Research Article

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Pretreatment of diabetic aged rats with combination of ginsenoside-Mc1 and silibinin protects liver from ischemia-reperfusion injury through an AMPK-dependent mechanism

Diyabetik yaşlı sıçanların ginsenoside-Mc1 ve silibinin kombinasyonu ile ön tedavisi, AMPK'ye bağlı bir mekanizma yoluyla karaciğeri iskemi-reperfüzyon hasarından korur

<https://doi.org/10.1515/tjb-2021-0010>Received January 14, 2021; accepted May 16, 2021;
published online June 3, 2021**Abstract**

Objectives: This study evaluated the protective efficacy of combination treatment with ginsenoside-Mc1 and silibinin against hepatic ischemia-reperfusion (IR) injury in diabetic-aged rats, and further explored AMPK's role in this protection.

Methods: A high-fat diet/streptozotocin was used to induce type-2 diabetes in aged rats (20–24 months). Diabetic-aged rats were pretreated with ginsenoside-Mc1 (10 mg/kg, IP) and silibinin (50 mg/kg, IP), alone or in combination, for 4 weeks before induction of hepatic IR injury.

Results: Induction of IR injury in diabetic-aged rats significantly elevated plasma levels of hepatic alanine and aspartate aminotransferases and negatively affected liver histology. Levels of 8-isoprostane, ROS production, Bax, and cleaved-caspase-3 expression were higher and manganese-superoxide dismutase

(MnSOD), glutathione, and Bcl2 and p-AMPK were lower in IR-receiving group. In comparison to individual treatments, the combination of ginsenoside-Mc1 and silibinin powerfully restored IR-induced changes in liver enzymes and histopathological indices, oxidative markers, AMPK, and apoptotic protein expressions. Inhibition of AMPK using compound-C in H₂O₂-stimulated HepG2 cells significantly abolished the protective effects of combination treatment.

Conclusions: Combination of ginsenoside-Mc1 and silibinin was superior to their alone usage in protecting hepatocytes of diabetic-aged rats from oxidative/apoptotic damages following IR injury, through an AMPK-mediated mechanism.

Keywords: aging; diabetes; ginsenoside compound-Mc1; liver ischemia reperfusion injury; oxidative stress; silibinin.

Öz

Amaç: Bu çalışma, diyabetik yaşlı sıçanlarda hepatik iskemi-reperfüzyon (IR) hasarına karşı ginsenoside-Mc1 ve silibinin ile kombinasyon tedavisinin koruyucu etkinliğini değerlendirdi ve AMPK'nin bu korumadaki rolünü daha da araştırdı.

Gereç ve Yöntem: Yaşlı sıçanlarda (20–24 ay) tip-2 diyabeti indüklemek için yüksek yağlı diyet/streptozotocin kullanıldı. Diyabetik yaşlı sıçanlar, hepatik IR hasarının

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indüksiyonundan önce 4 hafta boyunca tek başına veya kombinasyon halinde ginsenoside-Mc1 (10 mg/kg, IP) ve silibinin (50 mg/kg, IP) ile önceden tedavi edildi.

Bulgular: Diyabetik yaşlı sıçanlarda IR hasarının indüksiyonu, plazma hepatik alanin ve aspartat aminotransferaz seviyelerini önemli ölçüde yükseltti ve karaciğer histolojisini olumsuz etkiledi. IR alan grupta 8-izoprostan, ROS üretimi, Bax ve bölünmüş kaspaz-3 ekspresyon seviyeleri daha yüksekti ve manganez-süperoksit dismutaz (MnSOD), glutatyon ve Bcl2 ve p-AMPK daha düşüktü. Bireysel tedavilere kıyasla, ginsenoside-Mc1 ve silibinin kombinasyonu, karaciğer enzimlerinde ve histopatolojik indekslerde, oksidatif belirteçlerde, AMPK ve apoptotik protein ekspresyonlarında IR kaynaklı değişiklikleri güçlü bir şekilde restore etti. H₂O₂ ile uyarılan HepG2 hücrelerinde bileşik-C kullanılarak AMPK'nin inhibisyonu, kombinasyon tedavisinin koruyucu etkilerini önemli ölçüde ortadan kaldırdı.

