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Effect of Galectin-3 Inhibition on Hepatocyte Nuclear Factors 4α and 1α Expression in Liver after Acetaminophen Long-term Induced Toxicity

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Abstract

Acetaminophen (APAP) induced liver injury can occur through acute or repeated administration of high doses. Hepatocyte nuclear factors HNF4α and HNF1α are key transcription factors involved in regulating the expression of drug metabolism enzymes and are affected by proinflammatory cytokines. Meanwhile galectin-3 (Gal-3) is a β-galactoside-binding lectin implicated in the regulation of macrophage activation and secretion of inflammatory mediators. This study aimed to evaluate the impact of galectin-3 inhibition on *HNF4α* and *HNF1α* gene expression after long-term administration of APAP, and to investigate the resulted effect on APAP induced hepatotoxicity. The study was performed on thirty two Wistar rats divided equally into four groups; control group, modified citrus pectin (MCP) (Gal-3 inhibitor) receiving group, APAP receiving group, and APAP plus MCP receiving group. APAP induced inhibition of liver *HNF4α* and *HNF1α* gene expression, and elevation of liver Gal-3 and tumor necrosis factor- α levels (TNF-α), depletion of reduced glutathione and its maintaining enzymes, besides elevation in levels of serum liver function parameters and appearance of necrotic areas in the liver. Gal-3 inhibition preserved *HNF4α* and *HNF1α* gene expression, TNF-α and each of reduced glutathione and its maintaining enzymes near normal levels. It also significantly reduced APAP induced hepatotoxicity.

Keywords: *HNF4α; HNF1α;* Galectin-3; Inflammation; Acetaminophen long-term administration.

1. Introduction

Acetaminophen (APAP) induced liver injury is common, and usually leads to severe complications including liver failure. Long-term exposure to high subtoxic doses of APAP can cause severe liver injury like in case of acute toxic dose **[**1]. The primary metabolic pathways of APAP in liver are through conjugation with glucoronidate and sulfate in reactions mediated by uridine diphosphate-glucuronosyl transferases (UGTs) and sulfotransferases (SULTs). The conjugation resulted compounds are non-toxic and excreted in urine. When administrated at high dose, a portion of APAP is metabolized by cytochrome P450 enzyme CYP2E1 producing the highly toxic intermediate N-acetyl-p-benzoquinone imine (NAPQI). The highly reactive NAPQI covantely binds to cellular proteins forming cytotoxic protein adducts leading to hepatocellular necrosis. NAPQI can be reduced by GSH then excreted in bile, however APAP high dose leads to saturation of glucoronidation and sulfonation pathways and depletion of GSH exposing the hepatocytes to toxicity and necrosis [2, 3].

Hepatocyte nuclear factors (HNFs) are group of transcription factors that are important for hepatocyte proliferation and functionality [4, 5]. HNF4α and HNF1α are two important HNFs members involved in regulation of many UGTs, SULTs and CYP450 family enzymes in both liver and kidney [6 – 9]. HNF4α plays an important role during inflammation through regulation of acute-phase protein (APR) expression. However the inflammatory cytokines have dual effect on HNF4α, they may inhibit *HNF4α* expression and transcription function, reduce its DNA binding capacity and induce degradation of *HNF4α* mRNA and protein, or enhance HNF4α function. Furthermore HNF4α can inhibit IL-6/STAT3 inflammatory pathway [10, 11]. Proinflammatory cytokines levels increased after *HNF1α* gene knockdown in hepatocytes; meanwhile treatment of hepatocytes with proinflammatory cytokines inhibits *HNF1α* expression [12, 13].

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Galectin-3 (Gal-3) is a β-galactoside-binding lectin that regulates macrophage activation and inflammatory mediator production [14, 15]. It is an important modulator of both acute and chronic inflammation [16, 17]. Modified citrus pectin (MCP) is obtained from citrus fruit pectin via pH and temperature modifications, which break its bulky structure into shorter, unbranched, galactose-rich carbohydrate chains. MCP is able to tightly bind with Gal-3 carbohydrate recognition domain, which inhibits Gal-3 bioactivity [18, 19]. Inflammation seems to be a common factor between Gal-3 and both of *HNF4α* and *HNF1α*, so this study was conducted to evaluate the impact of galectin-3 inhibition on *HNF4α* and *HNF1α* gene expression levels and hepatotoxicity after long-term acetaminophen subtoxic high dose administration.

