The Anti-allergy Effects of Injinho-tang on the RBL-2H3 cells

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RBL-2H3 cells에서의 茵蔯蒿湯의 항 알레르기 효과

여경정・류지효・유선애・윤화정・고우신

茵蔯蒿湯은 한의학에서 여러 가지 질환에 사용되어 왔다. 특히 간경의 濕熱로 인한 질환에 습열을 제거함으로써證의 완화에 많은 응용이 되었다. 본 연구는 濕熱로 인한 질환중의 하나인 아토피 피부염에 그동안 응용이 적은茵蔯蒿湯을 실험적으로 응용함으로써 임상적인 가치를 가질 수 있는 지에 대한 기초연구를 진행하였다. 본 실험에 사용된 세포주는 rat leukemia (RBL-2H3) cells로茵蔯蒿湯은 알레르기와 관련된 사이토카인인 tumor necrosis factor (TNF)-α, interleukin (IL)-4를 용량의존적으로 억제하였지만 세포독성은 일으키지 않았다. 그리고 동물실험에서茵蔯蒿湯은 PCA반응에서 충분한 억제효과를 나타내었다. 또한 Compound 48/80으로 유도된 anaphylaxis shock도 용량의존적으로 억제함이 밝혀졌다.

Key words : Injinho-tang, Allergy, RBL-2H3 cell, cytokine, TNF-α

Introduction

Mast cells and basophils are critical participants in allergic diseases1). There cells express surface membrane receptors with high affinity and specificity for immunoglobulin (Ig)E, which is induced by IL-42). The interaction of antigen-bound IgE with surface membrane receptors causes the release of histamine, prostaglandins, leukotrienes, and cytokines3). These cytokine-induced reactions cause tissue inflammation, anaphylaxis, and scratching behaviors3,4).

Injinho-tang (IJHT) has been used as a traditional Oriental prescribable medicine. It is widely used as a medication for jaundice associated with liver inflammation5). This is a medicine preparation consisting of three herbs:
Artemisia capillaris Thunberg (茵薑), Rheum rhabarbarum (大黃), Gardenia jasminoides Ellis (梔子). Recent studies have reported that IJHT, A. capillaris extract (ACE), and scoparone isolated from A. capillaris have anti-inflammatory and analgesic activities. However, study on the anti-allergic effect of IJHT in the mast cells has not been identified.

Here, we investigated the anti-allergic activities of the IJHT on rat basophilic leukemia (RBL)-2H3 cells and allergic models. We found that IJHT inhibited degranulation, production and expression of pro-inflammatory cytokines from PMA and (or) A23187-induced RBL-2H3 cells. Therefore, Compound 48/80-induced systemic anaphylaxis and passive cutaneous anaphylaxis were inhibited by IJHT.

Materials and Methods

1. Reagents

Phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), p-nitro-phenyl-N-β-D-glucosaminide, anti-dinitrophenyl (DNP) IgE and DNP-human serum albumin (HSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco’s Modified Eagle's Medium (DMEM) containing L-glutamine (200 mg/l) and FBS were purchased from Hyclone (Logan, UT). TNF ELISA set (BD OptEIA™ Rat TNF ELISA Set), IL-4 ELISA set (BD OptEIA™ Rat IL-4 ELISA Set) were purchased from BD Biosciences (Franklin Lakes, NJ).

2. Preparation of Injinho-tang (IJHT)

Each of the IJHT was identified and authenticated by Professor, College of Oriental Medicine, Dongeui University (Busan, Korea) (Table 1). IJHT, a one day dose for human adults were boiled with distilled water at 100 °C, and the whole mixture is decocted until the volume is reduced by half. The extract water (400 ml) was filtered through 0.22 μm filter and the filtrate was freeze-dried (yield, 13.85 g) and kept at 4 °C. The dried filtrate was dissolved in phosphate buffered saline (PBS) and filtered through 0.22 μm filter before use.

Table 1. Prescription of Injinho-tang (IJHT)

<table>
<thead>
<tr>
<th>Herbs</th>
<th>Dose (One day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemisia capillaris Thunberg (茵薑)</td>
<td>40 g</td>
</tr>
<tr>
<td>Rheum rhabarbarum (大黃)</td>
<td>20 g</td>
</tr>
<tr>
<td>Gardenia jasminoides Ellis (梔子)</td>
<td>8 g</td>
</tr>
</tbody>
</table>

3. Animals

We purchased original stock of male ICR mice (5 weeks old) from Samtaco Bio Korea Inc. (Osan, Gyeonggi-do, Republic of Korea). The mice were housed six to eight per cage in a laminar air-flow room maintained at a temperature of 22 ± 1 °C and relative humidity of 55 ± 10 °C throughout the study.

