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Ferulic acid influences hepatic expression pattern of matrix metalloproteinases during alcohol and PUFA induced toxicity

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Abstract. – BACKGROUND AND OBJECTIVES: Alcoholic fibrosis and its end stage cirrhosis represent a major health problem worldwide. Liver fibrosis occurs when the rate of matrix synthesis exceeds matrix degradation. The degree of matrix remodeling depends on the ratio of active matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). The objective of the present work was to study the influence of ferulic acid, a polyphenolic compound, on the expression of MMPs and TIMPs during alcohol and heated polyunsaturated fatty acid (delta PUFA) induced liver toxicity in male albino Wistar rats.

MATERIALS AND METHODS: The levels of collagen, the activity of MMPs, the activity of TIMPs, the expression pattern of MMP were analyzed in liver.

RESULTS: The matrix metalloproteinase expression was found to be significantly increased in alcohol as well as delta PUFA treated rats and significantly decreased in alcohol + delta PUFA treated rats. The levels of TIMPs and the collagen were significantly increased in alcohol, delta PUFA and alcohol + delta PUFA groups. Administration of ferulic acid significantly decreased the levels of collagen, TIMPs and positively modulated the expression of MMPs.

CONCLUSIONS: Ferulic acid influences MMPs, TIMPs expression and effectively protects liver against alcohol and ΔPUFA induced liver fibrosis.

Key Words:
Ferulic acid, Liver fribrosis, Matrix metalloproteinase, Tissue inhibitor of matrix metalloproteinase, Collagen.

Introduction

Liver fibrosis is a common response to chronic liver injury that ultimately leads to liver cirrhosis. The development of fibrosis, and particularly cirrhosis, is associated with significant morbidity and mortality. Efficient and well tolerated antifibrotic drugs are lacking and current treatment is limited to liver transplantation. Hence, there is considerable imperative for the development of novel therapies for liver fibrosis[1]. Pathological accumulation of extracellular matrix (ECM) components especially collagen is a characteristic feature of fibrosis. The progressive fibrosis occurs when the rate of matrix synthesis exceeds matrix degradation. Matrix metallo proteinases (MMPs) are a family of structurally and functionally related zinc endopeptidases, which are capable of degrading all kinds of ECM protein components and are implicated in remodelling of tissues during fibrosis. Normally the degradative potential of MMPs is held in check by endogenous specific inhibitors, tissue inhibitors of metalloproteinases (TIMPs)[2]. Disruption of MMP/TIMP balance can lead to excessive accumulation of ECM components resulting in severe liver injury[1].

The MMPs are synthesized as inactive zymogens and activated by proteinase cleavage. They play a central role in many biological processes, such as embryogenesis, normal tissue remodelling, wound healing, angiogenesis, and in diseases such as atheroma, arthritis, cancer, and tissue ulceration[3]. Matrix degradation is carried out by a fine balance between activities of proteinases and their inhibitors. Controlled and limited proteolytic degradation of the ECM is essential for normal development, and maintenance of tissue homeostasis[4].

Deregulation of MMPs is associated with many degenerative diseases. Compelling evidence has documented the association of MMPs with liver fibrosis[5]. MMPs are expressed during the liver fibrogenesis and its expression increases with the progression of fibrosis. Hence, MMPs are considered as potential markers for assessing liver toxicity[5].

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Alcoholic liver disease is one of the most serious medical consequences of chronic alcohol abuse. Chronic alcohol consumption along with fried food can lead to wide range of liver disorders. A high fat diet primes the toxic effects of alcohol. Previous studies from our laboratory have demonstrated the toxic effects of ethanol and ΔPUFA (Δpolyunsaturated fatty acids)[6,7].

Ferulic acid, a natural phenolic compound, receives greater attention in research world due to its effective antioxidant properties. It is a ubiquitous plant constituent that arises from the metabolism of phenylalanine and tyrosine. Like other monophenolic compounds, ferulic acid is effective in scavenging free radicals[8]. Its protective effect on oxidative stress and hyperlipidemia during alcohol and ΔPUFA induced liver toxicity has already been proven in our laboratory[6]. Current study focuses on the influence of ferulic acid on MMPs, TIMPs and collagen levels- the fibrotic index, during alcohol and ΔPUFA induced hepatotoxicity.

