapitation. After removal and weighing the livers were placed in 10% formalin and 2.5% glutaraldehyde for histological processing.

Light microscopy

Liver samples were fixed in 10% formalin, routinely processed and embedded in paraffin wax. Sections of 5 micrometer thickness were cut and stained with haematoxylin and eosin (HE), Masson's trichrome for collagen, Toluidine blue for mast cells, Periodic acid schiff (PAS) for glycogen and by immunohistochemistry to demonstrate caspase-3, proliferating cell nuclear antigen (PCNA), nidogen (entactin) and smooth muscle α -actin (α -SMA) for evaluation of parenchymal and non-parenchymal cells. Photographs were taken using an Olympus BX 50 photomicroscope. Mast cells were counted in ten sections by using an eyepiece micrometer (OC-M, Olympus, Japan, X40) all portal areas in the section.

Electron microscopy

Liver tissues were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, tissues were postfixated with 2% osmium tetroxide in sodium phosphate buffer. Dehydration was accomplished by gradual ethanol series and tissues were embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were then viewed and photographed with a Zeiss 9EM.

Caspase-3, PCNA, nidogen, and α -SMA

The liver tissue samples were fixed in 10% neutral formaline for 24 h, embedded, and then serial sections (5 μ m) were collected on slides with polysine. After rehydrating, samples were transferred to 0.01 M citrate buffer (pH 6) and subsequently heated twice in a microwave oven for 5 min each time at 750 W. for antigen retrieval. After cooling for 20 min at room temperature, the sections were washed with phosphate buffer saline (PBS). To remove endogenous peroxidase activity, sections were kept in 3% H₂O₂ for 20 min and afterward washed with PBS. Sections were incubated with primary rabbit-polyclonal Caspase-3 antibody (NeoMarkers, Fremont, CA) and primary mouse-monoclonal PCNA antibody (Dako, Carpinteria, CA), primary mouse anti-smooth muscle Actin (anti- α -SMA Zymed Lab., Lab Vision, USD). Some sections were incubated with primary goat-polyclonal nidogen-1 and nidogen-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilution overnight at 4 °C. Negative control sections were treated with nonimmune serum diluted in the same manner. Labeling was visualized using the Universal LSAB kit (Dako, Carpinteria, CA) according to the manufacturer's instructions. Staining was completed with DAB Chromogen (Dako, Carpinteria, CA) for 1-2 min, and slides were counterstained with Harris's Haematoxylin, dehydrated and then cover-slipped with permount.

Detection of apoptotic cells

Apoptotic hepatocytes were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining using a commercial ready-to-use kit (Cat No MK500, Takara Bio Inc. Japan, In situ apoptosis detection kit) according to the manufacturer's recommendations. Randomly selected different areas of six sections per group at X 400 magnification were evaluated for the analysis of TUNEL staining. The apoptotic index (AI) was determined as the percentage of labeled cells (TUNEL positive) with respect to the total number of cells counted using the formula: AI = (Number of labeled cells/Total number of cells counted) x 100.¹⁹

Semiquantitative evaluation

Sections were evaluated with respect to Caspase-3, and PCNA localization in a semiquanti-

tative manner using a light microscope and selected areas were photographed. Two experienced observers unaware of the treatment were scored hepatocyte staining intensity as negative (-), weak (+), moderate (++) , strong (+++). HSCORE values of Caspase-3 and PCNA staining were obtained in a semiquantitative manner and included both intensity and distribution patterns of staining.²⁹ Ten different areas of six sections per group at X 400 magnification were evaluated for the analysis of immunohistochemical Caspase-3 and PCNA staining. Values were recorded as percentages of positively stained target cells in each of four intensity categories which were denoted as 0 (no staining), 1+ (weak), 2+ (moderate), 3+ (strong). For each tissue, an HSCORE value was derived by summing the percentages of cells that stained at each intensity category and multiplying that value by the weighted intensity of the staining, using the formula HSCORE = $\sum P_i (i + 1)$, where i represents the intensity scores and P_i is the corresponding percentage of the cells.

Statistical analysis

Comparisons of the HSCORE values and AI were performed by the One-Way ANOVA- Tukey's test. Number of mast cells were analysed by Mann-Whitney-U test. Data were expressed as mean \pm SD. P values less than 0.05 were considered to be statistically significant.

RESULTS

Light microscopy, electron microscopy and immunohistochemistry

Histologic findings of the groups to which only Enoxaparin (Group IV) and olive oil (Group V) were administered were close to the controls. In the CCL₄ administered group, wide areas of necrosis around the central vein, hepatocytes with picnotic and large nuclei and lipid accumulation were noted (Figures 1A and C). Engorgement of the sinusoids within the necrotic fields, hemorrhage and increase in inflammatory cells were observed (Figure 1B). Clusters of Kupffer cells (Figure 1D), apoptotic hepatocytes and mitotic hepatocytes (Figure 1C) were identified. In the Enoxaparin-treated group, enlargement of the sinusoids in some areas and persistence of lipid droplets in hepatocytes were observed though necrotic areas did not appear (Figures 1E

