Glycyrrhizin Represses Total Parenteral Nutrition Associated Hepatitis in Rats via Endoplasmic Reticulum Stress Suppression

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**Abstract**

Total parenteral nutrition (TPN) is an artificial way to support daily nutritional requirements bypassing the digestive system into the body but long-term TPN administration may cause severe liver dysfunction. Glycyrrhizin is an active component of licorice roots which has been widely used for the treatment of chronic hepatitis. The aim of this study is to investigate the hepatoprotective effect of glycyrrhizin on TPN-associated hepatitis in vivo. Liver dysfunction was induced by intravenous infusion TPN with a flow rate of 20 mL/kg/hr for 3 hours in Sprague Dawley rat. Glycyrrhizin was pretreated with a dose of 1, 3, and 10 mg/kg intravenously. After receiving TPN or saline (as control group) for 3 hours, the rats were sacrificed and the blood sample was collected for biochemical assay, and the liver tissue was removed for histopathological and immunohistochemical examinations. The results demonstrated that the aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB) and triglyceride (TG) were significantly increased in the TPN group without glycyrrhizin pretreatment and decreased in the glycyrrhizin-pretreated TPN group in a dose-dependent manner. The stained liver sections showed that the glycyrrhizin relieved the acute liver injury. The up-regulation of serum protein biomarkers of reactive nitrogen species, including nitrotyrosine and inducible NO synthase (iNOS), were attenuated by glycyrrhizin pretreatment. The endoplasmic reticulum (ER) stress factors such as phosphorylation of JNK1/2 and p38 MAPK and CHOP expression were decreased by glycyrrhizin pretreatment. In summary, our results suggest that glycyrrhizin declines TPN associated hepatitis factors may be through suppression of endoplasmic reticulum stress and reactive nitrogen stress.

Abbreviations: TPN: total parenteral nutrition; ER: endoplasmic reticulum (ER)

**1. Introduction**

Total Parenteral Nutrition (TPN) is a way to provide nutrients such as glucose, amino acids, lipids, added vitamins and dietary minerals to patients who are unable to sustain adequate nutrition by standard enteral means, such as patients suffering from cancer of gastrointestinal disorders. However, long-term TPN administration was found to increase the risks of hyperglycemia, hepatic inflammation, steatosis, insulin resistance and reduced immune responses [1, 2],among which hepatitis is one of the most commonly occurred complication [3, 4]. Although several factors, such as enteral feeding history, septic events, length of intestinal resection and prematurity/low birth weight, have been associated to high risk of TPN-induced hepatitis [5], the pathogenic mechanism is not yet fully understood [6, 7].

Three pathogenic pathways have been proposed to be involved in the TPN-induced chronic hepatitis:(1) Liver cell toxicity: this may be occurred as a result from the toxic effect of detergent-like bile salts [8, 9, 10, 11], a consequence of decreased bile excretion and gut hormone secretion due to reduced enteral feeding [10, 11]. According to Loff et al., TPN related hepatobiliary abnormalities in rabbits is mainly in liver acinar zone 3 area [20]. As zone 3 is composed of hepatocytes located around the hepatic venule, which involves mainly in the production of bile salt, the TPN related hepatic injury in zone 3 may be a typical feature for a direct toxic effect of the bile salt [12, 13].(2) Liver inflammatory reaction: this may a result triggered by the oxidative stress [14]. TPN is an important source of oxidants. Peroxides in TPN are derived mainly from the reduction of dissolved oxygen by electron donors such as vitamin C, amino acids, and lipids [15]. The peroxide-induced oxidative stress was found to be elevated in blood and liver after TPN administration in Guinea pigs [14]. Moreover, oxidative stress can trigger the expression of pro-inflammatory cytokines, which play essential roles in the inflammation cascade[16].Cytokines can be negatively regulated by various mechanisms, including the Suppressors Of Cytokines Signaling (SOCS) protein [17, 18]. The SOCS protein has been reported to be highly upregulated in response to pro-inflammatory cytokines (i.e., IFN]-γ, interleukin [IL]-1β, and IL-6) [17]. In particular, SOCS1 and SCOS3 proteins have been recognized to be the main modulators for the negative regulation of the cytokine-JAK–STAT pathway, and can thus be potentiated to induce anti-inflammation process [19]. (3) Hepatocyte apoptosis: this has been observed at 24 hours after TPN administration in a mouse model [20]. It may be initiated either by (i) activation of death receptors, e.g., Fas ligand, TNF-α, or (ii) dysfunction of mitochondria function, or (iii) endoplasmic reticulum (ER) stress as induced by accumulation of active caspases and destruction of chromatin (for review, see [21, 22]).

Recently, glycyrrhizin has been reported to exhibit hepatoprotective effects in treatment of viral hepatitis [23]. Glycyrrhizin is an active compound isolated from the root of licorice (Glycyrrhizaglara; Glycyrrhiza radix; Chinese name, Gancao). In tradition Chinese medicine, licorice is one of the most commonly-used herbs in treating liver disorders [24]. It is also used to reduce toxicity, to improve appetite or enhance the effectiveness of other ingredients in prescriptions [24, 25]. There is evidence for therapeutic application of glycyrrhizin in chronic hepatitis induced by virus, toxin, and ischemic-reperfusion injury [26]. In Japan, Stronger Neo-Minophagen C (SNMC), a commercially available glycyrrhizin-containing prescription, has been adopted in clinical practice for chronic viral hepatitis treatment for more than 30 years [27, 28].

