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# The suppressive effect of puerarin on atopic dermatitis-like skin lesions through regulation of inflammatory mediators in vitro and in vivo

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ABSTRACT

Atopic dermatitis (AD) is one of the common inflammatory immune disorders. Puerarin is the main isoflavonoid obtained from the root of Pueraria lobata and has been known have ameliorative effects on diverse inflammatory diseases. However, the effects of puerarin on AD have not been uncovered. 2,4-dinitrochlorobenzene (DNCB) was used to induce atopic dermatitis(AD)-like skin lesions on BALB/c mice for 17 days. Further, the BALB/c mice were orally administered puerarin. Puerarin ameliorated DNCB-induced AD-like symptoms in the mice by regulating skin thickness, degranulation of mast cells, and serum immunoglobulin E (IgE). Human keratinocytes (HaCaT cells) were also used to clarify the effects of puerarin on the secretion of pro-inflammatory cytokines. Puerarin inhibited the secretion of inflammatory cytokines and chemokines. The aim of this study was to investigate the protective and alleviative effect of puerarin on AD in vitro and in vivo. The results in this study indicated that puerarin ameliorates AD-like skin lesion and skin inflammation via regulation of various atopic and inflammatory mediators. Therefore, puerarin might be useful in treating AD and other skin diseases.



## 1. Introduction

Atopic dermatitis (AD) is a highly pruritic chronic inflammatory skin disease. The clinical symptoms of AD are known to relapse, and include, pruritic eczematous skin lesions, dry skin, skin hypersensitivity, and skin thickening [1,2]. Throughout the World, AD affects approximately 15–30% of children and about 2–10% of adults, and the prevalence rate of this disease is increasing annually [3]. AD is caused by a complex interaction between various factors, such as genetic factors, environmental factors, pharmacological disorders, skin barrier defects, and the immunological system [4]. Although the major cause of AD is still unknown, the onset of AD appears to be caused more accurately by allergic sensitization by environmental factors or allergens [1,5]. In early AD progression, extrinsic or environmental triggers damage the skin barrier. This damage aggravates immunoglobulin E (IgE)-mediated sensitization, serious forms of skin inflammation, and immune responses [6]. Moreover, AD develops owing to the imbalance between T-helper (Th)-1 cells and Th2 cells in the immune system [7]. Patients with AD commonly have increased levels of serum IgE and Th2-mediated inflammatory cytokines, such as interleukin (IL)-4, IL-5, and IL-13. Th2 cytokines directly affect skin cells, such as keratinocytes. The affected keratinocytes produce pro-inflammatory cytokines and chemokines that induce the invasion of immune cells into inflammatory skin lesions [8]. Chronic AD skin lesions are characterized by increased epidermal thickness and, activation and infiltration of mast cells. Furthermore, the expressions of several inflammatory mediators such as protease-activated receptor 2 (PAR2), nuclearfactor-κB (NF-κB), thymic stromal lymphopoietin (TSLP) and mitogen-activated protein kinases (MAPKs), are related to the severity of inflammation of AD [9].

Puerarin, one of the major isoflavonoid compounds, is isolated from the root of *Pueraria lobata* (known as Gegen in China), Gegen is widely used in traditional Chinese medicine. Until now,

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numerous medicinal properties of puerarin have been extensively identified [10]. It has been widely used in the treatment of cardiovascular and, neuronal diseases. Moreover, puerarin has been known to have anti-oxidant, anti-inflammatory, anti-cancer, and anti-diabetic effects and reduce the risk of diabetic complications. Furthermore, it has been used in alcoholic liver injury, bone formation, Parkinson's disease, Alzheimer's disease, and allergic inflammation. It is also used frequently to treat fever, diarrhea, emesis, and toxicosis.

However, there is no research on the effects of puerarin on AD in vivo and in vitro. Thus, the aim of our present study was to investigate the anti-inflammatory effect and the mechanism of puerarin on DNCB-induced AD in vivo and TNF-α/INF-γ-induced skin inflammations in vitro.

## 2. Materials and methods

### 2.1. Cell culture and reagents

Human keratinocytes, HaCaT, were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Hyclone), and 1% of 100 units/ml of penicillin-streptomycin, and incubated at 37 °C in a humidified 5% $CO_2$ incubator.

Puerarin (purity, >98%) was purchased from Sigma-Aldrich and dissolved in sterile PBS solution.

### 2.2. MTT assay

The MTT assay was used to investigate the cell viability of puerarin on the HaCaT cells. Briefly, cells in 100 μL culture medium were seeded at $1 \times 10^4$ cells/well in 96-well plates, and incubated for 24 h at 37 °C; puerarin was treated with several concentration (10 μM−100 mM). MTT solution was added to the well plates, and the cells were further incubated over 4 h at 37 °C. The crystallized formazan was dissolved in DMSO and read at 540 nm using a microplate reader.

