

Effect of Gamiseunggal-Tang on immediate type allergic reaction in mice

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Background & objectives: The herbal formulation, Gamiseunggal-Tang (G-Tang) has long been used for various allergic diseases. The mechanism of its action is largely unknown. We carried out this study to determine the effect of G-Tang on the mast cell-mediated anaphylactic reactions *in vivo* and *in vitro* murine models.

Methods: In this study, the effects of G-Tang on the mast cell-mediated anaphylactic reactions were examined by using the ear swelling, histamine assay, and ELISA method in murine model.

Results: Anal administration of G-Tang showed dose-dependent inhibitory activity on the compound 48/80-induced ear swelling response ($P<0.05$) and histamine release ($P<0.01$). G-Tang (0.001-0.1 g/kg) significantly inhibited passive cutaneous anaphylaxis ($P<0.05$) in mice. The production of tumour necrosis factor- α (TNF- α) was also significantly inhibited (about 47.4%, at 0.1 mg/ml, $P<0.01$) by treatment of G-tang in anti-dinitrophenyl IgE antibody-stimulated mast cells.

Interpretation & conclusion: Findings of our study showed that G-Tang inhibited immediate type allergic reaction in a murine model and may be beneficial in the treatment of allergic inflammatory diseases.

Key words Ear swelling response - gamiseunggal-Tang - mast cells - passive cutaneous anaphylactic reaction - tumour necrosis factor- α .

Gamiseunggal-Tang (G-Tang), a traditional oriental medicine, has been used for the treatment of acute and chronic allergic diseases (especially, urticaria) in Korea. It is clinically known that G-Tang has beneficial effect in the treatment of anaphylactic inflammation by enhancing the immune response and inhibiting anaphylactic inflammation¹. However, the mechanism of action of G-Tang is not clear.

In general, mast cell-mediated anaphylactic reaction is elicited by various stimulators including compound 48/80, concanavalin A and anti-IgE². Among the preformed and newly synthesized inflammatory substances released on degranulation of mast cells, histamine remains the best characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity³. Compound 48/80, the best-known polybasic histamine releaser, was discovered in the search for hypotensive agents. Its hypotensive effect was shown to be the result of histamine release⁴. Compound 48/80 also induces ear swelling in skin anaphylactic reaction model. Ear swelling test is a traditional predictive one for dermal sensitization in humans using mice⁵.

The secretory responses of mast cells can be induced by aggregation of their cell surface-specific receptors for IgE by the corresponding antigen⁶⁻⁸. It has been established that anti-IgE antibody induces passive cutaneous anaphylaxis (PCA) as a typical *in vivo* model for immediate hypersensitivity in anaphylactic reactions. Rat skin is considered as a useful site for studying PCA⁹. Although mast cells also store small amounts of cytokines in their granules¹⁰, these cells dramatically increase the production of tumour necrosis factor- α (TNF- α), interleukin-6 (IL-6) and other cytokines within 30 min after their surface Fc ϵ RI are cross-linked with specific antigen¹¹⁻¹⁴.

We undertook this study to evaluate the effect of G-Tang water extract on the compound 48/80 and the anti-dinitrophenyl (DNP) IgE antibody-induced skin anaphylactic reaction in murine model

by anal administration. We also investigated the influence of G-Tang water extract on anti-DNP IgE antibody-induced TNF- α production in mast cells.

Material & Methods

Materials: Compound 48/80, anti-DNP IgE, DNP-human serum albumin (HSA), *o*-phthaldialdehyde (OPA), evans blue, foetal bovine serum, α -minimum essential medium (MEM), Hank's balance salt solution (HBSS), 2,2'-Azino-bis (3 Ethylbenzthiazoline-6-sulphonic acid) tablet (ABTS) and metrizamide (density, 1.120 g/ml) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant TNF- α , biotinylated TNF- α and anti-murine TNF- α were purchased from R & D system Inc, USA.