In this study, we hypothesized that pretreatment of glycyrrhizin is able to alleviate the TPN-induced hepatitis. To elucidate this hypothesis, we used TPN-induced hepatitis rats as the animal model to investigate the hepatoprotective effects of glycyrrhizin upon TPN-induced acute liver dysfunction. We assessed the early stage of TPN related liver dysfunction obtained from biochemical, histopathological and immunohistochemical investigation. Our aim is to fill the knowledge gap in the molecular mechanism underlying the pharmacological effects of glycyrrhizin in treatment of TPN-induced hepatitis. We found that glycyrrhizin exhibits hepatoprotective activities in TPN-induced acute liver dysfunction through endoplasmic reticulum (ER) stresssuppression and pro-inflammatory cytokines attenuation.

**Materials and Methods**

2.1 Chemicals and Reagents

Glycyrrhizin, Reactive Nitrogen Species (RNS) scavenger (including nitrotyrosine and anti-iNOS), CHOP, Janus kinase (JNK) inhibitor (SP600125), and p38 inhibitor (SB203580) were purchased from Sigma (St. Louis, MO, USA). Total parenteral nutrition solution Kabiven™ was obtained from Fresenius Kabi AB, Sweden. Mouse monoclonal antibodies against GAPDH, β-actin, pro-inflammatory anti-TNF-α, IL-1β, IL-6 and nitrotyrosine were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit antibodies against human SOCS3, cleaved-caspase-3, phospho-p38 MAPK (Thr180/Tyr182), phospho-JNK1/2 (Thr183/Tyr185), cdk1Tyr15 and mouse monoclonal antibody cdk1 were purchased from Cell Signaling Technology (Beverly, MA, USA). The TdT-mediated dUTP Nick End Labeling (TUNEL) kits were from Roche (Germany). SDS, NP-40, sodium deoxycholate, protease inhibitor cocktails were purchased from Sigma (St. Louis, MO, USA).

2.2 Reagents and Drug preparation

Each 1440ml Kabiven® contains: glucose 11% 885 ml, amino acids/electrolytes(Vamin 18) 300ml, fat emulsion (Intralipid) 20% 255 ml. The total energy is 1038 Kcal in 1440 ml. The 200 mg rat received the TPN with infusion rate 20cc/kg/hr for three hours and the total infusion volume is 12 cc TPN, the energy is approximately 8.6 kcal.

The glycyrrhizin is was purchased from Stigma ( St. Louis. MO, USA) and we prepared the glycyrrhizin with three different treatment doses, 2mg GL in 10 cc normal saline(NS), 6 mg GL in 10cc NS, 20 mg GL in 10cc NS, then we use 1 cc of the mixture solution for the i.v. bolus pretreatment drug in 200 mg rats.

2.3 Animal Experiments and Drug Treatment

Adult male Sprague-Dawley rats weighing 200 ± 20 g were obtained from the National Yang-Ming University Animal Center, Taipei, Taiwan. The rats were speciﬁc pathogen-free and had free access to food (Laboratory Rodent Diet 5001, PMI Nutrition International LLC, MO, USA) and water. The rats were housed with a 12-h light and 12-h dark cycle. All animal experimental protocols involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC number: 981106) of National Yang-Ming University. Rats were anesthetized with urethane (1 g/kg, ip). All animals were equipped with left femoral vein for solution or drug administration.

Rats were intravenously infused with TPN via left femoral at a flow rate of 20 mL/kg/hr for 3 hours to induce acute liver injury. Rats were intravenously infused with normal saline ( sodium chloride 0.9% ) via left femoral at a flow rate of 20 mL/kg/hr for 3 hours as a control group. The three different dose of glycyrrhizin (1, 3 or 10 mg/kg) were pretreated intravenously before TPN infusion as three different dose of glycyrrhizin-pretreated groups. All glycyrrhizin was dissolved in 1 mL normal saline and i.v. bolus before TPN administration as pretreatment group. The TPN group also received equal volume normal saline 1 mL i.v. bolus before TPN administration. Every group had six animals respectively. Blood samples were collected through left femoral vein immediately and stored in heparin-coated capillary tubes for analysis of liver enzyme profiling and biochemical analysis. Liver tissue was collected and washed with normal saline and then fixed in 10 % neutral buffered formalin for histopathological examinations.

2.4 Biochemical analysis.

Blood samples collected from the animals were centrifuged at 3000 rpm for 15 minutes. The plasma samples were used for various biochemical analyses such as: aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB) and triglyceride (TG), which were performed by the Hitachi 902 Automatic Analyzer using the adapted reagents from Roche (Germany).

2.5. Histopathological evaluation.

The liver tissues were fixed in 10 % buffered formalin, embedded by paraffin and cut into 4 μm-thickness slides transversely from portal zone 1 to zone 3 area close to the terminal of hepatic venule tract for hematoxylin and eosin (H & E) staining. The rest of tissues were stored at -80 °C. The histopathological changes in the liver cell morphology were examined by BX51 light microscope (Olympus, Japan) at high power (200× magnification) for each slide. For quantitative purpose, two portal zones were randomly chosen in each slide with photos taken by Image-pro Plus medical image analysis system.The cell numbers were recorded. The normal hepatocytes were counted from 10 fields randomly chosen in liver sample in every group under 200× magnification light microscope. The means of normal cells were calculated per microscope field from six animals in each group.

2.6. Immunohistochemisty assay

Immunohistochemistry (IHC) stain was performed using a biotinylated secondary antibody (Vectastain Universal Elite ABC Kit). Monoclonal rabbit antibodies against human SOCS3 and cleaved-caspase 3 were diluted in a ratio of 1:100. The omission of primary antibodies was used as the negative control. For three slides, cytoplasm stained with brown was scored as positive. The expression of SOCS3 and cleaved-caspase 3 was quantitatively evaluated using Olympus Cx31 microscope with Image-pro Plus medical image analysis system. The digital images were captured using a digital camera (Canon A640). The positive area and optical density (OD) of SOCS3 and cleaved-caspase 3 positive cells were determined by measuring three randomly selected microscopic fields (400× magnification) for each slide. The IHC index was defined as average integral optical density (AIOD) (AIOD = positive area×OD/total area).