Materials and Methods

Animals

Male Wistar albino rats strain of body weight ranging 140-160g bred in Central Animal House, Rajah Muthiah Medical College, Tamil Nadu, India, fed on standard pellet diet (Agro Corporation Private Limited, Bangalore, India) were used for the study and water was given *ad libitum.* The standard pellet diet comprised 21% protein, 5% lipids, 4% crude fibre, 8% ash, 1% calcium, 0.6% phosphorus, 3.4% glucose, 2% vitamin and 55% nitrogen free extract (carbohydrates). It provides metabolisable energy of 3600Kcal.

The animals were housed in plastic cages under controlled condition of 12 h light/ 12 h dark cycles, 50% humidity and at 30 ± 2°C. The animals used in the present study were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council for Medical Research, Hyderabad, India and approved by the Animal Ethical Committee, Annamalai University.

Materials Used

Ethanol: Absolute ethanol (AR) was obtained from Hayman Private Limited, Witham, England.

Heated PUFA (ΔPUFA): Sunflower oil (Gold Winner) was subjected to heating at 180° C twice to produce heated (thermally oxidized) PUFA.

Ferulic acid: Ferulic acid was obtained from Sigma Chemical Company, Saint Louis, MO, USA.

TIMP-1 and TIMP-2 antibodies and secondary antibodies were obtained by Sigma Chemical Company, Saint Louis, MO, USA.

Experimental Design

The animals were divided into 8 groups of 6 rats each.

Group 1 (Normal): Control rats.
Group 2 (Alcohol): Rats given 20% ethanol [7.9 g/kg body weight][6] orally using an intragastric tube
Group 3 (ΔPUFA): Rats given high fat diet [15% heated sunflower oil] mixed with the diet
Group 4 (Alcohol + ΔPUFA): Rats given 20% ethanol + 15% heated sunflower oil.
Group 5 (Alcohol + FA): Rats given ferulic acid [20 mg/kg body weight] dissolved in 20% ethanol
Group 6 (ΔPUFA + FA): Rats given 15% heated sunflower oil + ferulic acid [20 mg/kg body weight] dissolved in distilled water
Group 7 (Alcohol+ ΔPUFA + FA): Rats given 15% heated sunflower oil + ferulic acid [20 mg/kg body weight] dissolved in 20% ethanol.
Group 8 (FA): Rats given ferulic acid [20 mg/kg body weight] dissolved in distilled water orally using an intragastric tube

The rats were maintained on isocalorific diet using glucose solution (total calories/day: 508K Cal/kg Body wt.). At the end of the experimental period of 45 days, rats were killed by cervical decapitation and liver tissue was removed, cleared off blood and immediately transferred to ice cold containers containing 0.9% NaCl for various experiments.

Biochemical Investigations

The liver was weighed and homogenized in appropriate buffer (10%) for the estimation of various biochemical parameters. The collagen was estimated by the method of Woessner[9]. Total MMP activity was assessed by multiwell zymography[10] and individual MMP expression was analysed by gelatin zymography[11]. Densitometric analysis was carried out using Gel/Chemi Doc. BioRad (Bio-Rad, Hercules, CA, USA) using software quantity one. MMP-2 and MMP-9, the major tissue remodeling gelatinases, were estimated by succinylation method[12]. The TIMP-1 and TIMP -2 were estimat-

ed by ELISA using antibodies in a VersaMax ELISA Microplate Reader (Molecular Devices Inc., Sunnyvale, CA, USA)[13].

Processing of Tissue for Histopathology

For histopathological study, two animals from each group were perfused with 10% formalin and the tissues were separated and stored in the same. They were later sectioned using a microtome, dehydrated in graded alcohol, embedded in paraffin section and stained with haematoxylin and eosin (H&E).

Statistical Analysis

Data were expressed as Mean ± S.D. Significant difference between groups was analyzed by analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). $p \leq 0.05$ was considered to be statistically significant.