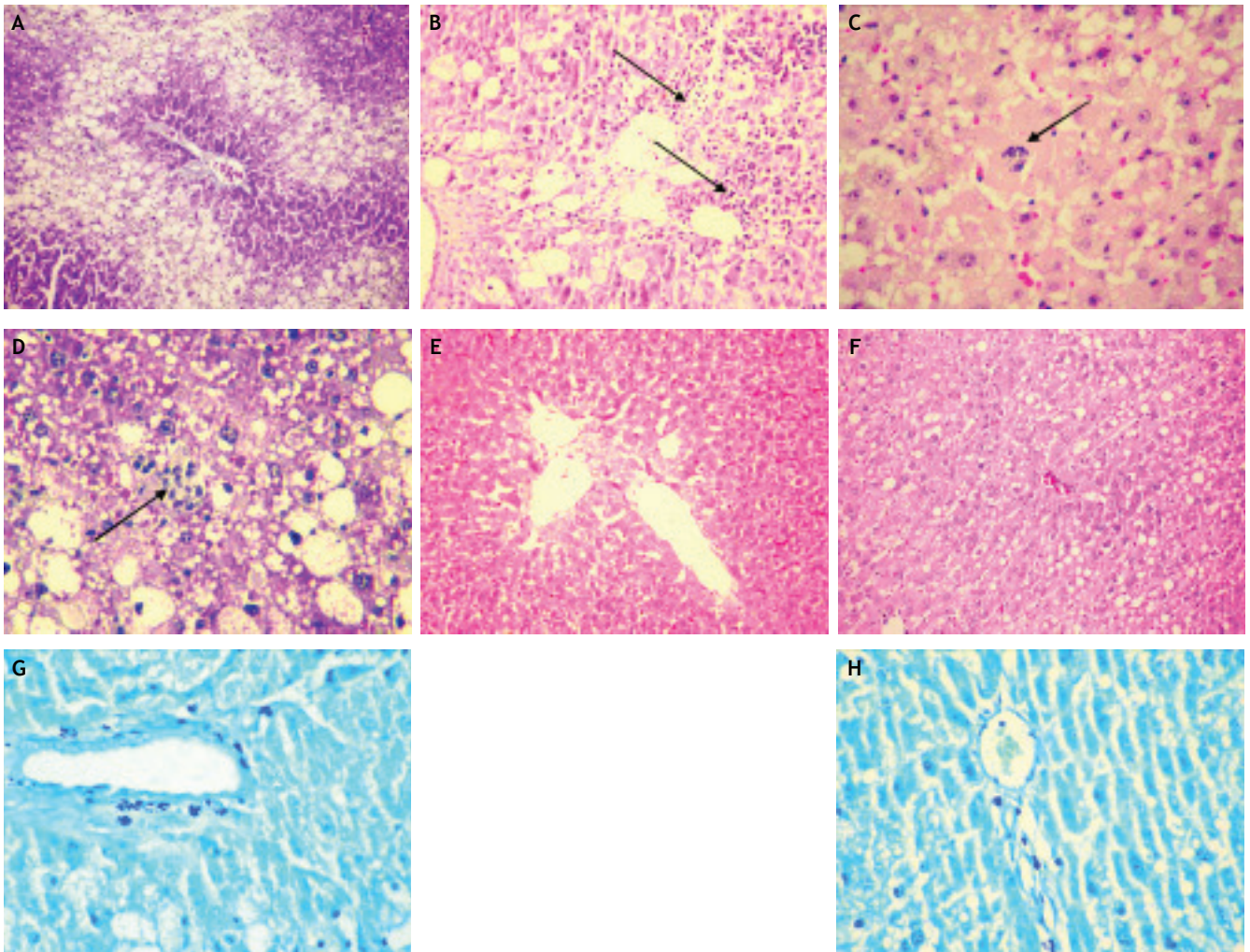


Figure 1. Masson's trichrome, HE and Toluidine blue stainings. CCl_4 group, wide spreading necrosis, Masson's trichrome x10 (A), increase in inflammatory cells (\rightarrow), HE x10 (B), mitotic figure (\rightarrow), HE x40, (C), activation of Kupffer cell (\rightarrow), Masson's trichrome x40 (D). Increase in mast cells, Toluidine blue staining X 40 (G). Enoxaparin prevented CCl_4 induced necrosis, HE x20 (E). Enoxaparin treatment caused vacuolization in the hepatocytes result of melting lipid droplets HE x20 (F). Enoxaparin prevented CCl_4 induced mast cell increase, Toluidine blue staining X 40 (H).

and F). Mast cells were seen around the portal area. CCl_4 exposure for four weeks also caused significant increase in the number of mast cells in portal area (Figure 1G) which was decreased by Enoxaparin ($P < 0.01$, Figures 1H and 2).

When the glycogen content of the hepatocytes was examined with respect to the controls, we found that staining of the cells around the necrotic areas was weaker and glycogen content was reduced (Figure 3A) in the CCl_4 -administered group whereas there were numerous PAS-stained hepatocytes in the Enoxaparin-administered group (Group III) and the staining intensity was comparable to the controls (Figure 3B). By the portal

areas and around the central veins in the CCl_4 -administered group, fibrosis was not detected. When the semi-thin sections were examined, necrosis, vacuolization in the hepatocytes, lipid accumulation and Kupffer cells were clearly identified (Figures 4A, B and C). TUNEL staining of the tissues demonstrated significant increase in the CCl_4 and significant decrease in the Enoxaparin-administered groups in the apoptotic hepatocytes compared to the controls (Figures 5 and 6).

