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The Potential Antifibrotic Effect of AMPK Activator(s) in Experimentally-Induced Hepatic Fibrosis: An Invivo Animal Study

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ABSTRACT

Background: Liver fibrosis is a serious health problem that might cause liver cirrhosis and hepatocellular carcinoma. Unfortunately, successful antifibrotic treatment is not yet available in clinical use. Interestingly, activation of adenosine monophosphate-activated protein kinase by phosphorylation (p-AMPK) has been known to attenuate liver fibrosis. Aspirin has a well-known antipyretic, antioxidant, and anti-inflammatory properties. Newly, it has also been recognized as an AMPK activator. Therefore, aspirin is worth to be currently investigated by evaluating its potential antifibrotic activity in CCl4-induced hepatic fibrosis in mice besides the possible mechanisms such as oxidative stress, inflammation, and AMPK activation.

Methods: Aspirin was administered orally at two doses (10 and 100 mg/kg) and silymarin was given as a reference agent at (100 mg/kg), three times weekly for 6 weeks. Liver fibrosis was induced using intraperitoneal CCl4 injection (0.5mL/kg in corn oil 1:4) two times weekly for 6 weeks. Then, hepatotoxicity parameters, histopathological findings, oxidative stress, inflammatory markers, p-AMPK concentration were assessed.

Results: CCl4 significantly increased serum hepatic aminotransferases, total bilirubin, total cholesterol, and triglycerides levels. This was accompanied by histopathological changes, significant depletion of reduced glutathione (GSH) content, increased lipid peroxidation and decreased catalase

(CAT) enzyme activity and a dramatic increase of inflammatory cytokines accompanied by decreased p-AMPK concentration. Conversely, aspirin co-treatment showed significant improvement in the histological abnormalities and hepatotoxicity parameters. Furthermore, aspirin resulted in significant amelioration of CCl4-induced oxidative stress as evidenced by restoring GSH content and CAT activity while reducing lipid peroxidation. Moreover, aspirin has a potent anti-inflammatory effect as verified by a significant decrease of proinflammatory cytokines as tumor necrosis factor and interleukins (IL-1β and IL-6). Furthermore, aspirin significantly enhanced AMPK activity compared with CCl4-intoxicated group.

Conclusion: Aspirin possesses a potential antifibrotic effect, which could be attributable – at least - to its antioxidant, anti-inflammatory, and AMPK activating properties.

Introduction

A global burden of disease (GBD) study estimates for liver cirrhosis reported that over 2 million deaths annually are caused by liver cirrhosis [1]. In 2015, there were more than (8 x 105) deaths from liver diseases worldwide. Therefore, liver diseases represent a considerable public health burden [2]. KSA, liver diseases showed 743 deaths and the need to perform liver transplants was 150 cases annually [3]. Liver fibrosis, which represents the common end of the majority of chronic liver diseases, is the process of the accumulation of fibrous connective tissue called the extracellular matrix (ECM) formed during inflammatory injury. In fact, it is a reversible process that is involved in almost all liver diseases. However, it may be transformed into irreversible liver cirrhosis, portal hypertension, and liver failure that usually necessitate liver transplantation [4].

Interestingly, studies showed that liver fibrosis could be reversed by cessation of liver injury and treating the underlying factors, such as biliary obstructions, fatty liver diseases, chronic alcoholism, Wilson's disease, cystic fibrosis, hemochromatosis, autoimmune hepatitis, medications such as methotrexate and antiepileptic drugs [5]. In addition, complete eradication of hepatitis C virus by interferon-α and antiviral drugs could result in fibrosis improvement [5]. When mild hepatocyte damage occurs with an intact fibrous framework of the lobules, regeneration happens, and the lobular pattern is preserved. In severe damage with a destroyed framework of lobules, liver cells are regenerated in the form of new nodules of liver tissue called regeneration nodules, as in liver cirrhosis [6]. In addition, chronic hepatic injury leads to progressive fibrosis as a compensatory mechanism, hindering normal hepatic function through fibrous tissue formation, which is considered a life-threatening condition [5].

Oxidative stress and inflammation were reported in all fibrotic disorders with chronic damage and remodelling. During liver injury, damaged hepatocytes release oxidants from their raptured mitochondria, leading to hepatocellular necrosis and the activation of inflammatory and profibrogenic mediators [7]. In this regard, acetylsalicylic acid (ASA, aspirin) is a weak organic acid belonging to the nonsteroidal anti-inflammatory drugs (NSAIDs) that is rapidly transformed into its active metabolite "salicylate". Both ASA and salicylate demonstrated strong antioxidant and anti-inflammatory activities through traditional inhibition of the cyclooxygenase (COX) enzyme [8]. It is an old drug having a new investigation as adenosine monophosphate-activated protein kinase (AMPK) activator activity [9,10,11,12] which could be a protective pathway against liver fibrosis [5]. Remarkably, targeting oxidative stress and inflammation has been reported to ameliorate experimental liver fibrosis [13].

The current study was carried out to evaluate the potential antifibrotic activity of ASA in carbon tetrachloride (CCl4) -induced hepatic fibrosis, besides the possible causal mechanisms with respect to oxidative stress, inflammation, and AMPK activity.