Results

Levels of Collagen

Figure 1 shows the levels of hydroxyproline and Figure 1A gives the levels of collagen. Both the levels of collagen and hydroxyproline were increased significantly in alcohol, ΔPUFA and alcohol + ΔPUFA groups. On treatment with ferulic acid (FA), there was a significant decrease. FA control group showed no significant change in the levels of hydroxyproline and collagen compared to normal.

Total Activities of MMPs

Figure 2 shows multiwell zymogram of the liver samples and Figure 2A gives the densitometric reading of the same. From the figures we could infer that the total activities of MMPs were increased significantly in alcohol and ΔPUFA groups when compared to normal and significantly decreased in alcohol + ΔPUFA group. In alcohol + FA and in ΔPUFA + FA groups, there was a significant decrease in MMP activities and in alcohol + ΔPUFA + FA there was a significant increase, when compared to untreated ones (groups 2, 3 & 4 respectively). FA treated group showed no change in MMP activities when compared to normal. The intensity of the light color is directly proportional to the activity of matrix metalloproteinases.

Expression Pattern of MMPs

Figures 3 and 4 gives gelatin zymogram of liver samples and IIIA & IVA gives the densitometric reading of the same. Four different kinds of MMPs; 130 KD, 92 KD, 72 KD & 45 KD were found to be expressed during alcohol and ΔPUFA ingestion and subsequent ferulic acid treatment. The activities of all four MMPs were found to be increased in alcohol and ΔPUFA groups and decreased in alcohol + ΔPUFA group when compared to normal. Treatment of FA to both alcohol and ΔPUFA groups significantly decreased the MMP activities whereas FA treatment to alcohol + ΔPUFA significantly improved the MMP activities when compared to corresponding untreated groups. FA control group showed no significant alterations in the pattern when compared to normal. The intensity of the light color is directly proportional to the activity of matrix metalloproteinases.

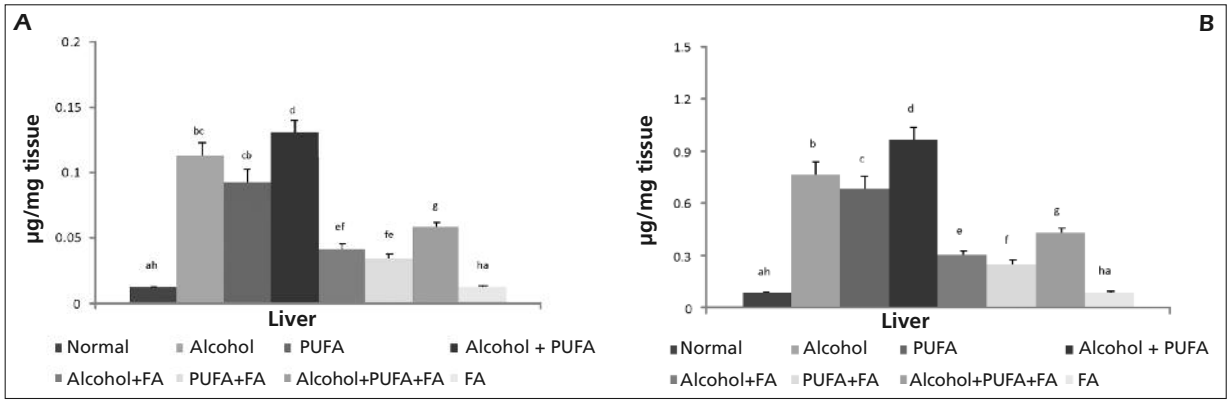

Figure 1. ***A,*** Levels of hydroxyproline. Values are Mean ± S.D. from 6 rats in each group. ANOVA followed by DMRT was used for comparison between the groups. Values not sharing a common superscript differ significantly at $p \leq 0.05$. ***B,*** Levels of collagen. Values are Mean ± S.D. from 6 rats in each group. ANOVA followed by DMRT was used for comparison between the groups. Values not sharing a common superscript differ significantly at $p \leq 0.05$.