α -SMA (Figures 7A, B and C) was comparable in the control and other groups. α -SMA was present around the vessels in the portal areas and did not stain the HSCs. Immunohistochemical labeling of

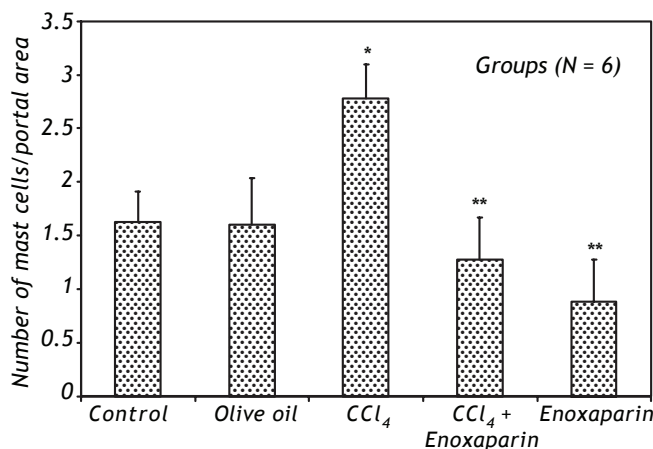


Figure 2. Number of mast cells in portal area. *Significantly different from control and olive oil groups ($P < 0.001$). **Significantly different from CCL₄ group ($P < 0.05$).

nidogen of groups were decreased in the bile duct and endothelial during acute hepatic CCL₄ injury (Figures 7D, E and F). When the fine structure was examined, mitochondria and rough endoplasmic reticula of the hepatocytes appeared normal in the control group (Figure 8A). In the CCL₄-administered group, on the other hand, the boundaries of the cells were indistinguishable, the organelles were disrupted and there were necrotic changes (Figure 8B) and apoptotic hepatocytes (Figure 8C). In the Enoxaparin-administered group, necrotic areas were less, but lipid deposits in the hepatocytes persisted (Figure 8D). In the necrotic areas, active hepatic stellate cells which transformed into myofibroblasts were not identified. Presence of increased collagen fibers was not observed around the sinusoids in the portal areas.

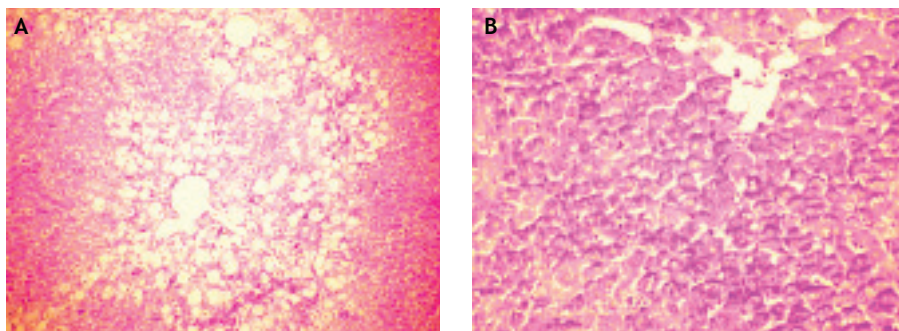


Figure 3. Liver tissues stained by PAS. In CCL₄ group weak staining in hepatocytes around necrotic areas x10 (A). Enoxaparin increased intensity of PAS staining x20 (B).

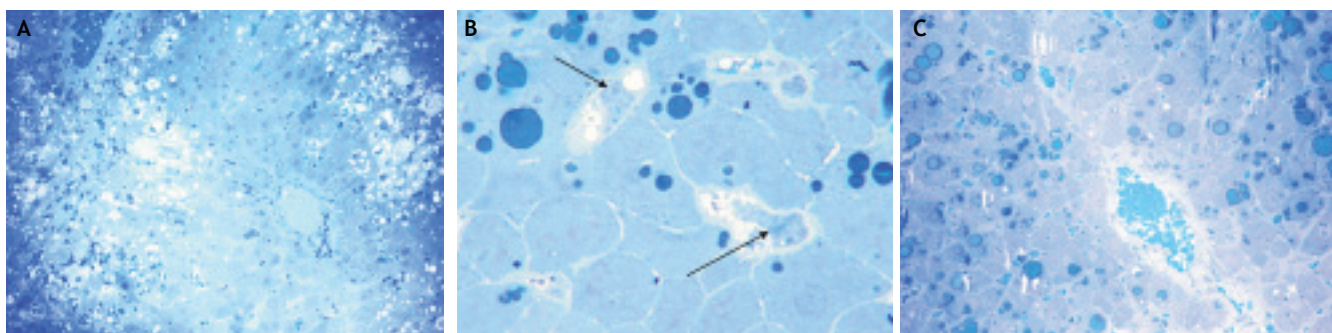


Figure 4. Semi-thin sections stained by Toluidin blue-Azur. In CCL₄ group necrotic fields and vacuolization in the hepatocytes x20 (A), active Kupffer cell (→) in the sinusoids x100 (B). Enoxaparin treatment x40 (C).

