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Glycyrrhizic Acid Protects against Thioacetamide-Induced Hepatic Fibrosis in Rats by Inhibition of HMGB1 and its crosstalk with Autophagy-Related Protein Beclin-1

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- Matrix Metaloproteinase-2 (MMP-2) and Tissue inhibitors of metalloproteinase (TIMP)

Abstract

Background: The pathogenesis of hepatic fibrosis (HF) involves hepatic stellate cells (HSC) activation to myofibroblasts that synthesize and secrete extracellular matrix (ECM). Autophagy has a role in the activation of quiescent HSCs. Beclin 1 regulates the formation of early autophagosome; however, its activity could be inhibited by binding to Bcl-2. The binding of the latter to Beclin 1 is mediated by a chromatin-associated nuclear protein; the High mobility group box 1 (HMGB1). This study aimed to analyze the effect of inhibiting Beclin 1 autophagy pathway using HMGB1 protein inhibitor, Glycyrrhizic acid (GA), on the progression of HF in a thioacetamide (TAA) induced rat model. **Methods:** HF was induced in 20 Wistar male rats; by TAA injection intraperitoneally twice weekly for eight consecutive weeks; divided into two random groups: untreated HF group and GA treated group. Ten rats served as controls. **Results:** The current work illustrated the inhibitory effect of GA on HF as evidenced by histopathological examination, reduction of oxidative stress, inflammatory and fibrotic markers like IL-1ß, TGF-β and α-SMA aligned with a downregulation in MMP-2 and TIMP expression. Inhibition of autophagy was also evident by decreased expression of both Beclin1 and HMGB1. **Conclusion:** GA acts as an inhibitor of the beclin-1 autophagy pathway and this could be a preventive therapy against development of HF.

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Introduction

Hepatic fibrosis (HF) is an excessive wound healing process complicating hepatic pathologies such as genetic disorders, infections and metabolic disorders [1].An imbalance in the production and degradation of extracellular matrix (ECM), which ultimately leads to excessive fibrosis and tissue architectural remodeling characterize HF.Stimulation of the hepatic stellate cells (HSCs), the main fibrogenic cell population, and other matrix producing cells by different mediators, inflammatory and profibrogenic, is an essential step in the fibrogenic process[2, 3]. HSCs lose cytoplasmic lipid droplets to transdifferentiate to activated myofibroblasts that synthesize and secrete ECM aiming at limiting the liver injury [4, 5]. In parallel, they synthesize different matrix metalloproteinases (MMPs) that degrade ECM, and specific tissue inhibitors of metalloproteinase (TIMP) to downregulate the activation of MMPs[6]. Ultimately, a rapid increase in ECM synthesis, coupled with increased TIMP activity results in the fibrotic scar.

One of the pathogenic mechanisms suggested in the fibrosis process is autophagy in HSCs which enables HSC activation by allowing the digestion of lipid droplets in quiescent HSCs, [7] and contributing to their transition to a myofibroblasts[8]. Inversely, different research speculated that autophagy prevents HF indirectly by regulating endothelial cell homeostasis and reducing inflammatory cytokines synthesis by macrophages and endothelial cells, resulting in limited hepatocyte injury[9]. Moreover, the oxidative and endoplasmic reticulum stress resulting from the accumulation of defective organelles and unfolded proteins is pronounced in case of disablement of autophagy[10]. Collectively, controversial studies argue whether autophagy is considered a hero or a criminal in HF.

One of the recent findings is a chromatin-associated nuclear protein; the High mobility group box 1 (HMGB1), that acts as a damage-linked molecular pattern and has an important role in sustaining autophagy[11]12]. In a stable homeostatic state, HMGB1 confines to the nucleus yet upon injury translocation to the cytosol and ECM occurs [13, 14]. HMGB1and Beclin 1 have a nearly homologous sequence [15], and Beclin 1 represents an evolutionarily regulatory step in early autophagosome formation and it interacts with several cofactors to induce autophagy. The activity of Beclin 1 could be downregulated by its binding to Bcl-2 or Bcl-XL [16]. Endogenous HMGB1 orients Beclin1 to autophagosomes by interfering with the Bcl-2 to Beclin1 interaction[15].

Until now, no specific treatment has been approved to be satisfactory for HF. Nowadays, the use of natural compounds to treat such refractory diseases is a relatively new concept in medicine. Glycyrrhizic acid (GA);active ingredient of licorice; is a natural extract that can be chemically synthesized. It blocks HMGB1 release into the extracellular space; acting as a direct inhibitor of HMGB1; thus, allowing Bcl-2 binding to Beclin1and inhibiting its activity [17]. Previous studies suggest that GA has anti-inflammatory, antiviral, antimicrobial, anti-oxidative, anticancer activities, immunomodulatory, hepatoprotective and cardio-protective effects [18, 19].

Objective

The current study was directed to define the action of HMGB1 protein inhibition by its specific inhibitor Glycyrrhizic acid on the progression of thioacetamide (TAA) induced fibrosis in rats.