Animals: The original stock of ICR mice and Wistar rats were purchased from the Dae Han Experimental Animal Center (Taejon, Korea) and the animals were maintained at the college of Pharmacy, Wonkwang University. The animals were housed five to ten per cage in a laminar air flow room maintained at a temperature of 22 $\pm$ 2°C and relative humidity of 55 $\pm$ 10 per cent throughout the study. All protocols were approved by the institutional animal care and use committee of Wonkwang University.

Preparation of G-Tang water extract: All extracts of G-Tang were prepared by decocting the dried prescription of herbs with boiling distilled water (100 g/l). The duration of decoction was about 3 h. The decoction was filtered, lyophilized, and kept at 4°C. The yield of extraction was about 10 per cent (w/w). The G-Tang water extract powder was dissolved in sterile saline (50 g/l). The ingredients of 80 g G-Tang include 8 g of *Pueraria thunbergiana* Benth, 4 g of *Cimicifuga heracleifolia* Kom, 4 g of *Paeoniae lactiflora* Pall, 4 g of *Glycyrrhiza uralensis* Fisch, 4 g of *Schizonepeta tenuifolia* var. *japonica* Kitag, 4 g of *Ledebouriella divaricata* Uekl, 4 g of *Forsythia koreana* Nakai, 8 g of *Scrophularia buergeriana* Miq, 12 g of *Arctium*

lappa L, 28 g of *Betula platyphylla* Suk. Var. *japonica* Sieb. The plants materials were obtained from Oriental drug store, Duckhyun Dang (Iksan, Republic of Korea) and identified by H.M. Kim, College of Pharmacy, Wonkwang University. Their voucher specimens have been deposited at the Herbarium at the College of Pharmacy, Wonkwang University.

Ear swelling response: Compound 48/80 (20 g/l) was freshly dissolved in saline and injected intradermally into the dorsal aspect of a mouse ear using a microsyringe with a 28-gauge hypodermic needle. Ear thickness was measured with a digimatic micrometer (Mitutoyo, Japan) under mild anaesthesia. Ear swelling response represented an increment in thickness above baseline control values. Ear swelling response was determined 40 min after compound 48/80 or vehicle injection. G-Tang (0.01 and 0.1 g/kg) was orally administered 1 h before the compound 48/80 injections (100 µg/site). The values obtained could be considered representing the effect of compound 48/80 rather than the effect of the vehicle injection (physical swelling), since the ear-swelling response evoked by physiologic saline returned to almost baseline thickness within 40 min.

Preparation of rat peritoneal mast cells (RPMCs): RPMCs were isolated as previously described¹⁵. In brief, rats were anaesthetized by ether and injected with 20 ml of Tyrode buffer B (137 mM NaCl, 5.3 mM glucose, 12 mM NaHCO₃, 2.7 mM KCl, 0.3 mM NaH₂PO₄ pH 7.4) containing 0.1 per cent gelatin (Sigma, USA) into the peritoneal cavity and the abdomen was gently massaged for about 90 sec. The peritoneal cavity was carefully opened and the fluid containing peritoneal cells were aspirated by a Pasteur pipette. Thereafter, the peritoneal cells were sedimented at 150 × *g* for 10 min at room temperature and resuspended in Tyrode buffer B. Mast cells were separated from the major components of rat peritoneal cells, *i.e.*, macrophages and small lymphocytes, according to the method described by Yurt *et al*¹⁶. In brief, peritoneal cells suspended in 1 ml of Tyrode

buffer B were layered on 2 ml of 22.5 per cent w/v metrizamide (density, 1.120 g/ml, Sigma, USA) and centrifuged at room temperature for 15 min at 400 × *g*. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml of Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose pH 7.4) containing 0.1 per cent bovine serum albumin. Mast cell preparations were about 95 per cent pure as assessed by toluidine blue staining. More than 97 per cent of the cells were viable as judged by trypan blue uptake.

