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Anti-Fibrotic Effects of Ganoderma Lucidum and Praziquantel on Liver Fibrosis of *Schistosoma japonicum*

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Abstract: Liver fibrosis is the main damage of *Schistosoma japonicum* (*S. japonicum*) in chronic and advanced stages. Praziquantel (PZQ) is currently used for all forms of schistosomiasis. Ganoderma Lucidum (GL) as a traditional chinese medicine has been used in many fields such as anti-oxidation, anti-tumorigenesis, immunoloregulation and anti-fibrosis. But the therapy of GL on schistosomiasis and its mechanism were not well revealed. To investigate the anti-fibrotic effects of GL and/or PZQ on *S. japonicum*-induced liver fibrosis, 60 Kunming mice were infected with *S. japonicum* repeatedly and treated with Normal Saline (NS), GL, PZQ, GL combined with PZQ, respectively. The results showed that GL treatment could relieve liver injury by decreasing serum Hyaluronic Acid (HA) and Laminin (LN) level and reducing spleen index ameliorating liver injury of infected mice. Researcher also found that the collagen 3 mRNA and TGF-a1 mRNA expression decreased in the mice of GL group and GL combined with PZQ could obtain much better results. Overall the findings revealed that GL can relieve liver fibrosis of *S. japonicum*especially when combined with PZQ.

Key words: *Schistosoma japonicum*, praziquantel, ganoderma lucidum, fibrosis, anti-oxidation

INTRODUCTION

Schistosomiasis remains a health problem in some parts of developing countries. *S. japonicum* causes hepatointestinal schistosomiasis with worms that develop in their host mesenteric systems and deposit their eggs in the hepatic periportal spaces. The pathology of chronic schistosomiasis is the egg-induced immune response resulting in tissue destruction as granuloma and the associated fibrosis formation.

GL and its extract such as polysaccharides and triterpenes have great effect in many fields such as immunoloregulation (Yeh *et al*., 2010), anti-oxidation (Sudheesh *et al*., 2010; Aydin *et al*., 2010), anti-tumor (Chang *et al*., 2009), anti-fibrosis (Wu *et al*., 2010; Wang *et al*., 2009) treatment of chronic hepatopathy (Shi *et al*., 2008), cardioprotection (Chu *et al*., 2011) and so on. GL can induce dendritic cells maturation to regulate immunological reaction (Jan *et al*., 2011; Meng *et al*., 2011) and its extract can relieve allergic itch through a peripheral action (Andoh *et al*., 2010). It was reported that GL can increase humoral and cellular immune responses to inhibit tumor growth and inhibit hepatoma cells tumorigenesis and metastasis *in vivo* and *in vitro* (Weng *et al*., 2009; Zhang *et al*., 2010). GL can also ameliorate rat liver fibrosis induced by carbon tetrachloride (Lin and Lin, 2006) probably by giving protection against hepatocellular necrosis upon its free-radical scavenging ability. Inspired by these predecessor studies of GL's therapeutic effects the aim to investigate the effect of GL on live fibrosis due to schistosomiasis. It is found that by dosing GL and/or PZQ to the mice infected by *S. japonicum* the liver injury can be ameliorated in the mouse samples.

MATERIALS AND METHODS

Parasites and animals: Cercarie of *S. japonicum* were gained from Oncomelania hupensis snails (Jiangsu Institute for Schistosomiasis Control, Wuxi, China). A total of 60 female Kunming mice (Jiangsu University, Zhenjiang, China) 22~26 g were gathered and used as the study samples and the animal studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Jiangsu University, China.

Establishment of infection models of schistosomiasis: Mice were divided into 5 groups randomly and each group has 12 mice. Group 1 is the uninfected control mice in group 2-5 were infected with 24 cercarie (8 cercarie

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1 week for consecutive 3 weeks). At the 10th week after infection mice in group 2-5 were conducted with intragastric administrations of 0.5 mL NS, 180 mg kg^{-1} GL 150 mg kg^{-1} PZQ or GL+PZQ once a day for 5 consecutive weeks, respectively. GL was bought from Chinese Academy of Agricultural Sciences (Beijing, China) and PZQ was purchased from Nanjing Pharmaceutical Factory Co., Ltd. (Jiangsu, China).

Mice were sacrificed under anaesthesia in 5 and 10 weeks after treatment. Livers spleens and serum were collected.

