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• BASIC RESEARCH •

Hepatoprotective role of *ganoderma lucidum* polysaccharide against BCG-induced immune liver injury in mice

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Abstract

AIM: **To examine the effect of *ganoderma lucidum* polysaccharide (GLP) on the immune liver injury induced by BCG infection, and investigate the relationship between degrees of hepatic damage and NO production in mice.**

METHODS: **Immune hepatic injury was markedly induced by BCG-pretreatment (125 mg· kg^{-1}, 2-week, iv) or by BCG-pretreatment plus lipopolysaccharide (LPS, 125 mg· kg^{-1}, 12-hour,iv) in mice *in vivo*.Hepatocellular damage induced by BCG-pretreated plus inflammatory cytokines mixture (CM), which was included TNF-a, IL-1b, IFN-g and LPS in culture medium *in vitro*. Administration of GLP was performed by oral or incubating with culture medium at immune stimuli simultaneity. Liver damage was determined by activity of alanine aminotransferase (ALT) in serum and in hepatocytes cultured supernatant, by liver weight changes and histopathological examination. NO production in the cultured supernatant was determined by the Griess reaction. Moreover, inducible nitric oxide synthase (iNOS) protein expression was also examinated by immunohistochemical method.**

RESULTS: **Immune hepatic injury was markedly induced by BCG or BCG plus inflammatory cytokines in BALB/c mice *in vivo* and *in vitro*. Under BCG-stimulated condition, augment of the liver weight and increase of the serum/supernatant ALT level were observed, as well as granuloma forming and inflammatory cells soakage were observed by microscopic analysis within liver tissues. Moreover, NO production was also increased by BCG or/and CM stimuli in the culture supernatant, and a lot of iNOS positive staining was observed in BCG-prestimulated hepatic sections. Application of GLP significantly mitigated hepatic tumefaction, decreased ALT enzyme release and NO production in serum/supernatant, improved the pathological changes of chronic and acute inflammation induced by BCG-stimuli in mice. Moreover, the immunohistochemical result showed that GLP inhibited iNOS protein expression in BCG-immune hepatic damage model.**

CONCLUSION: **The present study indicates that NO participates in immune liver injury induced by *Mycobacterium bovis* BCG infection . The mechanisms of protective roles by GLP for BCG-induced immune liver injury may be due to influence NO production in mice.**

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INTRODUCTION

Ganoderma lucidum polysaccharide (GLP) is an important pharmacological ingredient extracted from fruit bodies and mycelium of mushroom *Ganoderma Lucidum* (Fr.) Karst. It has been extensively documented that GLP can improve the damage induced by specific and nonspecific immunity responses[1,2]. In our laboratory recently studies, it was confirmed that GLP enhanced phagocytosis of intraperitoneal macrophage, inhibited the growth of implanted Sarcoma 180 and HL-60 tumor cells *in vitro*[3-5]. However, the regulating mechanism of GLP in the immune response remain unknown. On the other hand, hepatitis is a prevalent disease in the Chinese population. It has been recognized that the immune factors, such as virus/parasite infection, autoimmune stimuli, etc., were the dominant reasons of hepatic damage in hepatitis[6-10]. But the commonly used model of liver injury induced by chemicals does not accurately represent the clinical situation[11, 12]. Therefore, It is required that development of new therapy drugs depends primarily on the availability of animal models relevant to human hepatitis or hepatocellular immune damage.

In the recently studies, *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) infection has been proven to induce immune hepatic injury in rodent animal[13,14]. In this pathological model, the releases of hepatic endogenous cytokines, such as TNF-α, IFN-γ and IL-1β were observed *in vivo*[15-17]. Moreover, in our laboratory previously experiment, it has been observed that inflammatory cytokines including TNF-α and IL-1β stimulated NO production in the primary cultured rat hepatocytes *in vitro*[18], but the influence of GLP in this immune damage model and the exact function of NO production in the presence of inflammatory stimuli have not been elucidated yet. Therefore, the present study was performed to determine the effects of GLP on the BCG-stimulated immune liver injury *in vitro* and *in vivo*, and to investigate the possible mechanism of the influence induced by GLP in this immune response.