Methods and Materials

Ethical Considerations and Study Settings

This study protocol was registered, revised, and approved by the Unit of Biomedical Ethics and Research Committee of the Faculty of Medicine, King AbdulAziz University, Jeddah, Saudi Arabia. The study approval was obtained on October 28, 2018 and was given reference # 568-18. The study was conducted on 48 male albino SWR/J mice weighing 30-35 g, obtained from the animal house of King Fahd Medical Research Centre (KFMRC), housed and maintained in an air conditioned atmosphere at 22 ± 2°C, under a 12 hr light/dark cycle and provided with rodent diet and water ad libitum.

Study Design and Animal Grouping

Forty-eight male albino mice were randomly divided into six groups (8 animals/group) and treated for six weeks, ***Figure 1***. Group 1 served as the control and received corn oil (2 mL/kg i.p.) twice weekly and the cosolvent (propylene glycol: tween 80: water = 4:1:4) orally three times weekly on alternating days. Dosing volume of cosolvent was kept at 10 mL/kg animal weight [14]. Group 2 was injected CCl4 (0.5 mL/kg, 1:4 mixture with corn oil; i.p.) twice weekly [15] and the cosolvent (10 mL/kg). Group 3 was administered both CCl4 (0.5 mL/kg, 1:4 mixture with corn oil; i.p.) twice weekly and silymarin dissolved in the cosolvent at a dose of 100 mg/kg p.o. three times weekly on alternating days [16] to experimentally-induce liver fibrosis [17]. In groups 4 and 5, animals were administered both CCl4 twice weekly and aspirin dissolved in cosolvent at doses of 10 and 100 mg/kg, respectively, by oral gavage, three times weekly on alternating days. Group 6 (Aspirin alone) was given a high dose of aspirin (100 mg/kg p.o.) three times weekly and i.p. corn oil (2 mL/kg) two times weekly. The administered volume of propylene glycol (< 10 mL/kg) following the recommendations of Thackaberry et al., 2010 [18] and Gad et al., 2016 [19] to avoid any kind of toxicity or hepatoprotection. All drug solutions were freshly prepared before starting the experiments. All tested drugs and chemicals used are presented in ***Table 1***.

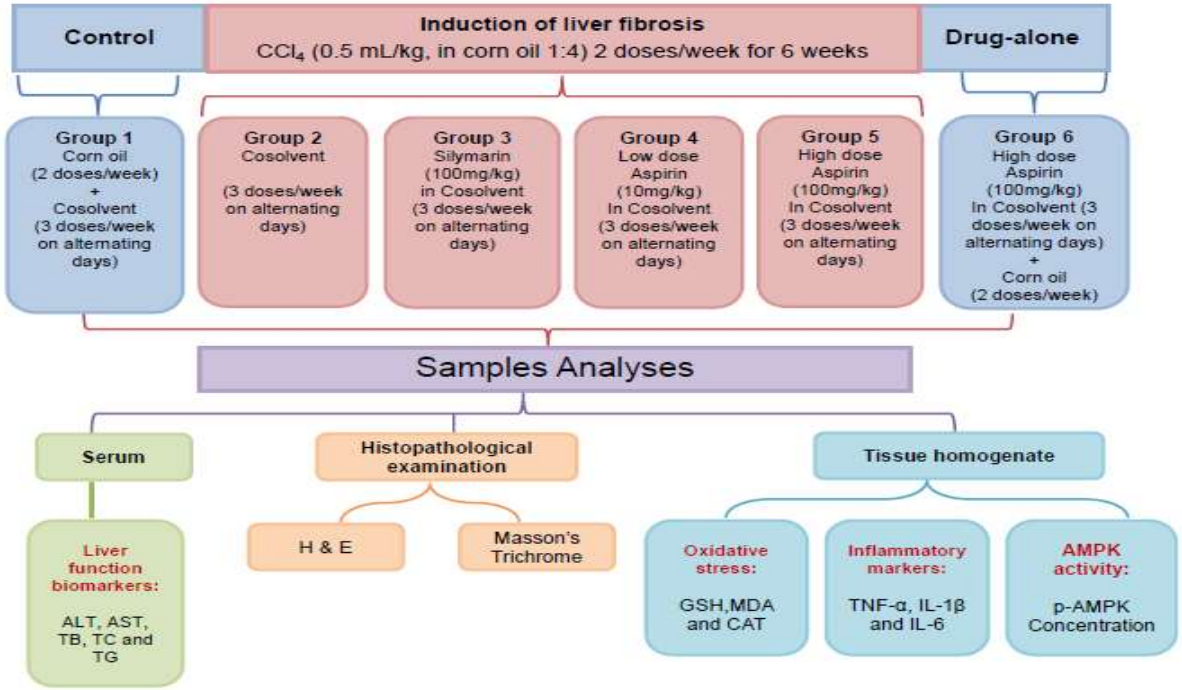

Figure 1: Illustrating the Grouping of studied Animals

Weight measurements were taken at the beginning of each week, and doses were adjusted accordingly. In order to rule out food interaction, food was withheld for 4 hours prior to oral gavage as well as 1 hour after gavage. For all groups, treatments by oral gavage were done on alternating days with CCl_4 or corn oil injection, and there were two days off every week. Also, mice were inspected one hour after each dose for abnormalities or signs of distress.