Histopathological analysis: After administrating with GL and/or PZQ for 10 weeks right lobes of the livers were gained and affixed in 4% paraformadehyde immediately for 48 h. Each mouse liver tissue was embedded in paraffin and sliced into 3 μm dewaxed and rehydrated then stained with Hematoxylin and Eosin (HE).

Spleen index: In 5 and 10 weeks after treatment, the spleen and its body of each mouse were weighed. The spleen index was obtained according to the ratio of spleen weight to body weight.

Chemiluminescence assay: In 5 and 10 weeks after treatment, mice sera were collected and analyzed the contents of HA and LN (Tigsun Diagnostics, Beijing, China). They were detected by chemiluminescent according to the manufacturers protocols.

Real-time PCR: In 5 and 10 weeks after treatment, mice liver tissues were homogenized in trizol the total RNAs were extracted and the cDNAs were synthesized using a reverse transcription kit (Invitrogen, California, USA). The reaction system included 10 μL 2×SYBGMix (Invitrogen, California, USA) 0.5 μL 10 μM of each primer 0.1 μL taq DNA polymerase and 1 μL cDNA. The PCR reaction was composed of one cycle of 6 min at 94°C followed by 40 cycles of 30 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C and a final extension for 10 min at 72°C. The relative expression of mRNA was normalized with β-actin and the results were expressed as fold amplification using Ct method (Provenzano and Mocellin, 2007). Each experiment was repeated 3 times. The primer sequences of forward and reverse were as follows: collagen 3: CTGGTCA GCCTGGAGATAAG, ACCAGGACTACCACGTTCAC; TGF-β1: CAAGTGTGGAGCAACATGTG, ATTCCGTCTC CTTGGTTCAG; β-actin: 5'-CACGAAACTACCTTCAAC TCC-3', 5'- CATACTCCTGCTTGCTGATC-3'.

Statistical analysis: We compared NS group vs. GL group and PZQ group vs. PZQ+GL group. Data were expressed as means±Standard Deviation (SD). Data were analyzed using two-tailed Students t-test when comparing two groups using GraphPad Prism software 5.0 (Graph Pad, San Diego, CA, USA). Statistical $p<0.05$ were considered statistically significant.

RESULTS

Histopathological findings: To judge whether GL had promoted the liver amelioration researchers observed histopathological changes at the 10th week after the treatment.

As shown in Fig. 1, mice in GL group had fewer inflammatory cells less fibrous tissue and smaller egg granuloma than those in NS group. Comparing with PZQ treated group administration of PZQ+GL could reduce liver injury notably by showing less collagen deposition and much fewer inflammatory cells infiltration.

Spleen weight index of infected mice: As shown in Fig. 2, the spleen index in GL group was 0.020±0.002 lower than that 0.023±0.003 in infected control group after 15 weeks of infection ($p<0.05$). Similarly, the spleen index was much lower in GL group than in infected control group after 20 weeks of infection ($p<0.01$). Compared with 0.009±0.003 in PZQ group the spleen index was much lower in PZQ+GL group (0.006±0.001, $p<0.05$) after 20 weeks of infection. These data suggests that spleen weight index reduces in mice of GL group and reduces more in mice of PZQ+GL group.

Serum HA and LN levels: The results in Fig. 3a showed that serum HA level in PZQ+GL group was less than in

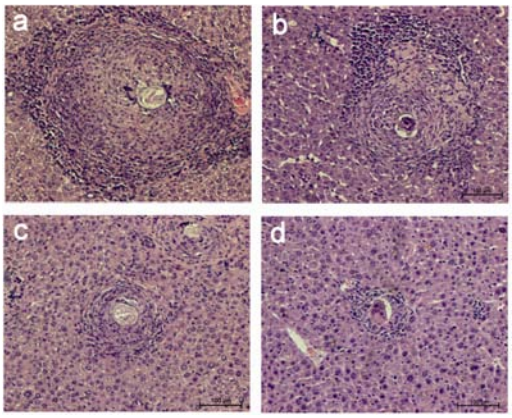

Fig. 1: Histopathological changes of injured livers at the 10th week after the treatment: a) NS control; b) GL group; c) PZQ group; d) PZQ+GL group. Scale bars: 100 μm