Materials & methods

Drugs and chemicals:

TAA and GA were purchased from Sigma-Aldrich Co. (Egypt). TAA was dissolved in 0.9% w/v saline solution for intraperitoneal injection, while GA was prepared in gum acacia solution for oral intragastric gavage.

Laboratory animals:

This study was conducted on 30 albino Wistar male rats weighing 150-180 grams. Rats were purchased from and housed in the animal research laboratory of Medical Physiology Department, Faculty of Medicine, Alexandria University, Egypt. One week before and during the whole period of the experiment, the rats were kept in plastic cages with an ambient temperature of (23±3) °C, stable air humidity and a natural day/night cycle. The animals had free access to standard rodent laboratory food and tap water. Proposal of the current study was approved by the institutional animal ethics committee at the Faculty of Medicine, Alexandria University (IRB code 00012098- FWA: No.00018699**;** membership in International Council of Laboratory Animal science organization, ICLAS). The serial registration number for this study was 0305088.

Experimental design:

One week after acclimatization, 20 rats received of TAA (200 mg/kg)by intraperitoneal injection twice weekly for eight consecutive weeks to induce HF[20], subsequently, these were randomly allocated into two groups (10 rats each): untreated HF group and GA treated group. Rats in GA treated group received intragastric GA at a dose of 150 mg/kg/day once daily for eight consecutive weeks parallel with TAA [21]. 10 healthy rats served as untreated controls and received an equal volume of saline (n = 10). Normal control and untreated HF groups received gum acacia solution orally by gastric gavage once daily.

Animal sacrifice and sampling:

After the experimental period (8 weeks), rats were anesthetized to collect blood samples from the rats' retro-orbital plexus. Blood was collected in plain non-heparinized test tubes for separation of serum by centrifugation at 3000 rpm for 15 minutes. The serum was then stored at -20^{o}C to be later used for assessment of liver functions. Then, rats were sacrificed, and liver samples were collected. Part of liver tissues was kept in 10% formaldehyde for histopathological examination. Another part of liver tissues was stored at -80^{o}C to be further homogenized for biochemical assessment.

Frozen hepatic tissues were washed with ice-cooled TBS, tris-HCl (10 mM), NaCl (133 mM) at pH 7.4, and 10% (w/v) was homogenized in lysis buffer NaCl (100 mM), EDTA (100 mM), Nonidet p-40 (0.5%), Na-deoxycholate (0.5%), Tris, pH 7.5 (10 mM) containing protease inhibitors cocktail. The homogenate was centrifuged for 10 min at 2000 g at 4 ^{o}Cthen the supernatant was allocated for protein concentration assessment using the method of Lowry et al [22].

Histopathological Examination:

Liver samples, fixed in 10% formaldehyde, were set in paraffin blocks then stained with hematoxylin and eosin (H&E) and examined under the light microscope (Leica, Germany).

Histological examination of all liver sections was done to assess the loss of normal architecture, and to quantify the mean size of the portal tract and the mean of the inflammatory infiltrate in 10 HPF. In addition, Masson's Trichrome staining was employed to quantify the percentage of the fibrosis. The quantification was done using Imagej, and to numerically assess the percentage area of fibrosis in each sample, color deconvolution v1.53 (Maryland, USA) was used[23, 24].

Immunohistochemical (IHC) staining and interpretation:

The Avidin-Biotin-Peroxidase methodwas performed on all sections for IHC staining. Alpha-smooth muscle actin (α-SMA) (Ready to use primary antibody, rat anti-human, monoclonal antibody, P0943; Leica Biosystems, USA) was added to each section using the Bond-Max fully automated immunostainer (Leica Biosystems, USA). In each IHC run, negative and positive controls were included where leiomyoma was used as the positive control for α-SMA antibody.The quantification of the IHC of α-SMA was done, in 10 representative fields in each slide, using Imagej, v1.53 (Maryland, USA).

Biochemical Studies

Assessment of liver functions biomarker:

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, total bilirubin and direct bilirubin were measured using commercial kits (Spectrum diagnostic, Egypt) by following the instruction protocol of kits [25, 26].

Assessment of oxidative stress markers

Malonaldehyde (MDA), xanthine oxidase (XO)and nitric oxide (NO) were assessed in the hepatic tissue homogenates. Colorimetric thiobarbituric acid reaction method was used to measure MDA, and the concentration was represented as (nmol/mg protein) [27]. The standard Griess reaction procedure was used to measure NO, and the level was expressed as μM /mg protein [28].Also, XO (EC 1.17.3.2) assay was carried out, and the activity was represented as (mol/h/mg protein) [29].

Assessment of antioxidant biomarkers

The reduced glutathione (GSH) levels (mg/mg protein) in liver tissue homogenate were determined by measuring the absorbance at 412, as described by Jollow et al[30]. Further, the evaluation of total-superoxide dismutase (SOD) (EC 1.15.1.1) and catalase (EC 1.11.1.6) activities was performed as previously described [31, 32].