Table 1: Materials Used in the Study

Drugs/Chemicals	Manufacturer Details
Aspirin "Acetyl salicylic acid"	Sigma-Aldrich Co., St Louis (Cat. # A2093, MO, USA)
Silymarin	Sigma-Aldrich Co., St Louis (Cat. # S0292, MO, USA)
Carbon Tetrachloride	Sigma-Aldrich Co., St Louis (Cat. # 289116, MO, USA)

Outcome Meassures

After six weeks, mice were anesthetized, and blood samples were collected from the retro-orbital plexus and allowed to clot. Sera were separated by centrifugation and used for the assessment of liver functions. Mice were then euthanized by cervical dislocation, and livers were rapidly dissected and washed with ice-cold saline. Representative hepatic tissues from 2 animals per group were kept in 10% formalin saline for histopathological examination. The remaining liver tissues were stored at -80 °C until biochemical analyses. Aliquotes from the frozen liver tissues were homogenized in ice-cold phosphate buffer solution (pH 8.0, 0.1 M) at 1:5 (w/v), using IKA T-25 Ultra-Turrax Digital High-Speed Homogenizer (Cole-Parmer Ltd., Mumbai, India) to obtain 20% homogenate just before analysis. The tested outcomes and kits used are summerized in ***Table 2***.

Table 2: Tested Outcomes and Kits Used

Outcome Measure	Kit
Liver Functions Serum Tests	
Alanine aminotransferase (ALT)	Alanine aminotransferase (ALT) kit: (Bio-diagnostic, Giza, Egypt) Catalog No.:AL1031
Aspartate aminotransferase (AST)	Aspartate aminotransferase (AST) kit: (Bio-diagnostic, Giza, Egypt) Catalog No.: AS1061
Total bilirubin (TB)	Total bilirubin (TB) kit (Bio-diagnostic, Giza, Egypt) Catalog No.: BR1111

Total cholesterol (TC)	Total cholesterol (TC) kit (Bio-diagnostic, Giza, Egypt) Catalog No.: CH1220
Triglycerides (TG)	Triglycerides (TG) kit (Bio-diagnostic, Giza, Egypt) Catalog No.: TR2030
Histopathological Examination of Hepatic Tissue	
Histopathologic Examination	Histopathologic Examination Hematoxylin and eosin (H & E), and Masson's trichrome.
Oxidative Stress	
Reduced Glutathione (GSH) Content	Biogiagnostics Catalog no. CR2510 (Giza, Egypt).
Lipid Peroxidation (MDA) Content	Biogiagnostics Catalog no. MD2528 (Giza, Egypt).
Catalase (CAT) Activity	Biogiagnostics Catalog no. CA2516 (Giza, Egypt).
Inflammatory Markersin Hepatic Homogenate	
TNF-α Concentration	Mouse TNF-α (Tumor Necrosis Factor Alpha) ELISA Kit (Elabscience Biotechnology Co., MO, USA) Catalog No.: E-EL-M0049
Interleukin-6 (IL-6) Concentration	Mouse IL-6 (Interleukin 6) ELISA Kit (Elabscience Biotechnology Co., MO, USA) Catalog No.: E-EL-M0044
Interleukin-1beta (IL-1β)	Mouse IL-1β (Interleukin 1 Beta) ELISA Kit (Elabscience Biotechnology Co., MO, USA) Catalog No.: E-EL-M0037
Phospho-Adenosine Monophosphate Activated Protein Kinase (p-AMPK) Concentration in Hepatic Homogenate	
p-AMPK	Mouse p-AMPK (Phosphorylated Adenosine Monophosphate Activated Protein Kinase) ELISA Kit (Elabscience Biotechnology Co., Mo, USA) Catalog No.: E-EL-M1361

Statistical Analyses

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's as a post-hoc test. Data are presented as mean ± SD and the level of significance was 0.05. All statistical analyses were performed using GraphPad Instat® software package (version 3.06). While GraphPad Prism® software (version 5) were used to sketch graphs (GraphPad software, LLC, San Diego, CA, USA).

Results

Liver Functions Parameters

As shown in ***Table 3***, serum levels of hepatic enzymes ALT and AST were significantly increased by four-folds in CCl4-challenged group compared to the control values. In addition, CCl4 raised significantly serum levels of metabolic biomarkers TB, TC and TG as compared to the control group. Co-administration of CCl4 with aspirin (10 and 100 mg/kg) were found to decrease the elevated levels of serum ALT and AST significantly when compared to CCl4-tested group. In particular, treatment with low dose aspirin (10 mg/kg) was found to significantly reduce the value of TB concentration but failed to make any significant changes in TC and TG levels as compared to CCl4-exposed group. However, treatment with high dose aspirin (100 mg/kg) resulted in significant decreases in TB, TC and TG levels in the corresponding CCl4-exposed group. Moreover, treatment with aspirin alone did not show any significant changes in hepatotoxicity parameters as compared to the control group.

Concurrent administration of the standard hepatoprotective drug "silymarin" with CCl4 was able to significantly ameliorate all hepatotoxicity markers, as compared to CCl4-exposed group.