2.7. Western blot analysis

After sacrifice, rats' livers were quickly removed, disassociated and homogenized in ice-cold lysis buffer (1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate and a protease inhibitor mixture comprised of phenylmethylsulfonyl fluoride, aprotinin, and sodium orthovanadate). The total cell lysate (50 μg of proteins) was separated by the SDS-polyacrylamide gel electrophoresis (PAGE) (12 % running, 4 % stacking) and analyzed by using designated antibodies and the Western-Light Chemiluminescent detection system (Bio-Rad, Hercules, CA), as previously described by Chiu et al.[29].

2.8. Statistical analysis.

Group data were represented as the mean ± standard deviation. The group effect was examined by one-way analysis of variance. Significant differences were established at P < 0.05. All statistical analysis was conducted using SPSS software.

**3. Results**

*3.1. Effect of glycyrrhizin on biochemical analysis*

To investigate the TPN effect on the biochemical markers of acute hepatitis for the experimental rats, TPN was intravenously infused via the left femoral vein of rats at a flow rate of 20 mL/kg/hr for 3 hours. The results demonstrated that the serum ALT and AST levels were significantly elevated in comparison to the control group (rats with normal saline infusion and without any glycyrrhizin pretreatment, Table 1). Of note, these enzymes were remarkably reduced in TPN rats pretreated with glycyrrhizin in a dose-dependent manner (1, 3 and 10 mg/kg, iv). The most significant effect occurred in the group of TPN rats with 10 mg/kg glycyrrhizin pretreatment, which ALT and AST concentrations in the serum were restored to the level of the saline-control group.

The total bilirubin (TB) also showed a dose-dependently decreasing response to glycyrrhizin pretreatment (1, 3 and 10 mg/kg, iv), but the effect of the lowest dose of glycyrrhizin (1 mg/kg, iv) was of no difference from the TPN rats without any glycyrrhizin pretreatment (Table 1). Similar dose-response patterns of glycyrrhizin pretreatment to TPN rats were observedon triglyceride (TG) examination (Table 1).

*3.2. Effect of glycyrrhizin on hepatic histopathological changes*

To investigate the hisotpathological changes, liver tissues were fixed in 10 % natural buffered formalin then stained with H & E. When comparing the liver cell morphology among control (CL), TPN, and TPN rats pretreated with 10 mg/kg glycyrrhizin, only CL and glycyrrhizin-pretreated group showed normal liver cell structure with well-defined cytoplasm and nucleus in the cells and ribbon-like hepatocyte arrangements (Figure 1A). On the contrast, the liver cells in the TPN rats, particularly in the zone 3 of the portal venule tract, appeared feather-like hepatocyte arrangements (open arrow Figure 1A). In addition, the hepatic parenchymal cells (hepatocytes) in the TPN rats demonstrated a sign of hepatic steatosis, represented by an excess accumulation of fat (triglycerides) (filled arrow in Figure 1A).

Quantification of pathological liver cells reveals that the number of normal hepatocytes showed that normal cell number was reduced in TPN rats (TPN vs. CL in Zone 1 (T=204±14; CL=408±12, *p*<0.05 ) and Zone 3 (T=160±19; CL=410±14, *p*<0.05), see Fig. 1B) and the incensement of cell numbers in pretreatment with glycyrrhizin 10mg/kg compared with TPN group was found. (GL+TPN vs. TPN in Zone 1 (T=204±14; GL=310±18, *p*<0.05) and Zone 3 (T=160±19; GL=344±19, *p*<0.05, see Fig. 1B).

*3.3. Effects of glycyrrhizin on the expression of Nitrotyrosine, iNOS and proinflammatory cytokines (*IL-1β, IL-6 and TNF-α*)*

After TPN administration, the serum protein biomarkers of reactive nitrogen species, nitrotyrosine and inducible NO synthase (iNOS) were increased in the TPN group compared with both control and three GL-pretreated groups (Figure 2A). Moreover, pretreatment with glycyrrhizin demonstrated a dose-dependent reduced expression of TNF-α, iNOS and nitrotyrosine proteins (Figure 2B). In particular, the highest dose (10 mg/kg) resulted in the most powerful inhibition of iNOS and nitrotyrosine (Figure 2 B). These results demonstrated that TPN administration increased production of proinflammatory cytokines, including IL-1β, IL-6 and TNF-α, which might be inhibited by pre-treatment of glycyrrhizin.

*3.4. Anti-apoptotic Effects ofglycyrrhizin*

To determine which kind of cell death was involved in the TPN related acute hepatitis, the apoptotic markers cleavage of caspase-3 in rat liver tissue of the groups of control group, TPN group and pretreatment group with glycyrrhizin were examined. **As shown in Figure 3,** the results demonstrated that pretreatment with glycyrrhizin prevented hepatocyte apoptosis in TNP related hepatitis rat model. The results revealed a distinct stained of cleavage casptase-3 within the hepatocyte in TPN group (Figure 3A). There was a significant and almost three-fold increase in hepatocyte apoptosis in the TPN group (11±1) compared with both control (3±1; *p*<0.05) and GL (10 mg/kg) pretreatment group (4±2; *p*<0.05) in cleavage casptase-3 in zone 1 area (Figure 2B). In zone 3 area, there was also about three-fold increase in hepatocyte apoptosis in TPN group (13±1) compared with both control (4±1; p <0.05) and GL (10 mg/kg) pretreatment group (5±1; p <0.05). These results consisted with Katz’s observation [30] that administration of TPN in mouse resulted in an increased hepatocyte apoptosis.