Determination of IL-1β, TIMP-1, MMP 2 and Beclin 1

ELISA measurement of interleukin - 1β (IL-1β)(MyBiosource Cat# MBS825017), TIMP (MyBiosource Cat# MBS175959), MMP-2 (MyBiosource Cat# MBS2515523) and Beclin 1 (MyBiosource Cat# MBS733192) was done in liver tissue homogenate of all rats. The manufacturer's protocol for commercial Kitwas followed for the analysis.

Western Blotting assessment of β-actin, Beclin1, α-SMA, TGF-ß and HMGB1

50 μg protein lysates were taken from each sample of the liver tissue homogenate to be mixed with 2X loading buffer (130 mMTris-HCl, pH 8.0, 30 % (v/v) Glycerol, 4.6 % (w/v) SDS, 0.02 % Bromophenol blue, 2 % DTT), boiled for 5 min then cooled at 4ºC. Separation was executed on 12 % SDS-PAGE mini-gel (1.6 ml H2O, 1.5 M Tris pH 8.8, 1.3 ml, 30 % Acrylamide acrylamide/bisacrylamide (29:1 mix in 100 ml) 2.0 ml, 10 % SDS, 10 % APS, TEMED 2 ml) and run at 120 V. Proteins transfer to a nitrocellulose membrane at 22 V was done overnight at 4ºC. The membrane was washed three times with TBST (50

mMTris, pH 7.5, 150 mMNaCl, 0.05 % Tween-20) and incubated in blocking buffer (TBST containing 5 % nonfat dry milk) for 1 h at room temperature and later incubated overnight in primary antibodies [β-actin (NB600-501), Beclin (mAb #3495), α-SMA (mAb #19245), transforming growth factor-beta (TGF-beta) (mAb #5544), and HMGB1 (#3935)] diluted with Tris-buffered saline containing Tween 20 (TBST) and 5% bovine serum albumin (BSA). After 3 times wash with TBST, the membrane was incubated for 1 h at room temperature with secondary antibody. The three times wash with TBST was repeated, then bands were detected by Alkaline phosphatase solution and quantified using ImageJ quantification software [33].

Statistical analysis

Mean ± standard deviation (SD) of data was calculated and statistical analyses were performed with IBM SPSS statistics, version 23.0 (IBM Inc.). One-way analysis of variance (ANOVA) followed by a LSD post-test for multiple comparisons analyses was done and P-value ≤ 0.05 was considered statistically significant.

Results

- **Liver function tests**

At the end of the experiment, liver function tests were assessed to detect the effect of TAA injury to the liver and the effectiveness of GA treatment. As shown in **Table 1,** TAA injection resulted in significant increase of ALT and AST by 1.6 and 1.8 folds to reach a value of (143.7± 19.3 IU/L), (249.2 ± 9.53 IU/L) when compared with control group (55.3 ± 7.9 IU/L), (88.6 ± 4.45 IU/L), respectively (P <0.001). While treatment with GA leadto significant reduction of both ALT and AST to reach a value of (87.8 ± 4.34 IU/L) and (111.3 ± 6.05 IU/L) (P <0.001). **(Table 1)**

Serum Albumin also was significantly decreased (P <0.001) in untreated HF group when compared with the control group (2.48 ± 0.23 g/dl), but treated HF and control group were not significantly different (P=0.01). There was significant increase in total bilirubin level in untreated HF group (0.88 ± 0.04 mg/dL) and treated HF group (0.72 ± 0.05 mg/dL) when compared with the control group (0.49 ± 0.04 mg/dL) (P< 0.001). **(Table 1).**

Table 1: Changes in liver function tests in the studied groups

	Control group	Untreated hepatic fibrosis	Treated hepatic fibrosis
ALT (u/l)	55.3 ± 7.9	143.7 ± 19.3**	87.8 ± 4.34**$^{\#\#}$
AST (u/l)	88.6 ± 4.45	249.2 ± 9.53**	111.3 ± 6.05**$^{\#\#}$
Albumin (g/dl)	3.69 ± 0.23	2.48 ± 0.23**	3.38 ± 0.28$^{\#\#}$
TB (mg/dl)	0.49 ± 0.04	0.88 ± 0.04**	0.72 ± 0.05**$^{\#\#}$
DB (mg/dl)	0.12 ± 0.05	0.28 ± 0.01**	0.17 ± 0.01**$^{\#\#}$

Data is represented as mean ± SD.**significantly different from control group (P<0.001).$^{\#\#}$significantly different from hepatic fibrosis group (P<0.001) (n=10). **Abbreviations**: ALT; Alanin Transaminase. AST; Aspartate aminotransferase, TB; total Bilirubin, DB; direct Bilirubin.

- **Histopathological and immunohistochemical results:**

TAA injected rats demonstrated complete loss of normal architecture, with 8 folds increase in percentage of fibrosis and intense chronic inflammatory cells infiltration. The portal tracts show heavy chronic inflammation, which was also evident by 5.4 folds increase in portal tract area. Immunohistochemistry by α-SMA, as a marker for HSCs activation, reveals increased amount of smooth muscle fibers by 9 folds (P<0.001) **(Figure 1 & 2)**.