MATERIALS AND METHODS

Reagents

Following reagents were purchased from Sigma Chemical Co.: collagenase (Type IV, 340 kU· g^{-1}), bovine insulin, and lipopolysaccharide (LPS, E.*coli*.0111:B4). Other materials were obtained from the following sources: kit for determining

serum and culture supernatant alanine transaminase (ALT) was from Beijing Institute of Biological Products (Beijing); *Mycobacterium tuberculosis* Bacille Calmétte-Guérin (BCG) vaccine was from the National Vaccine and Serum Institute (Beijing); human recombinant (rh) tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interferon-gamma (IFN-γ) were from Academy of Military Medical Sciences (Beijing), and Dulbecco' s modified Eagle' s medium (DMEM) from Gibco BRL; *Ganoderma lucidum* polysaccharide (GLP) was isolated from mycelium of *Ganoderma lucidum* and provided by the Department of Phytochemistry, College of Pharmacy, Beijing University[5]. For using immunohistochemistry, iNOS polyclonal antibody (rabbit anti-mouse immunoglobulin) was purchased from Beijing Zhong-Shan Biotechnology co., LTD.

Animals treatment and liver damage induction

Male BALB/c mice weighing 18-22g (6-8 weeks old), were provided by Experimental Animal Center, Beijing University. Immune hepatic injury was induced by intravenous injection of BCG (125 mg· kg^{-1}) for two weeks, or induced by LPS (125 μg· kg^{-1}) for 12 hours at BCG-pretreated 14day later[13,19]. Control group mice were treated by same volume of phosphate buffered saline (PBS). After animals were BCG-pretreated 7 days, the different concentrations (25 mg· kg^{-1}, 50 mg· kg^{-1}, 100 mg· kg^{-1} and 200 mg· kg^{-1}, respectively) of GLP were intragastric administered once at everyday within succedent one week. At immune stimulating 2 weeks later, mice were killed by cervical dislocation,blood was collected and centrifuged at 3000 rpm for 5 min. Serum was obtained at the supernatant for mensuration enzyme level. Liver samples were removed rapidly for histopathological and immunohistochemical examination.

Hepatocyte isolation and culture

Hepatocytes were harvested from control mice or BCG-pretreated for 2 weeks mice using an *in situ* collagenase perfusion technique[20]. After inhalation anesthesia, the abdomen of the animals was opened and shaved,the portal vein was exposed and cannulated. Then the liver was perfused at 37 ℃ *in situ* first with a calcium-free phosphate-buffered saline solution (PBS) with 6~8 mL/min velocity of flow. This perfusion was continued for 5 min, then it was switched to 0.5 g· L^{-1} collagenase and 10 g· L^{-1} bovine albumin in PBS buffer for 15 min. The liver was removed and the cells were combed gently in tissue culture medium. Hepatocytes were pelleted, washed, and separated from nonparenchymal cells by differential centrifugation at 50×g. Viability of cells exceeded 85 % as determined by trypan blue exclusion. Hepatocytes were plated onto 6-well plastic tissue-culture plates (1×10^9 cells· L^{-1} in each well). Medium in the control consisted of DMEM with L-arginine (0.5 mmol· L^{-1}), insulin (1 mmol· L^{-1}), Hepes (15mmol· L^{-1}), L-glutamine, penicillin, streptomycin, and 100 mL· L^{-1} low-endotoxin newborn calf serum. After overnight incubation, the medium was changed with a cytokines mixture (CM) containing LPS (10 mg· L^{-1}), IL-1β (10 KU· L^{-1}), TNF-α (500 KU· L^{-1}) and IFN-γ (100 KU· L^{-1}). Other experimental conditions included addition of GLP, at the different concentrations (50 mg· L^{-1} or 200 mg· L^{-1}), to the CM. After primary cultures were maintained for 24 h at 37 ℃ in 50 ml· L^{-1} CO_2, hepatocytes or cultured supernatants were collected for nitrite and ALT activity assays[21].