2. Materials and Methods

2.1. Chemicals

Acetaminophen (APAP) was purchased from Sigma Aldrich Co., St. Louis, Missouri, United States, and suspended in 20% Tween-80. Modified citrus pectin (MCP) was purchased from EcoNugenics Inc., Santa Rosa, CA, USA. Any other chemicals mentioned later were purchased from Sigma Aldrich Co., St. Louis, Missouri, United States unless reported otherwise.

2.2. Animals and ethical consideration

Thirty two male Wistar rats, weighing 90 –110 g each, were purchased from the National Research Centre (NRC), Cairo, Egypt. The animals were housed in the NRC animal house and maintained under standardized environmental conditions at 12 h light/dark cycle under a constant temperature of 25±1 ◦C. Rats were fed on basal diet [20, 21] and water was supplied ad libitum. Animals were acclimated to laboratory conditions one week prior to experiment beginning. All experimental procedures were performed according to guidelines of the institutional committee of animal's care and use of the NRC. The study protocol was approved by the ethical committee board of the NRC, with approval no. 17/140.

2.3. Experimental design

The rats were divided equally into four groups (8 rats per group). Group A: normal control. Group B: Rats received 1% MCP dissolved in drinking water daily for 4 weeks [22]. Group C: rats received oral APAP dose (1 g/kg b wt.) daily for 4 weeks [23]. Group D: rats received oral APAP dose (1 g/kg b wt.) and 1% MCP dissolved in drinking water daily for 4 weeks.

2.4. Collection of blood and liver samples

At the end of the experiment, the animals were anesthetized and blood samples were collected after 12 hours fasting using the orbital sinus technique [24]. Blood samples were left to clot in clean dry test tubes, and then centrifuged at 4000 rpm for five minutes. The clear supernatant serum was then separated and frozen at -20 ºC for the biochemical analyses. After blood collection, the rats were sacrificed by decapitation and the whole liver of each animal was rapidly dissected, thoroughly washed with isotonic saline and plotted. Small parts of each liver were cut and weighed for extraction of RNA and preparing liver tissue homogenate, the rest of each liver was fixed in formaldehyde buffer (10%) for histological examination.

2.5. Preparation of tissue homogenate

Liver tissue samples were homogenized in ice cold PBS buffer (pH 7.4, 4 ^{0}C) contains 100 mM Tris, 1 mM EDTA, 1% Triton X-100, and Protease inhibitor cocktail by using Fisherbrand 850 homogenizer, Pennsylvania, USA. Homogenates were centrifuged at 12,000 x g for 10 minutes using Sigma 2K15 centrifuge, Osterode, Germany. The supernatants were separated for parameters determination by ELISA. Meanwhile other liver tissue parts were homogenized in ice cold PBS buffer (pH 7.4, 4 ^{0}C) contains 1 mM EDTA, centrifuged; then supernatants were separated for determination of GSH, GR and GPx [25].

2.6. Biochemical analyses methods

Liver Galectin-3 (Gal-3) and tumor necrosis factor alpha (TNF-α) level were determined by ELISA technique using kit purchased from Elabscience Co., Texas, USA; Liver CYP2E1 level was determined by ELISA technique using kit purchased from Cusabio Co., Houston, USA according to methods described by the manufacturers and using Stat Fax 2100 Microplate Reader, Awareness Technology Inc., Florida, USA. Liver reduced glutathione (GSH) level and glutathione reductase (GR) and glutathione Peroxidase (GPx) activities were estimated using commercial colorimetric assay kits purchased from Biovision, Inc., California, USA based on the methods described by the manufacturer. Serum alanine and aspartate transaminases (ALT and AST), alkaline phosphatase (ALP) and total bilirubin were estimated using kits of Salucea BV Co., Haansberg, Netherlands according to the methods described by the manufacturer, on UVD-3500 spectrophotometer, Labomed Inc., California, USA.

2.7. RNA Isolation and reverse transcription

Liver tissue parts were homogenized using Tissuelyser and Qiagen Stainless beads (5mm) (Qiagen, Hilden, Germany), then manual extraction of total RNA from homogenized liver tissue was done using RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purity of extracted RNA was checked using NanoDrop2000™ spectrophotometer (Thermo Scientific, Massachusetts, USA). Complementary DNA (cDNA) was obtained from the extracted RNA using the High Capacity cDNA Reverse Transcription Kit™ (Applied Biosystems, Massachusetts, USA) according to the manufacturer's instructions on Veriti thermal cycler (Applied Biosystems) with conditions of 25 °C for 10 minutes, 37 °C for 120 minutes and 85 °C for 5 minutes to terminate the reaction.