4. Cells culture

RBL-2H3 cells were cultured in Dulbecco's
Modified Eagle’s Medium (DMEM) with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator with 5% CO₂. In all experiments, RBL-2H3 cells were treated for 1 h with the presence of the indicated concentrations of GST-G prior to stimulation with 50 nM PMA plus 1 μM A23187 in serum-free DMEM.

5. MTT assay

The cell viability of GST-G was assessed using the MTT assay10 in the remaining cells after Griess reaction. The MTT solution (0.5 mg/ml) was added to each well. After incubation for 2 h at 37 °C and 5% CO₂, the supernatant were removed and formed formazan crystals in viable cells were measured at 540 nm with a microplate reader. The percentage of cell viability was calculated against untreated cells. All experiments were performed in triplicate well.

6. β-hexosaminidase assay

β-hexosaminidase was measured in both supernatant and pellet fractions using a previously reported method11. Briefly, RBL-2H3 cells (3 × 10⁵ cells) were treated for 1 h with the presence of the indicated concentrations of GST-G prior to stimulation with 50 nM PMA plus 1 μM A23187 and incubated at 37 °C for 50 min. After stimulation, 50 μl of each sample was incubated with 50 μl of 1 mM ρ-nitro-phenyl-N-β-D-glucosaminide dissolved in 0.1 M citrate buffer, pH 5, in 96 well microtiter plate at 37 °C for 1 h. The reaction was terminated with 200 μl/well of 0.1 M carbonate buffer, pH 10.5. The plate was read at 405 nm in an ELISA reader. The inhibition percentage of β-hexosaminidase release was calculated using the following equation:

\[
\text{β-hexosaminidase release (\%)} = \frac{A_{405} \text{ of Sup}}{A_{405} \text{ of Sup} + A_{405} \text{ of pellet}} \times 100
\]

where is A₄₀₅ is absorption of measured at 405 nm and sup. is supernatant.

7. Enzyme-linked immunosorbent assay for pro-inflammatory cytokines (TNF-α, IL-4)

Each cytokines concentration in RBL-2H3 cells were measured with commercially available Rat TNF, IL-4 ELISA kit (BD Biosciences), according to the manufacture’s protocol. Color development was measured at 450 nm using an automated microplate ELISA reader.

8. Isolation of total RNA from cells and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated as per the manufacture’s instructions. Briefly, cells were lysed additional Trizol reagent (Invitrogen, Carlsbad, CA) and the cell lysate was passed through the pipette several times, 0.2 ml of chloroform was added per 1 ml of Trizol.
reagent. The tubes were shaken vigorously and incubated at room temperature for 2-3 min. The samples were centrifuged at 14,000 g for 20 min. The aqueous phase was transferred to a fresh tube and RNA was precipitated by the addition of 0.5 ml isopropanol. The RNA pellet was air-dried and resuspended in nuclease-free water. The concentration of RNA was estimated spectrophotometrically. Three microgram RNAs were reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI). Single stranded cDNA was amplified by PCR with primers (Table 2).

PCR amplifications were done in a 20 μl PCR PreMix (Bioneer Co., Korea) containing 10 mM Tris-HCl, 40 mM KCl, 1.5 mM MgCl2, 250 μM dNTP, 1 unit of Taq polymerase. Amplifications were carried out in a PCR machine (ASTEC PC802) using an initial denaturation at 95℃ for 5 min followed by 30 cycles (TNF-α: 35 cycles) of denaturation for 60 sec at 95℃, annealing for 60 sec at 52℃ and extension for 60 sec at 72℃. This was concluded with a final extension for 7 min at 72℃. Amplicons were separated in 1% agarose gels in 0.5× TBE buffer at 100 V for 30 min, stained with ethidium bromide and visualised under UV light. GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control to evaluate relative expressions of TNF-α and IL-4.

9. Compound 48/80-induced systemic anaphylactic shock

Mice (n=8) were given an intraperitoneal injection of the mast cells degranulator Compound 48/80 (8.0 μg/kg). IJHT was dissolved in saline and administered orally with a sonde 2 h before the injection of Compound 48/80. Mortality was monitored for 30 min after induction of anaphylactic shock.