*3.5. Effects of glycyrrhizin on phosphorylation of JNK1/2 and p38 as well as the CHOP expression*

**To investigate the molecular mechanism underlying** pharmacological effects of glycyrrhizin on preventing TPN-induced hepatocyte apoptosis, we studied several proapoptotic molecules expressed under endoplasmic reticulum (ER) stress. These molecules included Apoptosis Signal-regulating Kinase 1 (ASK1), c-Jun NH2-terminal Kinase (JNK1/2), p38 MAPK and CHOP proteins [21, 22, 31, 32]. As shown in Figure 4A, administration of TPN solution for 3 hours resulted in a remarkable increase in the phosphorylation of JNK1/2 and p38 MAPK as well as the expression of CHOP protein. Conversely, pretreatment with glycyrrhizin (1, 3, and 10 mg/kg) resulted in an observable reduce in the phosphorylation of JNK1/2 and p38 MAPK. In addition, glycyrrhizin also inhibited CHOP protein activation in dose-response manner (Figure 4B) and the strongest inhibition effect occurs at pretreatment with dosage of 10 mg/kg glycyrrhizin.

*3.6. Effect of glycyrrhizin on anti-inflammation by activation of SOCS3 in TPN related acute hepatitis in vivo*

To evaluate the anti-inflammatory effect of glycyrrhizin, the protein level of SOCS3 in rat liver was examined. SOCS3 is an inducible negative feedback regulator of inflammation. As shown in Figure 4A, the protein level was increased after 3 hours of TPN solution administration and pretreatment with glycyrrhizin can significantly increase the SOCS 3 protein level in a dose-dependent manner (Figure 4 B). The results demonstrated that endogenous inducible SOCS3 was induced after TPN administration to against the inflammation process. Furthermore, the SOCS3 protein showed over expression by pretreatment of glycyrrhizin (Fig4A), suggesting its efficacy in stopping the liver inflammatory cascade. We also investigate the immunochemical activation of SOCS3 in portal zone 1 and zone 3 close to terminal hepatic venule tracts (Figure 5A) and this has been validated by quantitative evaluation the SOCS3 protein intensity by the average integral optical density (Fig 5B).

**4. Discussion**

TPN is essential treatment when eternal feeding is impossible for patients on the cost of risks leading to parenteral nutrition associated liver disease (PNALD). As reviewed in the introduction section, the proposed causes of TPN-induced hepatitis are multi-factorials. Most studies of TPN related hepatitis investigated the late stage of TPN-related hepatitis; but understanding the mechanism and its interfering factor underlying the acute hepatic insults by TPN may provide valuable implications for practically preventing PNALD. Current study was motivated from this perspective and current results, from biochemical, histopathological as well as the immunohistochemical investigation, provide strong evidence suggesting that suppression endoplasmic reticulum stress and reactive nitrogen stress may have hepatoprotective effect on reducing development of PNALD. As shown in Table 1, elevation of ALT (Alanina aminotransferasa), AST (Aspartato aminotransferasa) and triglyceride in rat serum were observed after 3-h administration of TPN and normalization of ALT, AST and triglyceride after glycyrrhizin pretreatment with three different doses of 1, 3 and 10 mg/kg. Figure 1A also showed that server hepatocyte steatosis in TPN group. The liver is the main organ of glucose disposal in normal animals receiving 45% TPN-derived glucose [33]. Overconsumption of glucose is associated with increased accumulation of triglyceride in hepatocyte. As shown in Figure 1A, glycyrrhizin improved that. However, the difference between pretreatment group with GL dose 1 mg/kg and TPN group in total bilirubin revealed no statistically difference may indicated that adequate therapeutic dose was not being achieved and only influenced the concentrations of ALT and AST but not total bilirubin level (Table 1).A briefly summarize, current biochemical and pathological findings support the acute liver injury could be successfully induced by our TPN rat's model. Furthermore, the TPN-induced acute hepatic dysfunction could be prevented by pretreatment with glycyrrhizin in a dose-dependent manner.

According to a previous study, the hepatoprotective effect of glycyrrhizin in acute and chronic viral hepatitis patient is due to the cytoprotective effect through suppression of phospholipase A2 activity thereby reach the anti-cytotoxicity effect [34]. In addition, glycyrrhizin suppress TNF-α-induced cytotoxicity in the TNF-α-sensitive cell line in vitro and is considered to play an important pathogenic role in immune-mediatedcytotoxicity. This finding supports for explaining why GL can decrease the elevated transaminase levels in chronic viral hepatitis patients by inhibition of immune-mediated cytotoxicity against hepatocytes [35]. Furthermore, glycyrrhizin has anti-inflammatory effect. It attenuates inflammatory response by inhibition of nuclear factor kB (NF-kB) and PI3K [36]. Our data in Fig 2 and Tables 1 showed that TPN administration induced systemic proinflammationcytokines production and induce hepatitis. Glycyrrhizin can decreased these cytokines production and improve TPN related liver dysfunction.

Overproduction of nitric oxide and its derivative have been implicated as a cause of tissue damage by inflammation. Pro-inflammatory mediators NO is generated by iNOS and nitrotyrosine is considered as an indicator of the reaction during the inflammatory response [37, 38]. iNOS is produced by many different cell types in response to endotoxins and cytokines, such as gamma interferon, interleukin 1, and tumor necrosis factor alpha [39, 40]. Nitrosylation of tyrosine has been observed in cells derived from patients with several acute inflammatory [41]. In the other words, the detection of iNOS and nitrotyrosine may serve as an indicator of reactive nitrogen stress generated from NO [42]. In our study (Figure 2), we demonstrated that treatment with glycyrrhizin resulted in effective inhibition of NO production via suppressing expression of iNOS and nitrotyrosine protein. According to these result, we suggested that the therapeutic effect of glycyrrhizin in treatment of TPN related hepatitis is due in part to its inhibition of NO production via inhibit iNOS suppression, inhibition of TNF-α, iNOS and nitrotyrosine protein levels. In particular, pretreatment with glycyrrhizin (10 mg/kg) resulted in most powerful inhibition of iNOS and nitrotyrosine.

