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ORIGINAL ARTICLE

Protective effects of crocin and gallic acid on the liver damage induced by methylglyoxal in male mice: role of inflammatory factors

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ABSTRACT

Aim: This study aims to evaluate whether biochemical alterations caused by methylglyoxal (MG), improves by the administration of gallic acid (GA), crocin (Cr), and metformin (MT) in the liver.

Background: MG is produced naturally through various physiological processes, but high levels of MG cause inflammation in hepatocytes. Normal liver function is essential for maintaining glucose homeostasis. Gallic acid and crocin can reduce inflammation.

Methods: This experiment was done in 5 weeks. 50 male NMRI mice were randomly divided into 5 groups (n=10): 1) Control, 2) MG (600 mg/Kg/d, p.o.), 3) MG+GA (30 mg/kg/day, p.o.), 4) MG+Cr (60 mg/kg/day, p.o.), 5) MG+MT (150 mg/kg/day, p.o.). After one week of habituation, MG was administered for four weeks. Gallic acid, crocin, and metformin were administered in the last two weeks. Biochemical and histologic evaluations were assessed after plasma collection and tissue sample preparation.

Results: Gallic acid and crocin-received groups significantly reduced fasting blood glucose, total cholesterol, triglyceride levels, and elevated insulin sensitivity. Administration of MG exerted a marked increase in the levels of hepatic enzymes. Treatment with gallic acid, crocin, and metformin significantly decreased them. The altered levels of inflammatory factors in the diabetic group were significantly improved in the diabetic-treated groups. High levels of steatosis and red blood cells (RBCs) accumulation in the MG group markedly recovered in other treated mice.

Conclusion: Harmful effects of accumulated MG in the liver of diabetic mice were effectively attenuated by using gallic acid and crocin.

Keywords: Methylglyoxal, Gallic acid, Crocin, TNF-α, Liver.

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Introduction

Recent assessments have shown that the diabetic population will reach 629 million by 2045. It is striking when compared to 425 million in 2017 (1). Mainly, in recent decades the dramatic increase in type 2 diabetes (T2D) has occurred due to lifestyle changes, such as less physical activity and obesity growth (2). T2D is defined by hyperglycemia, insulin resistance (IR), and insulin secretion impairment, which can induce via an endogenous substance like MG (3). Additionally, T2D is contributed to the development of liver damage because of its ability to induce systemic inflammation. Changes in liver structure and functions due to inflammation induced by T2D have been reported by a previous study via evaluation of inflammatory mechanisms (4). Changes in liver cell function can be diagnosed by measuring key liver enzymes, including

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aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) (5). High-mobility group box 1 (HMGB1) induces different biological processes, such as inflammatory diseases (6). HMGB1 is released from necrotic cells and secreted by activated macrophages, mediating responses to infection, injury, and inflammation (7). Based on previous studies, HMGB1 indicates a pivotal role in liver damage induced by diabetes and carbon tetrachloride via inflammation of nuclear factor-kappa B (NF-κB) and tumor necrosis factor-alpha (TNF-α) activation (8, 9). In addition, another factor is autophagy-related genes 7 (ATG7), which plays a vital role in liver metabolic function and regeneration,; the absence of ATG7 showed hepatic regeneration disturbance after liver damage (10). A recent review study demonstrated that inflammation, steatosis, and liver damage quickly progressed by genetic deletion of ATG7 in mice (11).

MT is used as a hypoglycemic medicine by people with diabetes. In several studies, MT has indicated hepatoprotective properties in animal models of diabetes (12–14). Moreover, previous observations have revealed the interaction of MT with HMGB1 to inhibit its pro-inflammatory activity and the ameliorative effect of MT on liver autophagy by increasing the ATG7 expression level of aged mice (14, 15). According to a review study, MT can improve liver damage in nonalcoholic steatohepatitis and nonalcoholic fatty liver disease (16). Also, it was proven that metformin has a protective effect in streptozotocin-induced diabetic liver damage (17). Based on these results, MT has been selected as positive control medicine in this work.