Table 3 Effect of Aspirin on Hepatotoxicity Parameters in Mice Exposed to CCl4-induced Hepatic Fibrosis

	ALT (U/L)	**AST (U/L)**	**TB (mg/dL)**	**TC (mg/dL)**	**TG (mg/dL)**
Control	18.41± 2.95	31.98± 4.62	0.27 ± 0.04	74.21± 5.54	90.04 ± 7.08
CCl4 (0.5 mL/kg)	116.03a ±8.61	142.38a ±9.45	1.65a ± 0.13	153.08a ±9.63	196.21a ± 12
CCl4 + Sil. (100 mg/kg)	36.25a,b ±5.65	58.05a,b ±5.92	0.61 a,b± 0.08	95.41a,b ±7.83	124.45a,b± 6.69
CCl4 + ASA (10 mg/kg)	78.2a,b,c ±8.72	89.06a,b,c ±5.68	0.96a,b,c ±0.08	143.76a,c ± 6.89	183.98a,c ± 9.26
CCl4 + ASA (100 mg/kg)	41.06a,b ±4.40	65.46a,b ±4.69	0.67 a,b± 0.09	99.33a,b ± 7.71	140.45a,b,c± 6.85

ASA (100 mg/kg)	20.30b ± 3.73	30.94 b± 6.31	0.29b ± 0.03	76.44 b± 3.82	93.74b ± 7.94

Statistical analysis was carried out using one-way ANOVA followed by Tukey's as a post-hoc test

a: significantly different from the corresponding control at $p < 0.05$

b: significantly different from the corresponding CCl4-intoxicated group at $p < 0.05$

c: significantly different from the corresponding silymarin at $p < 0.05$

CCl4; carbon tetrachloride, Sil.; silymarin, ASA.; aspirin*Histopathological Examination of Hepatic Tissue*

Hematoxylin and Eosin Sections

The results showed that controls have exhibited normal histologic architecture, ***Figure 2A***, while the CCl4 challenged group has demonstrated dilated central vein with centrilobular congestion and degeneration in diffuse manner all over the hepatocytes and heavy inflammatory cells infiltration around portal vein as shown by arrows in ***Figure 2B***. Low dose aspirin group showed moderate dilatation of the central vein with centrilobular necrosis, ***Figure 2D***. Interestingly, sections taken from mouse liver treated with silymarin and a high dose of aspirin (100 mg/kg) presented restored hepatic parenchyma with an apparently normal histological structure of the central vein, except for some congestion in silymarin-treated mice, ***Figure 2C and 2E***. Aspirin only-treated group showed normal hepatocellular architecture, ***Figure 2F***.

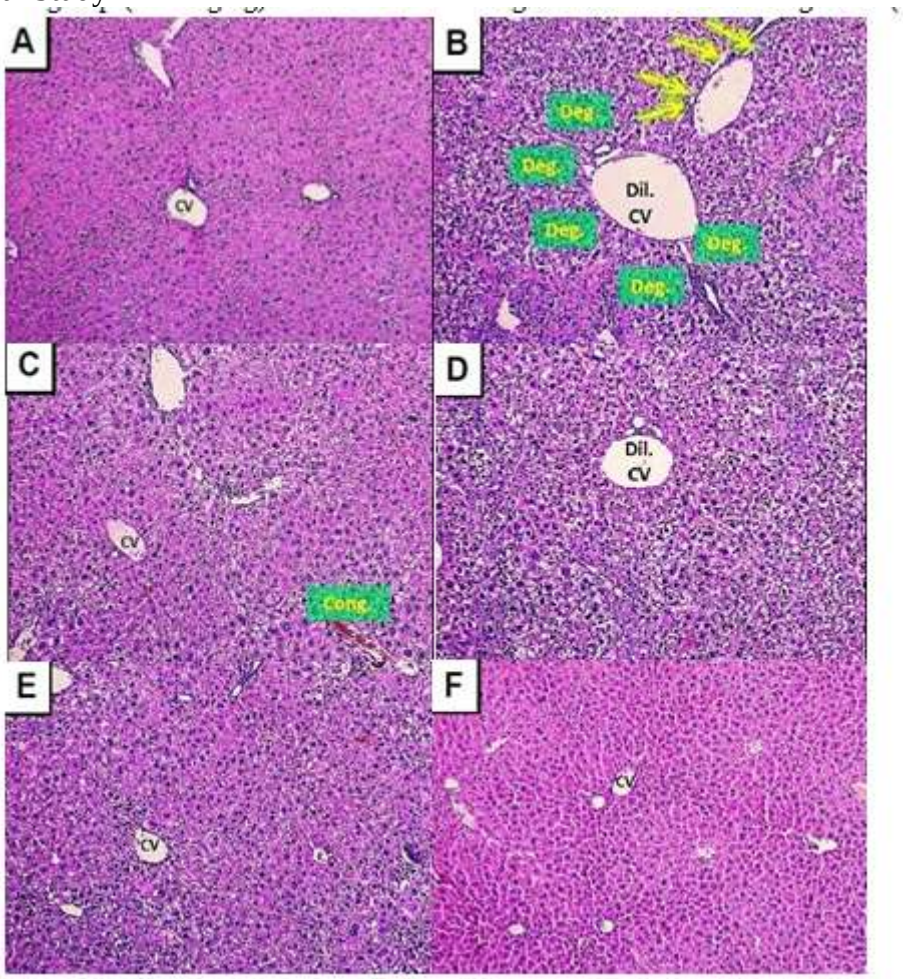

Figure 2 Representative photomicrographs of liver sections stained by H & E, X100 A) Control group showed normal hepatocellular architecture with normal histological structure of central vein. B) CCl4 group revealed dilated central vein with centrilobular degeneration (Deg.) all over the hepatocytes and extensive inflammatory cells infiltration around portal vein (arrows) c) silymarin-treated group (100 mg/kg) showed moderate degree of centrilobular congestion (Cong.) and degeneration. D) low dose aspirin-treated group (10 mg/kg) revealed dilated central vein with moderate centrilobular necrosis. E) high dose aspirin-treated group (100 mg/kg) revealed normal histological structure of central vein and hepatocytes. F) Aspirin only-treated group (100 mg/kg) showed normal hepatocellular architecture.