GA treatment restored the normal liver architecture and caused 75% reduction in percentage of fibrosis. The hepatocytes show normal size and shape with few cells show mild

ballooning. Most of the portal tracts were reduced by 65% of their area with mild inflammatory infiltrate. Immunohistochemistry by α-SMA revealed 72% reduction of smooth muscle fibers as compared to untreated HF group **(Figure 1 & 2)**.

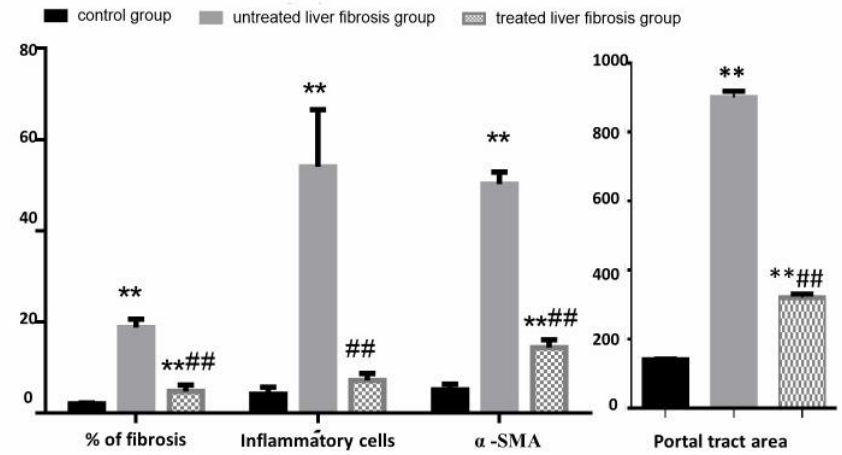

Figure 1: Graphical statistical analysis for the histopathological and immunohistochemical results in all studied groups
**significantly different from control group (P<0.001). ##significantly different from untreated hepatic fibrosis group (P<0.001) Abbreviations: α-SMA; Alpha- smooth muscle actin. Results are represented as mean ± SD.

