Suppression of Ethanol and Lipopolysaccharide-induced Liver Injury by Extracts of Hydrangeae Dulcis Folium in Rats

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Received November 1, 2002; Accepted May 26, 2003

In female SD rats that were injected with 4 g/kg BW ethanol p.o. followed by a 5 mg/kg BW lipopolysaccharide (LPS) i.v. injection, serum glutamic pyruvic transaminases (GPT) activity increased to about eight times that of normal rats. In this model, rats that had been fed a diet containing 1% Hydrangeae Dulcis Folium (HDF) extracts for 15 days showed significantly lower serum GPT activity (380.0 ± 58.2 IU/l) than the control group (3527.0 ± 774.1 IU/l). HDF’s efficacy was far superior to milk thistle in this model (2950.0 ± 915.9 IU/l).

When mouse macrophages were treated with HDF extracts at 50 μg/ml, TNF-α production induced by LPS was suppressed to about 10% of the control. Rat serum TNF-α levels induced by LPS was decreased to 58.7% of the control by administering 1000 mg/kg BW HDF extract p.o. These results indicate that HDF prevents alcohol-induced liver injury through the inhibition of TNF-α production.

Key words: Hydrangeae Dulcis Folium; lipopolysaccharide; ethanol; hepatitis; TNF-α

Hydrangeae Dulcis Folium (Amacha in Japanese) is a crude drug that is indigenous to Japan. It is made from the leaves and axis of the plant Hydrangea macrophylla SERINGE var. Thunbergii MAKINO by fermentation and drying. According to tradition, Hydrangea macrophylla SERINGE var. Thunbergii MAKINO is supposed to have been bred from Hydrangea macrophylla SER var. acuminata in the Edo period. Hydrangeae Dulcis Folium is known to be an antimicrobacterium,13 antulcer,14 antiallergy,2) and antioxidant.3) It also prevents lipid peroxidation of liver microsomes.

The Japanese Ministry of Health, Labor, and Welfare reported that 13% of liver cirrhosis cases were alcoholics, and in 1996, 59,000 patients were suffering from alcoholic hepatic diseases (alcoholic fatty liver 1.0, alcoholic hepatitis 40.0, alcoholic liver cirrhosis 4.0, unknown 14.0 (unit: thousand)).4) Chronic alcohol intake induces fatty liver, hepatitis, and liver cirrhosis. Moreover, no fewer alcoholic liver cirrhosis cases advanced to hepatic cancer. We recently reported that Hydrangeae Dulcis Folium (HDF) extracts showed a hepatoprotective effect on α-galactosamine-induced liver injury in rats (Nakagiri, R., and Hashizume, E., et al., in press). The aim of this study was to find whether or not Hydrangeae Dulcis Folium protects the liver from injuries caused by ethanol and lipopolysaccharides.

Long-term continuous intragastric enteral feeding of ethanol to the rats is needed to induce liver damage, and surgical expertise is required.15 Recently, Enomoto et al. reported a rat model of liver damage caused by ethanol and endotoxin (LPS) administration.6) Overdosage of ethanol increases gut permeability7) which can cause an influx of microflora-derived endotoxins into the blood.8) These endotoxins interact with a specific carrier, LPS-binding protein (LBP)9) to stimulate hepatic macrophages via the binding of the LPS-LBP complex to the CD14 receptor.10) These stimulations result in the release of proinflammatory mediators, e.g. TNF-α from the macrophages.11) Such endotoxin-mediated Kupffer cell activation is proposed as one of the major mechanisms of alcoholic liver injury.12)

After optimizing the protocol of inducing rat liver injury by ethanol and LPS administration, we evaluated HDF extracts in this model in comparison with milk thistle.13) To understand the possible mechanism of action, we examined HDF extracts on TNF-α production in vitro and in vivo.