Compound 48/80-induced histamine release: Purified mast cells were resuspended in Tyrode buffer A containing 0.1 per cent bovine serum albumin for the treatment of stimulators. Mast cell suspensions (2 × 10⁵ cells/ml) were pre-incubated for 10 min at 37°C before the addition with compound 48/80 (5 mg/l). The cells were pre-incubated with the G-Tang (0.001- 0.1 mg/ml) and then incubated (20 min) with compound 48/80. The reaction was stopped by cooling the tubes in ice. The cells were separated from the released histamine by centrifugation at 400 × *g* for 5 min at 4°C. Residual histamine in the cells was released by disrupting the cells with perchloric acid and centrifugation at 400 × *g* for 5 min at 4°C.

Assay of histamine release: The histamine content was measured by the OPA spectrofluorometric procedure¹⁷. The fluorescent intensity was measured at 438 nm (excitation at 353 nm) in a spectrofluorometer. The inhibition percentage of histamine release was calculated using the following equation:

$$\% \text{ Inhibition} = (a - b) \times 100/a$$

where 'a' is histamine release without G-Tang and 'b' is histamine release with G-Tang.

PCAR reaction: An IgE-dependent cutaneous reaction was generated by sensitizing the skin with an intradermal injection of anti-DNP IgE followed 48 h

later with an injection of DNP-HSA into the mice tail vein. The DNP-HSA was diluted in phosphate-buffered saline (PBS). The mice were injected intradermally with 100 ng of anti-DNP IgE into each of 4 dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. Forty eight hours later, each mouse received an injection of 200 μ l of the 1 : 1 mixture of 1 mg/ μ l DNP-HAS and 4 per cent Evans blue via the tail vein. One hour before this injection, G-Tang (0.001 – 0.1 g/kg) was orally administered. The mice were sacrificed 40 min after the intravenous challenge. The dorsal skin of mouse was removed for measurement of the pigment area. The amount of dye was then determined colorimetrically after extraction with 0.5 ml of 0.1N KOH and 4.5 ml of a mixture of acetone and phosphoric acid (with the ratio of 13 : 5), based on the method of Katayama, *et al*¹⁸. The absorbent intensity of the extraction was measured at 620 nm in spectrofluorometer, and the amount of dye was calculated with the Evans blue measuring line.

Assay of TNF- α production: To assess the effect of GTang in anti-DNP IgE-induced production of TNF- α , rat basophil leukemia (RBL)-2H3 cells and RPMCs were pretreated with various concentration of G-Tang (0.001 – 0.1 mg/ml) for 30 min prior to antigenic stimulation. TNF- α secretion was measured by a modified enzyme-linked immunosorbent assay (ELISA), as described previously¹⁹. The ELISA was devised by coating 96 well plates of rat monoclonal antibody (Ab) with specificity for TNF- α . Before subsequent steps in the assay, coated plates were washed with PBS containing 0.05 per cent Tween-20. All reagents used in this assay were incubated for 2 h at 37°C. Recombinant TNF- α was diluted and used as a standard. Six 10-fold serial dilutions starting from 5 ng/ml were used to establish the standard curve. Assay plates were exposed sequentially to biotinylated rat TNF- α , and avidine peroxidase, and ABTS substrate solution containing 30 per cent H₂O₂. The plates were read at 405 nm in spectrophotometer (Molecular Devices Corp., USA).

Statistical analysis: Statistical evaluation of the results was performed by Student's t-test and ANOVA with a Tukey post hoc test. $P < 0.05$ was considered as significant.

Results

G-Tang (0.1 g/kg) significantly inhibited ear swelling response induced by compound 48/80 (Table I). The inhibitory effect of G-Tang on compound 48/80-induced histamine release from RPMCs was studied. G-Tang dose-dependently inhibited compound 48/80-induced histamine release. Maximal inhibition rate of histamine release was 85 ± 4.2 per cent at 0.1 mg/ml (Fig.).