Up to now, many studies have been done to identify natural compounds with the most negligible side effects, accessibility, and effectiveness in the field of diabetes-induced liver damage. In recent years, using natural compounds instead of or together with chemical drugs (dual therapy) has become common in research. Gallic acid, a famous natural phenolic acid, has demonstrated gastrointestinal, cardiovascular, metabolic diseases, and kidney protection (18, 19). Other results also show that GA ameliorates liver damage complications associated with T2D and inflammation induced by nitrosodiethylamine via various signaling pathways (20–22). Crocin, the main bioactive component of saffron, demonstrates beneficial effects on various tissues. Based on a review study, Cr has anti-inflammatory effects on bowel diseases, gastritis, hepatitis, atherosclerosis, asthma, and depression (23). Notably, evidence reported that Cr ameliorates liver damage induced by diabetes (24), carbon tetrachloride (25), and methotrexate (26).

To our knowledge, no previous study has investigated whether GA and Cr had ameliorative effects on MG-induced diabetic liver damage in mice. Therefore, considering that GA and Cr are natural compounds with antioxidant actions, they may probably be diminished diabetic liver damage. The present study aimed to evaluate thethese compounds' effects concerning inflammation, autophagy biomarkers, and histological parameters in the liver of MG-induced diabetic mice.

Methods

Experimental Design

This study lasted five weeks (Figure 1). For this purpose, 50 male NMRI mice (four weeks old, 20-25 g) were purchased from Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. Animals were attributed by AJUMS experimental animal care guidelines under an ethics committee grantee No. (IR.AJUMS.ABHC.REC.1400.071). GA (Sigma St. Louis, MO), Cr (Sigma–Aldrich, USA), and MT (Solar, bio, South Korea) were purchased. During the experiment, the animals used water and rodent chow freely, and the room temperature was 25 °C, a 12 hr light/12 hr dark cycle, and humidity was 10%. All drugs were administered once a day orally by gavage. The dose of the drugs used in this study was obtained from previous research and our previous pilot study (18, 27). The animals habituated for one week. After that, MG was administered for four weeks. So, at the beginning of the fourth week, the mice were randomly divided into five groups (n=10), which included:

1. Control: orally received normal saline
2. MG (600 mg/kg, p.o): as a diabetic group (3)
3. MG+GA: diabetic mice received GA (30 mg/kg, p.o), (18, 28)
4. MG+Cr: diabetic mice received Cr (60 mg/kg, p.o), (27, 29)

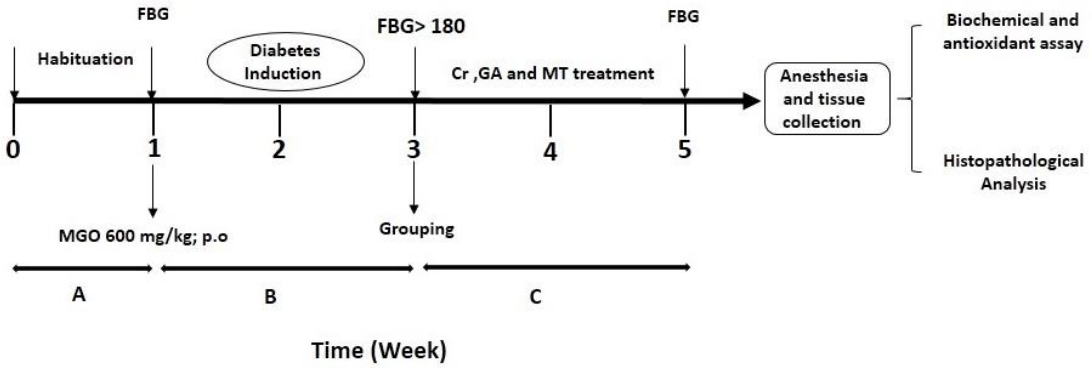

Figure 1. Diagrammatic representation of experimental protocol. A: Habituation, B: Diabetes induction by methylglyoxal (600 mg/kg), C: Administration of gallic acid (30 mg/kg, p.o), crocin (60 mg/kg, p.o), and metformin (150 mg/kg, p.o). At the end of 3th week, diabetic mice were selected based on their fasting blood glucose (FBG) levels and divided into Control: received normal saline, MG: diabetic with methylglyoxal administration (600 mg/kg), MG+Cr: diabetic + crocin 60 mg/kg, MG+MT: diabetic + metformin 150 mg/kg, lasted 2 weeks. Finally, biochemical and histological evaluations were assessed after anesthesia.