Materials and Methods

Materials. Lipopolysaccharides from the E. coli serotype B4 was purchased from Sigma-Aldrich Chemical, USA, for in vivo experiments, and from

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Abbreviations: LPS, lipopolysaccharide; LBP, LPS binding protein; GPT, glutamic pyruvic transaminases; HDF, Hydrangeae Dulcis Folium
Wako Pure Chemical, Osaka, Japan, for in vitro experiments. The assay kit for GPT was from Fujifilm Medical, Tokyo, Japan. Methylocellose MC #400 was from Nacalai tesque, Kyoto, Japan. Milk thistle extract, which contains silymarin 83%~ was purchased from Kenko Corporation, Tokyo, Japan. Saline was Otsuka normal saline from Otsuka Pharmaceutical, Tokyo, Japan.

**Hydrangeae Dulcis Folium extract.** One kg of *Hydrangeae Dulcis Folium* (Shihira Shoten, Tokyo, Japan) was extracted with 201 of water at 40°C, and the supernatant was removed. The residue was extracted again with 201 of 60% ethanol at 40°C, and the supernatant was filtered through a Miraclose sheet (Calbiochem). The supernatant of 60% ethanol extract was freeze-dried, and condensed in vacuo. Seventy grams of HDF extract was obtained.

**Fractionated Hydrangeae Dulcis Folium extracts.** *Hydrangeae Dulcis Folium* (Shihira Shoten, Tokyo, Japan) was extracted with 10 volumes of 70% acetone at room temperature, and this extract was applied to 30 ml of HP-20 in a column (Mitsubishi Chemical, Tokyo, Japan) and eluted with 60 ml of water, 33% methanol, 66% methanol, 100% methanol, and 100% acetone sequentially. The eluents except for the water eluent were divided into two fractions, the former 30 ml and the latter 30 ml eluents. These obtained 9 fractions (Fr.1–Fr.9) were condensed in vacuo.

**Animals.** Female SD rats aged 9 weeks (Japan SLC, Shizuoka, Japan), and male C3H/HeN mice aged 8–10 weeks (Charles River Japan, Kanagawa, Japan) were kept in an air-conditioned room at 22 ± 2°C, humidity 55% with a 12-h light (7:30–19:30) and dark cycle. All animal experiments were approved by the Animal Ethical Committee of Kyowa Hakko Kogyo (Tsukuba, Japan).

**Administration of ethanol and lipopolysaccharide.** Female SD Rats were fed a commercial diet (CE-2, CLEA Japan, Tokyo, Japan) for 5–7 days and randomly divided into groups of 6 animals each. All the rats except for those in the normal group were given ethanol (40% v/v ethanol) p.o., followed by the injection of LPS solution (5 mg/ml in saline) i.v. at 6, 12, 24, 36, or 48 hours after ethanol administration. Twenty-four hours later, blood was drawn from the vena cava of each rat under anesthesia with pentobarbital sodium (Nembutal, 1 ml/kg BW). The rats in the normal group were given saline instead of ethanol and LPS. Throughout the experiments rats were allowed free access to diets and water.

**Preparation and culture of macrophages.** Peritoneal macrophages were obtained from 8–10-week-old male C3H/HeN mice, as described elsewhere. The cells were seeded at a density of 1 × 10⁵ cells/well onto 96-well plates in a 200 μl Macrophage-SFM medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 50 μg/ml of the test samples, fractionated *Hydrangeae Dulcis Folium* extracts with methanol, water, and acetone as described in materials and methods. After 1 h, LPS was added to each well at a final concentration of 1 μg/ml. The cells were incubated for 20 h at 37°C in a humidified atmosphere of 5% CO₂, and 95% air. The culture supernatants were collected and kept at −80°C until assays.