Assay for hepatocellular enzyme release and NO production

As a marker of hepatocytes necrosis, activity of alanine aminotransferase (ALT) was spectrophotometrically measured using a determinating kit in serum and culture supernatants, at 520 nM in the presence of α-ketoglutarate, aspartate, NADH and malate dehydrogenase, as described[19]. The amount of NO production in the serum and the culture supernatants were determined as its stable oxidative product, nitrite, by an automated procedure based on the Griess reaction, as previously described[20].

Histopathological and immunohistochemical examination

Livers were removed, fixed overnight in 10 % buffered formalin, and paraffin-embedded. Six-micrometer sections were stained with hematoxylin-eosin for histological evaluation. Immunohistochemical staining for iNOS protein expression was carried out using rabbit polyclonal antibodies to iNOS on cryostat sections (five-micrometer). The sections were incubated with peroxidase-labeled rabbit anti-mouse immunoglobulin for 1 hour. After another wash in PBS, the sections were stained with AEC for several minutes to develop the color and washed in water. Each experiment was repeated two to three times with similar results. Three random sections of each liver were examined[19].

Statistics analysis

Data were presented with $\bar{x}\pm s$, Statistical analysis was performed using ANOVA. Differences were judged to be statistically significant when the *P* value was less than 0.05.

RESULTS

Effect of Ganoderma lucidum polysaccharide (GLP) on the liver weight and the activity of serum alanine transaminase (ALT) in BCG-induced immune hepatic injury in mice in vivo

Compared with the control of group, BCG-pretreatment markedly induced hepatic damage (Table 1). The augment of the liver weight and the serum ALT level were observed after BCG-administrated 2 weeks in mice ($P<0.01$). Furthermore, application of inflammatory lipopolysaccarides (LPS) for BCG-pretreated mice induced serum ALT activity further higher than that BCG-treated alone in mice ($P<0.05$), but the liver weights were not further increased than that BCG-stimulated only groups. On the other hand, under the presence of BCG stimuli conditions, administration of CLP decreased the liver weight within the range of 50 mg· kg^{-1} ($P<0.05$) to 200 mg· kg^{-1} ($P<0.01$), simultaneously, serum ALT release were significantly decreased by GLP treatment in a dose-dependent manner within the similar range of concentrations ($P<0.05$).

Table 1 Effect of Ganoderma lucidum polysaccharide (GLP) on the weight of liver and the activity of serum alanine transaminase (ALT) in BCG-induced immune hepatic injury in mice ($\bar{x}\pm s$)

Group	Liver weight (g)	ALT (U· L^{-1})
Control	0.99±0.16	22.03±10.99
BCG (125 mg· kg^{-1})	1.79±0.24^{b}	245.18±41.03^{b}
BCG (125 mg· kg^{-1}) + LPS (125 μg· kg^{-1})	1.84±0.14^{b}	285.88±23.81^{b,c}
BCG (125 mg· kg^{-1}) + GLP (25mg· kg^{-1})	1.78±0.20^{b}	236.86±27.94^{b}
BCG (125 mg· kg^{-1}) + GLP (50mg· kg^{-1})	1.57±0.18^{b,c}	189.81±43.99^{b,c}
BCG (125 mg· kg^{-1}) + GLP (100mg· kg^{-1})	1.28±0.20^{b,d}	178.78±13.16^{b,d}
BCG (125 mg· kg^{-1}) + GLP (200mg· kg^{-1})	1.41±0.43^{b,c}	208.18±27.93^{b,c}

$^{a}P<0.05$, $^{b}P<0.01$ compared with control. $^{c}P<0.05$, $^{d}P<0.01$ compared with BCG-pretreated group. n=9 mice (liver weight groups) or 10 mice (ALT groups).