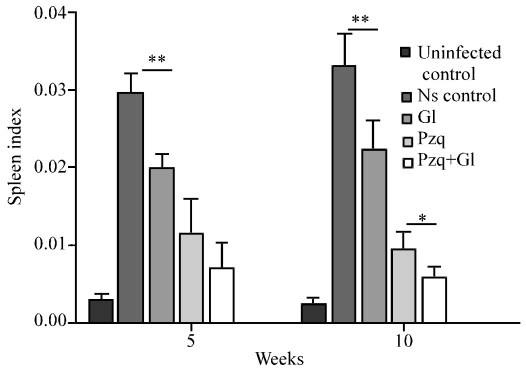

Fig. 2: Spleen index at 5th and 10th weeks after treatment. *p<0.05; **p<0.01

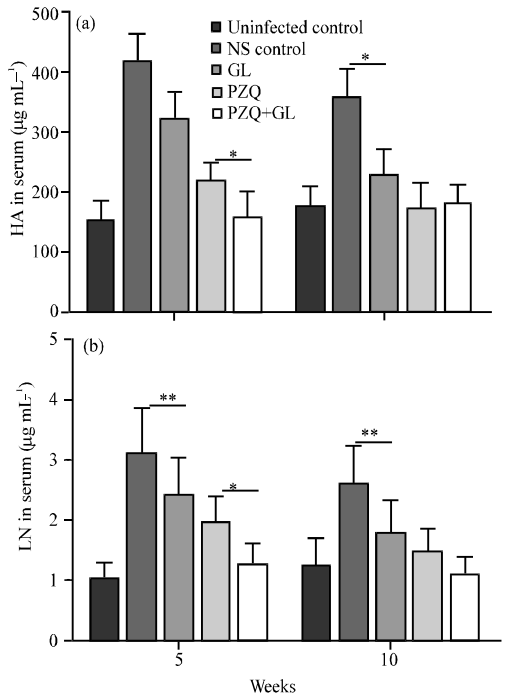

Fig. 3: Serum levels of HA and LN at 5 and 10th weeks after treatment. a) Serum HA level in each group (n = 6). *p<0.05; b) Serum LN level in each group (n = 6). *p<0.05; **p<0.01

PZQ+NS group after 15 weeks of infection (156.70±42.99 μg mL^{-1} vs. 219.50±28.32 μg mL^{-1} p<0.05). HA was much lower in GL group than in NS group after 20 weeks of infection (228.13±41.84 μg mL^{-1} vs. 356.30±47.50 μg mL^{-1}, p<0.05). As shown in Fig. 3b, LN in serum was less in GL group than in NS group after 15 and 20 weeks of infection (p<0.01).

After 15 weeks of infection, LN was much lower in PZQ+GL group (1.26±0.34 μg mL^{-1}) than that in PZQ group (1.97±0.42 μg mL^{-1}) (p<0.05). These data indicates that GL treatment reduced serum HA and LN levels.

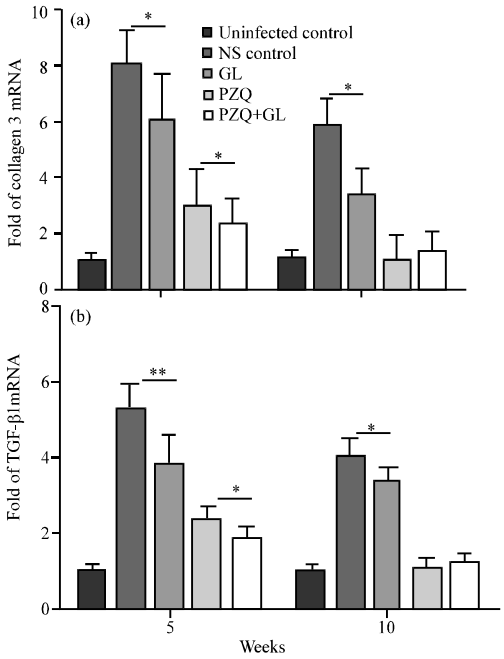

Fig. 4: Collagen 3 mRNA and TGF-β 1 mRNA expression in livers at 5th and 10th weeks after treatment: a) The expression of collagen 3 mRNA in liver tissues (n = 6). *p<0.05; b) The expression of TGF-β 1 mRNA in liver tissues (n = 6). *p<0.05; **p<0.01

Expression of collagen 3 and TGF-β1 mRNA: Liver fibrosis related gene expression was detected by real time PCR. As shown in Fig. 4a, collagen, 3 mRNA expression was lower in GL group (6.03±1.70) than in NS group (8.04±1.23) (p<0.05) and also lower in PZQ+GL group (2.31±0.90) than in PZQ+NS group (3.02±1.23) (p<0.05) after 15 weeks of infection. After 20 weeks of infection, collagen 3 mRNA expression decreased more in GL group (3.28±1.01) than in NS group (5.79±1.14) (p<0.05).