**Measurement of TNF-α levels.** A TNF-α sensitive cell line, murine fibrosarcoma L-929 (Dainippon Pharmaceutical, Osaka, Japan) was used for the assay of TNF-α production from the cultured macrophages, essentially as reported elsewhere. Absorptions of dissolved crystal-violet from the stained cells were read on a Model 3550 Microplate Reader (Nippon Bio-Rad Laboratories, Tokyo, Japan) using a 570-nm filter. TNF-α production was also measured with the ChemiKine Mouse TNF-α Sandwich ELISA Kit (Chemicon International, Temecula, CA, USA) and the (r)TNF-α rat ELISA system (Amersham Pharmacia Biotech, UK).

**Evaluation of Hydrangeae Dulcis Folium on the ethanol and lipopolysaccharide-induced liver injury model.** Rats were fed a commercial diet (CE-2) for 5–7 days and randomly divided into the groups of 6 animals each, followed by feeding with experimental diets for 2 weeks. The experimental diets contained 0.1, 0.3, or 1% *Hydrangeae Dulcis Folium* extracts or 1% milk thistle to the commercial diet CE-2, and the control diet contained 1% corn starch instead of HDF extracts or milk thistle. On the 14th day, all the rats except those in the normal group were given 4 g/kg BW ethanol (40% v/v ethanol, 12.67 ml/kg BW) p.o. once, between 9:00 and 10:00 AM. Six hours later, the rats were injected with 5 mg/kg BW LPS solution (5 mg/ml in saline, 1 ml/kg BW). Twenty-four hours after LPS injection, blood was drawn from the vena cava under anesthesia with pentobarbital sodium (Nembutal, 1 ml/kg BW). The rats in the normal group were given saline instead of ethanol and LPS. Throughout the experiments rats were allowed free access to diets and water.

**Evaluation of Hydrangeae Dulcis Folium in the rat sepsis model.** Rats were fed a commercial diet (CE-2) for 5–7 days and divided into groups of 3 animals each. They were starved from 9:00 AM, and at 1:00 PM they were administered with 100 or 1000 mg/kg BW HDF extracts dissolved in 0.5% methylocellose.
Female SD rats were given 4 g/kg of ethanol p.o. After 6, 12, 24, 36, and 48 hours, the rats were injected with 5 mg/kg of LPS i.v. Serum GPT activities were measured as described in Materials and Methods. Values are mean ± SEM (n = 6); different subscript letters (a, b) mean significant differences at \( p < 0.01 \) by Tukey’s test.

After two hours, they were injected with 20 \( \mu g/kg \) LPS (10 \( \mu g/ml \) in saline, 2 ml/kg BW) i.v. Blood was drawn after ninety minutes of LPS injection. Serum were prepared and kept at \(-80^\circ C\) until assay.

**Aminotransferase assay.** Serum was separated from whole blood by centrifugation at 3,000 rpm for 10 min. Serum was stored at \(-80^\circ C\) until measurement of the aminotransferase activity. The activities of alanine aminotransferase (ALT/GPT, EC 1.1.1.27) were measured using a commercial assay system, Fuji-DRICHEM 3500 (Fujifilm Medical, Tokyo, Japan).

**Statistical analysis.** The statistical significance in the differences in the activities of GPT and levels of TNF-\( \alpha \) against the normal or control group were evaluated by Tukey’s or Dunnnett’s tests.

### Results

**LPS injection time after ethanol administration**

The correlation between the severity of liver injury and the duration between ethanol administration and LPS injection was examined. Serum GPT activities of the groups the periods of which between ethanol administration and LPS injection was 6, 12, 24, 36, and 48 hours were 2310.0 ± 333.6 (IU/l), 1475.5 ± 488.1 (IU/l), 177.7 ± 79.6 (IU/l), 308.4 ± 187.1 (IU/l), and 204.2 ± 80.7 (IU/l), respectively. When the rats were injected with LPS six hours after ethanol administration, serum GPT activity showed the highest among the tested groups (Fig. 1). We measured the period from ethanol administration to LPS injection to be six hours in our protocol.

