

Suppressive Effects of *Perilla frutescens* on Mesangioproliferative Glomerulonephritis in Rats

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Leaves of *Perilla frutescens* var. *crispa* DECNE. (perilla, Labiatae) are used as a garnishing vegetable in East Asian countries as well as an herbal medicine prescribed in Kampo medicines such as Saiboku-to. A previous *in vitro* study revealed that a decoction of perilla leaves inhibits the proliferation of murine-cultured mesangial cells. In the present study, we evaluated the *in vivo* anti-proliferative effects of a perilla decoction using rat mesangioproliferative glomerulonephritis induced by an intravenous injection of rabbit anti-rat thymocyte serum (ATS). Leaves of perilla were boiled, and the decoction was orally administered to the rats as drinking water at doses of 100 and 500 mg/kg/d from the day of ATS-injection (day 0) to day 8, when rats were sacrificed. In the histological evaluation, the total number of glomerular cells, proliferating cell nuclear antigen (PCNA) positive cells, and macrophage/monocyte antigen-positive cells in the glomerulus, was significantly decreased in perilla-treated rats. A significantly lower level of proliferation was induced by the serum of the perilla-treated rats than by that of the controls. These results suggest that the perilla decoction suppresses the proliferation of mesangial cells *in vivo* by an inhibition of the glomerular infiltration of macrophage/monocytes and of the production of circulating growth factors.

Key words *Perilla frutescens*; glomerulonephritis; mesangial cell; Labiatae; proliferation; rosmarinic acid

Leaves of *Perilla frutescens* BRITTON var. *crispa* (perilla, Labiatae) are one of the most common herbs in Japan, used as an antidote for fish and crab meat or as a food colorant. Several studies on the pharmacological activities of the herb in relation to mental irritability,¹⁾ indigestion,²⁾ and food poisoning³⁾ have been reported. In traditional Japanese-Oriental Kampo medicine, perilla leaves are prescribed in Saiboku-to, which is occasionally used to treat bronchial asthma.⁴⁾ Perilla leaves are considered to play a significant role in the anti-asthmatic effects of Saiboku-to.⁵⁾

In previous *in vitro* studies, we have revealed the prominent inhibitory effects of Saiboku-to on the proliferation of cultured murine mesangial cells,⁶⁾ and have shown that a decoction of perilla leaves can also inhibit this proliferation,⁷⁾ suggesting that perilla leaves may be useful in preventing the progression of glomerulonephritis. In order to more fully observe the anti-nephritic effect of perilla, *in vivo* experiments were necessary. Nephritis induced by anti-thymocyte serum (ATS) is known as an experimental model for mesangial proliferative glomerulonephritis, referred to as thy-1 nephritis.⁸⁾ ATS contains antibodies against thy-1, originally an antigen of thymocyte and also appearing in rat glomerular mesangial cells. ATS selectively stimulates mesangial cells to proliferate, causing symptoms similar to human mesangioproliferative glomerulonephritis.⁸⁾ Thy-1 nephritis is characterized by initial mesangiolysis, platelet and monocyte/macrophage infiltration, and subsequent mesangial cell proliferation and mesangial matrix expansion.⁹⁾

In the present study, we evaluated the inhibitory effects of a perilla decoction on mesangial cell proliferation *in vivo*, using the ATS-induced glomerulonephritis model, and found that the oral administration of this perilla decoction suppresses the proliferation of glomerular cells.

MATERIALS AND METHODS

Preparation for Perilla Decoction Leaves of *Perilla frutescens* BRITTON var. *crispa* (strain No. 32) were cultivated in the Experimental Station for Medicinal Plants, Graduate School of Pharmaceutical Sciences, Kyoto University; the leaves were harvested in August, 1997. Voucher specimens are deposited in the herbarium of the Station (registration number: 2001). Strain No. 32 with purple leaves and the smell of perillaldehyde has been recommended in traditional Kampo medicine. Fresh leaves were boiled in 20 times weight of tap water for 1 h (extraction ratio: 5.7%), and the decoction was frozen at -20°C until administered to the rats. This decoction contained 8.5 (w/w)% of rosmarinic acid, which is the major active constituent to inhibit the proliferation of cultured mesangial cells *in vitro*.⁷⁾

Experimental Design Mesangioproliferative glomerulonephritis was induced in 6-week-old male Wistar rats (Shimizu Laboratory Materials, Kyoto, Japan) by the intravenous injection of rabbit ATS, which was prepared as earlier reported⁸⁾ and provided by Nippon Shinyaku Co. (Kyoto, Japan). Thirty rats were divided into five experimental groups ($n=6$, respectively): the normal group received injection of normal rabbit serum and treatment with tap water; the control group received injection of ATS (0.2 ml/kg b.w.) and treatment with tap water; the perilla low-dose and high-dose groups received injection of ATS and treatment with perilla decoction (100 and 500 mg/kg/d, respectively); the prednisolone (PSL) group received injection of ATS and treatment with PSL (Wako Pure Chemical Industry, Osaka, Japan, 2 mg/kg/d) used as a positive control. The perilla decoction and PSL were orally administered in drinking water *ad libitum* from the day of serum-injection (day 0) to day 8, respectively. All rats were sacrificed on day 8, when serum and kidneys were collected.