Cytokines are mediators of various biological processes including inflammation, apoptosis, necrosis, and fibrosis [43, 44]. Increased proinflammatory cytokines has been demonstrated in animal model of TPN [45, 46]. We also found that TPN administration increased production of proinflammatorycytokines, including IL-1β, IL-6 and TNF-α and glycyrrhizin decreased the production of these cytokines in dose-dependent inhibition (Figure 2). The JAK-STAT pathway is negatively regulated by various mechanisms including suppressors of cytokines signaling (SOCS) proteins. SOCS protein binds to JAK receptor, thereby suppressing further signals transduction [19]. Especially, SOCS1 and SOCS3 are the strongest inhibitors of JAKs. [19]. In addition, previous investigation demonstrated that SOCS3 protein over expressed in viral hepatitis B infected patient’s liver specimens and contributing to the severity of liver inflammation [47]. SOCS3 plays a protective role in cytokine mediated inflammatory process (Shouda et al., 2001) by inhibiting IL-6-medicated STAT3 activation [48]. Our results in Figure 5 showed that the endogenous inducible SOCS3 was increased after TPN administration as a negative regulator in TPN related hepatitis liver, moreover, pretreatment with glycyrrhizin with three different doses (Figure 4A and 4B) can enhance SOCS3 protein overexpression as a more effective for negative regulation the TPN related hepatitis. These findings indicate that the inhibitory effects of glycyrrhizin on hepatitis may exert SOCS3 expression. In summary, our study demonstrated that glycyrrhizin-inducted expression of SOCS3 contributing to inhibit inflammation and reactive nitrogen stress (RNS) production.

Hepatocytes are very susceptible to endoplasmic reticulum (ER) stress. The ER-stressed macrophages enhanced proinflammatory immune response, whereas ER-stressed hepatocytes became more susceptible to tumor necrosis factor-α-induced cell death [49]. In addition, infusions containing lipid and glucose activate stress kinases (JNK1/2 and p38 MAPK) and inhibited insulin signaling (PI3K) in rat’s liver. Furthermore, both glucose and lipids administration acutely increases ER stress in liver 12-h fasted rats [50]. The ER stress is known to induce apoptosis through the induction of CHOP protein and CHOP protein is an important molecular link of ER stress related hepatitis [51, 52]. Our data in Figure 3A and 3 B showed that cleavage caspase-3 is higher after TPN administration. This result indicated that hepatocyte apoptosis increased after TPN administration and pretreatment with glycyrrhizin with dose 10mg/kg can inhibit the apoptosis. In our present study, we found that glycyrrhizin pretreatment significantly inhibited phosphorylation of stress kinase JNK and p38 MAPK as well as CHOP protein activation thereby suppressed hepatocyte apoptosis in TPN rats (Figure 4A and Figure 4B).

Taken together, our data add new information about the molecular mechanisms by which glycyrrhizin inhibits inflammatory effects by reduction of iNOS, TNF-α, IL-1β, IL-6 and nitrotyrosine through activation of SOCS3. In addition, glycyrrhizin was found to have an anti-apoptosis effect in the TPN related hepatitis in rats through inhibited stress kinase JNK1/2, p38 MAPK phosphorylation and inactivated CHOP protein, thereby reduced ER stress. Taken together, we demonstrated a novel animal for early stage of TPN related hepatitis and our results revealed a possible mechanism of glycyrrhizin in protection of TPN related hepatitis (Figure 6). These findings may provide molecular evidence-based therapy for the use of glycyrrhizin in the prevention therapy of TPN related hepatitis in clinical practice.

**Conflict of interest**

We declare that we have no conflict of interest.

**Acknowledgments**

Funding for this study was provided in part by research grants from the National Science Council (NSC99-2113-M-010-001-MY3, NSC99-2628-B-010-008-MY3) Taiwan; the Tomorrow Medical Foundation, Taiwan; Chang Gung Memorial Hospital or Chang Gung University of Science and TechnologyChiayi Campus (grants CZRPG880253 and CMRPF6A0072).We thank Dr. Tim Baker and Dr. Bessy Hung for editingon this manuscript.