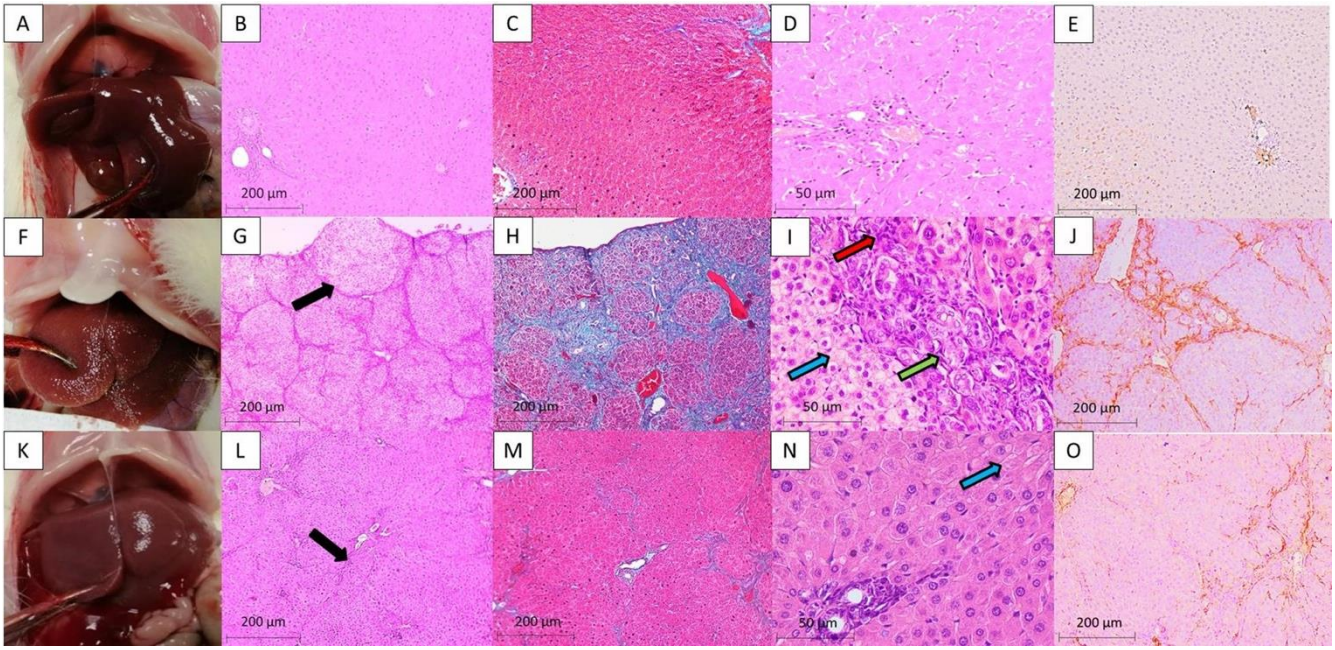

Figure 2: Gross and microscopic histopathological examination in all studied groups
Control group (A to E) shows normal liver architecture (A) (B C, 100x, 400x, H&E) Maisson's trichrome demonstrates the absence of abnormalfibrosis and the normal portal tracts size (D, 100x). α-SMA reveals few normal positive cells(E, 400x)
Untreatedhepaticfibrosis group (F to J)depictingenlargement of the liver size withloss of the normal architecture and replacement by firmcirrhotic variable sized nodules (F) liver tissue with complete loss of the normal architecture and cirrhotic variable sized nodules surrounded by intense chronic inflammatory cells and fibrous tissue bands (black arrow) (G, 100x, H&E). Masson's trichrome stain highlights the massive fibrosis around the cirrhotic nodules (H, 100x). The hepatocytes show severe ballooning and degenerative changes (blue arrow) with expanded portal tracts, proliferating bile ducts (green arrow) and chronic inflammation (red arrow) (I, 400x, H&E) α-SMA reveals increased positive cells (J, 400x)
GA treated hepatic fibrosis group (K to O) reveals liver with normal size and architecture (K) the microscopic picture shows preserved architecture and normal portal tracts with few areas depicting mild periportal fibrous expansion (black arrow)(L, 100x, H&E). Masson's trichrome stain highlights the mild residual periporal fibrosis (M, 100x) The hepatocytes demonstrate mild and focal ballooning (blue arrow) (N, 400x, H&E). α-SMA reveals decreased positive cells compared to the induced group (O, 100x).

- **Marker for oxidative stress and antioxidant activity:**

As shown in **Figure 3**, TAA injection twice weekly (200 mg/kg, i.p.) led to a profound imbalance between oxidants and antioxidants in hepatic tissues. The reactive oxygen and nitrogen species significantly increased in untreated HF rats compared the controls; as illustrated by an increase of 4.2 folds in MDA, a marker of lipid peroxidation, 7.6 folds increase in NO and 2 folds increase in XO. Meanwhile, all antioxidant enzymes activities concomitantly decreased. GSH, a key antioxidant, was reduced by 82.5 %, SOD reduced by 76.4% and catalase was reduced by 73.9% (P <0.001). **(Figure 3)**

Meanwhile, treatment with GA (150 mg/kg/day for 8 weeks) allowed significant decrease of reactive oxygen and nitrogen species and rebalanced the antioxidant system of the liver tissue in comparison to the untreated HF group (P <0.001). GA caused a reduction in MDA by 79%, NO by 88% and XO by 85%. As such, the antioxidant system was restored. GSH, SOD and catalase were elevated by 4.7, 3.8 and 1.9 folds, respectively when compared to the untreated HF group. It is worth mentioning that no significant difference between treated HF and control group regarding MDA, NO, GSH and SOD was noted. **(Figure 3)**

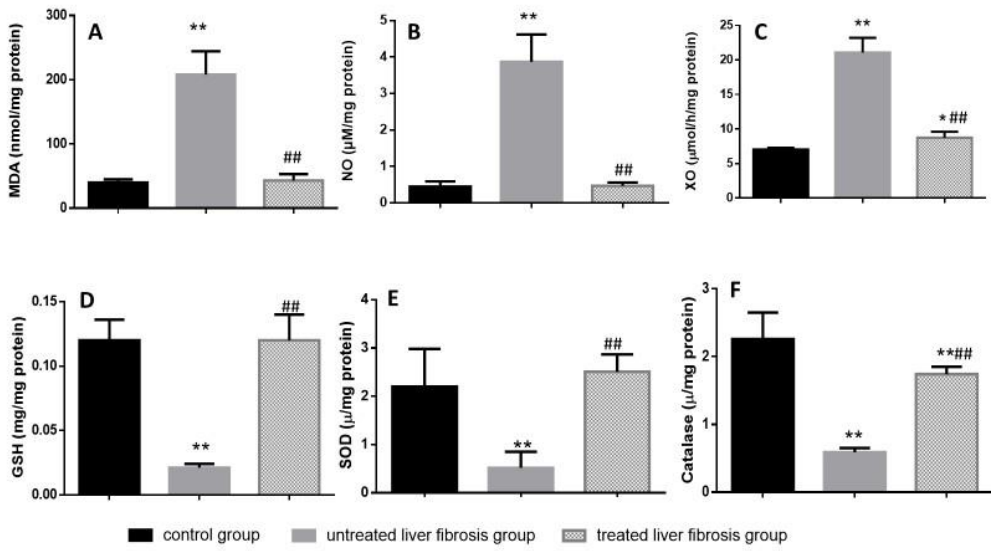

Figure 3: Graphical statistical analysis for oxidant (A-C) - antioxidant (D-F) markers in all studied groups

* Significantly different from control group (P<0.05), ** significantly different from control group (P<0.001), ##Significantly different from untreated hepatic fibrosis group (P<0.001). **Abbreviations**: MDA; Malondialdehyde, NO; Nitric oxide, XO; Xanthine oxidase, GSH; Glutathione, SOD; superoxide dismutase. Data are presented as means ± SD using one-way ANOVA test (n=10).