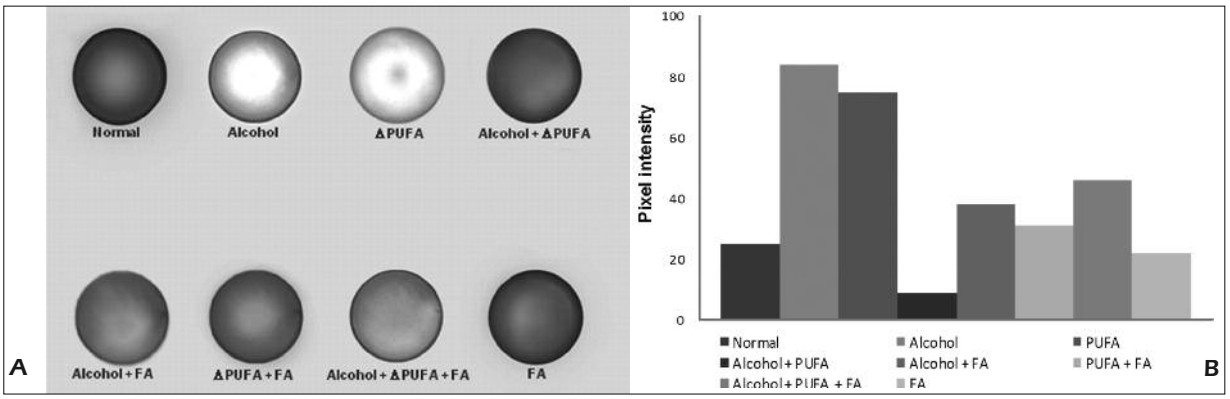

Figure 2. ***A,*** Multiwell zymogram of liver samples of FA treated rats. The intensity of the light color is directly proportional to the activity of matrix metalloproteinases. ***B,*** Densitometry of the multiwell zymogram of liver samples of FA treated rats.

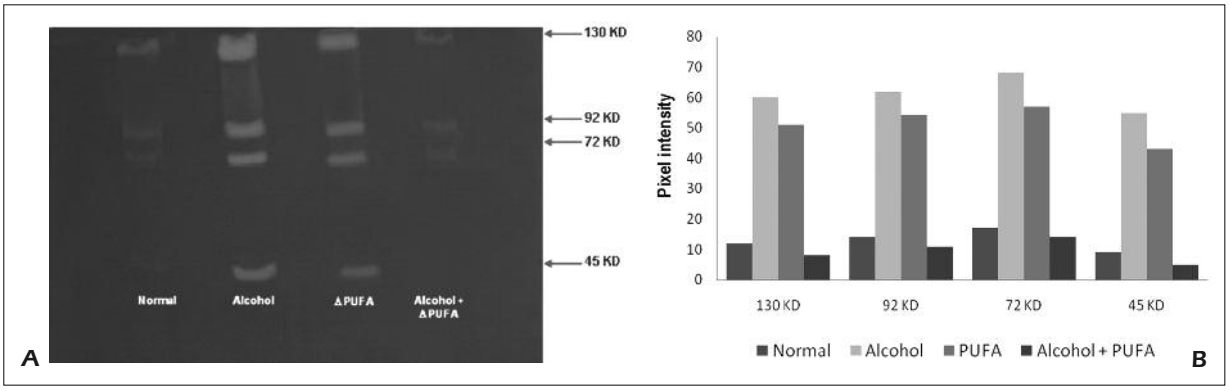

Figure 3. ***A,*** Zymogram of liver samples in normal and in alcohol and/or ΔPUFA toxicity. The intensity of the light color indicates extent of cleavage of gelatin by MMPs. ***B,*** Densitometry of the zymogram of liver samples in normal and in alcohol and/or ΔPUFA toxicity.