5. MG+MT: diabetic mice received MT (150 mg/kg, p.o), (30)

Animals were anesthetized by combining ketamine 10% + xylazine 2% (90 +10 mg/kg, respectively) to collect liver tissues. The liver was then washed with normal saline, and a piece of the liver was removed for histological evaluation. Also, the isolated tissue samples were frozen in liquid nitrogen and stored at -80 °C for biochemical evaluation.

Induction of type 2 diabetes mellitus

According to a previous study, MG can induce T2D, and IR is one of the adverse consequences of MG accumulation (3). In this study, MG dissolved in normal saline. The mice were gavaged by MG for four weeks, once a day. At the beginning of the fourth week, fasting blood glucose (FBG) was measured after six hours of fasting from the tail vein. Then, animals with FBG above 180 mg/dl were used as diabetic mice (27). At this time, the grouping of animals began for drug treatment.

Biochemical assays

Determination of diabetic variables

Plasma insulin concentration was measured by ELISA assay kits. Blood glucose levels were measured by a glucometer (Elegance CT-X10, convergent technologies, Germany). Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was calculated by the following formula:

HOMA-IR: Insulin (μIU/mL) × FBG (mg/dL) / 405

Lipid profile measurement

The plasma level of total cholesterol (TC) and triglyceride (TG) were assessed using commercial kits (Pars Azmoon, Iran) and the auto-analyzer method.

Determination of hepatic enzymes and inflammatory factors

The liver tissue was homogenized in ice-cold Tris–HCl buffer (0.1 M, pH 7.4, ratio 1:4 w/v), centrifuged for 15 min at 10,000 g, and supernatants were used for assessment of TNF-α, NF-κB, ATG7, and HMGB. AST, ALT, and ALP plasma levels were measured using an Autoanalyzer device (BT3000, Italy) and biochemical assay kits (Pars Azmoon, Iran). Inflammatory factors were determined by ELISA kits (ZellBio Gmbh, Germany).

Histology assessment

The mouse liver was removed immediately and fixed in a 10% formalin solution. Then isolated liver was dehydrated in graded alcohol concentrations and embedded in paraffin. Sections 4-6 μm were prepared and stained with hematoxylin and eosin (H&E). Six microscopic images per animal were prepared and used to assess steatosis, congestion of erythrocytes, and infiltration of inflammatory cells using a digital research microscope (BMZ-04- DZ, Behin Pajouhesh ENG. CO., Iran). A mean of 10 fields was considered for each slide and was read in a “blind” fashion.

Statistical analysis

The data were analyzed by GraphPad Prism 9 and represented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used for statistical analysis comparison between

different groups, followed by post hoc high significant difference (HSD) tests (with Bonferroni correction method), P<0.05.

Results

Effects of GA, Cr, and MT on FBG, HOMA-IR, TC, and TG

The levels of FBG significantly increased in the MG group and markedly decreased in the antioxidant-treated mice (P<0.001). Meanwhile, evaluation of HOMA-IR indicated that insulin sensitivity decreased, as disclosed by increased HOMA-IR in the MG group (P<0.001). This variable decreased in all antioxidant-receiving mice (P<0.001). Elevated TG and TC levels in the MG group were remarkably reduced as a result of treatment with all antioxidants for TG (P<0.001), as well as GA (P<0.05), Cr (P<0.05), and MT (P<0.01) for TC. Comparison between treated groups showed that MT had a better effect than GA in improving TG level (P<0.05), (Figure 2).

Effects of GA, Cr, and MT on liver enzymes alterations

The levels of AST and ALT increased in the MG group (P<0.001). Administration of GA (P<0.001), Cr (P<0.01), and MT (P<0.001) improved the AST levels. The ALT levels in GA (P<0.01), Cr, and MT (P<0.05) groups were lower than in the MG group. Increased levels of ALP in the MG group (P<0.001) were markedly decreased in all diabetic-treated groups (P<0.001), (Figure 3).