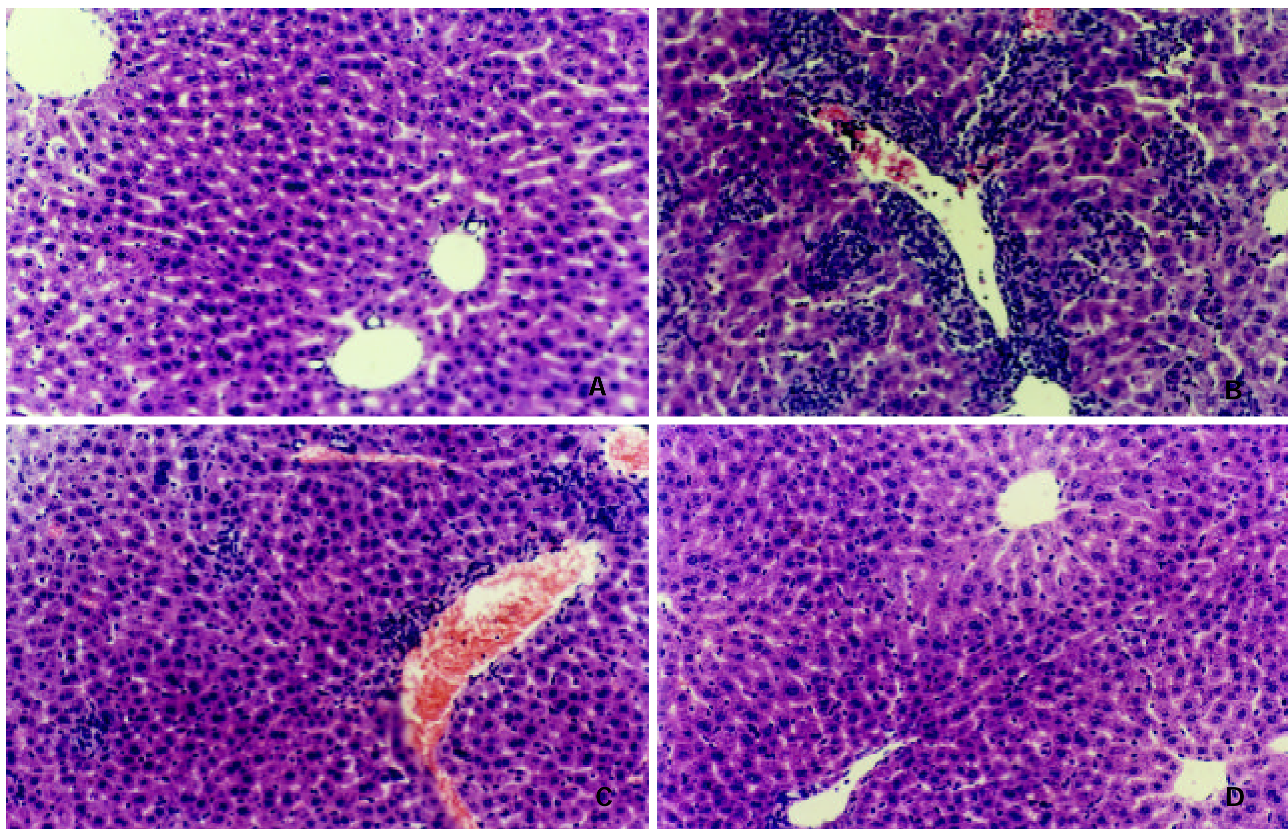

Figure 1 Histological changes of BCG-induced immune hepatic injury in the presence or absence of *Ganoderma lucidum* polysaccharide (GLP) in mice. Hematoxylin and eosin.Mice were treated with (A) control, (B) Bacille Calmette-Guérin (BCG, 125 mg· kg $^{-1}$, 2 weeks) , (C) BCG plus lipopolysaccarides (LPS, 125 μg· kg $^{-1}$, 12hr), (D) BCG plus GLP (100 mg· kg $^{-1}$), as described in Materials and Methods. (Original magnification 200×)