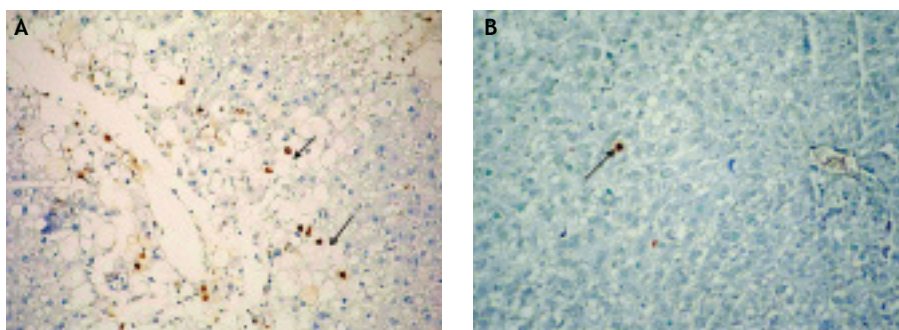


Figure 5. Apoptotic cells by TUNEL immunolabelling. In CCL₄ group increased apoptotic hepatocytes (→) (A), Enoxaparin treatment decreased number of apoptotic hepatocytes (→) (B).

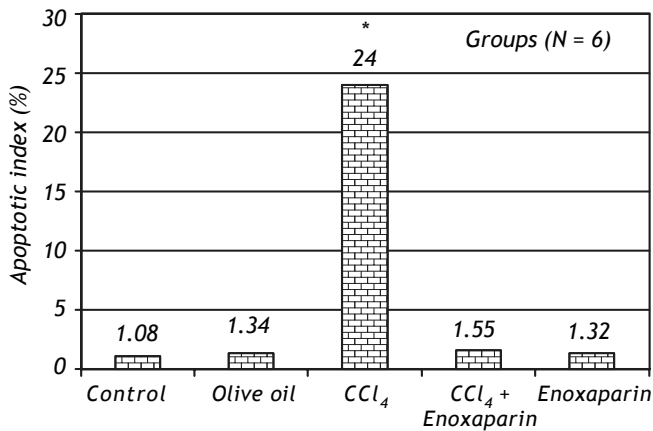


Figure 6. Apoptotic hepatocyte index of groups. *Significantly different from all other groups ($P < 0.0001$).

HSCORE assessment

Semi-quantitative HSCORE values gathered during the assessments of the groups in terms of Caspase-3, PCNA, and nidogen are shown in table I, data are expressed as mean \pm standard deviation. The CCl₄-administered group, in comparison to the all other experimental groups, depicted significant increase in Caspase-3 (28.88 ± 3.42), and PCNA (205.60 ± 24.27) values in hepatocytes ($P < 0.05$). Increases in PCNA, which is a marker of proliferation, and Caspase-3, which plays a key role in apoptosis, suggest that CCl₄ increases mitotic activity and apoptosis in hepatocytes at the same time. HSCORE values were significantly lower in the group

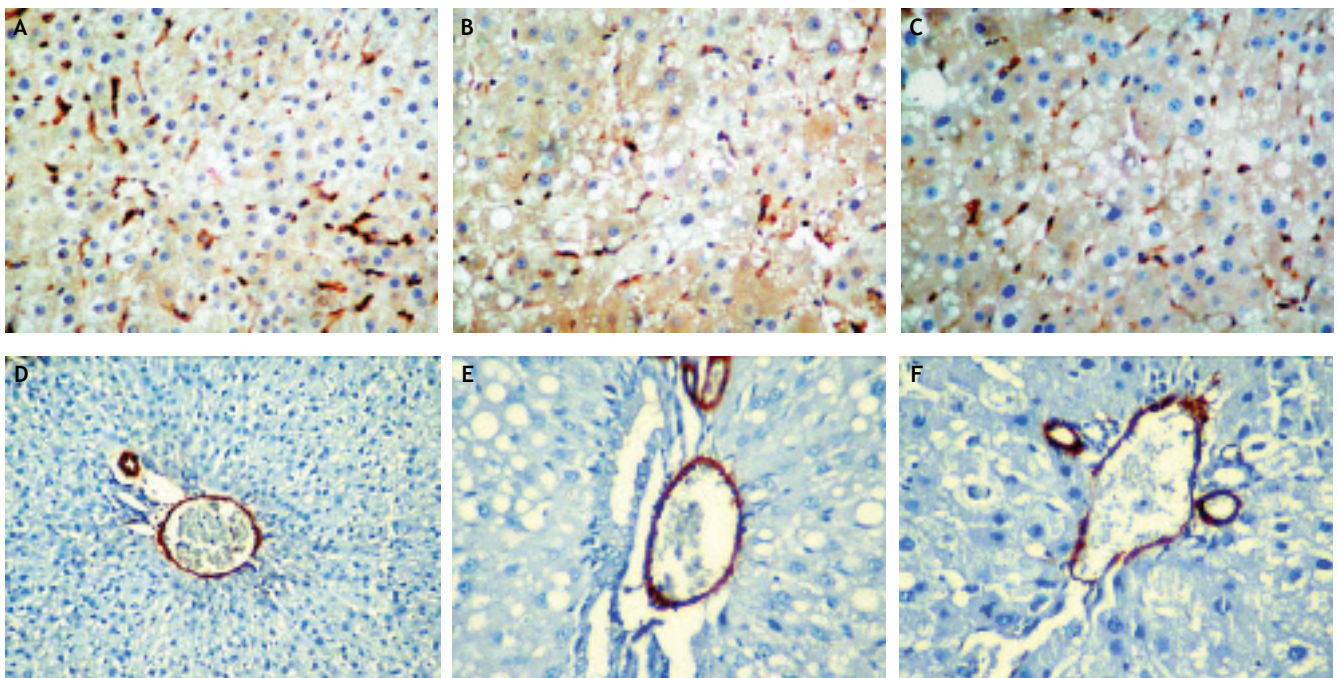


Figure 7. Immunostaining of Nidogen (A, B and C) ve α -SMA [D x 20, E x 40, F x 40]. Control group (A and D). CCl₄ group (B and E). Enoxaparin treatment (C and F).