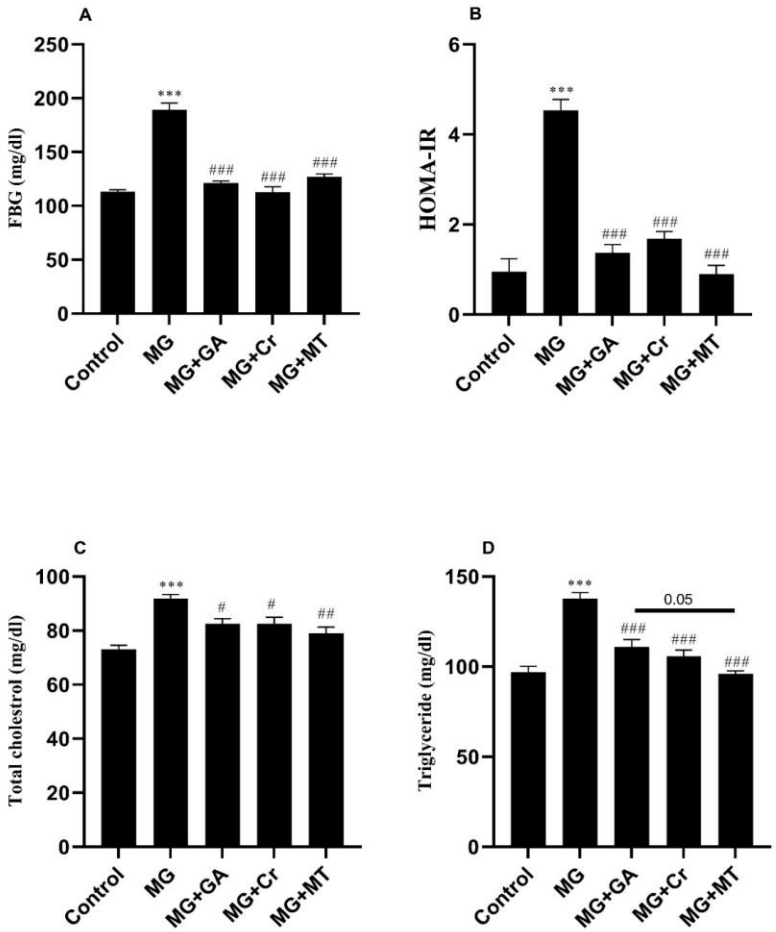

Figure 2. Effects of crocin, gallic acid, and metformin on fasting blood glucose (FBG), insulin resistance index (HOMA-IR), and lipid profile indexes. MG: diabetic with methylglyoxal administration (600 mg/kg), MG+GA: diabetic + gallic acid 30 mg/kg, MG+Cr: diabetic + crocin 60 mg/kg, MG+MT: diabetic + metformin 150 mg/kg. Data are presented as mean ± SEM, n=10 in each group. * Significant difference with control; # significant difference with MG; 1 symbols P<0.05; 2 symbols P<0.01, 3 symbols P<0.001.