Sonuç: Ginsenoside-Mc1 ve silibinin kombinasyonu, AMPK aracılı bir mekanizma yoluyla, diyabetik yaşlı sıçanların hepatositlerini IR hasarını takiben oksidatif/apoptotik hasarlardan korumada tek başına kullanımlarından daha üstündü.

Anahtar Kelimeler: ginsenoside bileşik-Mc1; silibinin; Karaciğer iskemi reperfüzyon hasarı; oksidatif stres; Yaşlanma; Şeker hastalığı.

Introduction

It was confirmed that diabetes mellitus and aging have a synergistic effect in disturbing many vital organ functions over time. Aged individuals with diabetes develop life-threatening diseases including cardiovascular, neurodegenerative, hepatic, and renal diseases, resulting in far disability even worse than usual, and increased mortality rate [1]. It is well documented that the interaction of aging and diabetes contributes to the development of a vicious cycle between oxidative stress and inflammation, which leads to the production of excessive reactive oxygen species (ROS) and subsequent activation of pro-apoptotic mediators, resulting in chronic complications associated with liver dysfunction [2, 3]. The occurrence of liver damage via increased oxidative stress and apoptosis has been associated with the progression of aging and diabetes in various experimental and clinical situations [4–6]. Besides, the liver tissue is usually subjected to ischemia/reperfusion (IR)-induced injury after liver transplantation, hemorrhagic shock, and hepatic tumor resection, leading to liver failure as a surgical complication. Most

pathophysiological mechanisms of IR injury are the same as those of diabetes and thus, the occurrence of hepatic IR-related complications increases in the presence of diabetes in aged subjects [7]. Therefore, finding out new targets and therapies as well as understanding the underlying mechanisms of hepatocyte protection against IR-mediated injury in the aged liver need further experimental researches.

Ginseng has been traditionally used in the world, especially in Asia, for boosting energy levels as well as for its beneficial effects against oxidative stress and inflammatory conditions, cancer, obesity, hypertension, hypercholesterolemia, diabetes, and aging [8–11]. Ginsenoside compound-Mc1 is one of the main constituents derived from ginseng extracts, which is more pharmacologically active because of being deglycosylated [12, 13]. The growing evidence has shown that ginsenosides have anti-oxidative and anti-apoptosis effects, which activate different cell signaling pathways related to the type of organs, cause increased glutathione (GSH) and manganese superoxide dismutase (MnSOD) and create a balance between oxidants and antioxidants in the cells [14–16]. Moreover, silibinin is an active ingredient of the medicinal plant silymarin (milk thistle) [17–20], and has a worldwide reputation for the treatment of liver disease as a powerful antioxidant [19]. Its protective effect on diabetes and cancer has also been reported [17, 19]. Clinical and experimental studies have proven the anti-oxidative, and anti-inflammatory effects of silibinin via multi-cell signaling pathways [19, 21]. Some reports have demonstrated that the combined treatment of silibinin with other protective compounds is more effective on liver damage than when used lonely [22]. However, the effect of silibinin or ginsenoside compound-Mc1 have not been evaluated in diabetic and aged animals yet.

AMP-activated protein kinase (AMPK) is an important energy sensor that regulates metabolic homeostasis in many situations with disturbed energy metabolism such as IR injury, diabetes, and aging [23]. The evidence suggests that the phosphorylation of AMPK provides protective impacts on hepatic IR injury via preserving cellular energy and reducing oxidative stress, inflammatory reactions, and hepatocyte apoptosis [23]. Interestingly, administration of ginsenoside Mc1 to H9c2 cardiomyocyte significantly increased the level of phosphorylated AMPK and protected the mice heart against IR insults [14]. In addition, silibinin has promisingly repressed the pathogenesis of the non-alcoholic fatty liver disease, *in vitro* and *in vivo*, through the activation of AMPK [24]. Thus, AMPK might become a promising target for the combination of ginsenoside Mc1 and silibinin in hepatic IR injury.