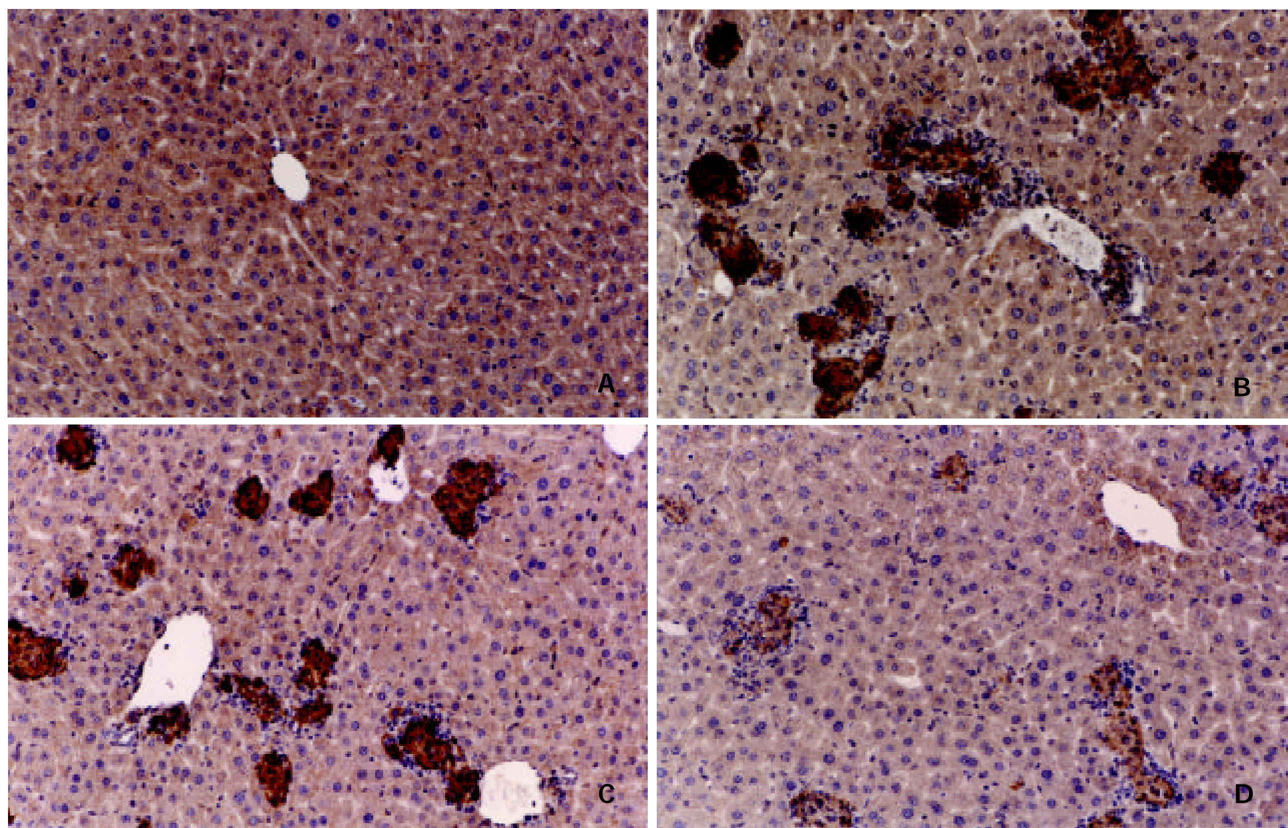

Figure 2 Immunohistochemical examination of inducible nitric oxide synthase (iNOS) protein expression stimulated by BCG in the presence or absence of *Ganoderma lucidum* polysaccharide (GLP) in mice. (Original magnification 200×). Mice were treated with (A) control, (B) Bacille Calmette-Guérin (BCG, 125 μg· kg $^{-1}$, 2 weeks) , (C) BCG plus lipopolysaccarides (LPS, 125 mg· kg $^{-1}$, 12hr), (D) BCG plus GLP (100 mg· kg $^{-1}$), as described in Materials and Methods.