Masson's Trichrome sections:

Masson's trichrome staining was done to evaluate collagen distribution in the fibrotic liver, which is expressed in blue. All hepatic sections of the control and aspirin-only treated groups revealed a normal distribution of collagen fibers in the hepatic tissue , ***Figure 3A and 3F***. However, in the CCl4-exposed group, extensive deposition of collagen fibers around the central vein as well as interlobular was observed all over the hepatic tissue with dilated and congested central vein, ***Figure 3B***. Silymarin and low dose aspirin treated groups (10 mg/kg) revealed a moderate degree of collagen fibers distribution, ***Figure 3C and 3D***. Remarkably, mice that have been treated with a high dose of aspirin (100 mg/kg) demonstrated a minimal degree of collagen fibers in hepatic tissue, ***Figure 3E***.

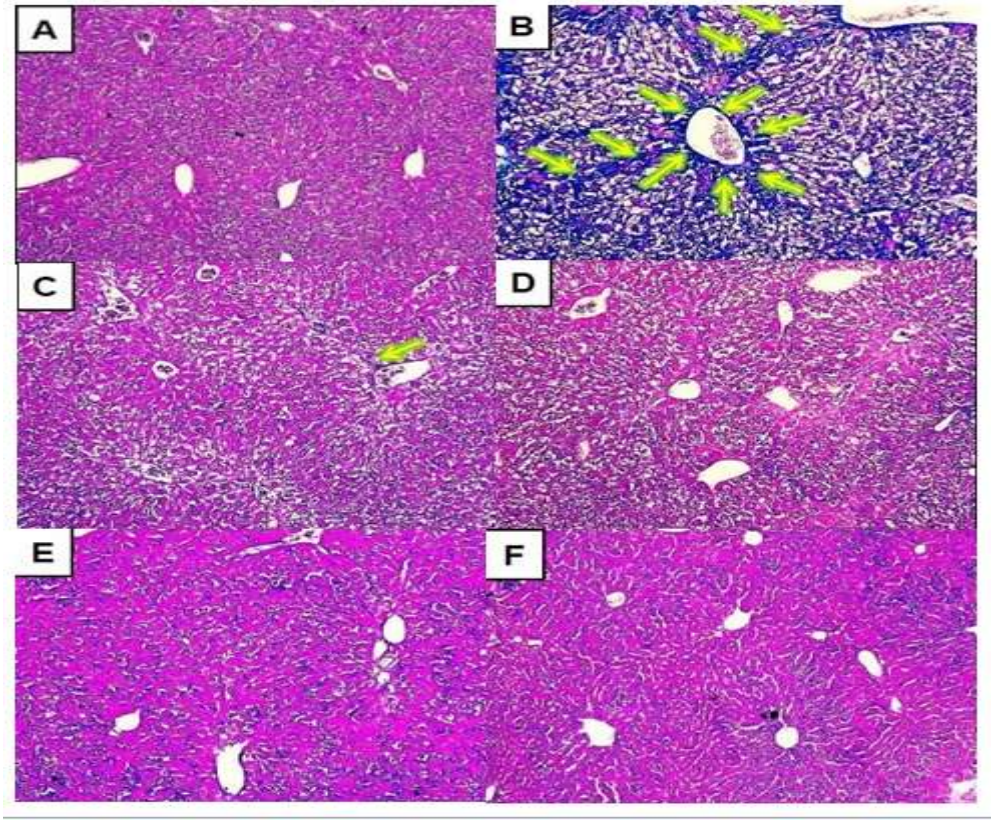

Figure 3 Representative photomicrographs of liver sections stained by Masson's trichrome X100 A) The control grshowed aowed normal distribution of collagen in hepatocytes. B) CCL4 group revealed extensive collagen deposition in diffuse manner all over hepatocytes with dilated and congested central vein (arrows). C) silymarin-treated group (100mg/kg) showed moderate degree of collagen fibers. D) low dose aspirin-treated group (10 mg/kg) revealed moderate degree of collagen fibers. E) high dose aspirin-treated group (100 mg/kg) revealed minimal degree of collagen fibers. F) Aspirin only-treated group (100 mg/kg) showed normal degree of collagen fibers.

Oxidative Stress Markers

Reduced Glutathione Content in Liver Tissue (GSH)

Results revealed that oxidative stress was evident in liver homogenate in CCl4-exposed mice, as assessed by severe depletion of GSH content when compared to control group. Conversely, concurrent treatment of aspirin (at 10 & 100 mg/kg) with CCl4 significantly improved GSH content in liver tissue as compared to CCl4 group, in a dose-related manner. Treatment with silymarin was able to normalize the GSH contents so that no significant change from the control group was detected. Similarly, aspirin-only treated group has shown normal GSH content when compared to control group, ***Table 4***.