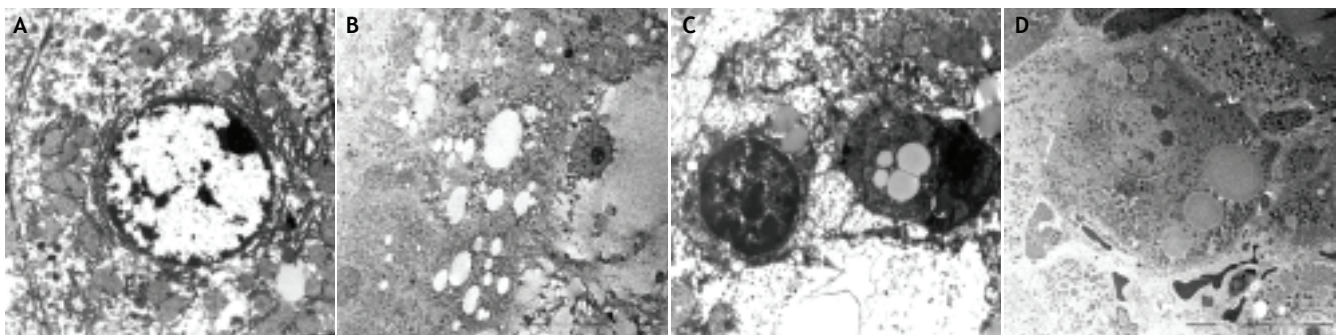


Figure 8. Images of electron microscopy. Control group (A). In CCl₄ group necrotic areas (B), apoptotic hepatocytes (C). Enoxaparin treatment (D).

Table 1. HSCORE of Caspase-3 and PCNA immunostainings (Mean \pm SD).

Groups (n = 6)	Caspase-3 (Hepatocyte)	PCNA (Hepatocyte)	Nidogen (Bile duct)
Control	3.60 \pm 1.67	7.60 \pm 3.29	400 \pm 0.00
Olive oil	2 \pm 1.41	8.40 \pm 2.61	400 \pm 0.00
CCl ₄	28.88 \pm 3.42*	205.60 \pm 24.27*	300 \pm 0.00*
CCl ₄ + Enoxaparin	17.6 \pm 3.91*	153.60 \pm 30.8*	400 \pm 0.00
Enoxaparin	10.88 \pm 1.19	14.8 \pm 3.63	400 \pm 0.00

* Significantly different from all other groups (P <0,05).

treated with Enoxaparin than the CCl₄-administered group (P < 0.05) but significantly higher than the group that received only Enoxaparin and the controls. Enoxaparin also prevented decreasing nidogen expression in bile ducts (Table 1).

There was no significant difference in body and hepatic weight between the experimental groups and controls.

DISCUSSION

The liver injury model with CCl₄ was used to study effect of Enoxaparin at early stage of chronic liver injury. Enoxaparin was beneficial on hepatocytes both for preventing apoptosis and improving regeneration which may be the mechanisms of Enoxaparin for preventing liver fibrosis. However, rats did not developed liver fibrosis with this experimental model, and so chronic treatment of Enoxaparin should be studied to settle role of Enoxaparin on preventing liver fibrosis.

In the normal adult liver, hepatocytes are in a quiescent state and turn over very slowly [1–2 times/year]. However, the liver regenerates very quickly during acute toxic liver injury in rodents, a similar process occurs at a somewhat slower rate in larger animals and in humans.³⁰ There are two stages of hepatic injury, namely regeneration stage and fibrosis stage. In the regeneration stage, cells of the original type appear in the injured tissue. In the fibrosis stage, normal parenchyma is replaced by connective tissue and ECM accumulation resulting with scar formation. HSCs play important and deterministic roles at this stage. The recent studies have focused on preventing fibrosis by reducing the activated HSCs.^{1,31}

CCl₄ has long served as a model for study of hepatotoxicity with centrilobular hepatic necrosis. It is accumulated in hepatic parenchyma cells and metabolized to CCl₃ by liver cytochrome P450-dependent monooxygenases. Metabolic activation of CCl₄

to free radicals, namely trichloromethyl and trichloromethyl peroxy radicals, is reported to enhance lipid peroxidation and protein oxidation leading to liver injury.^{16,19} Acute injury as a result of single dose of oral, intraperitoneal or subcutaneous CCl₄ administration is usually reversible and full regeneration can be achieved.^{2,8} Fibrosis and pseudolobulation occur when the duration of administration is prolonged. The number of α -SMA (+) cells increases in the fibrotic areas. These changes are reported to recover with Vitamin-A administration.²⁰