Effect of Ganoderma lucidum polysaccharide (GLP) on the pathohistological changes in BCG-stimulated hepatic tissues in mice in vivo

As shown in Figure 1, oppositing with the results of control group, BCG-stimulated group were observed markedly changes of liver histologic structure (Figure 1-B), for example, infiltration within liver lobules by inflammatory cells, extensive hepatocytes hypertrophy, nuclear narrow, and granulation and vacuolization of the hepatocyte cytoplasm were observed in the liver section. Moreover, treatment with BCG plus LPS for mice resulted in more severe histological changes including thrombosis in the central hepatic vein and hemorrhage in the liver parenchyma(Figure1-C). Granulomas formation, a marker of chronic hepatitis fibrosis' were significantly increased by BCG-stimulated hepatic tissues (Tabel 2, $P<0.01$). But in the presence of BCG condition, the result showh that LPS was not triggered more the granuloma forming, on the contrary, triggered more fearful hepatic tissues hemorrhage (Figure 1 B-C).

On the other hand, the results of histological examination shown that GLP (100 mg· kg $^{-1}$) alleviated hepatic damage in BCG-induced acute inflammation, such as markedly decrease of infiltration within liver lobules by inflammatory cells, nuclear narrow,etc. in the observed liver section (Figure 1-D). Moreover, granulomas formation were also decreased by GLP treatment at concentration range from 100 mg· kg $^{-1}$ to 200 mg· kg $^{-1}$,($P<0.01$).

Table 2 Effect of Ganoderma lucidum polysaccharide (GLP) on the granuloma formation (numbers/microscopic view) in BCG -pretrested mice hepatic histological slides. ($\bar{x}\pm s$)

Croup	Granulomas
Control	0
BCG (125 mg· kg $^{-1}$)	64.67±4.97^{b}
BCG (125 mg· kg $^{-1}$) + LPS (125 μg· kg $^{-1}$)	54.40±4.93^{b}
BCG (125 mg· kg $^{-1}$) + GLP (50mg· kg $^{-1}$)	60.00±4.24^{b}
BCG (125 mg· kg $^{-1}$) + GLP (100mg· kg $^{-1}$)	4.00±1.22^{b,d}
BCG (125 mg· kg $^{-1}$) + GLP (200mg· kg $^{-1}$)	36.80±5.81^{b,d}

$^{a}P<0.05$, $^{b}P<0.01$ compared with control. $^{c}P<0.05$, $^{d}P<0.01$ compared with BCG-pretreated group. n=5 microscopic views.

Effects of Ganoderma lucidum polysaccharide (GLP) on the ALT activity and NO production induced by BCG in the presence or absence of cytokines mixture (CM) in primary cultured mice hepatocytes in vitro

The result of this part of experiment shown that inflammatory cytokines increased NO production and ALT release into the supernatant in the primary cultured hepatocytes prestimulated by BCG ($P<0.01$, Table 3). In the absence of cytokines condition, addition of CLP only had not influence on the activity of ALT enzyme and NO production in BCG-pretreated cultured supernatant ($P>0.05$). Whereas, in the presence of inflammatory cytokines plus BCG prestimuli condition, ALT activity and NO production were markedly inhibited by application of GLP ($P<0.01$).

CM (Cytokines mixture):IL-1β 10 KU· L^{-1},TNFα 500 KU· L^{-1}, and IFNγ 100 KU· L^{-1} plus LPS 10 mg· L^{-1}; Cultured hepatocytes were harvested from control group, BCG-prestimulated group *in vivo*, and BCG plus CM-stimulated group *in vitro*, respectively, in the absence or presence of GLP for 24 h; Amount of nitrite and activity of ALT in the supernatant were assayed 24 h after start of stimulation *in vitro*.