2.8. Evaluation of HNF4α and HNF1α mRNA levels by qRT-PCR

Levels of HNF4α and HNF1α gene expression were evaluated by using Taqman assays (ID no. Rn04339144_m1 and Rn00562020_m1 respectively) and Taqman gene expression master mix (Applied Biosystems). Expression levels of target genes were normalized to the endogenous control beta actin (ID no. Rn00667869_m1). QuantStudio 12K Flex Real time PCR equipment was used with cycle conditions: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Relative expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method and represented as fold change.

2.9. Histopathological examinations and quantitative measurement of liver damaged areas

The liver of rats of different groups were removed and fixed in 10% formal saline, 5 µm thick paraffin sections were stained with haematoxylin and eosin stains [26] and examined by digital light microscope, Leica Qwin 500, Leica Microsystems, Wetzlar, Germany. Quantitative measurement of liver damaged areas was carried out using the device accompanied Image Analyzer software. Five non overlapping fields were chosen from each liver sample of the groups treated with APAP alone or accompanied with MCP, and the mean values were obtained.

2.10. Statistical analysis

Statistical analysis was performed using SPSS version 21.0. Data obtained in the present work are represented as average mean ± standard deviation. Statistical analysis was evaluated using the ANOVA test and Tuckey post-hoc for multiple comparisons between groups. P values less than 0.05 were considered statistically significant [27].

3. Results

3.1. Biochemical analyses results

Acetaminophen (APAP) repetitive administration led to toxic effects on hepatocytes represented by significant ($p<0.05$) increase in serum activities of alanine and aspartate aminotransferase (ALT and AST), alkaline phosphatase (ALP) enzymes and level of total bilirubin (Fig. 1).

Liver levels of galectin-3 (Gal-3) and tumor necrosis factor-alpha (TNF-α) showed significant ($p<0.05$) elevation along with significant ($p<0.05$) depression in levels of CYP2E1, reduced glutathione (GSH), and activities of glutathione reductase (GR) and glutathione peroxidase (GPx) compared to the control group (Fig. 2 & Fig. 3). Meanwhile supplement of modified citrus pectin (MCP) in conjunction with APAP administration restored the mentioned parameters near to control group's levels with a significant ($p<0.05$) change in comparison with the group received APAP only. There was no significant difference between control and MCP only treated groups.

3.2. Hepatocytes nuclear factors HNF4α and HNF1α quantitative gene expression

Acetaminophen long-term administration led to significant ($p<0.05$) decrease in both HNF4α and HNF1α mRNA levels in comparison with control group. However inhibiting Gal-3 resulted in significant improvement in HNF4α and HNF1α mRNA levels displayed as a significant ($p<0.05$) increase compared to the group received APAP only, and no significant difference compared to the control group (Fig. 2).