**Dose-dependent effects of the LPS injections on the increase of serum aminotransferase in rats**

We tested the dose-dependence of LPS on the increase of serum GPT in rats with or without ethanol administration to find whether ethanol contributed to the liver damage (Fig. 2). In ethanol-given groups, serum GPT activities of 0.5, 1.5, and 5 mg/kg LPS injected groups were 947.5 ± 312.1, 3783.0 ± 1011.4, and 5545.0 ± 1855.9, respectively. On the other hand, in non-ethanol-given groups, serum GPT activities of 0.5, 1.5 and 5 mg/kg LPS were 58.5 ± 16.2, 85.8 ± 38.7, and 304.0 ± 121.9 (IU/l) (Fig. 2).

**Effects of Hydrangeae Dulcis Folium extract on the body weights and food intakes of the rats**

Among experimental groups, there were no significant differences in rats’ body weights and food intakes (Table 1).
Effects of Hydrangeae Dulcis Folium extract on ethanol and LPS-induced liver injury

Female SD rats were fed experimental diets containing Hydrangeae Dulcis Folium extracts at 0, 0.1, 0.3, or 1% to the commercial diet CE-2 for 15 days. Rats were treated with ethanol and LPS on day 14, and serum GPT activities were measured as described in materials and methods. Values are mean ± SEM (n = 6). *p < 0.05 by Dunnett’s test vs 0% group.

Comparison between Hydrangeae Dulcis Folium extract and milk thistle

Female SD rats had been fed experimental diets containing 1% HDF extract or 1% milk thistle for 15 days, followed by the injections of ethanol and LPS. Serum GPT levels of the control group, the HDF group, and the milk thistle group were 3527.0 ± 774.1, 2950.0 ± 915.9, and 380.0 ± 58.2 (IU/l) respectively. This clearly shows that the effect of HDF extract is much stronger than that of milk thistle extract (Fig. 4).

Effects of Hydrangeae Dulcis Folium extract on LPS-induced TNF-α production from isolated macrophages

L-929 cells as known to be damaged by TNF-α. The production of TNF-α from macrophages was measured by evaluating the viability of L-929 cultured in the medium supplemented with the culture supernatant of macrophages that were cultured in the presence of 50 μg/ml of the fractions of HDF extract and 1 μg/ml LPS. As shown in Fig. 5, Hydrangeae Dulcis Folium fractions of 5, 6, and 9 increased the viability of L-929, suggesting that these fractions inhibited TNF-α production from LPS-stimulated macrophages.

Effects of Hydrangeae Dulcis Folium extract on LPS-induced production of TNF-α in vivo

TNF-α in the rat serum was increased by LPS injection to the value of 28.3 ± 1.8 (pg/ml) in the control group. Serum TNF-α in untreated normal rat was undetectable. In the rats given 1000 mg/kg BW of the HDF extract, the TNF-α level after LPS injection was 16.6 ± 4.8 (pg/ml), which was significantly lower than that of the control group. These results show HDF extracts suppressed TNF-α production in vivo (Fig. 6).
suggest that the major mechanism of action of its antioxidative activity. The results of milk thistle and lately, immune-related genes for cytokines have relating ethanol metabolism have been researched, from activated Kuppfer cells. **Folium** is supposed to suppress the harmful influence of “influxed” endotoxin. **Hydrangeae Dulcis Folium** is thought to be a suppression of NF-κB and of lipopolysaccharide i.v. induced liver injury.16) That the administration to female rats of ethanol p.o. and of lipopolysaccharide i.v. induced liver injury. Enomoto et al. reported that the administration to female rats of ethanol p.o. and of lipopolysaccharide i.v. induced liver injury.16) When we followed their protocol, the serum GPT activities of the control by feeding a 1% HDF extract-containing diet. Milk thistle, a known hepatoprotective agent, was not significantly effective in this model. The active compound of milk thistle is known to be silymarin. The mechanism of action of silymarin is thought to be a suppression of NF-κB by its antioxidative activity. The results of milk thistle suggest that the major mechanism of action of **Hydrangeae Dulcis Folium** in this model is other than its antioxidative activity. Rats were directly injected with endotoxin, and therefore **Hydrangeae Dulcis Folium** is supposed to suppress the harmful influence of “influxed” endotoxin. **Hydrangeae Dulcis Folium** may protect Kupffer cells from endotoxin and/or may inhibit inflammatory mediator transduction from activated Kupffer cells.