**References:**

1. W. T. Chance, S. Sheriff, R. Dayal et al., "The role of polyamines in glucagon-like peptide-2 prevention of TPN-induced gut hypoplasia," *Peptides*, vol. 27, no. 4, pp. 883-892,2006.
2. B. Stoll, D. A. Horst, L. Cui et al., "Chronic parenteral nutrition induces hepatic inflammation, steatosis, and insulin resistance in neonatal pigs," *J Nutr*, vol. 140, no. 12, pp. 2193-2200,2010.
3. M. Raman and J. P. Allard, "Parenteral nutrition related hepato-biliary disease in adults," *ApplPhysiolNutrMetab*, vol. 32, no. 4, pp. 646-654, 2007.
4. I. S. Sandhu, C. Jarvis and G. T. Everson, "Total parenteral nutrition and cholestasis," *Clin Liver Dis*, vol. 3, no. 3, pp. 489-508, viii, 1999.
5. V. J. Kumpf, "Parenteral nutrition-associated liver disease in adult and pediatric patients," *NutrClinPract*, vol. 21, no. 3, pp. 279-290, 2006.
6. M. Cavicchi, P. Beau, P. Crenn, C. Degott and B. Messing , "Prevalence of liver disease and contributing factors in patients receiving home parenteral nutrition for permanent intestinal failure," *Ann Intern Med*, vol. 132, no. 7, pp. 525-532,2000.
7. W. Luman and J. L. Shaffer, "Prevalence, outcome and associated factors of deranged liver function tests in patients on home parenteral nutrition," *ClinNutr*, vol. 21, no. 4, pp. 337-343,2002.
8. A. F. Attili, M. Angelico, A. Cantafora, D. Alvaro and L. Capocaccia , "Bile acid-induced liver toxicity: relation to the hydrophobic-hydrophilic balance of bile acids," *Med Hypotheses*, vol. 19, no. 1, pp. 57-69,1986.
9. B. S. Tomar, "Hepatobiliary abnormalities and parenteral nutrition," *Indian J Pediatr*, vol. 67, no. 9, pp. 695-701, 2000.
10. A. S. Brinkman, S. G. Murali, S. Hitt et al., "Enteral nutrients potentiate glucagon-like peptide-2 action and reduce dependence on parenteral nutrition in a rat model of human intestinal failure," *Am J PhysiolGastrointest Liver Physiol*, vol. 303, no. 5, pp. G610-622,2012.
11. A. K. Jain, B. Stoll, D. G. Burrin, J. J. Holst and D. D. Moore , "Enteral bile acid treatment improves parenteral nutrition-related liver disease and intestinal mucosal atrophy in neonatal pigs," *Am J PhysiolGastrointest Liver Physiol*, vol. 302, no. 2, pp. G218-224,2012.
12. S. Loff, B. Kranzlin, M. Moghadam et al., "Parenteral nutrition-induced hepatobiliary dysfunction in infants and prepubertal rabbits,"*PediatrSurgInt*, vol. 15, no. 7, pp. 479-482, 1999.
13. R. Shamir, I. Zahavi, Z. Bar-Sever et al., "Total parenteral nutrition-associated cholestasis after selective damage to acinar zone 3 hepatocytes by bromobenzene in the rat," *Life Sci*, vol. 52, no. 4, pp. 371-376,1993.
14. W. Elremaly, T. Rouleau and J. C. Lavoie , "Inhibition of hepatic methionine adenosyltransferase by peroxides contaminating parenteral nutrition leads to a lower level of glutathione in newborn Guinea pigs," *Free RadicBiol Med*, vol. 53, no. 12, pp. 2250-2255, 2012.
15. S. Laborie, J. C. Lavoie and P. Chessex , "Paradoxical role of ascorbic acid and riboflavin in solutions of total parenteral nutrition: implication in photoinduced peroxide generation," *Pediatr Res*, vol. 43, no. 5, pp. 601-606, 1998.
16. H. Knowles, Y. Li, and A.L. Perraud, " The TRPM2 ion channel, an oxidative stress and metabolic sensor regulating innate immunity and inflammation, " *Immunol Res*, vol 55, no. 1-3,pp. 241-8,2013.
17. C. E. Egwuagu, C. R. Yu, M. Zhang et al., "Suppressors of cytokine signaling proteins are differentially expressed in Th1 and Th2 cells: implications for Th cell lineage commitment and maintenance," *J Immunol*, vol. 168, no. 7, pp. 3181-3187, 2002.
18. C. R. Yu, R. M. Mahdi, S. Ebong et al., "Cell proliferation and STAT6 pathways are negatively regulated in T cells by STAT1 and suppressors of cytokine signaling," *J Immunol*, vol. 173, no. 2, pp. 737-746, 2004.
19. A. Yoshimura, M. Suzuki, R. Sakaguchi, T. Hanada and H. Yasukawa, "SOCS, Inflammation, and Autoimmunity," *Front Immunol*, vol. 3, p. 20, 2012 .
20. Y. Tazuke, R. A. Drongowski, I. Btaiche, A. G. Coran and D. H. Teitelbaum, "Effects of lipid administration on liver apoptotic signals in a mouse model of total parenteral nutrition (TPN),"*PediatrSurgInt*, vol. 20, no. 4, pp. 224-228, 2004.
21. K. F. Ferri and G. Kroemer, "Organelle-specific initiation of cell death pathways," *Nat Cell Biol*, vol. 3, no. 11, pp. E255-263, 2001.
22. H. C. Wang, W. Huang, M. D. Lai and I. J. Su, "Hepatitis B virus pre-S mutants, endoplasmic reticulum stress and hepatocarcinogenesis," *Cancer Sci*, vol. 97, no. 8, pp. 683-688, 2006.
23. U. A. Ashfaq, M. S. Masoud, Z. Nawaz and S. Riazuddin,"Glycyrrhizin as antiviral agent against Hepatitis C Virus," *J Transl Med*, vol. 9, p. 112, 2011.
24. H. L. Liao, T. C. Ma, Y. C. Li, J. T. Chen and Y. S. Chang, "Concurrent use of corticosteroids with licorice-containing TCM preparations in Taiwan: a National Health Insurance Database study," *J Altern Complement Med*, vol. 16, no. 5, pp. 539-544,2010.
25. M. N. Asl and H. Hosseinzadeh, "Review of pharmacological effects of Glycyrrhiza sp. and its bioactive compounds," *Phytother Res*, vol. 22, no. 6, pp. 709-724,2008.
26. A. Del Prete, A. Scalera, M. D. Iadevaia et al., "Herbal products: benefits, limits, and applications in chronic liver disease," *Evid Based Complement Alternat Med*, vol. 2012, p. 837939, 2012.