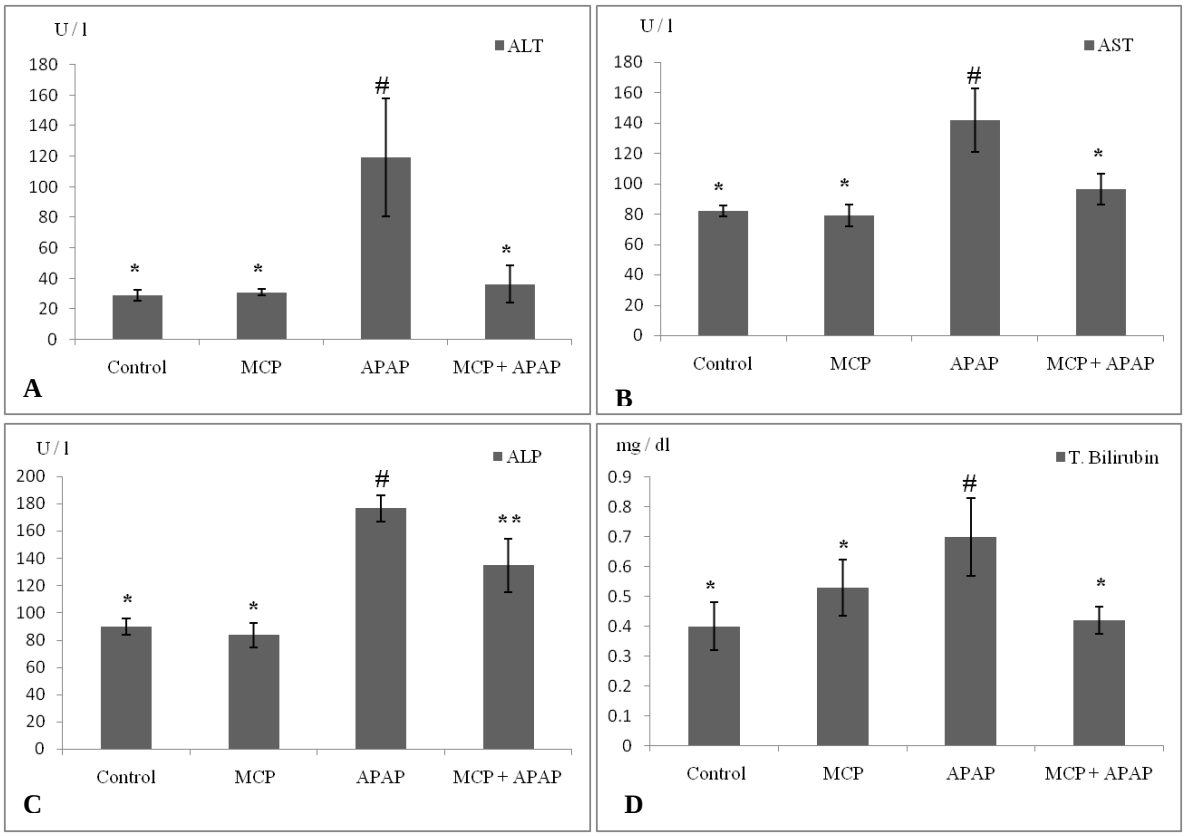

Figure 1: Serum levels of (**A**): Alanine transaminase, (**B**): Aspartate transaminase, (**C**): Alkaline phosphatase and (**D**): total bilirubin. All data are represented as mean ± standard deviation (error bars). Values with different symbols (*, #, **) are significantly different at $P<0.05$ using ANOVA test. APAP: acetaminophen. MCP: modified citrus pectin.

3.3. Histopathological analyses results

Acetaminophen long-term administration induced liver injury represented by disturbance of lobular structure, extensive hepatocyte necrosis and degeneration. Meanwhile MCP administrated group showed normal liver architecture almost like the control group. Administration of MCP along with APAP reduced the later induced toxic effect, which was observed through significant ($p<0.05$) decrease in hepatocyte necrotic areas (Fig. 4 & Fig. 5).

4. Discussion

In this work, we studied the effect of acetaminophen (APAP) subtoxic high dose long-term administration on rat liver hepatocyte nuclear factors *HNF4α* and *HNF1α* genes expression levels. Also we studied the effect of inhibiting galectin-3 (Gal-3), by using modified citrus pectin (MCP), on hepatic *HNF4α* and *HNF1α* genes expression levels and reducing the hepatotoxic effect of APAP long-term administration.

APAP high dose can induce hepatorenal toxicity in Wistar rats including hepatocellular necrosis and inflammation [22, 28 - 31**]**. Furthermore APAP subtoxic high dose repetitive administration induces hepatic injury and inflammation [23]. Additionally, this study focuses on proinflammatory cytokines as a probable link between Gal-3 and the mentioned HNFs.

Long-term administration of APAP led to significant increase in serum alanine and aspartate transaminases (ALT and AST), alkaline phosphatase (ALP) activities and total bilirubin level when compared with normal control group, which agrees with *Ozcelik et al.* [31] and *Ilavenil et al.* [32]. N-acetyl-p-benzoquinone imine (NAPQI) is a toxic metabolite of APAP that is accumulates after saturation of non-toxic pathways of APAP metabolism and excretion and reduced glutathione depletion. NAPQI covalently binds to critical hepatocellular proteins leading to necrosis, oxidative stress and tissue injury. ALT, AST and ALP are normally found inside hepatocytes, especially ALT which is more specific to hepatocytes. The elevated serum levels of these parameters are indication for hepatocytes injury and necrosis, which agrees with our histopathological results. Elevated total bilirubin is related to impairment in liver function of up-taking bilirubin from blood due to hepatocellular necrosis and bile duct damage [33]. Inhibiting Gal-3 led to