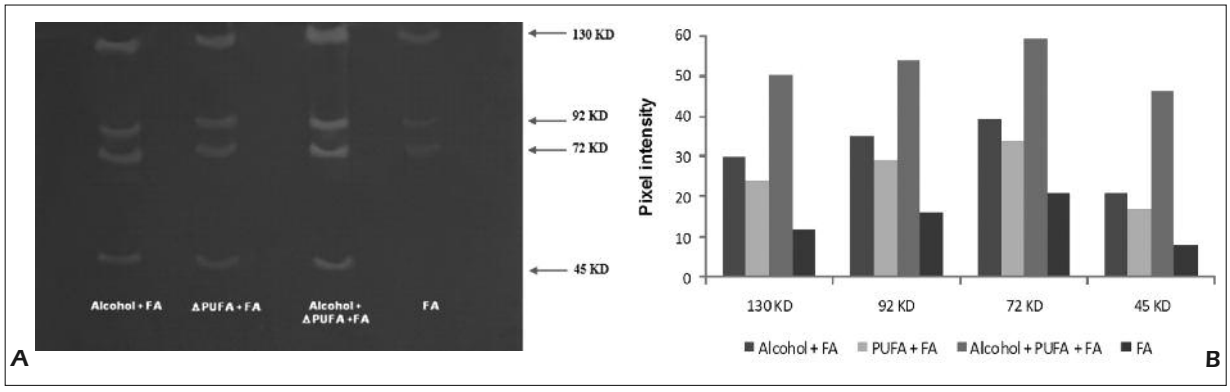

Figure 4. ***A,*** Zymogram of liver samples during FA treatment. The intensity of the light color indicates extent of cleavage of gelatin by MMPs. ***B,*** Densitometry of the zymogram of liver samples during FA treatment.

Activities of MMP-2 and MMP-9

Figure 5 shows the changes in the activities of MMP-2 (72 KD) and MMP-9 (92 KD). The activities of MMP 2 and MMP 9 were significantly increased in alcohol and ΔPUFA groups and decreased in alcohol + ΔPUFA group when compared to normal. On treatment with FA there was a significant decrease in MMP activities in alco-

hol and ΔPUFA groups and significant increase in alcohol + ΔPUFA groups compared to corresponding untreated groups.

Changes in the Levels of TIMP-1 and TIMP-2

The levels of both TIMP-1 and TIMP-2 were increased significantly in alcohol, ΔPUFA and alcohol + ΔPUFA groups (Figure 6). Administration of FA significantly depleted the levels of TIMPs. FA control group showed no significant change in TIMPs activities compared to normal.

Discussion

Liver fibrosis results from chronic damage to the liver characterised by excessive accumulation of ECM proteins. The accumulation of ECM proteins distorts the hepatic architecture by forming a fibrous scar, and the subsequent development of nodules of regenerating hepatocytes[1]. In the acute phases of liver injury, as liver fibrosis progresses, there is increased expression of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs)[2]. In the present study, we estimated the levels of collagen, MMPs and TIMPs in alcohol and ΔPUFA induced toxicity and subsequent treatment.

In this study, the levels of collagen and the expression of the MMPs were found to be increased in alcohol and ΔPUFA treated groups. The increased expression of MMPs may be for breaking down and repair the excessive collagen that is deposited during alcohol and ΔPUFA intake. Ethanol causes a number of physiological and biochemical changes in liver which include formation of toxic byproduct (acetaldehyde)[14], increasing the cellular redox state, induction of cytochrome P450 2E1 (CYP2E1)[15], formation of 1-hydroxyl ethyl free radical and endotoxin derived Kupffer cells[16], which in turn produces tumour necrosis factor[17]. The net effect is a vicious cycle of damage and progression of disease leading to increased activation of hepatic stellate cells (HSC) and increased accumulation of collagen resulting in fibrosis. Heated PUFA causes increased susceptibility of membrane to lipid peroxidation and due to toxic metabolites produced during heating of PUFA, there is an increased damage of membrane and fibrosis[18]. Hence, the levels of collagen were increased during alcohol and ΔPUFA ingestion.

We can give two possible explanations for increased activity of MMPs during alcohol and ΔPUFA ingestion. First, MMPs over expression may be induced by the increased amount of type IV collagen and other matrix proteins with compensatory homeostatic mechanism against excessive deposition of matrix compounds. Second explanation is the induction of MMPs by cytokines transforming growth factor β and tumor necrosis factors (TGF β and TNF α) and other inflammatory mediators during liver injury[19].