10. Passive cutaneous anaphylaxis (PCA)

IgE-dependent cutaneous reaction was generated by sensitizing the skin with the 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 PBS. The mice were injected intradermally with 300 ng of anti-DNP IgE into each of three dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-in-soluble red marker. Forty-eight hours

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Oligonucleotide sequences (5’ to 3’ direction)</th>
<th>Expected size</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>CGTCTACTCCTCAGGCCC TCCACTCAGCGATGACATT</td>
<td>226 bp</td>
<td>NM012675</td>
</tr>
<tr>
<td>IL-4</td>
<td>AACACTTTGAAACCGTACGATGACATT</td>
<td>330 bp</td>
<td>X16058</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGCCAAAAGGTTCCATTGCGGACTGTTGGTT</td>
<td>201 bp</td>
<td>NM017008</td>
</tr>
</tbody>
</table>
later, each mouse received an injection of 300 μl of 1:9 mixture of 10 mg/ml DNP-HSA in PBS and 4 % Evans blue via the tail vein. Two hours before this injection, IJHT was administered orally with a sonde. The mice were sacrificed 30 min after the intravenous challenge. The dorsal skin of the mouse was removed for measurement of the pigment area. The amount of dye was then determined colorimetrically after extraction with 0.5 ml of 1 N KOH and 4.5 ml of a mixture of acetone and phosphoric acid (in a ratio of 5:13, v/v), based on the method of Katayama et al.¹². The absorbance of the extract was measured at 620 nm in a spectrophotometer, and the amount of dye was calculated using an Evans blue calibration curve.

11. Statistical analysis

Data is presented as the mean ± SE (standard error) of at least three separate experiments. Comparisons between two groups were analyzed using Student’s t-test. P values less than 0.05 considered be statistically significant.

Results

1. Effect of IJHT on degranulation and cell viability in RBL-2H3 cells

Inhibitory effects of IJHT on the release of β-hexosaminidase from RBL-2H3 cells were evaluated by the methods, as described in Materials and Methods. The release of β-hexosaminidase decreased significantly with all concentrations of IJHT. The inhibition rate of β-hexosaminidase release were 66.2 % with a dose of 0.5 mg/ml, 80.26 % with a dose of 1.0 mg/ml, and 92.23 % with a dose of 2.0 mg/ml. (Fig. 1).

![Fig. 1. Effect of IJHT on degranulation in RBL-2H3 cells.](image)

Cells were treated with the indicated concentration of IJHT. Degranulation was assessed by β-hexosaminidase release into the supernatant. β-hexosaminidase released into the medium is presented as mean ± SE (n=4). *** P < 0.005; significantly different from the stimulated group.

![Fig. 2. Effect of IJHT on the cell viability in RBL-2H3 cells.](image)

Cell viability was evaluated by MTT assay. Data represent the mean ± SE of three independent experiments.
2. Effect of IJHT on the cell viability

The cell viability effect of IJHT on RBL-2H3 cells was evaluated by MTT assay. IJHT concentrations from 1.0 \( \mu g/ml \) to 2.0 \( \mu g/ml \) had no effect on cell survival (Fig. 2). These results suggest IJHT inhibits A23187-induced TNF-\( \alpha \) and IL-4 production in RBL-2H3 cells without effect on the cell viability in each condition.

3. Effect of IJHT on secretion of TNF-\( \alpha \) and IL-4 in RBL-2H3 cells

To assess the effect of IJHT on the secretion of pro-inflammatory cytokines, RBL-2H3 cells were treated with various concentrations of IJHT for 8 h. The levels of TNF-\( \alpha \) and IL-4 were analyzed by ELISA. Treatment with IJHT significantly inhibited cytokines secretion in RBL-2H3 cells (Fig. 3A, 3B). When RBL-2H3 cells were treated only A23187 the cells secreted 334.02 ± 10.78 pg/ml TNF-\( \alpha \) and 1046.54 ± 22.11 pg/ml IL-4. Whereas, RBL-2H3 cells were pre-treated with 2.0 \( \mu g/ml \) IJHT, the cells secreted 43.28 ± 0.56 pg/ml TNF-\( \alpha \) and 460.75 ± 9.54 pg/ml IL-4.

4. Effect of IJHT on expression of TNF-\( \alpha \) and IL-4 in RBL-2H3 cells

Also to assess the effect of IJHT on the pro-inflammatory cytokines (TNF-\( \alpha \) and IL-4) expression, these cytokines expression decreased significantly by IJHT in dose-dependent manner (Fig. 4). In contrast to TNF-\( \alpha \) and IL-4, the level of GAPDH mRNA expression remained the same under these conditions.
5. Effect of IJHT on Compound 48/80-induced systemic anaphylaxis

To determine the contribution of IJHT in anaphylactic reaction, we used an in vivo model of systemic anaphylaxis. As a mast cell degranulator, Compound 48/80 (8.0 ng/ml) was used. After the injection of Compound 48/80, the mice monitored for 30 min, after which the mortality rate was determined. The period for observation of mortality was based on the control mice that had died in 30 min by Compound 48/80. An oral administration of saline as a control resulted in a fatal reaction in 100 % of group. When IJHT was orally administered at the dosed of 1.0 and 2.0 g/kg 2 h before Compound 48/80 injection, the mortality was inhibited (Table 3).