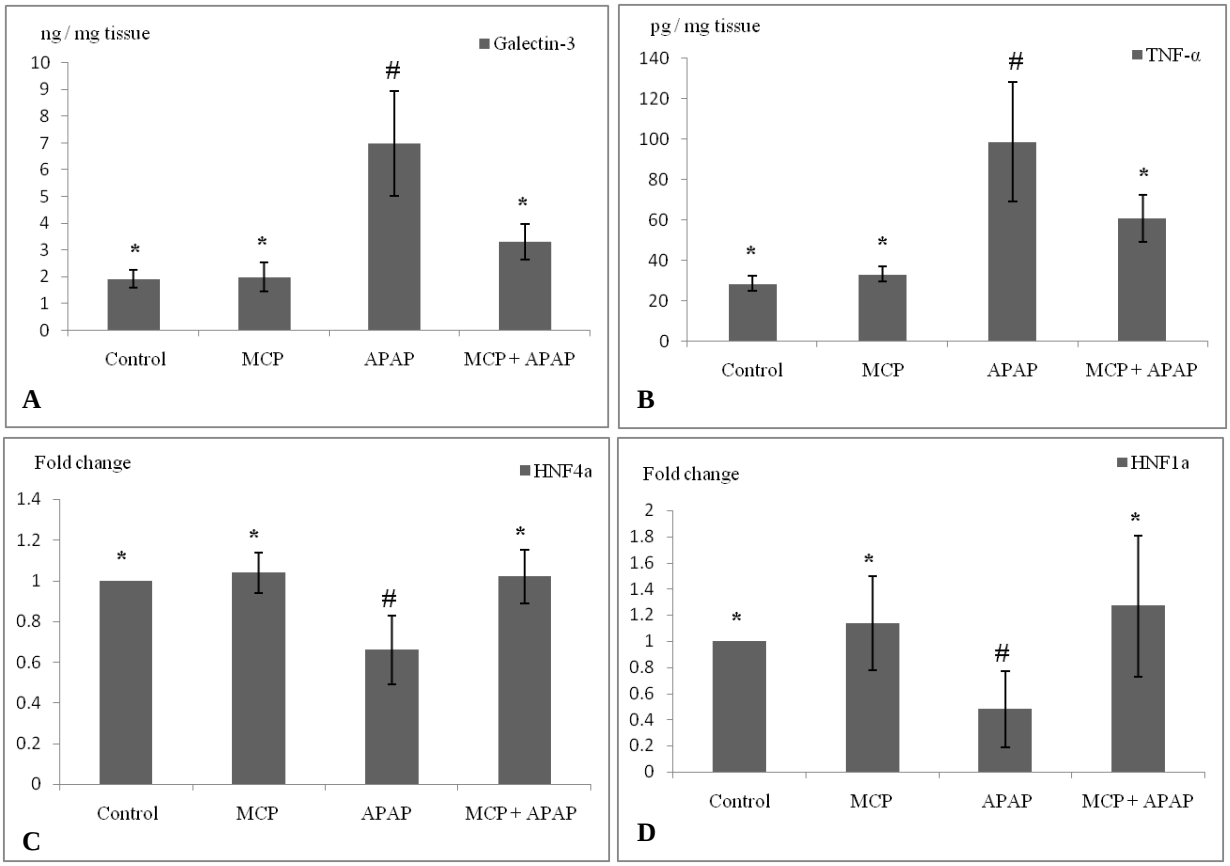

Figure 2: Levels of hepatic (**A**): galectin-3, (**B**): TNF-α, (**C** and **D**): *HNF4α* and *HNF1α* gene expression fold change. All data are represented as mean ± standard deviation (error bars). Values with different symbols (*, #) are significantly different at $P<0.05$ using ANOVA test. APAP: acetaminophen. MCP: modified citrus pectin.

significant decrease in ALT, AST, ALP activities and total bilirubin level, beside an observed improvement in liver histology compared to the group received only APAP. This probably due to reduced glutathione (GSH) and glutathione reductase (GR) which were shown having significantly higher levels in MCP treated group in comparison with the APAP only receiving group. Since GSH binds to NAPQI forming non-toxic conjugate, and GR is the enzyme converting oxidized glutathione to the reduced form to act as antioxidant molecule, so both GSH and GR act like defence system against NAPQI induced toxicity.

Levels of liver Gal-3 and tumor necrosis factor-alpha (TNF-α) showed significant elevation after APAP long-term administration which is in agreement with *Dragomir et al.* [14] and *Hinson et al.* [34]. APAP induces liver tissue injury which stimulates recruitment of macrophages to injury site. Macrophages in turn start to secrete Gal-3 and pro-inflammatory cytokines including TNF-α in extracellular matrix inducing inflammation. Macrophages with Gal-3 expressed on its surface were found to be of higher number in injured tissue than in normal one, they act in an autocrine or paracrine pathway to induce monocyte migration and activation and stimulates cytokine expression [16, 17, 35, 15]. MCP administration significantly reduced liver Gal-3 and TNF-α levels in comparison with the untreated group. This agrees with *Kolatsi-Joannou et al.* [23] who reported that MCP reduced Gal-3 expression level in experimental acute kidney injury. This can be explained as a direct effect on Gal-3 bioactivity. Gal-3 recruits other macrophages and monocytes to injury site, where they release more Gal-3 and inflammatory cytokines in a feedback loop effect [35]. So inhibiting Gal-3 bioactivity is suggested to inhibit Gal-3 and proinflammatory cytokines mutual secretion loop and sequentially reduced Gal-3 and TNF-α levels.