Table 3 Effects of Ganoderma lucidum polysaccharide (GLP) on the alanine transaminase (ALT) activity and nitrite (NO_2^-) production induced by BCG-prestimulating in the presence or absence of cytokines mixture (CM) in primary cultured mice hepatocytes in vitro ($\bar{x}\pm s$)

Group	ALT (U· L^{-1})	NO_2^- (μmol· L^{-1})
Control	11.52±1.41^{b}	1.41±0.72^{a}
BCG	17.87±3.41	3.52±1.72
BCG + GLP (50 mg· L^{-1})	21.30±2.87	3.95±1.27
BCG + GLP (200 mg· L^{-1})	18.03±2.24	3.24±1.08
BCG + Cytokines Mixture (CM)	46.34±4.17^{b}	13.53±5.58^{b}
BCG + CM + GLP (50 mg· L^{-1})	23.98±6.33$^{a, d}$	4.11±2.26^{d}
BCG + CM + GLP (200 mg· L^{-1})	20.61±3.74^{d}	3.49±1.38^{d}

$^{a}P<0.05$, $^{b}P<0.01$ compared with BCG-pretreated group; $^{c}P<0.05$, $^{d}P<0.01$ compared with BCG+CM group. n=7 mice . (3 wells for each treatment in each experiment).

Effect of Ganoderma lucidum polysaccharide (GLP) on the inducible nitirc oxide synthase (iNOS) protein expression in BCG-stimulated mice hepatic tissues in vivo

To confirm the possible mechanism about hepatoprotective role of GLP against BCG-stimulated in mice, the correlativity between iNOS expression and immune hepatic damage were investigated. As shown in the results of immunohistochemistry, compared with control group mice, there was a lot of iNOS positive brown stained agglomerate observed in BCG-stimulated hepatic section (Figure 2 A-B). But consisted with the results of granuloma forming , there were not more the iNOS expression induced by LPS in the presence of BCG stimuli condition (Figure 2-C). On the contrary, treatment of GLP significantly inhibited iNOS protein expression under similary BCG-stimulated condidtion (Figure 2-D).

DISCUSSION

In the present experiment, the results shown that the administration of GLP was effective against acute and chronic hepatic inflammation induced by BCG-immunostimuli in mice. Administration of GLP significantly decreased serum or supernatant ALT level in BCG-caused acute inflammatory response *in vivo* and *in vitro*. Histological changes, such as hemorrhage and necrosis in hepatic lobules, inflammatory infiltration of lymphocytes and kupffer cells around the central vein, were simultaneously improved by the treatment of GLP. These results were consistent with that GLP showed anti-inflammatory and antioxidative activities in the previous other laboratory observed results[22]. Moreover, pathohistological examination also showed that GLP decreased the granuloma formation, which is popularly considerd as the first step of fibrillar repair in the chronic inflammatory process[23-26]. This result suggested that GLP may be not only as an anti-inflammatory agent, but also may be used as an antifibrotic therapy for hepatocirrhosis.

To investigate the possible mechanisms of the hepatic protective effect of GLP in the immune-stimulated condition, we further detected NO production in primary cultured hepatocytes and iNOS protein expression in the BCG-stimulated hepatic tissues[27-30]. The results shown that GLP alone had no effect on the production of NO in the cultured hepatocytes. In the presence of BCG condition, cytokines

mixture (CM) including TNF-α, IFN-γ, and LPS, significantly increased the NO production. When combined with GLP, this effect had been remarkably reversed. At the same time point, GLP also attenuated the increase of ALT activity in inflammatory cytokines-stimulated hepatocytes *in vitro*. It has been recognized that NO is produced by cNOS and/or iNOS in mice liver[31-37]. The results of immunohistochemistry shown that GLP effect on NO production is mainly through iNOS under immunological stimuli condition. The results of this study suggested that although the exact mechanism of action of GLP on such macrophage/lymphocyte properties of granulomas remain unknown, nevertheless, it might be related to NO production induced by cytokines[38-42]. Therefore, inhibition of NO production is partly the mechanisms of GLP protective effect on the immunological injured liver.

In summary, the present study indicates that NO participates in immune liver injury induced by *Mycobacterium bovis* BCG infection. Furthermore, the mechanisms of protective roles by GLP for BCG-induced immune liver injury in mice may be due to influence NO production. However, further study is needed to understand the exact mechanisms of the antihepatotoxic activity and the free radical scavenging activity of GLP. The clinical applicability of GLP remains to be established.

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