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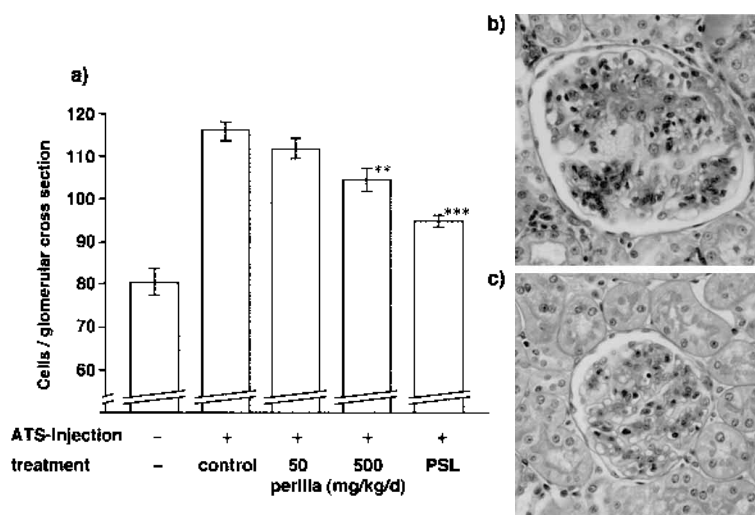


Fig. 1. (a) Effects of a Perilla Decoction on Total Glomerular Cell Number in Mesangioproliferative Glomerulonephritis Induced by ATS

Forty glomerular cross sections in a kidney specimen, and hematoxylin-positive nuclei were counted. Total cell number was determined as the mean cell number in 40 glomeruli. Values are represented as mean \pm S.E. ($n=6$). PSL; prednisolone (2 mg/kg/d). ** $p<0.01$ and *** $p<0.001$ compared to control.

(b), (c) Microscopic Photographs of Glomerulus in Control Group (b) and Perilla (500 mg/kg/d)-Treated Group (c)

Marked enlargement of glomerular size with mesangial cell proliferation and matrix expansion was recognized in the control rats, which were slightly suppressed by perilla-treatment. PAS-staining ($\times 400$).

Urinary Evaluation Urinary albumin from 24-h urine samples collected on day 4 after the serum injection was measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Nephra[®], Philadelphia, PA, U.S.A.).

Histological Evaluation of Renal Tissue Kidney tissues were fixed in Dubosque-Brazil solution, and embedded in paraffin. Sections (4 μ m) were stained with hematoxylin-eosin. The average number of cells in a glomerular cross section was evaluated by counting hematoxylin-positive nuclei in 40 glomeruli.

Immunohistological Evaluation of Renal Tissue Kidney tissues were fixed in 10% neutralized buffered formalin, and embedded in paraffin. Sections (4 μ m) were stained with anti-proliferative cell nuclear antigen (PCNA) using a DAKO EPOS anti-rat PCNA/HRP kit (DAKO Corp., Carpinteria, CA, U.S.A.), and with anti-rat monocyte/macrophage antigen (ED-1, Chemicon International Inc., Temecula, CA, U.S.A.) using an ABC Elite-peroxydase staining kit (Vector laboratories, Inc., Burlingame, CA, U.S.A.). The average number of ED-1 or PCNA positive-cells in a glomerular cross section was evaluated by counting the cells in 40 glomeruli in each section.

Measurement of DNA Synthesis in Cultured Murine Mesangial Cells DNA synthesis in cultured murine mesangial cells was measured in the same manner as that of our previous study.¹⁰ Mesangial cells isolated from Balb/c mice were cultured in a 3 : 1 mixture of Dulbecco's modified Eagle medium and an F12 nutrient mixture (Gibco BRL, Gaithersburg, MD, U.S.A., respectively), supplemented with a 17% fetal calf serum (FCS, Biowhittaker, Walkersville, MD, U.S.A.), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Gibco BRL) in 5% CO₂ at 37°C. After seeding mesangial cells into 24-well plates (2 \times 10⁴ cells/well) and incubating the cells for 96 h, the culture medium was replaced with an FCS-free medium. Quiescent cells were stimulated with a medium containing 5% serum of each rat, and subsequently incubated for 24 h. One μ Ci/well [³H]thymidine (NEN Prod-

ucts, Boston, MA, U.S.A.) was added to the culture medium for the last 4 h of incubation. The radioactive thymidine incorporated into the DNA was measured as the precipitate of 5% trichloroacetic acid, using a scintillation counter (Aloka, Tokyo, Japan). The experiment was conducted in duplicate.