Therefore, based on the above, it is hypothesized that the combined usage of compounds can significantly block the interfering effect of aging and diabetes on liver protection against IR injury. For this purpose, using diabetic aged rats and hepatocyte HepG2 cells, we investigated the effect of ginsenoside Mc1 and silibinin combination therapy on liver protection from IR-induced oxidative stress and apoptosis and further explored the role of AMPK in this protection.

Materials and methods

Animals

The 20–24 month-old male Wistar rats (300 ± 25 g) were used in this study. The rats were housed in an animal room with free access to food and water and maintained at 22 ± 2 °C on a 12 h light/dark cycle according to the standard guidelines. All steps of the experiment were carried out after approval by the Ethical Commission of Affiliated Hospital of Hebei University (ethic number: HEBEI201907A).

Establishment of diabetes

A high-fat diet and low dose (35 mg/kg) streptozotocin method was used to induce type 2 diabetes in animals. Following acclimation of rats for 2 weeks, they fed with a high-fat regimen (which 62% calories were from fat) for 4 weeks and then injected intraperitoneally with streptozotocin (dissolved in 0.1 mol/L citrate buffer, pH 4.5). After 72 h, the diabetic rats were diagnosed when their fasting plasma glucose levels were 250 mg/dL or more. The diabetic period lasted for 10 weeks.

Animal grouping

The aged diabetic rats of matched age were randomly divided into five groups, $n=6$ in each, including: (1) diabetic aged rats or DAR group (rats received no IR injury), (2) diabetic aged rats received hepatic IR injury; as DARIR group, (3) diabetic aged rats received ginsenoside compound-Mc1, 10 mg/kg, by intraperitoneal injection at intervals of 2 days for 4-week before induction of IR injury [14]; as DARIR + GCM group, (4) diabetic aged rats received silibinin, 50 mg/kg, administered by intraperitoneal injection every day for 4-week before induction of IR injury [25]; as DARIR + SIL group, and (5) diabetic aged rats received a combination of ginsenoside compound-Mc1 and silibinin; as DARIR + GCM + SIL group. The drugs were dissolved in dimethyl sulfoxide, and DAR and DARIR groups received dimethyl sulfoxide ($<0.1\%$) intraperitoneally to reduce the vehicle effect.

Liver ischemia reperfusion (IR) injury

At the end of the diabetic period, each animal was heparinized (500 IU/kg) and anesthetized with sodium pentobarbital (50 mg/kg), intraperitoneally. Following midline laparotomy, the ligaments of the liver were removed and then all structures of the portal triad of the left

and median hepatic lobes were occluded for 60 min to induce a 70% hepatic ischemia. Intestinal blood flow through the right and caudate lobes was allowed to prevent mesenteric congestion. After 1 h, the occluded blood flow was reestablished to allow 6-h reperfusion. Finally, at the end of reperfusion, the blood and liver samples were collected, and rats were humanely sacrificed [7].

Determination of plasma level of hepatic enzymes

To measure plasma levels of liver function enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), blood sampling was done before removal of the livers. The plasma of the collected samples was separated using a centrifuge. Thereafter, hepatic enzymes levels were assayed using the rat-specific enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions (Abcam, USA).

Hepatic histological assessment

The collected liver tissues from the middle lobe were fixed in 10% formalin for at least 24 h, then the samples were dehydrated with a sequence of ethanol solutions, embedded in paraffin, cut into 5 μ m sections, and stained with Hematoxylin and Eosin (H & E) dye. Slides were blindly observed under a light microscope. The following indices were assessed: intercellular edema, inflammatory cell infiltration, sarcoplasmic vacuolation, and eccentric nucleus. For semi-quantitative evaluation of these indices the following scoring system was used: (–): normal tissue, (+): mild change, (++) : moderate change, (+++) : severe change and the total score is calculated by averaging all scores of each group. The severity of tissue damage was estimated based on the presence or absence of the sum of these changes in different animal groups and expressed in percentages.