In the present study, CCl₄ was administrated to rats every other day for 4 weeks caused necrosis, lipid peroxidation reduction in glycogen in the hepatocytes, inflammation and activation of Kupffer cells but not fibrosis. As a result, we did not observe HSCs stained by α -SMA. The number of mast cells in portal area also increased in early stage of liver injury by induced CCl₄ which is parallel with other studies.¹³⁻¹⁵ Mast cells have a significant role in tissue destruction and remodelling in liver injuries by releasing vasoactive, nociceptive and pro-inflammatory mediators. Matsunaga, *et al.*, found that in livers with chronic diseases, the densities of tryptase+ mast cells, chymase+ mast cells, and S-100+ nerve fibers in the stroma were significantly higher than of the ones in normal livers. They observed a significant increase of tryptase+ and chymase+ mast cells and also innervation in correlation with degree of fibrosis.¹⁴

CCl₄ induces massive necrosis in the liver and consequent oxidative stress activates caspase-3 and increases apoptosis. Increase in apoptotic hepatocytes has been described in hepatic fibrosis and viral hepatitis.^{9,21,27,32} Although apoptosis of HSCs shown to have a peak at 64h,⁹ in another study apoptosis started to increase at 2 weeks and made a peak at 4 weeks.²¹ The recent study evaluated only the apoptosis of hepatocytes which increased in a dose-dependent fashion. Apoptosis scores were found to be

16.76 \pm 1.1 and 25.55 \pm 1.96 after the single CCl₄ doses of 20 and 50 μ l/kg, respectively.¹⁹

In addition to apoptosis, increase in PCNA-labeling was also noted in the hepatocytes of CCl₄-administered rats.³³ PCNA score was reported as 6.4 \pm 1 in a control group while that was 19.7 \pm 2.0 a day after CCl₄ administration.³⁴ TUNEL method was used to demonstrate increase in apoptotic hepatocytes and Caspase-3 immune-staining was used to identify hepatocytes that will undergo apoptosis. PCNA score as an indicator of proliferation was increased significantly in the groups with necrosis as opposed to the control group. There was an 8-fold increase in apoptosis and 27-fold increase in proliferation in comparison to the control group. In acute injury, the response of the hepatocytes is significantly more prominent and cells prepare for mitotic division. On one hand, there is elimination of hepatocytes affected from CCl₄ by apoptosis and, on the other hand, there is an increase in the proliferation of un-affected hepatocytes.

In the normal adult liver, basal lamina is present in the bile ducts, arteries and veins, but not in sinusoids. Laminin, nidogen (entactin), and Type IV collagen increase/accumulate and a basement membrane-like structure forms in the space of Disse during hepatic regeneration and fibrosis, although an organized basal lamina does not develop.^{35,36}

Immunohistochemical labeling of nidogen of groups were decreased in the bile duct and endothelial during acute hepatic CCl₄ injury. Since HSCs were not activated after 4 weeks of CCl₄ administration, nidogen expression and collagen were not increased. Presence of α -SMA staining only around the vessels and absence of HSCs lend also support to this result. Thus, this liver injury model used in the present study was an early stage of chronic damage.

Numerous drugs and herbal substances have been tried in the treatment of hepatic injury and fibrosis. To date, there is no routinely used treatment modality. Both *in-vitro* and *in-vivo* experimental studies showed heparin has anti-fibrotic activity in liver and also other organs.^{8,17,22-26} Heparin has been shown to inhibit growth of HSCs which play important roles in fibrosis.³⁷ Signal transduction pathway of Heparin is related to activities of extracellular signal regulated kinase (ERK) and down regulation of activator protein 1 (AP-1).²⁶

The effects of LMWHs were investigated in cholestatic liver injury model and Nadroparin and Enoxaparin have been shown to decrease serum bilirubine level, alleviate degeneration and reduce fibrosis.²⁷ Enoxaparin has been shown to have both

anti-inflammatory and antifibrotic activities. Enoxaparin reduced hepatic fibrosis in thioacetamide-induced cirrhotic rats.²⁵ Harada, *et al.*, showed that, dalteparin, a LMWH, protects liver from ischemia/reperfusion-induced injury by inhibiting leukocyte activation. They suggested that therapeutic effect of Dalteparin might be independent of its anticoagulant activity but dependent on its capacity to enhance endothelial production of prostacyclin via cyclooxygenase-1 activation.³⁸

Recently, Dalteparin sodium was used in CCl₄-induced fibrosis for 7 weeks and dose-dependent increases in PCNA score of hepatocytes and in apoptosis of cultured HSCs were observed.⁸ They found that, necrosis around the central vein and inflammatory cell infiltration in the middle zone occurs 24 h after CCl₄ administration which was not prevented by Dalteparin. There was an increase in PCNA (+) labelled hepatocytes and the increase in non-parenchymal cells appeared 72 h later. Dalteparine diminished α -SMA staining and fibrotic bands formation which occurred 7 weeks after CCl₄ administration. It has been reported that dalteparine inhibits the profibrogenic response due to chronic CCl₄ administration, stimulates Hepatocyte Growth Factor (HGF), down-regulates Transforming growth factor (TGF)-beta and directly inhibits the proliferation of the HSCs.⁸ Heparin functions as a hepatotrophic factor by inducing production of HGF.^{10,11} *In vitro* studies showed that addition of heparin to cultures of human fibroblasts, leukemic cells, and umbilical vein endothelial cells stimulates HGF production to 3-6-fold higher levels than seen in the absence of heparin. Administration of heparin to rats increased blood HGF to 2.5-5 fold higher levels than controls given saline alone. Consequently, a remarkable enhancement of *in-vivo* liver regeneration was induced after 30% partial hepatectomy.²⁵ Additionally, Shi, *et al.*, showed anti-fibrotic effect of LMWH in clinical trial involving 34 patients with chronic hepatitis.²⁸