Statistical Analysis Values are represented as mean \pm S.E. The statistical significance between the control and the perilla-treated groups was determined by one-way analysis of variance (ANOVA). The statistical significances between the normal and control groups, and between the control and PSL groups were determined by the Student's *t*-test. A difference of $p<0.05$ was considered statistically significant.

RESULTS

The perilla decoction was orally administered to the rats with an intravenous injection of ATS. When the rats were sacrificed on day 8, no significant differences in the weights of the body, heart, liver, spleen or kidneys were observed among rats of the normal, control, and perilla-treated groups. PSL-treated rats significantly reduced their body weight (control, 232.8 \pm 6.9; PSL, 207.0 \pm 4.2, $p<0.01$; g).

In this animal model, proteinuria is maximum on the 4th day after the injection of ATS.¹¹ In control rats, the amount of urinary albumin was significantly increased, while it was slightly decreased in the perilla-treated rats in a dose-dependent manner (normal, 44 \pm 12; control, 1239 \pm 585; low dose, 893 \pm 772; high dose, 610 \pm 136; PSL, 4903 \pm 3718; albumin μ g/d). These data were evenly varied, and there was no statistical significance.

Figure 1 shows the total cell number in the rat glomerulus. ATS-treatment significantly increased the cell number in the glomerulus on day 8 ($p<0.001$), while the cell number was significantly decreased in a dose-dependent manner in the perilla-treated rats ($p<0.01$).

To evaluate the proliferating glomerular cells and the infiltration of monocytes/macrophages in glomeruli, we counted

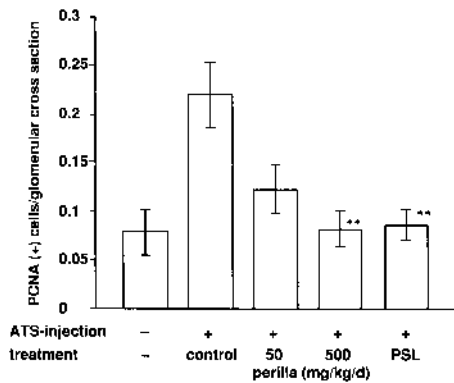


Fig. 2. Effect of a Perilla Decoction on the Proliferating Glomerular Cells in Mesangioproliferative Glomerulonephritis Induced with Anti-thymocyte Serum

The proliferative cell nuclear antigen (PCNA)-positive cell number of a rat was determined as the average of positive cell numbers in 40 glomerular cross sections. Values are represented as mean ± S.E. ($n=6$). PSL; prednisolone (2 mg/kg/d). ** $p<0.01$ compared to control.

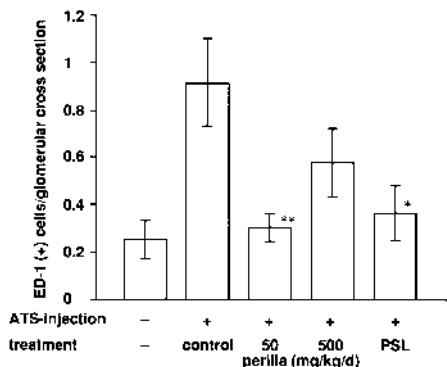


Fig. 3. Effect of a Perilla Decoction on Monocyte/Macrophage Infiltration in Mesangioproliferative Glomerulonephritis Induced with Anti-thymocyte Serum

The ED-1 positive cell number of a rat was determined as the average of positive cell numbers in 40 glomerular cross sections. Values are represented as mean ± S.E. ($n=6$). PSL; prednisolone (2 mg/kg/d). ** $p<0.01$ compared to control.

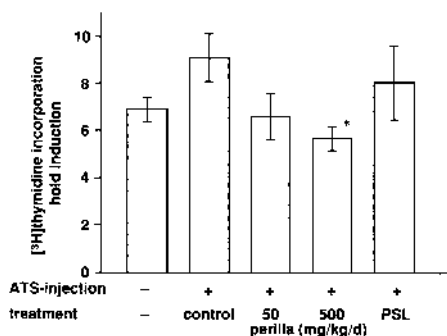


Fig. 4. Effect of the Serum of Perilla-Treated Rat on Cultured Murine Mesangial Cell Proliferation

Cultured mesangial cells were incubated with a medium containing 5% serum of the rat for 24 h. [^3H]Thymidine was pulsed for the last 4 h. The values are represented as mean ± S.E. of the fold induction ([^3H]thymidine incorporation with/without the serum). PSL; prednisolone (2 mg/kg/d). * $p<0.05$ compared to control.

the number of PCNA-positive and ED-1-positive cells. As shown in Fig. 2, perilla treatment significantly diminished the number of PCNA-positive cells in a dose-dependent manner ($p<0.01$). Figure 3 shows the ED-1-positive cell numbers in the rat glomerulus. The number of ED-1-positive cells in per-

illa-treated rats was significantly, but not dose-dependently, decreased ($p<0.01$).