Measurement of oxidative stress markers

The samples isolated from the middle lobe of the liver was homogenized in a PBS buffer containing protease inhibitor cocktail (Sigma-Aldrich, USA), including AEBSF (104 mM), Aprotinin (80 μ M), Bestatin (4 mM), E-64 (1.4 mM), Leupeptin (2 mM) and Pepstatin A (1.5 mM). The supernatants were removed from the homogenates and quickly frozen at -70 °C. Protein content of supernatants was detected using the Bradford method. The concentrations of 8-isoprostane level as a marker of lipid peroxidation, as well as glutathione (GSH) and manganese superoxide dismutase (MnSOD), as the major intracellular antioxidants, in each supernatant were measured using the rat specific Enzyme-linked immunoassay (ELISA) kits according to the kit instructions (MyBioSource, CA, USA). The relative protein levels of each sample were normalized by the amount of total protein.

ROS production in liver tissue

A commercial kit of 2,7-dichloro-dihydrofluorescein diacetate (DCFDA) assay was used for the determination of hepatic intracellular ROS production. The liver tissue (50–100 mg) was incubated with 20 μ M DCFDA (Sigma-Aldrich, USA) for 1 h at 37 °C. DCFDA, a fluorescent dye, measures hydroxyl, peroxy, and other ROS activity within the cell and its organelles. During incubation, DCFDA is

oxidized by ROS into a highly fluorescent compound, 2', 7'-dichloro-fluorescein (DCF). DCF fluorescence was detected by a fluorometric method. An increase in the fluorescent intensity indicated an increased ROS production.

Determination of protein expression by Western blotting

Equal amounts of proteins from each sample were separated in 10% SDS-PAGE and transferred onto a PVDF membrane. After blocking for 1-h with TBS-T (10 mM Tris-HCl at pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 5% fat-free milk, the membrane was incubated with primary antibodies against BAX, Bcl2, cleaved-caspase 3, total-AMPK, and p-AMPK proteins (Cell Signaling, USA) and detected with the enhanced chemiluminescence (ECL, Amersham) reagents. The intensity of the bands was calculated using Image J software (NIH, USA) and normalized to each sample based on the intensity of β -actin (Cell Signaling, USA) as the internal control.

Evaluating the role of AMPK in the protective effects of combination therapy in H₂O₂-treated HepG2 cells

In another series of studies, the hepatocyte cell lines (HepG2 cells) were used to evaluate the role of AMPK in the combination therapy. HepG2 cells were cultured (95% O₂ and 5% CO₂, 37 °C) in DMEM medium containing 10% FBS, 100 U/mL penicillin/streptomycin. HepG2 cells were pre-incubated with the combination of ginsenoside-Mc1 and silibinin (50 μ mol/L for both) with or without the AMPK inhibitor, compound C (5 μ M) for 24 h and stimulated with exposing to H₂O₂ (600 μ M) for 2 h. This part of the study included four groups (N=6) including (1) HepG2 cells treated with H₂O₂ as Control group, (2) H₂O₂-treated HepG2 cells received ginsenoside-Mc1 and silibinin as GCM + SIL group, (3) H₂O₂-treated HepG2 cells received compound C as CC group, and (4) H₂O₂-treated HepG2 cells received ginsenoside-Mc1, silibinin, and compound C as GCM + SIL + CC group.

Statistical analysis

All values were expressed as means $\pm$ standard deviation. Comparison of the differences of parameters between groups were performed through one-way ANOVA followed by Tukey post hoc test utilizing SPSS v16. Histological injury severity was analyzed using Kruskal-Wallis test. A $p < 0.05$ was considered statistically significant.