The level of expression of MMPs was decreased in alcohol + ΔPUFA treated groups compared to

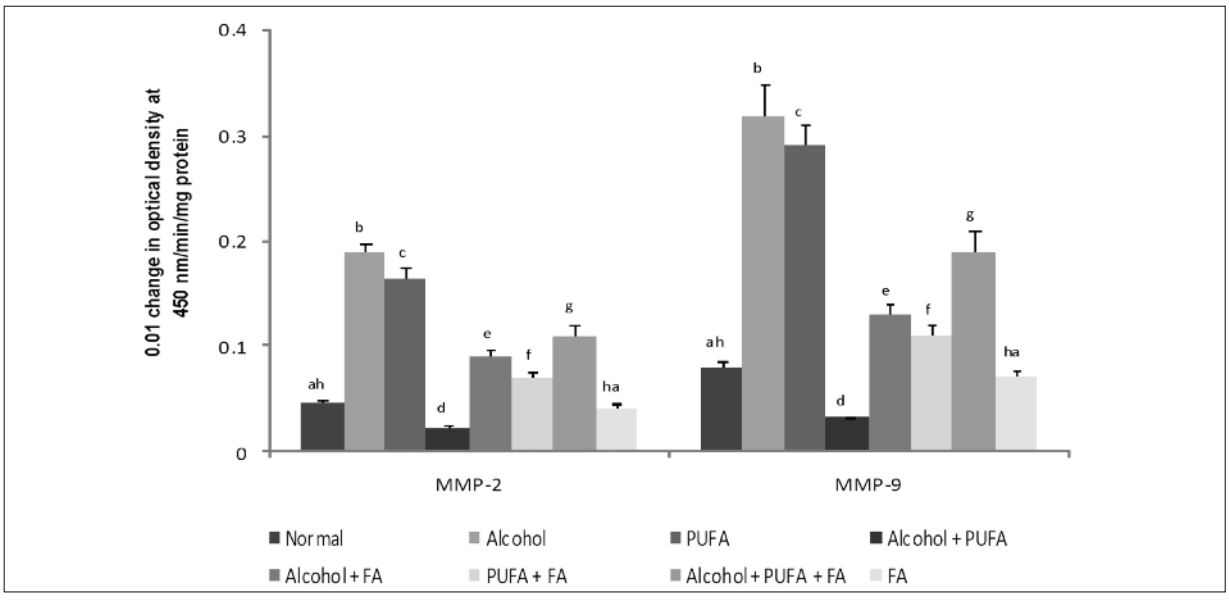

Figure 5. Activities of MMP-2 and MMP-9. Values are Mean ± S.D from 6 rats in each group. ANOVA followed by DMRT was used for comparison between the groups. Values not sharing a common superscript differ significantly at $p \leq 0.05$.