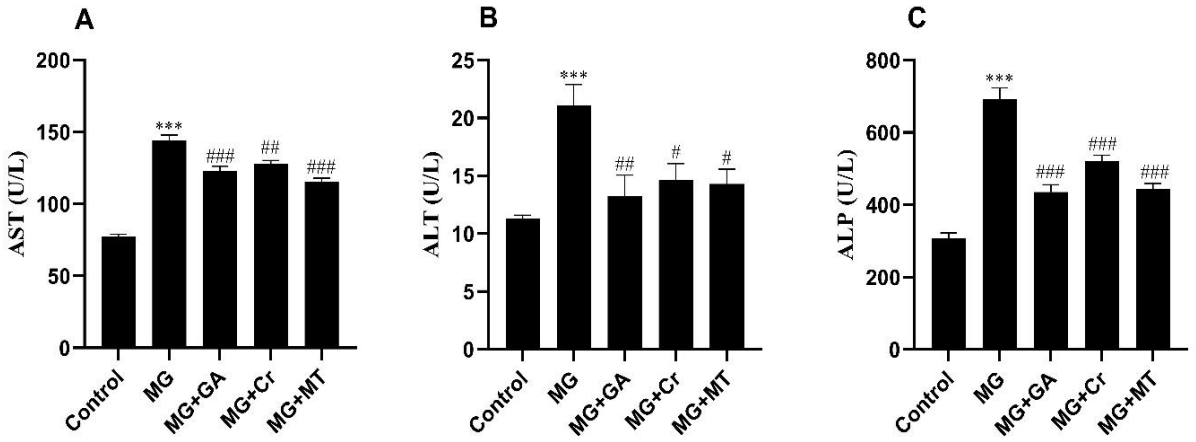

Figure 3. Effects of crocin, gallic acid, and metformin on liver enzymes. MG: diabetic with methylglyoxal administration (600 mg/kg), MG+GA: diabetic + gallic acid 30 mg/kg, MG+Cr: diabetic + crocin 60 mg/kg, MG+MT: diabetic + metformin 150 mg/kg. Data are presented as mean ± SEM, n=10 in each group. * Significant difference with control; # significant difference with MG; 1 symbols P<0.05; 2 symbols P<0.01, 3 symbols P<0.001.

Effects of GA, Cr, and MT on inflammatory factors

A comparison of inflammatory factors between control and MG groups exhibited that TNF-α, NF-κB, and HMGB1 remarkably increased, and the levels of ATG7 decreased in the MG group (P<0.001). The levels of TNF-α dramatically decreased in the liver of diabetic-treated groups (P<0.001). Also, the levels of NF-κB decreased in GA (P<0.01), and those that received Cr and MT (P<0.001). In addition, Cr and MT efficiently reduced the levels of NF-κB compared to GA (P<0.001). There were significant differences among the MG group and diabetic-treated mice when checking ATG7 levels, as, GA (P<0.001), Cr (P<0.05), and MT (P<0.01) effectively improved the level of this variable. Furthermore, in the evaluation of HMGB1 levels, the ameliorative effects of GA (P<0.05), Cr (P<0.05), and MT (P<0.01) were observed (Figure 4).

Effects of GA, Cr, and MT on liver histology

There was an increase in inflammatory cell inflammation in the MG group (P<0.001), and treatment with MT remarkably reduced it (P<0.001). There was no significant effect between GA and Cr in evaluating this parameter. Indeed, MT has a better effect on reducing inflammatory cell inflammation than GA and Cr (P<0.001). Accumulation of RBCs markedly increased in the MG group (P<0.001), and administration of GA (P<0.01), Cr (P<0.001), and MT (P<0.05) reduced it. There was no steatosis in the control group, while it increased sharply in the MG group (P<0.001). Administration of GA, Cr, and MT showed a significant reduction in the percentage of steatosis (P<0.001). In general, MT had a better effect on reducing inflammation, and Cr had a better effect on RBCs accumulation. The efficacy of GA, Cr, and MT was similar in evaluating steatosis (Figures 5A and 5B).

Discussion

Acceptable evidence has illustrated that T2D lead to the progression of diabetic complications in various tissue (31). Studies have shown that MG can activate extensive pro-inflammatory cytokines and is involved in the pathogenesis of inflammation and diabetes (18, 32, 33). Evidence has acknowledged that T2D leads to liver morphology changes and promotes liver damage such as inflammation, steatosis, and hepatocyte degeneration (8, 14, 21). The present study increased glycemic indices, liver enzymes, and plasma levels of TG and TC. Also, liver histological changes such as inflammation, RBC accumulation, and steatosis in MG-treated mice were observed.