Effect of G-Tang on PCA: Another way to test anaphylactic reactions is to induce PCA reaction. Anal administration of G-Tang significantly inhibited the PCA reaction in a dose-dependent manner (Table II, $P < 0.05$). Maximal effective concentration of G-Tang was 0.1 g/kg.

Effect of G-Tang on TNF- α production from RBL-2H3 cells and RPMCs: The constitutive and inducible production of TNF- α was demonstrated by ELISA

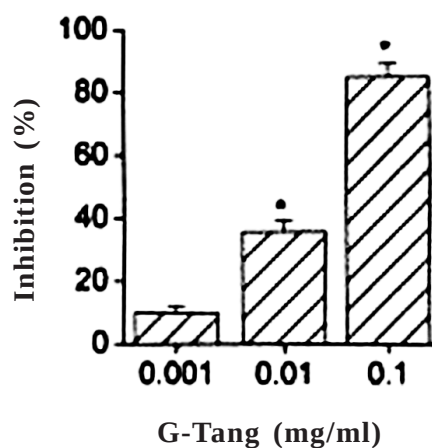


Fig. Effect of G-Tang on compound 48/80-induced histamine release from rat peritoneal mast cells (RPMC). RPMC (2×10^5 cells) were preincubated with G-Tang (0.001 – 0.1 mg/ml) at 37°C for 30 min prior to addition of compound 48/80 (5 mg/l). Each data point represents the mean $\pm$ SEM of three experiments. * $P < 0.01$: compared to control.

Table I. Effect of G-Tang on compound 48/80-induced ear swelling response in mice

G-Tang (g/kg)	Compound 48-80 (100 µg/site)	Thickness of ear (mm)	Inhibition (%)
None (Saline)	+	1.223 ± 0.09	–
0.01	+	1.080 ± 0.19	11.7 ± 10
0.1	+	0.805 ± 0.22	34.2±9*

G-Tang was anally administered 1 h prior to the compound 48/80 (100 µg/site) injections (n=9). Thickness of ear is the difference between before and after of compound 48/80 injection. Values are presented as mean ± SEM of three independent experiments

$P < 0.05$ compared to saline

Table II. Effect of G-Tang on the 24 h passive cutaneous anaphylaxis (PCA) in mice

Treatment	G-Tang (g/kg)	Inhibition (%)
None (Saline)	–	–
G-Tang	0.001	20.0 ± 2.3
	0.01	41.4 ± 5.6*
	0.1	53.8 ± 4.5*

G-Tang was anally administered 1 h prior to the challenge with antigen (n=9). Each amount of dye is presented as the mean ± SEM of three independent experiments

* $P < 0.05$ compared to saline

Table III. Effects of G-Tang on IgE-mediated TNF-α production from RBL-2H3 cells and RPMCs

G-Tang (mg/ml)	TNF-α production (ng/ml on RBL-2H3 cells)	TNF-α production (ng/ml) on RPMCs
None (Saline)	2.30 ± 0.56	0.34 ± 0.01
0.001	1.90 ± 1.18	0.30 ± 0.00*
0.01	1.46 ± 0.78*	0.29 ± 0.01*
0.1	1.21 ± 0.76*	0.28 ± 0.01*

IgE-stimulated mast cells (RBL-2H3 cells, 3×10^5 ; RPMCs, 1×10^5) were incubated for 30 min in the absence or presence of G-Tang before stimulation with DNP-HSA (100 ng/ml). TNF-α released into the medium is presented as the mean ± SEM of three independent experiments

* $P < 0.05$ compared to saline

method. Anti-DNP IgE significantly enhanced TNF-α production compared with media control on RBL-2H3 cells and RPMCs ($P < 0.05$). However, in anti-DNP IgE-stimulated cells, TNF-α production

decreased by treatment of G-Tang (Table III). Maximal inhibitory effect of G-Tang was shown in 0.1 mg/ml.