Liver *HNF4α* and *HNF1α* relative expression levels decreased significantly due to APAP long-term administration. APAP toxicity induces tissue injury and inflammation. Proinflammatory cytokines are reported to have inhibitory effect on *HNF4α* expression level and transcription activity [10, 11]. Similarly, *Qian et al.* [13] reported that proinflammatory cytokines have inhibitory effect on *HNF1α* expression level in hepatocytes. Moreover, HNF4α positively regulates *HNF1α* expression, so down regulation of *HNF4α* can negatively affect *HNF1α* expression [10]. MCP administration restored

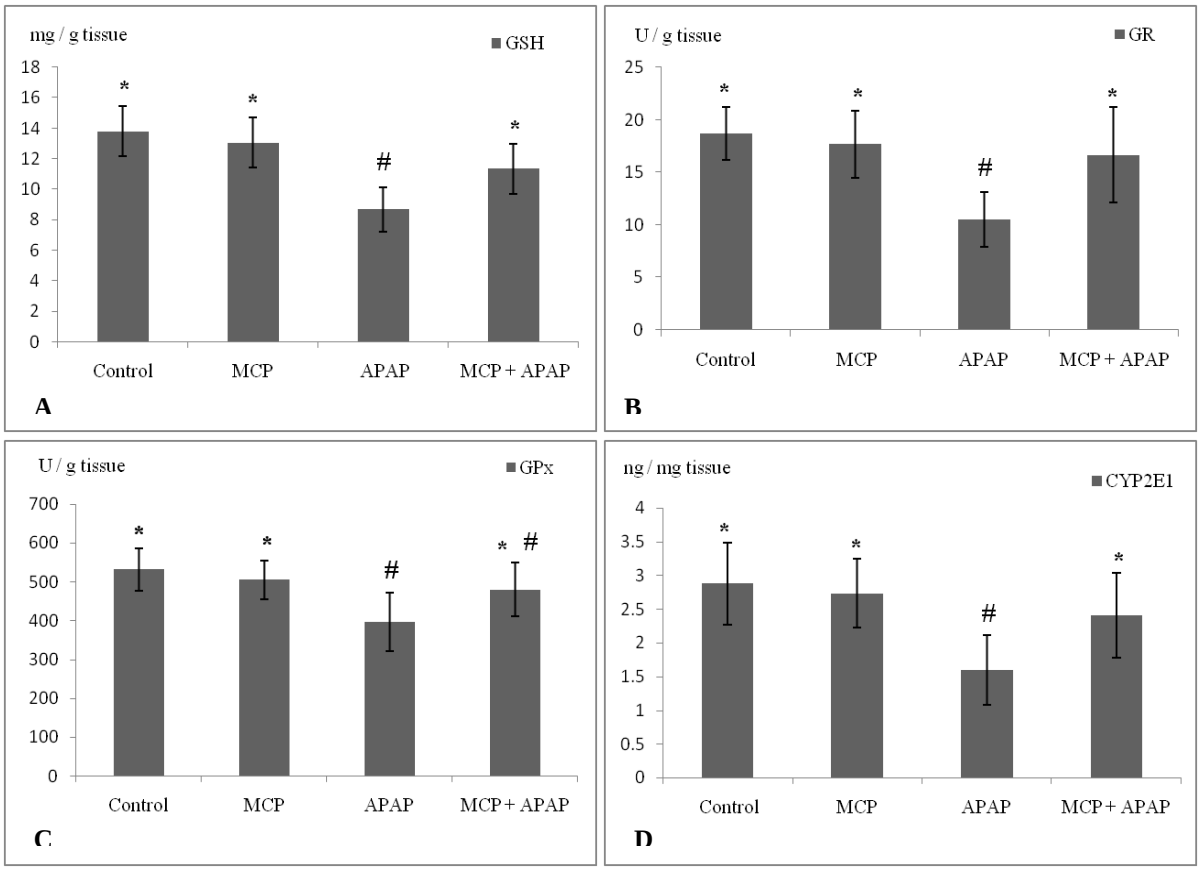

Figure 3: Levels of hepatic (**A**): GSH, (**B** and **C**): GR and GPx activities, (**D**): CYP2E1. All data are represented as mean ± standard deviation (error bars). Values with different symbols (*, #) are significantly different at $P<0.05$ using ANOVA test. APAP: acetaminophen. MCP: modified citrus pectin.