As shown in Fig. 4b, TGF-β1 mRNA expression in GL group was much lower than that in NS group after both 15 weeks (3.86±0.72 vs. 5.29±0.64, p<0.05) and 20 weeks (3.42±0.31 vs. 4.08±0.43, p<0.05) of infection. TGF-β1 mRNA expression was lower in PZQ+GL group than in PZQ+NS group after 15 weeks of infection (p<0.05). These data suggests that GL reduce expression of collagen 3 and TGF-β1 mRNA.

DISCUSSION

The aim of this study was to observe whether GL could relieve hepatic fibrosis of *S. japonicum* infected mice. The main damages of hepatic schistosomiasis are the granuloma formation around the eggs in the acute

stage and liver fibrosis in the chronic and advanced stages. It is characterized by excessive secretion and deposition of Extracellular Matrix (ECM) components such as types I and III collagen fibronectin and proteoglycan (Abdulmonem Al-Hayani, 2011). Activated Hepatic Stellate Cells (HSC) play an acute role in the ECM secretion and Transforming Growth Factor-β1 (TGF-β1) is the most important cytokine to activate HSCs (Zhao *et al*., 2011; Chuang *et al*., 2011).

It was reported (Wang *et al*., 2009) that GL could inhibit the proliferation of hepatic stellate cells by blocking the PDGF receptor to reduce collagen deposition. Lin and Lin (2006) found in their study that GL protects hepatic cells by eliminating the free radical to relieve rat liver fibrosis induced by carbon tetrachloride. PZQ as the main anti-parasite drug can be used as anti-fibrosis drug as previously reported (Liang *et al*., 2011; El-Lakkany *et al*., 2012).

Based on these research findings we developed mice hepatic pipe-stem fibrosis of schistisomiasis with repeated infection. We then administrated mice with GL in intragastric manner for 5 consecutive weeks. Spleen weight index (Rakotonirina *et al*., 2010) serum parameters as HA and LN (Parsian *et al*., 2010) as fibrosis markers all decreased after the GL treatment. Researchers demonstrated that mice treated with GL significantly ameliorated liver fibrosis of schistosomiasis in both 5 and 10 weeks after treatment.

To further confirm the reversal of fibrosis we detected the collagen 3 mRNA and TGF-β1 mRNA in livers. They all decreased after GL treatment indicating that GL could inhibit fibrosis related genes expression. TGF-β1 is considered as a major pro-apoptosis and pro-fibrosis factor which is shown to induce hepatocytes apoptosis and fibrosis formation (Tache *et al*., 2011). It can promote HSC activation and proliferation as well as collagen deposition in the liver (Rygiel *et al*., 2008; Soliman *et al*., 2010).

Lang *et al*. (2011) reported that TGF-β1 siRNA can inhibit the expression of TGF-β1 and attenuate rat fibrosis induced by CCl4. Thus, the degression of TGF-β1 reflected the amelioration of liver injury (Subeq *et al*., 2011). The results indicated that GL relieved liver fibrosis perhaps by inhibiting TGF-β1 expression. In the study, PZQ was therapeutically administrated as drug control. Mice with schistosomiasis were administrated with PZQ for 5 consecutive weeks then showed relief in liver fibrosis as previously reported (Liang *et al*., 2011). PZQ administrated with GL together could gain even better results than the single administration with GL or PZQ. This suggested that GL could be used in combination with PZQ to fight liver fibrosis of schistosomiasis.

CONCLUSION

In this study, researchers have demonstrated that GL could relieve liver injury of schistosomiasis by reducing sera HA and LN level decreasing collagen deposition inhibiting collagen 3 mRNA and TGF-β1 mRNA expression and promoting liver amelioration thus it can relieve *S. japonicum* induced liver fibrosis. PZQ administrated together with GL could gain even better results in terms of anti-fibrosis in liver. The underlying mechanism will be explored in the future.

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