The serum from each rat induced DNA synthesis in cultured murine mesangial cells. This inducible effect of the control rat serum was stronger than that of normal rat serum. A significantly lower level of proliferation was induced by the serum of perilla-treated rats than by that of the controls, while there was no difference in proliferation for those administered the serum of PSL-treated rats ($p<0.05$, Fig. 4).

DISCUSSION

Thy-1 nephritis is an animal model of mesangioproliferative glomerulonephritis frequently used for the screening of anti-nephritic drugs. The mechanisms of this model are considered to be divided into the following two phases.^{8,12} The first phase is characterized by complement-dependent mesangiolysis occurring within 1–3 h of the ATS-injection.¹³ In the second phase, excessive proliferation of the surviving mesangial cells is stimulated by inflammatory mediators such as the platelet derived growth factor¹⁴ and the transforming growth factor- β ,¹⁵ and mesangial matrix expansion is exhibited.⁹ Although detailed anti-nephritic mechanisms were not shown, the present study revealed the suppressive effects of a perilla decoction on mesangioproliferative glomerulonephritis *in vivo*.

In this model, cytokines derived from infiltrated glomerular macrophages/monocytes play an important role in the proliferation of mesangial cells.^{14,15} Therefore, a perilla decoction would suppress mesangioproliferative glomerulonephritis not only by a direct inhibition of the proliferation of mesangial cells, which has been shown in an *in vitro* experiment,⁷ but also by an inhibition of macrophage/monocyte infiltration into glomeruli following the production of inflammatory cytokines. In this context, ED-1-positive glomerular cells were decreased in perilla-treated rats in the present study. Furthermore, a significantly lower level of proliferation was induced by the serum of perilla-treated rats than the control serum in cultured mesangial cells, suggesting that perilla suppresses the production of circulating cytokines or growth factors. Previous study has indicated that orally administering a perilla leaf extract to mice suppresses the production of tumor necrosis factor- α .¹⁶ This effect of perilla may contribute to anti-nephritic action as shown in the present study. The inhibitory effects of a perilla decoction on the glomerular infiltration of macrophages/monocytes, however, did not show dose-dependency. In this study, the day of sacrifice was determined as 8 d after ATS-treatment, since the proliferation of mesangial cells was at its strongest at this time.¹¹ Therefore, the time of sacrifice could have been too late for accurately evaluating the glomerular infiltration of macrophages/monocytes. Further experiments conducting sequential sacrifices after ATS-injection and perilla-treatment are needed to evaluate in detail the anti-nephritic mechanisms of a perilla decoction.

In thy-1 nephritis, mesangiolysis following the proliferation of mesangial cells is involved in a complement system.¹² Rosmarinic acid, which is the representative compound for the anti-nephritic effects of a perilla decoction in *in vitro* experiments,⁷ has an anti-complement effect through its inhibition of C3 convertase¹⁷ and C5 convertase,¹⁸ as

well as the covalent attachment to activated C3b.¹⁹⁾ These results strongly suggest that the anti-nephritic effect of the perilla decoction in the present study was dependent on the anti-complement effect of rosmarinic acid. However when the kidney specimens in this study were stained with anti-rat C3 or C1q antibodies, there was little deposition of complements identified on the glomeruli of each group. In thy-1 nephritis, the glomerular complement deposits appear 30 min after and then disappeared 48 h after ATS-treatment,²⁰⁾ suggesting that complement-dependent inflammation occurs at a very early phase in this model. Since the perilla decoction was orally administered *ad libitum* to the rats after ATS-injection, it is possible that insufficient active constituents in perilla decoction were circulating in the rat body just after ATS-treatment and therefore did not affect such early complement-dependent inflammatory events. It is suggested that the *in vivo* anti-proliferative effects of a perilla decoction might not be dependent on the suppression of mesangiolysis by the inhibitory effects of rosmarinic acid on complements in the first phase, but on the direct inhibition of active constituents in the perilla decoction on the proliferation or suppression of cytokine production in the second phase.

In conclusion, the present study shows that the oral administration of a decoction of perilla leaves results in anti-proliferative effects on glomerular cells in rat mesangioproliferative glomerulonephritis. Taken together with our previous *in vitro* study showing that a perilla decoction can inhibit the proliferation of cultured murine mesangial cells,⁷⁾ it is suggested that *P. frutescens* is a promising agent for preventing mesangioproliferative glomerulonephritis.

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