HNF4α and *HNF1α* relative expression levels close to the normal control levels. Since inflammatory cytokines are reported to have inhibitory effect on *HNF4α* and *HNF1α* expression, therefore it can be concluded that inhibiting Gal-3(by using MCP) reduced inflammation and might contribute in restoring normal levels of *HNF4α* and *HNF1α* expression.

Hepatic reduced glutathione (GSH) level decreased significantly due to APAP long-term administration which coincides with *O'Brien et al.* [36]. The decline in GSH level is because of an irreversible covalent binding occurs between NAPQI and GSH molecules leading to GSH depletion [3, 31]. Inhibiting Gal-3 resulted in significant increase in liver GSH level in comparison with the untreated group received only APAP. *Zhang et al.* [37] reported that HNF4α affect GSH production and *HNF4α* gene deficient mice suffered reduction in GSH production. Although HNF4α and HNF1α activate expression of cytochrome p450 enzymes which mediate the oxidation of APAP to produce the toxic metabolite NAPQI, they also promote expression of phase II detoxifying enzymes including uridine diphosphate-glucuronosyl transferases (UGTs) and sulfotransferases (SULTs) that consist the principle pathways of APAP non-toxic metabolism and excretion, especially UGT1A9 [6, 7, 8, 38, 39]. Since *HNF4α* and *HNF1α* expression levels were higher in MCP treated group; so this can be mainly assumed as the reason behind the higher GSH level.

In the same way APAP long-term administration reduced both glutathione reductase (GR) and glutathione peroxides (GPx) activities in the liver. GPx activity was reported to decrease significantly after exposure to high doses of APAP **[**34], and the conjugates of GSH with NAPQI have inhibitory effect on GR activity [40]. Moreover, HNF4α deficiency leads to decrease in GPx activity [41]. MCP treated group showed significant increase in GR and improvement in GPx activities compared to APAP receiving group. These improvements can be related to the positive effect of MCP on *HNF4α* expression level and activity preservation through inhibiting Gal-3 mediated inflammation as mentioned before. Restoration of *HNF4α* expression could have enhanced GPx expression and lessens the probability of GSH-NAPQI conjugates formation through promoting UGTs and SULTs expression which enhances the non-toxic metabolic pathways of APAP.