6. Effect of IJHT on PCA

Local injection of anti-DNP IgE followed by an intravenous antigenic challenge was performed. Anti-DNP IgE was injected into dorsal skin sites. After 48 h, all animals were injected intravenously with DNP-HSA containing Evans blue dye. When IJHT was orally administered to the mouse at the doses of 1.0 and 2.0 g/kg, the PCA was significantly inhibited (Fig. 5). The inhibition rate were 49.57 % at 1.0 g/kg IJHT and 65.38 % at 2.0 g/kg IJHT.

Discussion

Allergy is a hypersensitive response caused by IgE-dependent mast cell activation. The

![Fig. 5. Effect of IJHT on PCA reaction in mice. IJHT was administered orally with 1.0, 2.0 g/kg once a day for 7 days before the challenge with antigen. Data represents the mean ± SE from 6 mice. *** P < 0.005; significantly different from saline.](image)

<table>
<thead>
<tr>
<th>Dose(g/kg)</th>
<th>Compound 48/80 (8.0 ng/kg)</th>
<th>Mortality (%)</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None(saline)</td>
<td>+</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>IJHT 1.0</td>
<td>+</td>
<td>37.5</td>
<td>62.5</td>
</tr>
<tr>
<td>IJHT 2.0</td>
<td>+</td>
<td>12.5</td>
<td>87.5</td>
</tr>
<tr>
<td>IJHT 2.0</td>
<td>-</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Note. Group of mice (n=8/group) were orally pre-treated with 500 μl of saline of IJHT at various doses 2 h before the intraperitoneal (i.p.) injection of Compound 48/80. Mortality (%) within 30 min following Compound 48/80 i.p. injection is represented as:
(number of dead mice ÷ total number of experimental mice) × 100
high-affinity IgE-receptor (FcεRI) is expressed primarily by mast cells. Cross-linking of IgE-bound FcεRIIs by a multivalent antigen (allergen) on mast cells induces the release of biologically active mediators: the performed mediators stored in the cytoplasmic granules, including histamine and β-hexosaminidase, and the newly synthesized mediators, such as leukotrienes and cytokines. The β-hexosaminidase assay has been widely used to monitor RBL-2H3 mast cell degranulation. In the present study, we found that IJHT inhibited degranulation from PMA plus A23187-induced RBL-2H3 cells (Fig. 1). However, IJHT had no effect on cell survival at all concentrations (Fig. 2).

In recent years, it has been established that activated mast cells synthesize and release various cytokines and chemokines, of which the mast cell-derived TNF-α is probably of particular importance in causing allergic inflammation. In addition, mast cells have also been shown to produce IL-4. The IL-4 induces IgE production in B lymphocytes. In previous report, IJHT inhibited TNF-α, IL-6, and monocyte chemoattractant protein-1 (MCP-1) secretion and nitric oxide (NO) release in LPS-stimulated RAW 264.7 macrophage cells. Similarly, in the present study shows that IJHT significantly suppressed pro-inflammatory cytokines (TNF-α and IL-4) secretion and mRNA expression in activated RBL-2H3 cells (Fig. 3, 4).

Intracellular calcium, cAMP, and histamine release in murine mast cells were effectively stimulated by positively charged substances such as Compound 48/80. It is usually supposed that the most potent secretagogues include the synthetic Compound 48/80 and polymers of basic amino acids. An appropriate amount of Compound 48/80 has been used as a direct and convenient reagent to study the mechanism of anaphylaxis reaction. The mortality induced by Compound 48/80 was dose-dependently reduce by IJHT (Table 3).

The secretory response of mast cells can be induced by aggregation of their cell surface-specific receptors for IgE (FcεRI) by the corresponding antigen (allergen). It has been established that the anti-DNP antibody induces PCA reactions as a typical model for immediate hypersensitivity. PCA is a very effectiveness way to test skin allergic reactions, which has been successfully applied by Kim et al, in murine model with some agents. In the present study, we utilized PCA for testing protection effect from IgE-mediated local allergic reaction. Fig. 5 shows the inhibitory effect of IJHT on PCA reaction.

This study to demonstrated the anti-allergic activities of IJHT were designed to use an animal model and rat mast cell lines in vitro. We have shown that IJHT can modulate the allergic reaction induced by PMA plus (or) A23187 in RBL-2H3 cells, IJHT suppressed degranulation, production and expression of pro-inflammatory cytokines genes from stimulated RBL-2H3 cells. And Compound 48/80-induced systemic anaphylaxis and IgE-mediated PCA reaction were inhibited dose-dependently by IJHT. The results of this
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study suggests that IJHT significantly inhibited the release of inflammatory mediators, and immediate-type allergic reactions, so IJHT could be used as an anti-allergic medicine.

References

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