Lipid peroxides concentration in liver tissue (MDA)

CCl4-exposed group presented significant 3-fold elevation of MDA concentration when compared to control group. In contrast, co-administration of aspirin (10 & 100 mg/kg) demonstrated significant decreases in MDA concentration by about 22.5% and 49.2%, respectively; as compared with CCl4-challenged group. On the other hand, silymarin-treated group was not statistically different than control group and was able to diminish the effect of CCl4 on MDA level by 71.2%, in comparison to the CCl4-exposed group, ***Table 4***.. Again, aspirin-only treated mice did not demonstrate any significant difference from the control group

Catalase enzyme activity in liver tissue (CAT)

Exposure to CCl4 resulted in a significant decrease in CAT enzyme activity by 53% compared to control animals. Low dose aspirin (10 mg/kg) did not show any significant difference in CAT activity compared to the CCl4 group, while high dose aspirin (100 mg/kg) revealed a significant increase in CAT enzyme activity by 41% as compared to CCl4-intoxicated mice. Notably, silymarin was able to increase CAT activity even higher than the control group. Furthermore, aspirin alone group demonstrated non-significant change from the control group, ***Table 4***.

Table 4 Effect of aspirin on liver contents of reduced glutathione (GSH), lipid peroxides (MDA) and catalase enzyme (CAT) activity in mice exposed to CCl4.

	GSH (nmol/mg pr)	**MDA (nmol/mg pr)**	**CAT (U/mg pr)**
Control	0.27 ± 0.03	25.33 ± 3.97	85.9 ± 6.48
CCl_4 (0.5 mL/kg)	0.03_{a} ± 0.01	76.25_{a} ± 5.10	40.21_{a} ± 5.63
CCl_4 + Sil. (100 mg/kg)	0.28_{b} ± 0.03	21.93_{b} ± 4.06	$98.26_{a,b}$ ± 7.23
CCl_4 + ASA (10 mg/kg)	$0.08_{a,b,c}$ ± 0.01	$59.1_{a,b,c}$± 3.72	$46.33_{a,c}$ ± 3.67
CCl_4 + ASA (100 mg/kg)	$0.14_{a,b,c}$ ± 0.01	$38.53_{a,b,c}$ ± 4.14	$56.78_{a,b,c}$ ± 6.04
ASA (100 mg/kg)	0.27_{b} ± 0.02	26.96_{b} ± 3.04	76.35_{b} ± 6.23

Statistical analysis was carried out using one-way ANOVA followed by Tukey's as a post-hoc test

a: significantly different from the corresponding control at $p < 0.05$

b: significantly different from the corresponding CCl4-challenged group at $p < 0.05$

c: significantly different from the corresponding silymarin at $p < 0.05$

CCl4; carbon tetrachloride, Sil.; silymarin, ASA.; aspirin, pr; protein

Inflammatory markers

Tumor necrosis factor (TNF-α) concentration in hepatic homogenate

As shown in ***Table 5***, CCl4 significantly elevated TNF-α concentration by 60%, compared to the control group. Conversely, ASA co-treatment at both low and high doses (10 & 100 mg/kg) was able to normalize the concentration of TNF-α, similar to that in the control mice. Interestingly, ASA (100 mg/kg) caused further nonsignificant reduction of TNF-α concentration from the control value. On the other hand, silymarin showed slight but significant improvement in TNF-α concentration by about 17%, when compared to CCl4-challenged group.

Interleukin 6 (IL-6) and Interleukin 1-beta (IL-1β) concentrations in hepatic homogenate

Other inflammatory cytokines (IL-6 & IL-1β) were also assessed, as displayed in ***Table 5***. In fact, similar pattern of activities was detected with both cytokines. Briefly, CCl4 exposure caused significant (two-fold) increases in their concentrations, as compared to the corresponding control group. Furthermore, silymarin was able to significantly diminish IL-6 & IL-1β concentrations by 34.2% and 30.6%; respectively, as compared to CCl4-exposed group. Again, both doses of ASA (10 & 100 mg/kg) significantly reduced hepatic concentrations of IL-6 & IL-1β from CCl4-challenged group, that became within the normal range similar to the control values. Moreover, aspirin alone group showed reduction in IL-1β and IL-6 concentrations, so that no significant differences from the corresponding control group were detected.

Table 5 Effect of aspirin on concentrations of interleukin one beta (IL-1β), interleukin six (IL-6), and tumor necrosis factor alpha (TNF-α) in mice subjected to chronic CCl4 intoxication

	TNF-α (ng/mg protein)	IL-6 (pg/mg protein)	IL-1β (pg/mg protein)
Control	0.88± 0.10	71.79± 7.75	11.46±1.70
CCl4 (0.5 mL/kg)	1.41a ± 0.20	168.57a ± 17.47	23.59a ± 2.53
CCl4 + Sil. (100 mg/kg)	1.20a,b ± 0.14	110.93a,b ± 14.82	16.37a,b ± 1.43
CCl4 + ASA (10 mg/kg)	0.88b,c ± 0.08	72.79b,c ± 5.74	12.80b,c ± 1.84
CCl4 + ASA (100 mg/kg)	0.71b,c ± 0.09	67.65b,c ± 7.51	8.5b,c ± 1.88
ASA (100 mg/kg)	0.68b ± 0.07	60.69b ± 8.79	9.54b ± 2.06