According to a previous study, elevated levels of TG and TC that characterize diabetic dyslipidemia are associated with IR (34). Moreover, the accumulation of lipids in the liver strongly relates to hyperglycemia and IR (22, 35). Thus, adjusting the TG and TC levels effectively prevents diabetes and its complications (10, 22). Present results showed that GA and Cr remarkably

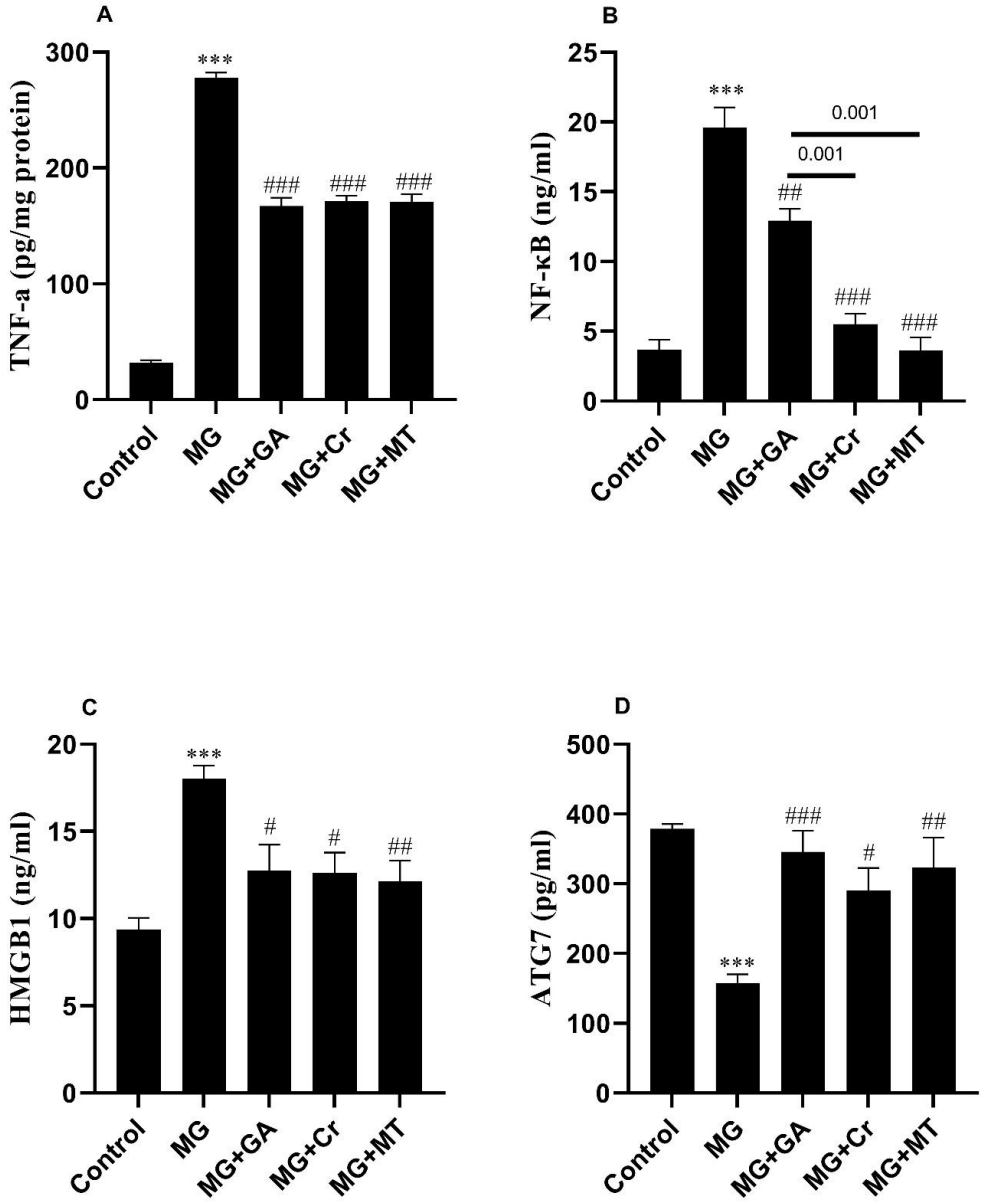

Figure 4. Effects of crocin, gallic acid, and metformin on inflammatory factors. MG: diabetic with methylglyoxal administration (600 mg/kg), MG+GA: diabetic + gallic acid 30 mg/kg, MG+Cr: diabetic + crocin 60 mg/kg, MG+MT: diabetic + metformin 150 mg/kg. Data are presented as mean ± SEM, n=10 in each group. * Significant difference with control; # significant difference with MG; 1 symbols P<0.05; 2 symbols P<0.01, 3 symbols P<0.001.