Results

Liver enzyme levels and histopathological findings

In the present study, we investigated the effect of combination therapy with ginsenoside Mc1 and silibinin on liver function enzymes and the level of hepatocyte oxidative stress and apoptosis in diabetic aged rats and hepatocyte HepG2 cells. The plasma levels of ALT and AST enzymes in diabetic aged rats showed a significant increase compared

with their levels in the DAR group (diabetic aged rats without IR injury) ($p < 0.01$). Administration of ginsenoside-Mc1 to diabetic aged rats significantly decreased both hepatic enzymes compared with the DARIR group ($p < 0.05$) (Figure 1A and B). However, silibinin alone did not affect the plasma levels of these enzymes. In addition, the combination of both treatments significantly reversed the IR-induced increase in both ALT and AST levels in comparison with DARIR group ($p < 0.01$).

The hepatic histologic results showed severe changes in intercellular edema, inflammatory cell infiltration, sarcoplasmic vacuolation, and eccentric nucleus in hepatic tissue of diabetic aged rats subjected to DARIR injury (Figure 2A and B and Table 1). These results designated that induction of IR in these rats accompanied by significant liver structural damages in line with increased dysfunction indicated by elevated levels of hepatic enzymes. These histopathological changes were significantly improved following pretreatment of diabetic aged rats with ginsenoside-Mc1 or silibinin, especially when they being combined (Figure 2 and Table 1).

Expression of apoptotic proteins in hepatic tissue

The expression of apoptotic proteins in the hepatic samples of diabetic aged rats was assayed by the Western blotting technique. Induction of IR injury in these rats led to a significant increase in protein expression of Bax ($p < 0.05$) and cleaved-caspase 3 ($p < 0.01$), and a significant decrease in protein expression of Bcl2 ($p < 0.05$) compared with the controls (Figure 3A and B). Silibinin alone did not affect the expression of apoptotic proteins, and ginsenoside-Mc1 alone significantly reduced the expression of Bax and cleaved-caspase 3 ($p < 0.05$), without any effect on the Bcl2 level as compared with the IR group. However, combination treatment significantly downregulated the expression of Bax and cleaved-caspase 3 not only in comparison with DARIR group ($p < 0.001$), but also in comparison with their levels in individual treatments ($p < 0.05$ for both) (Figure 3A and B). Also, the expression of Bcl2 was significantly increased following combination treatment ($p < 0.01$) (Figure 3C).

Oxidative stress in hepatic tissue

The levels of ROS, lipid peroxidation marker, and antioxidant enzymes were measured by the fluorometric and ELISA methods to appraise the levels of hepatic oxidative stress. The levels of intracellular ROS production ($p < 0.001$)

Figure 1: Plasma levels of hepatic enzymes. (A) ALT: alanine aminotransferase; (B) AST: aspartate aminotransferase. The data were expressed as mean $\pm$ SD. N=6 for each group. ** $p < 0.01$ as compared with DAR group; # $p < 0.05$ and ## $p < 0.01$ as compared with DARIR group. DAR, diabetic aged rat; DARIR, diabetic aged rat subjected to ischemia-reperfusion; GCM, ginsenoside compound-Mc1; SIL, silibinin.

Figure 2: Histopathological changes and severity of hepatic tissues damages. (A) DAR group; (B) DARIR group; (C) DARIR + GCM group; (D) DARIR + SIL group; and (E) DARIR + GCM + SIL group. N=6 for each group. 40 $\times$ magnification. DAR, diabetic aged rat; DARIR, diabetic aged rat subjected to ischemia-reperfusion; GCM, ginsenoside compound-Mc1; SIL, silibinin.

Table 1: Effects of intervention on the severity of hepatic histologic alteration in experimental groups.