Statistical analysis was carried out using one-way ANOVA followed by Tukey's as a post-hoc test

a: significantly different from the corresponding control at $p < 0.05$

b: significantly different from the corresponding CCl4-challenged group at $p < 0.05$

c: significantly different from the corresponding silymarin at $p < 0.05$

CCl4; carbon tetrachloride, Sil.; silymarin, ASA.; aspirin

Concentration of Mouse Phosphorylated Adenosine Monophosphate-Activated Protein Kinase (p-AMPK) in Hepatic Homogenate

Under the current experimental conditions, AMPK activity was assessed by measuring the concentration of its phosphorylated (active) form in mouse hepatic homogenate using a species-specific ELISA kit. As shown in (Table 4 and Figure 9), CCl4 exposure revealed marked decreased concentration of p-AMPK by about 50%, compared to the control group. Furthermore, silymarin treatment significantly improved p-AMPK concentration, increasing its concentration by about 43% compared to CCl4-intoxicated group. Conversely, treatment with low dose of ASA (10 mg/kg) failed to cause any significant improvement of p-AMPK concentration in comparison to the diseased group. On the other hand, ASA at the high dose (100 mg/kg) was able to significantly elevate the concentration of p-AMPK by 53% compared to CCl4-challenged group. Remarkably, the Aspirin-only treated group has the same activity as the control group and did not show any significant difference.

Table 6 Effect of aspirin on mouse concentration of phosphorylated adenosinemonophosphate- activated protein kinase (p-AMPK) in CCl4-induced hepatic fibrosis

	p-AMPK (pg/mg protein)
Control	14.83 ± 1.20
CCl_4 (0.5 mL/kg)	7.50_a ± 0.83
CCl_4 + Sil. (100 mg/kg)	$10.72_{a,b}$ ± 1.26
CCl_4 + ASA (10 mg/kg)	$6.95_{a,c}$ ± 1.14
CCl_4 + ASA (100 mg/kg)	$11.45_{a,b}$ ± 1.41
ASA (100 mg/kg)	15.11_b ± 2.34

Statistical analysis was carried out using one-way ANOVA followed by Tukey's as a post-hoc test

a: significantly different from the control at $p < 0.05$

b: significantly different from the CCl4-challenged group at $p < 0.05$

c: significantly different from silymarin at $p < 0.05$

CCl4; carbon tetrachloride, Sil.; silymarin, ASA; aspirin, p-AMPK; phosphorylated adenosine monophosphate- activated protein kinase

Discussion

Progressive liver fibrosis is a serious health problem worldwide, induced by a variety of chronic hepatic injury [20]. Fortunately, it is believed to be reversible through the apoptosis of activated hepatic stellate cells (HSCs) and degradation of extracellular matrix (ECM) proteins [21,22]. Thus, it is urgent to develop antifibrotic therapy that can reverse liver fibrosis [23]. However, up to now, there is no treatment for liver fibrosis available for clinical use [24]. Both ASA and salicylate demonstrated strong

antioxidant and anti-inflammatory activities through traditional inhibition of COX enzyme [8]. Furthermore, recent studies reported that ASA inhibits cardiac fibrosis, having a potential cardioprotective effect [25]. Therefore, the aim of current study was to evaluate the potential antifibrotic effect of ASA in CCl4-induced hepatic fibrosis besides the possible causal mechanisms, with respect to oxidative stress, inflammation and AMPK activity.

It is currently recognized that hepatotoxin CCl4 exposure is the most experimentally-used inducer of liver fibrosis, hepatocellular inflammation, and necrosis [26]. The hepatic cytochrome CYP P450 oxygenase system of the endoplasmic reticulum converts CCl4 into trichloromethyl free radical (CCl3). Unless neutralized by radical scavengers, these radicals in turn abstract hydrogen from other lipid molecules, thus propagating the process of lipid peroxidation. Moreover, these reactive radicals bind covalently to a variety of biological molecules, leading to membrane lipid peroxidation, abnormal protein function, damage to mitochondria and nuclei, release of inflammatory mediators from activated inflammatory cells, impairing physiological functions of hepatocytes and to cell necrosis [27,28]. In the presence of an oxygen molecule, CCl3 is transformed into the trichloromethyl peroxy radical (CCl3OO), which is more reactive and shorter lived than CCl3 radical (Mico and Pohl, 1983). Unless neutralized by radical scavengers, these radicals in turn abstract hydrogen from other lipid molecules, thus propagating the process of lipid peroxidation. Moreover, these reactive radicals bind covalently to a variety of biological molecules leading to membrane lipid peroxidation, abnormal protein function, damage of mitochondria and nuclei, release of inflammatory mediators from activated inflammatory cells, impairing physiological functions of hepatocytes and to cell necrosis [27,28].

Based on the current study, CCl4 induced hepatotoxicity is evidenced by significant elevations of hepatotoxicity parameters like: ALT, AST, TB, TC, and TG. It has been reported that chronic administration of CCl4 significantly raises AST and ALT levels, reflecting liver inflammation and necrosis, accompanied by histopathological changes in hepatic architecture [29]. That is also in harmony with previous studies indicating the ability of CCl4 to elevate TC & TG in addition to increased liver weight and hepatic hypertrophy [30,31].