decreased TG and TC serum levels, indicating their ability to improve lipid metabolism and reduce IR. Also, partial recovery of liver inflammation, RBCs accumulation, and steatosis further confirmed the improvement effects of GA and Cr that agree with previous results (22, 26). Liver enzyme amelioration is another helpful effect of GA and Cr. AST, ALT, and ALP are intracellular enzymes of hepatocytes; therefore, their leakages into the circulation are crucial for diagnosing liver damage (36). Current findings exhibited the ability of GA and Cr to modulate liver enzymes in diabetic mice.

Research showed that HMGB1 is an important mediator of inflammation and is expressed and released by damaged or activated immune cells when liver damage occurs; then, it activates the NF-κB and finally leads to inflammation (8, 14). In the present study, the levels of HMGB1 and NF-κB expression in the liver

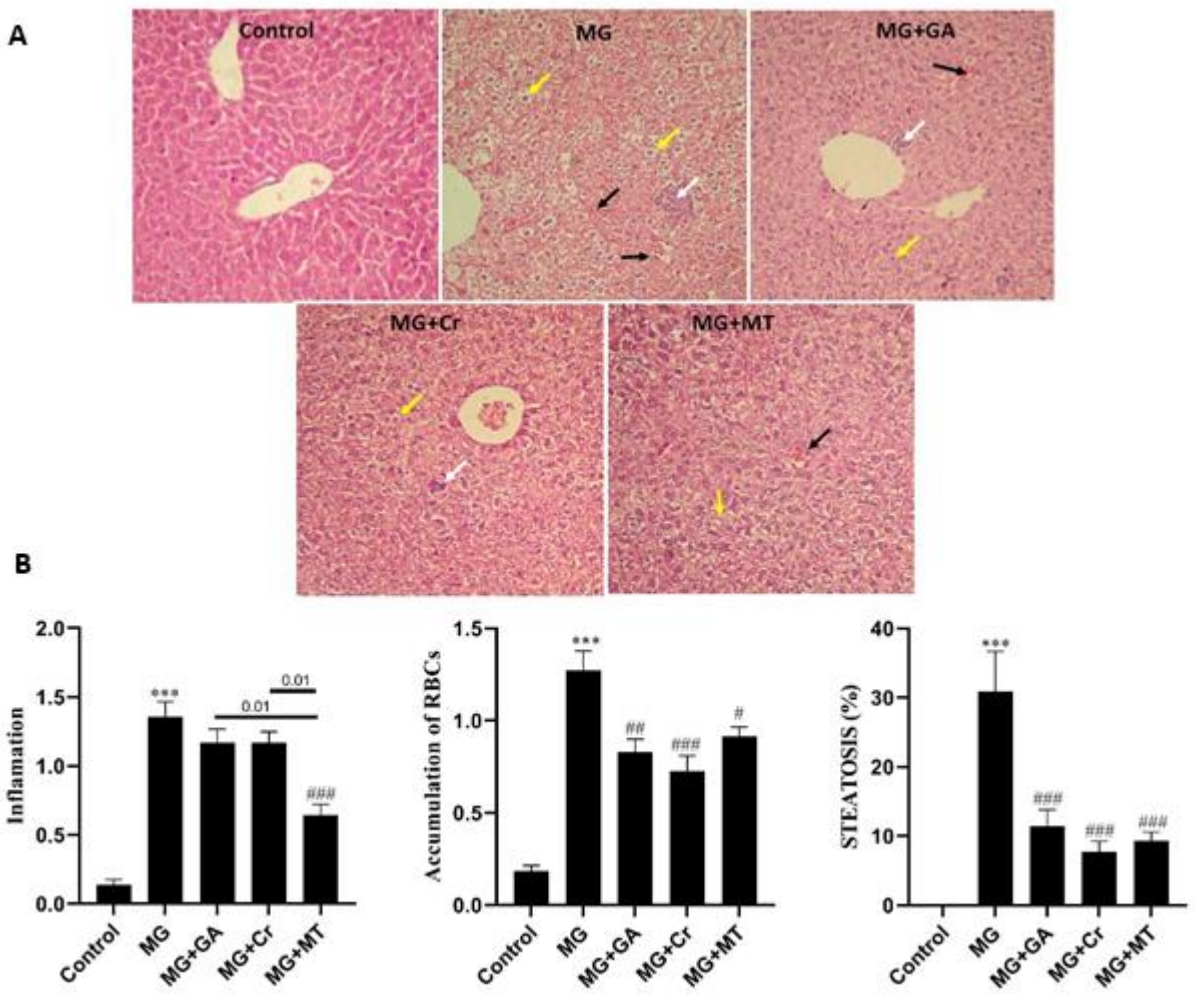

Figure 5. Effects of crocin, gallic acid, and metformin on liver histology. White arrows: Inflammation, Black arrows: Accumulation of red blood cells, yellow arrows: Steatosis, Scale bar: 250 µm. MG: diabetic with methylglyoxal administration (600 mg/kg), MG+GA: diabetic + gallic acid 30 mg/kg, MG+Cr: diabetic + crocin 60 mg/kg, MG+MT: diabetic + metformin 150 mg/kg. Data are presented as mean ± SEM, n=10 in each group. * Significant difference with control; # significant difference with MG; 1 symbols P<0.05; 2 symbols P<0.01, 3 symbols P<0.001.

tissues of MG-receiving mice were higher than in the control. These findings indicated that inflammation occurred in the liver of diabetic mice, while treatment with GA and Cr restored these alterations and inhibited the inflammatory response.

Basal secretion of liver TNF-α strikingly is increased during liver damage (37), which can induce liver cell apoptosis by caspase-3 activation (38). Besides, TNF-α affects the NF-κB signaling pathway and initiates liver inflammation (37). Consistent with this, we observed an enhancement of liver TNF-α levels in the MG group. Administration of GA and Cr decreased TNF-α levels and alleviated the inflammatory response in the liver of diabetic mice. Notably, the inhibitory effects of GA and Cr on TNF-α and HMGB1 levels were equal; but the effect of Cr on NF-κB was stronger than GA.