27. F. Stickel and D. Schuppan , "Herbal medicine in the treatment of liver diseases," *Dig Liver Dis*, vol. 39, no. 4, pp. 293-304, 2007.
28. M. Makuuchi, N. Kokudo, S. Arii et al., "Development of evidence-based clinical guidelines for the diagnosis and treatment of hepatocellular carcinoma in Japan," *Hepatol Res*, vol. 38, no. 1, pp. 37-51,2008.
29. Y.T. Yeh, S.S. Hur, J. Wang et al., "Matrix Stiffness Regulates Endothelial Cell Proliferation through Septin 9, " *PLoS ONE*, vol7, no.10, pp.e46889, 2012
30. M.S. Katz, K.A. Thatch, and M.Z. Schwartz, “Dose variation of hepatocyte growth factor and its effects on an animal model of TPN-induced liver injury," [*J Surg Res*](http://www.ncbi.nlm.nih.gov/pubmed/20691983##), vol 163,no. 2, pp.294-8, 2010.
31. C. J. van der Kallen, M. M. van Greevenbroek, C. D. Stehouwer and C. G. Schalkwijk , "Endoplasmic reticulum stress-induced apoptosis in the development of diabetes: is there a role for adipose tissue and liver?," *Apoptosis*, vol. 14, no. 12, pp. 1424-1434, 2009.
32. N. Farley, G. Pedraza-Alva, D. Serrano-Gomez et al.,"p38 mitogen-activated protein kinase mediates the Fas-induced mitochondrial death pathway in CD8+ T cells," *Mol Cell Biol*, vol. 26, no. 6, pp. 2118-2129, 2006.
33. O. P. McGuinness, C. Donmoyer, J. Ejiofor, S. McElligott and D. B. Lacy, "Hepatic and muscle glucose metabolism during total parenteral nutrition: impact of infection," *Am J Physiol*, vol. 275, no. 5 Pt 1, pp. E763-769, 1998.
34. W. Jimenez, J. Claria, V. Arroyo and J. Rodes, "Carbon tetrachloride induced cirrhosis in rats: a useful tool for investigating the pathogenesis of ascites in chronic liver disease," *J GastroenterolHepatol*, vol. 7, no. 1, pp. 90-97, 1992.
35. M. Yoshikawa, Y. Matsui, H. Kawamoto et al., "Effects of glycyrrhizin on immune-mediated cytotoxicity," *J GastroenterolHepatol*, vol. 12, no. 3, pp. 243-248, 1997.
36. T. C. Kao, M. H. Shyu and G. C. Yen, "Glycyrrhizic acid and 18beta-glycyrrhetinic acid inhibit inflammation via PI3K/Akt/GSK3beta signaling and glucocorticoid receptor activation," *J Agric Food Chem*, vol. 58, no. 15, pp. 8623-8629, 2010.
37. X. Q. Wei, I. G. Charles, A. Smith et al., "Altered immune responses in mice lacking inducible nitric oxide synthase," *Nature*, vol. 375, no. 6530, pp. 408-411, 1995.
38. A. K. Nussler and T. R. Billiar, "Inflammation, immunoregulation, and inducible nitric oxide synthase," *J LeukocBiol*, vol. 54, no. 2, pp. 171-178, 1993.
39. D. Minc-Golomb, I. Tsarfaty and J. P. Schwartz, "Expression of inducible nitric oxide synthase by neurones following exposure to endotoxin and cytokine," *Br J Pharmacol*, vol. 112, no. 3, pp. 720-722, 1994.
40. A. Ialenti, A. Ianaro, S. Moncada and M. Di Rosa, "Modulation of acute inflammation by endogenous nitric oxide," *Eur J Pharmacol*, vol. 211, no. 2, pp. 177-182, 1992.
41. N. Riteau, P. Gasse, L. Fauconnier et al., "Extracellular ATP is a danger signal activating P2X7 receptor in lung inflammation and fibrosis," *Am J RespirCrit Care Med*, vol. 182, no. 6, pp. 774-783,2010.
42. C. K. Fan, Y. H. Lin, C. C. Hung, S. F. Chang and K. E. Su, "Enhanced inducible nitric oxide synthase expression and nitrotyrosine accumulation in experimental granulomatous hepatitis caused by Toxocaracanis in mice," *Parasite Immunol*, vol. 26, no. 6-7, pp. 273-281, 2004.
43. N. V. Gorbunov, S. J. McFaul, A. Januszkiewicz and J. L. Atkins , " Pro-inflammatory alterations and status of blood plasma iron in a model of blast-induced lung trauma,"*Int J ImmunopatholPharmacol*, vol. 18, no. 3, pp. 547-556, 2005.
44. M. Di Giannantonio, S. Frydas, D. Kempuraj et al., "Cytokines in stress,"*Int J ImmunopatholPharmacol*, vol. 18, no. 1, pp. 1-5, 2005.
45. Y. Feng, M. W. Ralls, W. Xiao et al., "Loss of enteral nutrition in a mouse model results in intestinal epithelial barrier dysfunction," *Ann N Y AcadSci*, vol. 1258, pp. 71-77, 2012.
46. Y. Feng, J. E. McDunn and D. H. Teitelbaum, "Decreased phospho-Akt signaling in a mouse model of total parenteral nutrition: a potential mechanism for the development of intestinal mucosal atrophy," *Am J PhysiolGastrointest Liver Physiol*, vol. 298, no. 6, pp. G833-841, 2010.
47. B. Koeberlein, A. zurHausen, N. Bektas et al., "Hepatitis B virus overexpresses suppressor of cytokine signaling-3 (SOCS3) thereby contributing to severity of inflammation in the liver," *Virus Res*, vol. 148, no. 1-2, pp. 51-59, 2010.
48. A. Sasaki, H. Yasukawa, T. Shouda et al., "CIS3/SOCS-3 suppresses erythropoietin (EPO) signaling by binding the EPO receptor and JAK2," *J BiolChem*, vol. 275, no. 38, pp. 29338-29347, 2000.
49. J. Liu, F. Ren, Q. Cheng, L. Bai et al., "Endoplasmic reticulum stress modulates liver inflammatory immune response in the pathogenesis of liver ischemia and reperfusion injury," *Transplantation*, vol. 94, no. 3, pp. 211-217, 2012.
50. G. Boden, W. Song, X. Duan et al., "Infusion of glucose and lipids at physiological rates causes acute endoplasmic reticulum stress in rat liver," *Obesity (Silver Spring)*, vol. 19, no. 7, pp. 1366-1373, 2011.
51. T. Kamiya, H. Nishihara, H. Hara and T. Adachi, "Ethanol Extract of Brazilian Red Propolis Induces Apoptosis in Human Breast Cancer MCF-7 Cells through Endoplasmic Reticulum Stress," *J Agric Food Chem*, vol. 60, no. 44, pp. 11065-11070, 2012.
52. H. Malhi and R. J. Kaufman, "Endoplasmic reticulum stress in liver disease," *J Hepatol*, vol. 54, no. 4, pp. 795-809, 2011.