In the present study, necrosis around the central vein of the hepatic tissue was found markedly improved in rats treated with Enoxaparin, which is a LMWH. Enoxaparin prevented increase of mast cells which was induced by CCl₄. Decrease in the number of activated Kupffer cells and increase in the number of PAS positive-stained hepatocytes were also observed. By preventing activation of Kupffer cells, release of pro-inflammatory agents decrease and tissue damage can be hindered. This beneficial effect of Enoxaparin was also evident on apoptosis and HS-CORE of PCNA. Enoxaparin did not totally prevent

proliferation of hepatocytes. Even though increased apoptosis and PCNA improved with Enoxaparin, it was still higher than the control group.

CONCLUSION

Enoxaparin, a low molecular weight heparin, prevented inflammation in acute phase and possibly reduced the activation of Kupffer cells and mast cells, and demonstrated a protective role against necrosis and apoptosis. LMWH is cheap and safe, can be an option for treatment of liver injury if the further studies also confirm its beneficial effects on chronic liver injury.

ABBREVIATIONS

- **CCl₄**: Carbon tetrachloride.
- **PCNA**: Proliferating cell nuclear antigen.
- **ECM**: Extracellular matrix.
- **HSC**: Hepatic stellate cell.
- **LMWH**: Low-molecular-weight heparin.
- **HE**: Haematoxylin and eosin.
- **PAS**: Periodic acid schiff.
- **α-SMA**: Smooth muscle α-actin.
- **PBS**: Phosphate buffer saline.
- **TUNEL**: Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.
- **AI**: Apoptotic index.
- **AP-1**: Activator protein 1.
- **ERK**: Extracellular signal regulated kinase.
- **HGF**: Hepatocyte growth factor.
- **TGF**: Transforming growth factor.

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REFERENCES

1. Wynn TA. Cellular and molecular mechanisms of fibrosis. *J Pathol* 2008; 214: 199-210.
2. Palmes D, Spiegel HU. Animal models of liver regeneration. *Biomaterials* 2004; 25: 1601-11.
3. Lotersztajn S, Julien B, Teixeira-Clerc F, Grenard P, Mallat A. Hepatic Fibrosis: Molecular Mechanisms and Drug Targets. *Annu Rev Pharmacol Toxicol* 2005; 45: 605-28.
4. Svegliati-Baroni G, Di Sario A, Casini A, Ferretti G, D'Ambrosio L, et al. The Na⁺/H⁺ exchanger modulates the fibrogenic effect of oxidative stress in rat hepatic stellate cells. *J Hepatol* 1999; 30: 868-75.
5. Shi J, Aisaki K, Ikawa Y, Wake K. Evidence of hepatocyte apoptosis in rat liver after the administration of carbon tetrachloride. *Am J Pathol* 1998; 153: 515-25.
6. Park SW, Lee CH, Kim YS, Kang SS, Jeon SJ, Son KH, Lee SM. Protective Effect of Baicalin Against Carbon Tetrachloride-Induced Acute Hepatic Injury in Mice. *J Pharmacol Sci* 2008;106:136-43.
7. Liu J, Tan H, Sun Y, Zhou S, Cao J, Wang F. The preventive effects of heparin-superoxide dismutase on carbon tetrachloride-induced acute liver failure and hepatic fibrosis in mice. *Mol Cell Biochem* 2009; 327: 219-28.
8. Abe W, Ikejima K, Lang T, Okumura K, Enomoto N, Kitamura T, Takei Y, et al. Low molecular weight heparin prevents hepatic fibrogenesis caused by carbon tetrachloride in the rat. *J Hepatol* 2007; 46: 286-94.
9. Lee JI, Lee KS, Paik YH, Park YN, Han KH, Chon CY, Moon YM. Apoptosis of hepatic stellate cells in carbon tetrachloride induced acute liver injury of the rat: analysis of isolated hepatic stellate cells. *J Hepatol* 2003; 39: 960-66.
10. Matsumoto K, Nakamura T. Heparin functions as a hepatotrophic factor by inducing production of hepatocyte growth factor. *Biochem Biophys Res Commun* 1996; 227: 455-61.
11. Sakiyama R, Fukuta K, Matsumoto K, Furukawa M, Takahashi Y, Nakamura T. Stimulation of Hepatocyte Growth Factor Production by Heparin-derived Oligosaccharides. *J Biochem* 2007; 141: 653-60.
12. Tore F, Tuncel N. Mast cells: target and source of neuropeptides. *Curr Pharm Des* 2009; 15: 3433-45.
13. Jeong DH, Lee GP, Jeong WI, Do SH, Yang HJ, et al. Alterations of mast cells and TGF-β1 on the silymarin treatment for CCl₄- induced hepatic fibrosis. *World J Gastroenterol* 2005; 11: 1141-48.
14. Matsunaga Y, Kawasaki K, Terada T. Stromal mast cells and nerve fibers in various chronic liver diseases: relevance to hepatic fibrosis. *Am J Gastroenterol* 1999; 94: 1923-32.
15. Takeshita A, Shibayama Y. Role of mast cells in hepatic remodeling during cholestasis and its resolution: relevance to regulation of apoptosis. *Exp Toxicol Pathol* 2005; 56: 273-80.
16. Abraham P, Cathrine WG. Oxidative damage to the lipids and proteins of the lungs, testis and kidney of rats during carbon tetrachloride intoxication. *Clin Chim Act* 1999; 289: 177-9.
17. Fýrat T, Kükner A, Töre F, Ergür BU. The effect of low molecular weight heparin on kidney tissue of rats exposed to carbon tetrachloride. *Erciyes Medical J* 2009; 31: 293-303.
18. Wernke MJ, Schell JD. Solvent and malignancy. *Clin Occup Environ Med* 2004; 4: 513-27.
19. Das RK, Hossain U, Bhattacharya S. Protective effect of diphenylmethyl selenocyanate against CCl₄-induced hepatic injury. *J Appl Toxicol* 2007; 27: 527-37.
20. Rivera-Espinoza Y, Muriel P. Pharmacological actions of curcumin in liver diseases or damage. *Liver Int* 2009; 29: 1457-66.
21. Priya S, Sudhakaran PR. Curcumin-induced recovery from hepatic injury involves induction of apoptosis of activated hepatic stellate cells. *Indian J Biochem Biophys* 2008; 45: 317-25.
22. Deepa PR, Varalakshmi P. The cytoprotective role of a low-molecular-weight heparin fragment studied in an experimental model of glomerulotoxicity. *Eur J Pharmacol* 2003; 478: 199-205.
23. Günther A, Lübke N, Ermert M, Schermuly RT, Weissmann N, Breithecker A, Markart P, et al. Prevention of bleomy-