Groups	Intercellular edema	Inflammatory cell infiltration	Sarcoplasmic vacuolation	Eccentric nucleus
Control	–	+	+	–
IR	+++	+++	+++	+++
IR + GCM	++	++	++	++
IR + SIL	++	++	++	++
IR + GCM + SIL	+	+	+	+

N=6 in each group. (–): normal, (+): mild change, (++) moderate change, (+++) severe change. IR, ischemia-reperfusion; GCM, ginsenoside compound-Mc1; SIL, silibinin.

and 8-isoprostane ($p < 0.01$) in hepatic tissues (as the indicators of oxidative stress) were significantly augmented following hepatic IR induction in diabetic aged rats as compared with control rats; however, the levels of endogenous antioxidants MnSOD ($p < 0.01$) and GSH ($p < 0.001$) were significantly lessened in DARIR group (Figure 4A–D).

Although both individual treatments significantly increased antioxidant levels when compared with DARIR group ($p < 0.05$), none of them significantly reduced oxidative marker 8-isoprostane, and only ginsenoside-Mc1 alone significantly reduced ROS production ($p < 0.05$). Nevertheless, combination therapy not only reduced the

Figure 3: Pro- and anti-apoptotic proteins expression in liver tissue. (A) Bax; (B) cleaved caspase 3; (C) Bcl2; and (D) representative immunoblots. The data was expressed as mean $\pm$ SD. N=6 for each group. ** $p < 0.01$ and *** $p < 0.001$ as compared with DAR group; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ as compared with DARIR group. \$ $p < 0.05$ and & $p < 0.05$ as compared with DARIR + GCM and DARIR + SIL groups, respectively. DAR, diabetic aged rat; DARIR, diabetic aged rat subjected to ischemia-reperfusion; GCM, ginsenoside compound-Mc1; SIL, silibinin.

IR-induced ROS overproduction and 8-isoprostane elevation ($p < 0.01$), but also recovered the levels of antioxidants MnSOD and GSH ($p < 0.001$) in comparison to DARIR group. Moreover, the reduction in ROS production and 8-isoprostane levels in the combination group was significantly greater than those of individual treatments ($p < 0.05$) (Figure 4A and B). These results indicated that combined treatment was more effective than alone treatments in reducing oxidative stress.

The levels of AMPK expression in hepatic tissue

The expression of total and phosphorylated forms of AMPK was evaluated by Western blot. There were no significant differences in total AMPK expression between groups (Figure 5A). Nonetheless, the p-AMPK/t-AMPK ratio was decreased significantly in DARIR group compared with the controls ($p < 0.001$) (Figure 5B). The p-AMPK/t-AMPK ratio was significantly increased to some extent in ginsenoside-Mc1 received group ($p < 0.05$), but silibinin alone had no significant impact. Finally, combination of both compounds significantly and more potently upregulated the

expression of p-AMPK in comparison to DARIR group ($p < 0.001$) and silibinin alone group ($p < 0.05$).

The role of AMPK in the protective effects of combination therapy in H_2O_2 -treated HepG2 cells

To evaluate the role of AMPK in the protective effect of combination therapy with ginsenoside-Mc1 and silibinin, the H_2O_2 -exposed HepG2 cells were treated with compound C as a specific inhibitor of AMPK. The results showed that combination treatment significantly reduced ROS production, increased MnSOD levels, downregulated the cleaved-caspase 3 expression ($p < 0.01$), and upregulated the p-AMPK expression ($p < 0.001$) in H_2O_2 -exposed HepG2 cells in comparison to the untreated control group (Figure 6A–C). Inhibition of AMPK by administration of compound C reversed the effects of combination treatment on hepatocyte ROS, MnSOD, and cleaved-caspase 3 and p-AMPK expression levels (Figure 6). Results from the animal experiments and HepG2 cells suggest that the hepatoprotective effect of combined treatment may be associated with an AMPK-dependent mechanism, leading to the amelioration of liver IR injury.