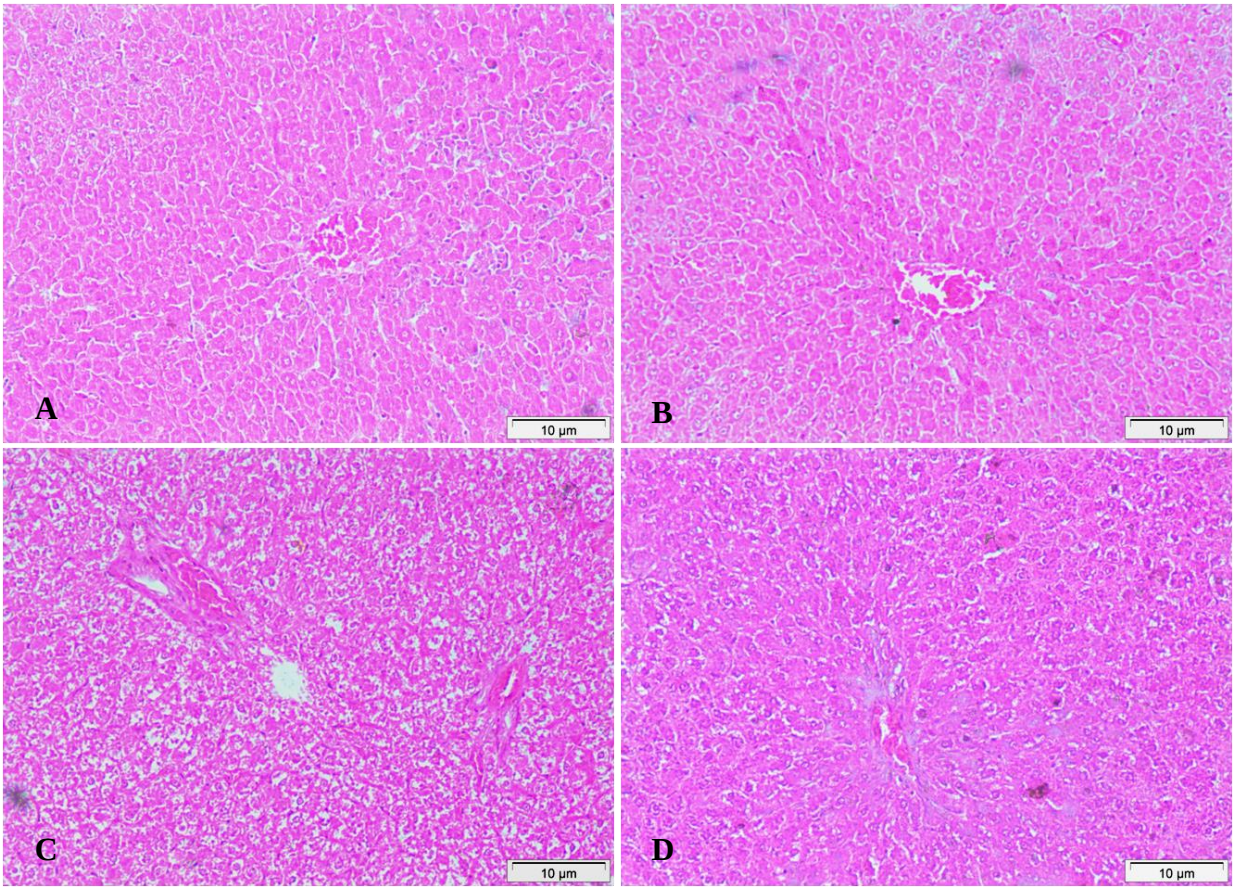

Figure 4: Photomicrographs of liver cross sections from: (**A**): control group, showing normal architecture of hepatic lobule and normal hepatocytes, (**B**): group received modified citrus pectin, showing normal hepatic architecture. (**C**): group received acetaminophen, showing disturbance of lobular structure, large areas of focal necrosis and degeneration (**D**): group received modified citrus pectin and acetaminophen, showing notable decrease in necrotic areas. (H&E stains, Scale bar: 10 µm).

APAP long-term administration significantly reduced hepatic CYP2E1 level compared to the control group, which agrees with *Papackova et al.* [42]. This can be related firstly to the induced inflammation, because inflammatory cytokines were reported to inhibit CYP2E1 expression level [43]. Secondly to the reduction occurred in *HNF1α* expression level, which regulates CYP2E1 expression [38]. MCP adminisrtation caused significant increase in CYP2E1 level compared to the group received only APAP. This can be related to the higher expression level of hepatic *HNF1α* and reduced inflammation in the MCP treated group versus the untreated one.

Acetaminophen administration led to great damage to the rat liver. It induced focal necrosis accompanied with degeneration of hepatocytes, which resulted in disturbance of hepatic lobule structure. These results agree with *Eakins et al.* [29] and *Mahmoud et al.* [30]. This also confirms our biochemical analyses results. Administration of MCP along with APAP resulted in an improvement in the hepatic tissue architecture, which is represented by significant reduction in areas of necrotic damage and

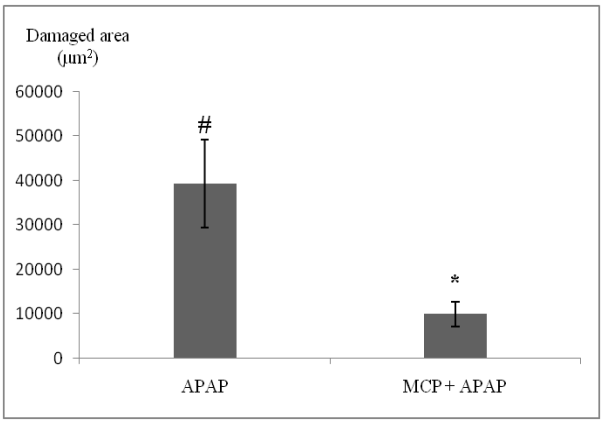

Figure 5: Liver damaged area per five non overlapping fields per rat in groups treated by APAP only or accompanied with MCP. The data are represented as mean ± Standard deviation (error bars). Values with different symbols (*, #) are significantly different at $P<0.05$ using ANOVA test. APAP: acetaminophen. MCP: modified citrus pectin.

Degeneration compared with the APAP only receiving group (Fig. 5). This result supports our biochemical analyses results.

5. Conclusion

In conclusion, APAP subtoxic high dose long-term administration induced hepatic necrosis and inflammation in Wistar rats, which probably had role in reducing *HNF4α* and *HNF1α* gene expression levels. Inhibiting Gal-3 during APAP administration resulted in reduction of inflammation and restoration of *HNF4α* and *HNF1α* gene expression closer to normal control levels. This might had contributed in improving other hepatic function and antioxidant parameters which were negatively affected by APAP intoxication. So inhibiting Gal-3 could be helpful against long-term APAP induced liver injury.

Conflicts of interest

There are no conflicts of interest.

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