Discussion

Our study showed that oral administration of G-Tang inhibited compound 48/80-induced ear swelling response and anti-DNP IgE-induced PCA reaction in murine model. G-Tang also inhibited compound 48/80-induced histamine release and anti-DNP IgE –induced TNF-α production.

It is believed that stimulation of mast cells with compound 48/80 initiates the activation of a signal transduction pathway, which leads to histamine release. Compound 48/80 and other polybasic compounds are shown to activate G proteins^{20,21}. Chadi *et al*²², reported that compound 48/80 activated mast cell phospholipase D (PLD) via heterotrimeric GTP-binding proteins²². They identified recombinant G (β2r2) subunit markedly synergized PLD activation by compound 48/80 in permeabilized RBL-2H3 cells. Study on the compound 48/80-induced histamine release in murine mast cell was a good experimental model²³. Our results indicated that mast cell-mediate immediate type allergic reactions were inhibited by G-Tang. Tasaka *et al*²⁴ showed that the compound 48/80 increased the permeability of the lipid bilayer membrane by causing a perturbation of the membrane. So, it is possible to hypothesize that G-Tang might act on the lipid bilayer membrane affecting the prevention of the perturbation being induced by compound 48/80.

The G-Tang administered mice were protected from IgE-mediated local allergic reaction. This was supported by the effect of G-Tang on PCA. The mechanism of the protection against anti-DNP IgE, is not clear at present. It is conceivable that G-Tang inhibits the initial phase of immediate type allergic reactions, probably through interference with the degranulation system. Our data demonstrated that G-tang inhibited anti-DNP IgE-induced TNF-α production from RBL-2H3 cells. The effect

of G-Tang on mast cell cytokine production *in vivo* and the relative importance of mast cells as a source of TNF- α during inflammatory and immune responses are important areas for future studies.

G-Tang consists of 10 different herbs. Each one of the 10 medicinal herbs has a different effect; isoflavones, isolated from *Pueraria thunbergiana*, inhibited the prostaglandin E2 production in 12-O-tetradecanoylphorbol 13-acetate (TPA)-stimulated macrophages²⁵. Ferulic acid and isoferulic acid, which are the main active components of the rhizoma of *Cimicifuga heracleifolia*, have been used as an anti-inflammatory drug frequently in Japanese traditional medicine²⁶. *Paeoniae lactiflora* has a protective effect on endothelial cells and their function²⁷. Glycyrrhizin, isolated from *Glycyrrhiza uralensis*, has a hepatoprotective activity²⁸. *Schizonepeta tenuifolia* inhibited substance P-induced itch response²⁹. The butanol fraction of the aqueous extract of *Forsythia koreana* inhibited nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) gene expression in interferon-gamma plus lipopolysaccharide-stimulated macrophages³⁰. Phenylpropanoids isolated from *Scrophularia buergeriana* have a neuroprotective effects against glutamate-induced neurodegeneration³¹. *Arctium lappa* has an anti-inflammatory and radical scavenge effects³². Even though those herbs are frequently used to treat the various diseases, these effects are not the same with G-Tang. This prescription was also composed on the basis of the theory of oriental medicine to maximize its efficacy.

The amounts of oriental medicine for adult Korean (average body weight 60 kg) are about 100-150 g. The yield of powdered extraction is commonly about 5 per cent (w/w). Therefore, an adult Korean (average body weight 60 kg) takes 5-7.5 g at one time. From this, the dose of G-Tang for an adult person can be 0.1 g/kg. The dose range of 0.001-0.1 g/kg (0.001 mg/ml - 0.1 mg/ml) used in this study was therefore chosen to see the dose-dependency.

In conclusion, the results obtained in the present study provide evidence that G-Tang inhibited immediate type allergic reactions *in vivo* and *in vitro* in a murine model. To our knowledge, this is the first report of such selective inhibition of mast cell-mediated immediate type allergic reaction by G-tang.

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