- cin-induced lung fibrosis by aerosolization of heparin or urokinase in rabbits. *Am J Respir Crit Care Med*. 2003; 168: 1358-65.
24. Tsukada S, Enomoto N, Takei Y, Hirose M, Ikejima K, Kitamura T, Sato N. Dalteparin sodium prevents liver injury due to lipopolysaccharide in rat through suppression of tumor necrosis factor-alpha production by Kupffer cells. *Alcohol Clin Exp Res* 2003; 27: 7-11.
 25. Assy N, Hussein O, Khalil A, Luder A, Szvalb S, Paizi M, Spira G. The beneficial effect of aspirin and enoxaparin on fibrosis progression and regenerative activity in a rat model of cirrhosis. *Dig Dis Sci* 2007; 52: 1187-93.
 26. Li W, Zhang J, Huang Q, Zhu H, Zhang X. Long-term administering low anticoagulant activity heparin can lessen rat hepatic fibrosis induced by either CCl4 or porcine serum injection. *Hepatol Res* 2006; 36: 115-23.
 27. Abdel-Salam OME, Baiuomy AR, Ameen A, Hassan NS. A study of unfractionated and low molecular weight heparins in a model of cholestatic liver injury in the rat. *Pharmacol Res* 2005; 51 :59-67.
 28. Shi J, Hao JH, Ren WH, Zhu JR. Effects of heparin on liver fibrosis in patients with chronic hepatitis B. *World J Gastroenterol* 2003; 9: 1611-4.
 29. Seval Y, Akkoyunlu G, Demir R, Asar M. Distribution patterns of metalloproteinase (MMP)-2 and-9 and their inhibitors (TIMP-1 and TIMP-2) in the human decidua during early pregnancy. *Acta Histochem* 2004; 106: 353-62.
 30. Alison MR, Islam S, Lim S. Stem cells in liver regeneration, fibrosis and cancer: the good, the bad and the ugly. *J Pathol* 2009; 217: 282-98.
 31. Ramadori I, Dudas J. Physiology and pathophysiology of liver inflammation, damage and repair. *J Physiol pharmacol* 2008; 59: 107-17.
 32. Sola S, Amaral J, Aranha M, Ster C, Rodrigues C. Modulation of hepatocyte apoptosis: cross-talk between bile acids and nuclear steroid receptors. *Curr Med Chem* 2006; 13: 3039-51.
 33. Donthamsetty S, Bhave US, Mitra MS, Latendresse JR, Mehendale HM. Nonalcoholic fatty liver sensitizes rats to carbon tetrachloride hepatotoxicity. *Hepatology* 2007; 45: 391-403.
 34. Zhang LM, Liu DW, Liu JB, Zhang XL, Wang XB, Tang LM, Wang LQ. Effect of naked eukaryotic expression plasmid encoding rat augments of liver regeneration on acute hepatic injury and hepatic failure in rats. *World J Gastroenterol* 2005; 11: 3680-5.
 35. Neubauer K, Saile B, Ramadori G. Liver fibrosis and altered matrix synthesis. *Can J Gastroenterol* 2001; 15: 187-93.
 36. Kikkawa Y, Mochizuki Y, Miner JM, Mitaka T. Transient expression of laminin α_1 chain in regenerating murine liver; restricted localization of laminin chains and nidogen-1. *Exp Cell Res* 2005; 305: 99-109.
 37. Yuan TX, Zhang JS, Zhang YE, Chen Q. Culture of rat liver Ito cells and the observation of inhibitory effect of heparin on Ito cells. *Shanghai Yíke Daxue Xuebao* 1996; 23: 90-3.
 38. Harada N, Okajima K, Uchiba M. Dalteparin, a low molecular weight heparin, attenuates inflammatory responses and reduces ischemia-reperfusion-induced liver injury in rats. *Crit Care Med* 2006; 34: 1883-91.