Figure 4: Oxidative/antioxidative markers in liver tissue. (A) ROS production assessed by DCFDA dye; (B) 8-isoprostane; (C) MnSOD; and (D) glutathione or GSH levels. The data was expressed as mean $\pm$ SD. N=6 for each group. ** $p < 0.01$ and *** $p < 0.001$ as compared with DAR group; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ as compared with DARIR group. \$ $p < 0.05$ and & $p < 0.05$ as compared with DARIR + GCM and DARIR + SIL groups, respectively. DAR, diabetic aged rat; DARIR, diabetic aged rat subjected to ischemia-reperfusion; GCM, ginsenoside compound-Mc1; SIL, silibinin.

Figure 5: AMPK protein expression in liver tissue. (A) total-AMPK (t-AMPK); (B) phosphorylated-AMPK (p-AMPK); and (C) representative immunoblots. AMPK, AMP-activated protein kinase. The data was expressed as mean $\pm$ SD. N=6 for each group. *** $p < 0.001$ as compared with DAR group; # $p < 0.05$, and ### $p < 0.001$ as compared with DARIR group. & $p < 0.05$ as compared with DARIR + SIL group. DAR, diabetic aged rat; DARIR, diabetic aged rat subjected to ischemia-reperfusion; GCM, ginsenoside compound-Mc1; SIL, silibinin.

Figure 6: AMPK inhibition reversed the effect of combination therapy in H_2O_2 -stimulated HepG2 cells. (A) ROS production; (B) MnSOD; (C) cleaved caspase 3; (D) p-AMPK; and (E) representative immunoblots. The data was expressed as mean $\pm$ SD. $N=6$ for each group. ** $p<0.01$ and *** $p<0.001$ as compared with Control group; and # $p<0.05$ as compared with GCM + SIL group. GCM, ginsenoside compound-Mc1; SIL, silibinin; HepG2, human hepatoma cell line; CC, compound C.

Discussion

This study reported that the pre-treatment of diabetic aged rats with a combination of ginsenoside compound-Mc1 and silibinin was superior to their monotherapy in the protection of hepatic tissue against IR injury and subsequent oxidative stress and apoptosis. This protection of combination therapy was achieved through an AMPK-dependent mechanism.

Hepatic IR injury delays the recovery of patients undergoing liver resection and transplantation. Elderly patients have a higher risk of undergoing liver surgery than younger individuals. Having diabetes, as accompanying comorbidity, aggravates IR injury in aged subjects [7]. In this situation, numerous interfering factors including hyperglycemia, chronic oxidative stress, apoptosis, and inflammation lead to extensive abnormalities in metabolism and the activity of intracellular signaling kinases. All of them may ultimately attenuate the efficacy of therapeutic modalities [3, 4]. Thus, the multifactorial pathophysiology of hepatic IR injury in an aged diabetic patient requires a multimodal and combinational therapeutic

approach. In consistent with this scenario, this study showed that IR injury led to ROS overproduction and elevated levels of oxidative stress and apoptosis in the liver of diabetic aged rats, which was associated with high plasma levels of the hepatic enzymes (ALT and AST), and the abnormal histological changes. Treatment of these rats with ginsenoside-Mc1 alone or silibinin alone, although corrected the effect of IR injury on liver parameters, these effects were not consistent. However, the combination of them had a powerful hepatoprotective impact by inducing antioxidative and anti-apoptotic effects.

Emerging studies indicate that ginsenoside compounds and silibinin, the main constituents derived from ginseng extracts and silymarin respectively, have a potent antioxidant effect, using alone or in combination with other drugs [14, 18, 21, 26]. Similar to our findings, preconditioning of rats with ginsenoside-Rg1 [27] or ginsenoside-Rb1 [28] (the major components of the root and stem of ginseng) significantly reduced the infarct volume after cerebral [27] or cardiac [28] IR injury, respectively, through down-regulating the protease-activated receptor-1 and upregulating endothelial nitric oxide synthase (eNOS) and nitric