The present findings indicate that co-treatment of animals with ASA (10 and 100 mg/kg) or silymarin significantly lowered the rise of serum ALT and AST activities. This was in accordance with [8], who reported that aspirin had anti-inflammatory and antioxidant properties and could protect against hepatic fibrosis induced by several chemicals, as well as improve microscopic histological analysis. Notably, our results found that higher doses of ASA (100 mg/kg) significantly improved the metabolic function as established by the plasma concentration of TB, TC and TG. Moreover, histopathological examination of collagen fibers using Masson's trichrome staining was able to preserve the normal hepatic architectural parenchyma. ASA oral LD50 in mice was found to be 1170 mg/kg [32], thus, the tested doses were in the safe range and compatible with published literature [33]. These findings are consistent with the previous study of ***Yoshida et al. 2014***, [34] indicating that long-term ASA supplementation significantly reduces hepatotoxicity indices and inhibits the progression of fibrosis.

ASA therapy, in a dose-related manner, significantly improved GSH contents and CAT activity while decreasing MDA concentration. This was supported by the evidence that ASA could protect against the sequence of oxidative stress that intensifies the inflammatory response of CCl4-induced liver fibrosis. These findings were in line with the previous study of ***Chávez et al. 2012,*** [8] that showed the promising potential of ASA at a dose of (100 mg/kg) to improve the liver fibrosis and oxidative damage by its protective effects against CCl4. Moreover, an earlier study demonstrated that ASA

showed significant reduction of lipid peroxidation revealed by decreased MDA concentration and elevated CAT enzyme activity in liver homogenates [35].

It is well-known that chronic administration of CCl4 significantly induces the inflammatory cytokines TNF-α, IL-6 and IL-1β concentrations in tissue homogenates which means a severe inflammatory response [24]. To our knowledge, treatment with ASA revealed a strong anti-inflammatory effect by significantly diminishing the elevated concentrations of TNF-α, IL-6 and IL-1β in hepatic tissue homogenates, so that no significant changes from the control mice were observed. Interestingly, this amelioration was achieved even by treatment with low dose ASA (10 mg/kg).

As a cellular energy sensor, AMPK is a heterotrimeric protein complex that consist of a catalytic α-subunit (α1and α2), a scaffolding β-subunit (β1 and β2) and a regulatory γ-subunit (γ1, γ2 and γ3) [10]. It plays an important role in maintaining the balance between anabolic and catabolic processes for cellular energy hemostasis in response to metabolic stress by stimulating fatty acid and glucose uptake and oxidation when cellular energy is low [36,37].

It is well known that chronic inflammation is a risk factor of chronic diseases such as liver fibrosis, diabetes, obesity and cancer. AMPK was found to be inhibited in such chronic diseases and its activation could be beneficial for treatment [38]. Previous studies had suggested that AMPK protects the liver [5,39] and different organs such as lungs [40], heart [41], kidneys [42], and muscles [43] against fibrosis. It is dysregulated in chronic diseases like inflammation, obesity, cancer and diabetes. Based on its important role in pathology and physiology, it is emerging as one of the most promising targets for the treatment and prevention of these metabolic diseases [38]. The most well-known mechanisms of AMPK activation are phosphorylation at threonine-172 (T172) of the α-subunit and AMP and/or ADP binding to γ-subunit [44], while ATP competitively inhibits the binding of both AMP and ADP to the γ-subunit, which indicates that AMPK is a sensor of AMP/ATP or ADP/ATP ratios [38].

In the present study, phosphorylated-AMPK concentration significantly dropped in the CCl4-exposed group as compared to the control one. This was in line with previous study of ***Kumar et al. 2014,*** [45] in which chronic administration of CCl4 induced fibrosis and decreased the activity of AMPK. On the other hand, treatment with ASA high dose (100 mg/kg) or silymarin significantly raised the concentration of phosphorylated-AMPK as compared to the diseased group. Consistent with these findings, ASA has been proven to activate the AMPK signalling pathways in-vivo, resulting in phosphorylation of AMPK in hepatocytes of mice [9].

Importantly, it has been reported in in-vitro study that ASA is a direct AMPK activator in mouse colorectal cancer cells (RKO, SW480, and HCT116) [12] and human HepG2 (human hepatocarcinoma cell line [11]. This effect could be largely attributed to its antioxidant and anti-inflammatory effects. Moreover, ***Yoshida et al. 2014,*** [34] and ***He et al. 2015,*** [11] reported that ASA administration promotes AMPK activation significantly in time-dependent and dose-dependent manners. These findings add further support to the value of ASA as an antifibrotic and a candidate for primary prevention of liver fibrosis via AMPK signalling mechanism.

Conclusion

The current study presents evidence for the antifibrotic effects of ASA. The potential mechanisms for its antifibrotic activity against liver fibrosis include: It defends oxidative stress through restoring GSH content and CAT activity as well as ameliorating lipid peroxidation. In addition, it attenuates liver inflammation by reducing the expression of proinflammatory cytokines IL-6, IL-1β, and TNF-α. Moreover, it directly activates AMPK, which revealed its protective role against liver fibrogenesis.

Recommendations

Since hepatic fibrosis is an extremely complicated disease, further investigation of ASA antifibrotic mechanisms on a molecular level is required. Finally, more studies are reasonable in order to establish the clinical applicability of ASA treatment in patients with active fibrogenesis liver diseases.

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