### 2.3. Real-time PCR (polymerase chain reaction)

The dorsal skin tissues were collected in each mouse at the end of the experiment. The HaCaT cells were harvested after reagent treatment. Total RNA was isolated from the skin tissues and cells using Trizol reagent. The cDNA was synthesized using the Super Script TM III kit, according to the manufacturer's protocol. The mRNA expression was quantitatively determined using Power SYBR Green PCR Master Mix (Applied Biosystems). The primer sequences used for real-time PCR are shown in Table 1.

### 2.4. Western blot analysis

The HaCaT cells were pre-treated with puerarin for 2 h and stimulated with TNF-α/IFN-γ (each 10 ng/ml) for 30 min during incubation at 37 °C. The total proteins were extracted from the cells using a lysis buffer. The lysate was subjected to SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were probed with primary antibodies at 4°C overnight. After antibody probing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody. The membranes were visualized using an enhanced chemiluminescence detection kit (Millipore, MA). Membranes were exposed to Fusion Fx gel documentation system (Vilber Lourmat, Marne-la-Vallee).

### 2.5. Animals

Four-week-old male BALB/c mice weighing 20 ± 2 g were purchased from SAMTAKO Bio Korea. The mice were housed in stainless steel cages in a controlled environment with a temperature of 22 ± 2 °C and 55 ± 5% humidity under a 12 h light/12 h dark cycle. They were fed a standard laboratory diet and water ad libitum. The research was carried out in accordance with the ethical regulations with the approval of the Animal Experiment Ethics Committee of Chonbuk National University (Confirmation No. CBNU 2018-007).

### 2.6. DNCB-induced AD-like skin lesion

The animals were randomly divided into six groups of five mice each: Normal group: normal control group with a vehicle treatment; DNCB-induced group: negative control group, mice sensitized with DNCB and treated with water; P 10 group: mice sensitized with DNCB and treated with 10 mg/kg puerarin; P 30 group: mice sensitized with DNCB and treated with 30 mg/kg puerarin; P 50 group: mice sensitized with DNCB and treated with 50 mg/kg puerarin; and dexamethasone group: positive control group, mice sensitized with DNCB and treated with 1 mg/kg dexamethasone.

AD was induced in the mice by treatment with DNCB (Sigma-Aldrich). On day 0, the hair on the dorsal skin of the mice in all groups was shaved using an electric razor. During days 1−3, 200 μL of 1% DNCB dissolved in acetone/olive oil (4:1,v/v) was applied once daily to the dorsal skin of the mice for 3 days to induce dermatitis in the DNCB-induced, puerarin groups, and dexamethasone groups. The normal group received a vehicle treatment (only acetone/olive oil). After this initial sensitization treatment, the mice were dorsally treated with 0.5% DNCB in acetone/olive oil at 2 days of intervals for 2 weeks. The puerarin groups (10 mg/kg, 30 mg/kg, and 50 mg/kg) and dexamethasone group (1 mg/kg) were orally administered with their respective drug concentrations daily for 2 weeks from day 4. The mice were sacrificed on day 17 of the experiment.

### 2.7. Measurement of dorsal skin thickness and clinical score of the AD-like skin lesions

The dorsal skin thickness of each mouse was measured thrice using a micrometer (Mitutoyo, Japan) on the last day of the experiment. The severity of AD was evaluated as the average of the individual skin symptom scores assessed as 0 (none), 1 (mild), 2 (median) and 3 (severe) for each mouse on the last day of the experiment.

### 2.8. Histological analysis

At the end of the experiments, the tissue specimens from the dorsal skin of each mouse were obtained, fixed in 10% formalin,

**Table 1**
Primers utilized for real-time PCR.

| Gene | Forward | Reverse |
|---|---|---|
| hIL-5 | GCTAGCTCTTGGAGCTGCCT | CTTCAGTGCACAGTTGGTGA |
| hIL-6 | CTCCACAAGCGCCTTCGGTC | TGTGTGGGGCGGCTACATCT |
| hTNF-α | TTGGAGTGATCGGCCCCCAG | ACAGGCTTGTCACTCGGGGTT |
| mTNF-α | TAGCCAGGAGGGAGAACAGA | TTTTCTGGAGGGAGATGTGG |
| m IL-1β | CTCCATGAGCTTTGTACAAGG | TGCTGATGTACCAGTTGGGG |
| mIL-6 | GACAACCACGGCCTTCCCTA | GGTACTCCAGAAGACCAGAGGA |
| mIL-4 | ATGGGTCTCAACCCCCAGCTA | TGCATGGCGTCCCTTCTCCT |
| mIL-31 | GCATAAAGCAGGCATCTGGG | ACTGTGTTCTCGCTCAACAC |
| GAPDH | GTTAGGAAAGCCTGCCGGTG | GCATCACCCGGAGGAGAAATC |

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