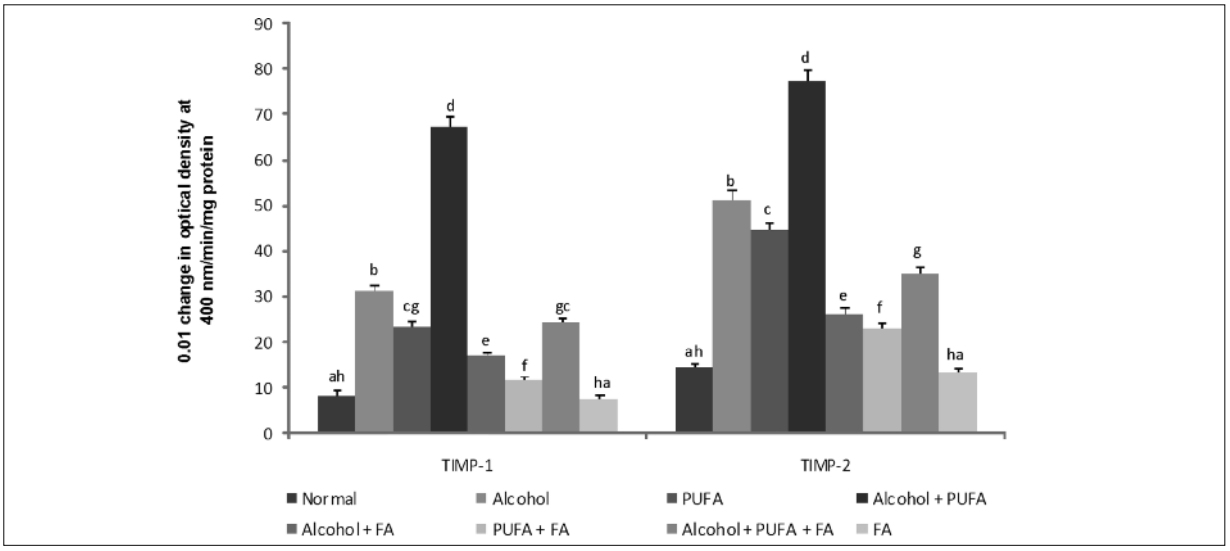

Figure 6. Levels of TIMP-1 and TIMP-2. Values are Mean ± S.D from 6 rats in each group. ANOVA followed by DMRT was used for comparison between the groups. Values not sharing a common superscript differ significantly at $p \leq 0.05$.

alcohol and ΔPUFA treated groups. Intake of alcohol along with fried foods increases the toxicity of the liver. This in turn causes increased induction of CYP2E1 and severe liver injury. The decreased expression of MMPs in alcohol + ΔPUFA group suggests final stage of liver cirrhosis. Possible explanations for this include decreased procollagen gene expression and biosynthesis, decreased activation of pro MMPs or specific inhibition of native MMPs[20]. It has reported that following a single toxic liver injury, MMPs increase during early phase of liver injury, maximum during inflammation and diminish at end stage of fibrosis[5]. From this we can conclude that increased MMPs level in alcohol and ΔPUFA in our study correlates with inflammation, whereas decreased MMPs level in alcohol + ΔPUFA describes advanced fibrosis.

In our study we observed increased levels of TIMPs in alcohol, ΔPUFA and in alcohol + ΔPUFA treated rats which indicates the progression of fibrosis. TIMPs inhibit the action of collagen degradation enzyme and play a key role in increased ECM deposition. An increase of TIMPs expression is discussed as one of the pathogenic mechanisms of liver fibrosis[2].

Administration of ferulic acid significantly decreased the fibrotic changes by decreasing the levels of collagen and TIMPs and positively modulating the activities of MMPs in alcohol, DPUFA and alcohol+DPUFA groups. This can be attributed to effective antioxidant property of the drug. Ferulic acid is able to scavenge a variety of ROS that are produced during pathological conditions[8], and thus prevent the activation of stellate cells by reactive oxygen species (ROS). Moreover phenolic compounds are known to inhibit the inflammatory cytokines: tumor necrosis factors and interleukins[21]. Ferulic acid being a phenolic compound might inhibit such molecules which are involved in fibrosis formation. It has been proven that ferulic acid protects liver through reduction of oxidative damage and inflammatory signaling pathways[8].

The expression of MMPs was decreased in alcohol+DPUFA group. This might be due to their increased utilization as a result of severe hepatic injury and thus depleting their levels. Studies have demonstrated that there is a decrease in MMP activities in the later progressive stage of liver fibrosis[22]. This might be due to increased expression of MMP inhibitors and other antiproteases which steadily overwhelm the effects of MMPs. In the present study it can be observed that in alcohol + DPUFA group, the activities of MMPs declined in response to increased TIMPs levels. Thus in case of alcohol + ΔPUFA, ferulic acid might act by reducing markers of HSC activation (collagen and TIMPs) and by promoting fibrillar matrix degradation through elevation of MMPs activity.

Histopathological observations showed significant pathological changes in the liver consistent with the results. In our study, in alcohol administered rats we observed sinusoidal dilatation, congestion and thickening of blood vessels, characteristics of extensive oxidative stress induced damage and microvesicular type fatty changes, the main

feature of alcoholic fatty liver. Changes occurred in this group were similar in severity to that described previously[23,24]. ΔPUFA treated rats showed vascular congestion, portal inflammation and fatty changes around central vein, which can be clearly attributed to the oxidative stress induced by ΔPUFA. Increased ingestion of PUFA alters the fatty acid composition of membrane[25], thereby making them more susceptible to lipid peroxidation.

Conclusions

In light of the results obtained, it appears that ferulic acid acts as antifibrotic agent primarily by inhibiting the activation of stellate cells during early stage of damage and by deregulating collagen, TIMPs and promoting matrix degradation during severe stage of injury. These qualities are ideal for the fibrosis reversing agents. Hence, ferulic acid can be used as effective antifibrotic agent for liver fibrosis.

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