According to a review study, autophagy as a bulk catabolic process has a protective function allows lipid droplet degradation, and its impairment could contribute to steatosis in the liver. Moreover, the absence of ATG7 leads to impaired insulin signaling, and its restoration ameliorates the function of insulin in the liver of animals with ATG7 deficiency (39). In addition, autophagy defects compromise liver cell survival, leading to TNF-α elevation, and, eventually, hepatocellular carcinoma (40). Also, the absence of Atg7 disturbs liver regeneration after damage (10). Regarding the relation between ATG7 and liver steatosis, and in agreement with previous research (39),

in our study, decreased levels of ATG7 raised liver steatosis in the diabetic group.

Nonetheless, GA and Cr administration noticeably prevented the excessive reduction of ATG7. However, GA and Cr attenuated the size and number of lipid droplets and liver steatosis extent in diabetic mice. Here, the effects of GA and Cr on liver steatosis reduction were similar. Little studies reported the effects of GA and Cr on the levels of liver ATG7; however, we observed that GA and Cr could simulate the effect of ATG7 on steatosis and adjust it.

Conclusion

Accumulation of MG, which occurs in T2D, triggers inflammatory factors and causes damage to hepatocytes. Cr and GA have anti-diabetic and anti-inflammatory effects. Our data showed that GA and Cr modulate the levels of ATG7, HMGB1, NF-κB, and TNF-α in the liver and thus exert considerable hepatoprotective effects. These findings indicated that GA and Cr are inhibitory in the progression of liver steatosis and inflammation in diabetic mice. Evaluation of other supporting molecular mechanisms and the exact signaling pathways, such as gene or microRNA expression, will be helpful in providing better results.

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Conflict of interests

The authors have no conflict of interest in this study.

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