- **Changes in inflammatory and fibrotic markers:**

- **Upregulation of Il-1ß (ELISA) and TGF-ß (Western blot) in HF and their reduction after GA treatment**

Il-1ß and TGF-ß were upregulated in TAA induced HF group, while GA treatment of led to significant down regulation of both markers without any significant difference versus the control group. Increased production of IL-1β is associated with

several auto-inflammatory syndromes besides its modulation of cell differentiation, proliferation, apoptosis and matrix production being a multipotent growth factor. The upregulation of Il-1ß and TGF-ß by 56% and 42% in untreated HF rats then their reduction after treatment by 36% and 46% indicates their association with the development and resolution of HF. **(Figure 4 A & Figure 5).**

- **Upregulation of MMP-2 and TIMP-1 in HF and their reduction after GA treatment**

HF is a pathophysiological state where ECM accumulates within the injured tissue due to an imbalance in its synthesis and degradation. This was evident in our results by significant increase in TIMP in untreated HF rats to reach mean value of (69.48 ± 4.63 pg/mg protein) as compared to control rats (34.44 ± 3.23 pg/mg protein) (P<0.001). However, treatment with GA caused its reduction to (33.02 ± 2.97 pg/mg protein) to be not significantly different from control group. TIMP-1 is known to block matrix metalloproteinase (MMP) activity thus inhibiting ECM degradation in addition to promoting the longevity of activated HSC, a major source of ECM **(Figure 4 C)**.

On the other hand, MMP-2 is an enzyme capable of degrading ECM molecules especially type IV and V collagens. In our study, injection of TAA twice weekly resulted in significant increase of MMP-2 (138.41 ± 19.95 pg/mg protein) as compared to the normal control group (34.18 ± 5.89 pg/mg protein). Conversely, GA treated HF group demonstrated a significant reduction of MMP-2 expression (43.11 ± 3.47 pg/mg protein) as compared to untreated HF group (P<0.001) **(Figure 4 D)**. This elevation of MMP-2 in the fibrotic model reflects the increase in myofibroblastic cells and suggests that the degrading activity of MMP-2 may not be sufficient degrade fibrosis in ECM.

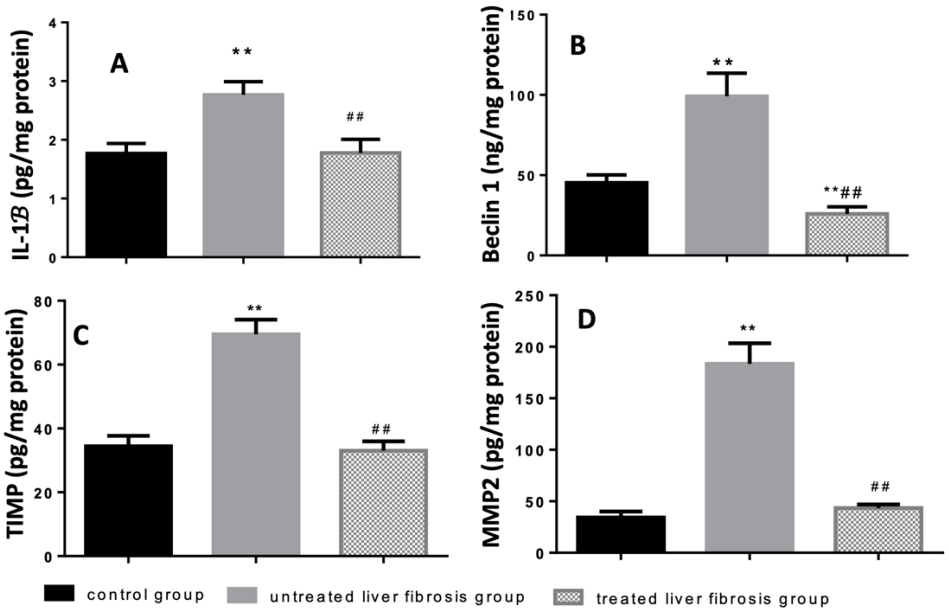

Figure 4: Changes in inflammatory and fibrotic markers in all studied groups

** Significant versus control group (P<0.001), ## Significant versus untreated HF group, (P<0.001). **Abbreviations**: IL-1ß: interleukin-1ß, TIMP: tissue inhibitor of metalloproteinases, MMP 2: matrix metalloproteinase 2. Data are presented as means ± SD using one-way ANOVA test (n=10).

- **Changes in Autophagy pathway:**

Our results showed that TAA insult resulted in 73% increase in HMGB1 as compared to control group. Meanwhile, GA treatment resulted in 37% reduction of HMGB1 as compared to untreated HF rats (P<0.001), highlighting the role of HMGB1 in HF. **(Figure 5)**.

Beclin 1 is increased during periods of cell stress; a key step in mediating autophagy. Beclin 1 has increased significantly in untreated HF rats when compared with the control group (P<0.001). However, Beclin 1 level in GA treated group was significantly reduced compared to untreated HF group (P<0.001) **(Figure 4 B& Figure 5)**.