In alcoholic liver disease, various kinds of enzyme relating ethanol metabolism have been researched, and lately, immune-related genes for cytokines have come to be noticed.17) TNF-α is one of these cytokines proven to be closely correlated to the initiation and promotion of alcoholic hepatitis. Association of a polymorphism of the tumor necrosis factor promoter region with susceptibility to alcoholic steatohepatitis was indicated.18) Long-term ethanol feeding caused liver injury in wild-type mice but not in TNF-R1 knockout mice, suggesting that TNF-α plays an important role in the development of early alcohol-induced liver injury via the TNF-R1 pathway.19) Neutrophils derived from alcoholic hepatitis patients were more responsive to endotoxin stimulation, and produced more TNF-α than those from healthy people.20) Many patients with alcoholic liver diseases tend to show endotoxiaemia, and their plasma endotoxin and serum cytokine levels are shown to correlate with the severity of liver disturbance.21) Oral antibiotic treatment prevents elevated aspartate aminotransferase activities and reduced hepatic pathological damage in rats exposed to ethanol.22) Acute ethanol administration increases intestinal permeability before pathological changes occur, and the influx of endotoxin is supposed to be accelerated.23) All these facts suggest close relationship among ethanol, hepatitis, and TNF-α. Thurman et al. proposed the endotoxic theory as a major mechanism of alcoholic liver injury. Gut permeability is increased by the taken ethanol and the microflora-derived endotoxin may leak into the bloodstream. These endotoxins activate Kupffer cells to induce nitric oxide,24) superoxide25) and cytokines such as TNF-α, which activate nuclear translating factors such as NK-kB,26) finally leading to liver damage. In this study HDF extracts inhibited TNF-α production both in vitro and in vivo, suggesting that this effect could involve the action of the hepatoprotective mechanism.

We used female rats because they are known to be more sensitive to ethanol and LPS. It is reported that estrogen increases blood endotoxin levels and the CD14 production from Kupffer cells.27) Such hormone-dependent differences in the production of CD14, NF-kB, and TNF-α were supposed to be some of the reasons why females are more sensitive to ethanol than males as seen in humans.28)

Suppression of ethanol and LPS-induced liver injury by **Hydrangeae Dulcis Folium**, in addition to our previous studies on β-galactosamine-induced liver injury, indicates that **Hydrangeae Dulcis Folium** may suppress liver damage by its antioxidative, antibacterial, gut-protective, and anti-TNF-α effects. Injection of anti TNF-α antibody improves hepatic injury in chronically alcohol-given rats.29) Anti TNF-α antibody also showed their validity against rheumatoid arthritis and Crohn’s disease.30) **Hydrangeae Dulcis Folium** may improve these TNF-α related diseases like rheumatoid arthritis, osteoporosis, and other inflammatory diseases by inhibiting TNF-α production. Further studies are needed to clarify the

**Fig. 6.** Effects of **Hydrangeae Dulcis Folium** Extract on LPS-Induced Production of TNF-α in vivo.

Rats were orally administered with **Hydrangeae Dulcis Folium** extracts at 100 or 1000 mg/kg BW. After two hours they were injected with 20 μg/kg LPS (10 μg/ml in saline, 2 ml/kg BW) i.v., then blood was drawn ninety minutes later. Serum TNF-α levels were measured as described in Materials and Methods. Values are mean ± SEM (n = 8–10). *p < 0.05 by Dunnett’s test.
References


4) Japanese Ministry of Health, Labor and Welfare report No. 112 of the total number of the patients in 1996.