**Figure legends**

Figure1.Effect of glycyrrhizin (GL) on liver histological examination in Total Parenteral Nutrition (TPN) rats. H&E stained histological examination of livers were revealed to portal area (zone1) and terminal hepatic venules area (zone3). (A) saline infusion as control(I); TPN infusion without treatment(II); and GL pretreatment with dosage 10 mg/kg before TPN infusion(III) staining of zone 1(left column) and zone 3 (right column) in liver. The liver pathology in control group showed normal hepatocyte structure and arrangement. The TPN nontreated group showed damage of liver structure as indicated by infiltration of monocytes (open arrow head), steatosis (filled arrow) and the presence of necrotic cells (open arrow). Pretreatment with GL 10 mg/kg indicated normalization of the damaged hepatocyte. (B) Evaluation of pathological examination was quantitative. The normal hepatocytes were counted from 10 fields randomly (200×magnification) of each liver sample and values represent the mean of mean ± SD of 6 rats. \**p*<0.05(TPN versus control group); #*P*<0.05 (between TPN and GL-pretreated +TPN group).

Figure2. Relative intensities of the RNS oxidants and acute inflammation cytokines of rat’s serum. (A) Representative Western blot analysis of rat’s serum inflammation cytokine proteins: nitrotyrosine, iNOS, IL-1β, IL-6 and TNF-α.TPN group had high levels of oxidative stress and inflammation. (B) The relative density was calculated by dividing the density of protein by the density of all the matched spots in the respective gel. The expression of RNS oxidants and inflammation proteins in serum were higher in TPN group compared with control group and pretreatment groups. GAPDH served as the internal control. Protein levels were quantified by densitometric analysis with the control being set at 100%. Data depicted in bar graph were the mean ± SD of three independent measurements. (n=6 in each group).\**p*<0.05 versus control group ; #*p*<0.05 between pretreatment groups and TPN group.

Figure3.Immunohistochemical change of cleavedcaspase-3 staining in rat’s liver in different liver zonal area; TPN versus pretreatment GL group (A) Pretreatment GL with 10 mg/kg dosage before onset of TPN administration. TPN group (I) and GL group pretreatment group (II) animals received an equal volume of solvent.( Group (I) normal saline 1cc i.v. bolus before TPN adminstration ; pretreatment group (II) GL 10 mg/kg in normal saline 1cc i.v. bolus before TPN adminstration) Representative photographs immunostained with antibodies against cleaved caspase-3 staining showed, respectively.(B) Quantitative of immunohistochemical change of cleavedcaspase-3. Average integrated optical density (AIOD) of positive stained area was evaluated from three randomly selected observation fields of each liver sections. Data were expressed as mean ± SD (n=6/group). \**p*<0.05 versus control group. #*p*<0.05 TPN versus GL 10mg/kg pretreatment group.

Figure4. Effect of glycyrrhizin (GL) on liver histological SOCS3 and apoptotic pathway protein expression of rats’ liver. (A) Western blot analysis of protein related to SOCS3 and apoptotic pathway in control group,TPN group and pretreated with GL groups. (B) Evaluation of nuclear protein SOCS3 and CHOP expression and MAPK signaling protein expression were quantitative.The TPN group was associated with a significant increase in SOCS3 protein expression, and decrease SCOS3 protein expression in GL pretreatment groups. The total phosphorylated levels of p38 MAPK and JNK proteins were higher in TPN groups compared with control and pretreatment groups. The CHOP protein also had the same result.Equal amounts of protein from total cell lysate of liver pretreated with GL in rats were analyzed. The β-actin served as the internal control. Protein levels were quantified by densitometric analysis with the control being set at 100%. Data depicted in bar graph were the mean ± SD of three independent measurements (n=6 in each group).\**p*<0.05 versus control group. #*p*<0.05 between pretreatment groups and TPN group.

Figure5. Effect of glycyrrhizin (GL) on liver cytokine-inducible SOCS3 protein. (A)Immunohistochemical staining for SOCS3 in rat’s liver was analyzed in different liver zonal area. Control group (I); Rats with TPN infusion (II); GL pretreatment (10mg/kg) (III) staining of portal and terminal hepatic venues zones. (B) Quantitative of immunohistochemical SOCS3 by average integrated optical density (AIOD). Positive stained area was evaluated from three randomly selected observation fields of each liver section. Data were expressed as mean ± SD (n=6/group). \**p*<0.05 versus control group. #p<0.05 TPN versus GL 10mg/kg pretreatment group.

Figure6. Schematic presentation of the signaling pathways involved in glycyrrhizin (GL)-inhibited TPN liver apoptosis and inflammation in rats. The effect of GL on the scavenger of RNS, which involved in SOCS3 activation and thus inhibited inflammatory cytokines release. GL also resulted in liver cell apoptosis inhibition via CHOP protein inactivation. GL also inhibit JNK and p38 MAPK protein phosphorylation and inhibited liver cell apoptosis.