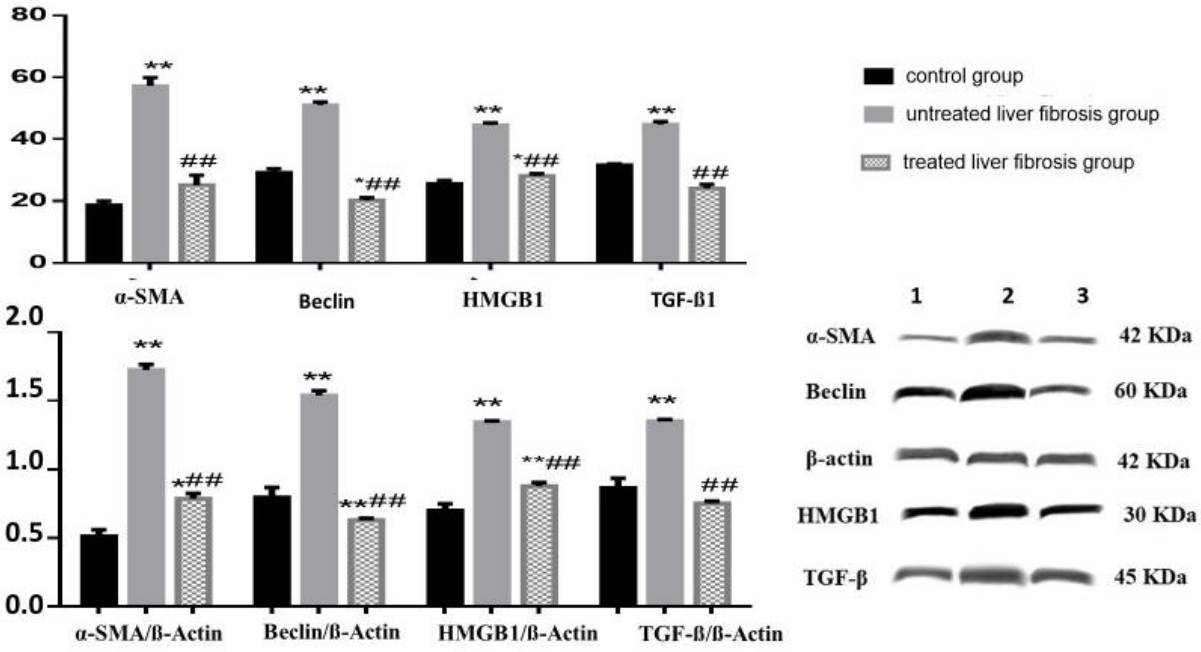

Figure 5: Western blot results of α-SMA, Beclin 1, HMGB1 and TGF-ß in all studied groups

* Significantly different from control group (P<0.05), ** Significant versus control group, (P<0.001), ##significant versus untreated HF group (P<0.001). **Abbreviations:** α-SMA: α smooth muscle actin, HMGB1: high mobility group box 1, TGF-ß: transforming growth factor ß. Data are presented as means ± SD using one-way ANOVA test (n=10).

Discussion

A multitude of chronic liver diseases of different etiology ultimately cause injury to the liver, where the wound-healing response leads to HF which eventually progresses towards advanced liver cirrhosis and might end in hepatic carcinoma. Currently, no effective therapies are approved to treating advanced HF besides transplantation; which is a costly approach not readily available to all patients[34]. Consequently, the search for an effective safe anti-fibrotic therapy has been the focus of variable research. The goal of the present study was to analyze the potential anti-fibrotic effect of HMGB1 inhibitor, GA, in removing the fibrotic scar through inhibiting autophagy.

In the present study, the induction of HF after TAA administration was evident by marked increase in liver enzymes (ALT and AST) with significant histopathological changes including complete loss of the normal architecture showing cirrhotic variable sized nodules surrounded by intense chronic inflammatory cells and fibrous tissue bands. In addition, immunohistochemistry and western blotting for α-SMA revealed increased

amount of smooth muscle fibers. TAA also led to increase in oxidative stress markers and decrease in antioxidant enzymes. This oxidative stress is associated with increase in pro-fibrotic marker TGF-ß1. TGF-β1 was found tostimulate autophagy parallel to its established role in HF pathways[35]. Increased ECM synthesis and fibrosis was evident by 216% increase in α-SMA, 101% increase in TIMP and 304% increase in MMP-2 as compared to the control group. TIMP-1 is known to block MMP-2 activity, thus inhibiting matrix degradation. Additionally, TIMP-1 promotes the survival of activated HSCs and inhibits their apoptosis [36, 37]; evidenced by the notable increase in TIMP mRNA and protein levels during fibrosis [38, 39]. However, MMP levels remain relatively stable or augment slightly[40], excluding MMP-2.The aforementioned levels dramatically increase during fibrogenesis and this fact is explainedby its involvement in normal liver architecture degradation in pathological sates[40, 41]. The concomitant increase in TIMP-1 and MMP-2 expression is a result of activated HSCs, which are the source of this increase [40-42].