oxide (NO), leading to increased SOD and decreased malondialdehyde concentrations. Ginsenoside-Mc1 is more pharmacologically active because of being deglycosylated [12, 13]. Hong et al. [14] reported the cardioprotective, anti-oxidative, and anti-apoptotic effects of ginsenoside-Mc1 in high-fat diet-induced obese mice via regulating AMPK and Bax proteins, followed by increased superoxide dismutase-2 and decreased caspase-3 activity. Another study indicated that the endoplasmic reticulum stress-mediated hepatocyte damage and insulin resistance were alleviated through the suppression of c-Jun N-terminal kinase (JNK) following the treatment of HepG2 cells and diet-related obese mice with ginsenoside-Mc1 [29]. Moreover, administration of silibinin to the obese mice and HepG2 cells reduced free-radical insults by activating the SIRT1/AMPK pathway [24]. Silibinin also protected the liver against IR injury through anti-oxidative and anti-apoptotic mechanisms [30, 31].

In the present study, we tested the most effective doses of ginsenoside-Mc1 and silibinin reported in previous IR injury situations in young and healthy animals [23, 32]. However, the considerable hepatoprotection was observed in the combination of them. Both aging and diabetes are the main comorbidities of IR injury, which may negatively influence the protective influences of monotherapy with ginsenoside-Mc1 or silibinin. Combining them, however, augmented their efficacy to protect the hepatic tissue and significantly reduced ROS production, lipid peroxidation (8-isoprostane), and pro-apoptotic proteins Bax and caspase-3 and significantly increased endogenous antioxidants MnSOD and GSH as well as anti-apoptotic protein Bcl2. Some previous reports have demonstrated that the combined treatment of silibinin with other hepatoprotective compounds is more effective on liver damage suppression than when used alone. For example, Rasool et al. [22] investigated the effect of concomitant treatment of silymarin and glycyrrhizin (the main active ingredient of licorice root) on liver damage induced by hepatotoxin carbon tetrachloride in rats. They found a greater hepatoprotective effect with combination therapy against oxidative liver damage accompanied by increased SOD and GSH levels. Furthermore, their combination therapy effectively decreased ALT and AST, and profoundly recovered the histopathological changes such as degenerated hepatocytes, cellular necrosis, and dilated hepatic sinusoids [22].

In our study, similar to the previous report [22], a partial synergism was observed in the effect of combination therapy with ginsenoside-Mc1 and silibinin via reduction of oxidative/apoptotic markers. Administration of compound C to the HepG2 cells abolished this synergistic effect, indicating that this protection was accomplished through phosphorylating and activating AMPK in hepatocytes. The AMPK plays the

main role in regulating energy metabolism especially in metabolic-related conditions and disorders such as aging and diabetes [23, 24]. Activation of AMPK also reduces oxidative stress and apoptosis in several ways. To explore the contribution of this kinase in the protective effects of combination therapy in H_2O_2 -stimulated HepG2 cells, we observed that inhibition of AMPK by compound C considerably reversed the effect of combination therapy on reducing hepatic ROS level, and expression of pro-apoptotic cleaved-caspase 3, and increasing antioxidant MnSOD levels. This finding supports that the anti-oxidative and anti-apoptotic impacts of combination therapy in hepatic IR setting of diabetic aged rats are AMPK-dependent. However, numerous other mechanisms such as the activation of protein kinase C pathway, endoplasmic reticulum stress, and mitochondrial function may play a causal role in this protection [4, 21, 29, 33], which requires further studies.

Conclusion

In conclusion, the hepatoprotective effects of ginsenoside-Mc1 and silibinin combination on IR-induced liver injury in diabetic aged rats were greater than those of their monotherapy. Their co-administration prevented IR injury through the reduction of oxidative stress and pro-apoptotic proteins as well as upregulation of antioxidative and anti-apoptotic proteins. Activation of AMPK was the important mechanism of combined treatment-induced hepatoprotection.

Research funding: Hebei Provincial Government-funded Project for provincial Clinical Medical Talents in 2017.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflict of interest.

Informed consent: Not applicable.

Ethical approval: All steps of the experiment were carried out after approval by the Ethical Commission of Affiliated Hospital of Hebei University (ethic number: HEBEI201907A).

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