To examine the biological effect of GA on HF, 150 mg/kg/day GA was administered intragastric once daily for eight weeks concomitant with TTA injection. The histopathological examination of liver sections revealed restoration of normal hepatic architecture after GA treatment; noticeably decreasing the severity of HF. In addition, Masson's trichrome stain highlighted the few residual fibrous tissue bands and immunohistochemistry by α-SMA revealing 56% reduction of α-SMA, a well-established biomarker of activated HSCs. In addition, the AST and ALT levels decrease after GA treatment indicates a possiblehepatoprotective effect on the fibrogenesis process. Moreover, reduction of proinflammatory cytokines TGF-β and IL-1ß was evident which indicates resolution of inflammation. In addition, GA had inhibited the expression of TIMP-1 and MMP-2 in hepatic tissue, compared with the control group. Many studies revealed that a decrease in TIMP-1 protein levels during resolution of fibrosis, correlated with decreased numbers of activated HSCs [37].

We further investigated the possible mechanism by which GA as an HMGB1 inhibitor modulated the fibrotic process.Autophagosomes are cytosolic vesicles enclosing self-digested cytoplasmic material formed during a self-digestion process, termed "Autophagy"[43]. It was not until the study of Zhu et al [44], that the autophagy role in HSC activation was established. In 2011, Thoen et al study[45] was the first to illuminate that HSCs activation was associated with an autophagic flux and suppressed by an autophagy inhibitor. This findingsupported the belief that autophagy is involved in HSCs activation. To the best of our knowledge, HMGB1 has a role in both, fibrosis and autophagy. We speculate that HMGB1 inhibition may ameliorate HF by regulating the activity of Beclin1 autophagy pathway. Becilin1 has been involved in pathogenesis of HF as a study done by Chen et al showed that Knock-down of Beclin1 in HSCs resulted in the inhibition of α-SMA, TIMP-1 and Collagen I and the autophagy markers protein expression which protects against fibrosis [46].

In support of the proposition of the effect of GA on autophagy, our western blot analysis results indicated that autophagy has increased in liver tissues following TAA-induced injury. The expression of Beclin 1 and HMGB1 was 76% and

73% higher in HF model compared with the control. This increase could be attributed to the increase in oxidative stress as ROScan be regarded as signal leading to the induction of autophagy, apoptosis and necrosis. Furthermore, ROS signals are essential for HMGB1 translocation and upregulation of autophagy [47]. GA treated rats have decreased the level of Beclin 1 and HMGB1 by 61% and 37% as compared to untreated HF rats to be insignificantly different from control group.

HMGB1, an extracellular damage associated molecular pattern molecule (DAMP), is a Beclin 1-binding protein essential in autophagy maintenance. It is a chromatin associated nuclear protein, owning a sizeable sequence similarity with Beclin 1 in yeast, mice and human. Endogenous HMGB1 replaces Bcl-2 in the Bcl-2/Beclin 1 reaction, redirecting the pathway to autophagosomes. Correlation of liver HMGB1 protein expression and the fibrogenesis in patients with chronic hepatitis C virus infection and primary biliary cirrhosishas been noted in several studies[48]. Compared to healthy controls, the serum levels of HMGB1 wereincreased in these patients, proposing protein secretion as an explanation. These findings were validated by replication in different mouse models of HFincluding TAA administration, cholestasis and NASH induced by feeding a methionine- and choline-deficient diet. Overall, an essential role was attributed to HMGB1 in HF as attested by these findings[48, 49]. In a subsequent study, Ge et al [48]proved that HMGB1 stimulated HSCs migration in vitroand in vivo, a key event in the progression of HF. To further demonstrate their theory, the authors performed an in vivo ablation of HMGB1in HSCs whichreduced fibrosis about 25%. HMGB1 could result in ECM accumulation since it led to stimulation of α-SMA expression and inhibition of the activity of the collagen-degrading MMP-2, as found by Kao et al [50]. The role of HMGB1 has been emphasized since neutralization of HMGB1 has protected against HF whereas injection of HMGB1 promoted HF.

On the other hand, two studies [51, 52]could not place HMGB1 in HF pathogenesis. They speculated that chronic injury responses such as fibrosis, regeneration, and inflammation were not significantly affected by liver specific HMGB1 deficiency. It rather downregulated ductular/progenitor cell expansion and hepatocyte metaplasia.

HMGB1 can be a promising therapeutic target in HFor nonetheless a biomarker as it is definitely involved in in fibrosis progression, still the contribution of the various isoforms as well as their exact role in fibrosis regression and the different receptors involved during the resolution phase remain open questions and may help detangling some aspects of the role of HMGB1 in HF.

Conclusions

Our work showed that GA acts as an inhibitor of the autophagy pathway and could be a prophylactic agent against the development of HF in patients with chronic liver disease. We demonstrated the direct inhibition of HMGB1 was the molecular basis of its anti-fibrotic effect, leading to the inactivation of the Beclin1 signaling pathway and ultimately the inhibition of autophagy in HSCs. This information highlights a new strategy to prevent HF through inhibiting the Beclin1 pathway.

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Conflicts of Interest

None of the authors has any potential conflicts of interest to disclose.

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