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Guest Editors: Wansu Park, Salih Mollahaliloglu, Vitaly Linnik, and Han Chae

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# Editorial Board

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*Editorial*

# Challenge of Innovative Technology: How to Improve Efficiency of Korean Medicine?

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Advancement in science inevitably brings about the development of medicine. With the state-of-the-art technology, Western medicine continuously innovates. Nevertheless, there are still global health issues to be resolved, that is, chronic pain disease, chronic inflammatory disease, autoimmune disease, chronic sprain, myofascial pain syndrome, ischemic stroke, dementia, Parkinson's disease, arteriosclerosis, obesity, metabolic disease, type 2 diabetes, vertigo, allergy, infertility, dysmenorrhea, endometriosis, degenerative arthritis, and cancer. Additionally, new emerging infectious diseases such as *E. bola*, MERS, and bird flu as well as antibiotic resistant infections have become a threat to humans. Thus, not less than other traditional and integrative medicines, Korean Medicine (KM) is also challenged to innovatively cope with modern health issues. In 2014, the National Health Insurance of Korea covered 55% of total cost which patients (over 13 millions) paid for medical services provided by KM facilities including hospitals and clinics. More detailed, accurate, and valuable data regarding KM (including clinical and basic study) are needed not only to deal efficiently with modern health issues but also to bring about the innovation of KM services. To improve efficiency and accuracy in KM, modern technology and bioscience are indispensable. The innovation of practice and evolvement in KM help people overcome diseases and live healthily.

Our special issue, which had opened for 6 months in the second half of 2015, focused on challenge of innovative technology: how to improve efficiency of KM.

An article by D.-H. Kim et al. described that they took the TEM images of a cross section of the primo vessel (PV) inside a lymph vessel; the TEM study reveals the loosely distributed collagen fibers with plenty of empty spaces and the lumens with the endothelial nuclei; it turns out to be very similar to the ultrastructure of the PVs observed on the surfaces of internal organs; it also shows how compactly the PV is surrounded with lymphocytes.

J.-H. Kim et al. reported that the identification of *Atractylodes* rhizomes samples, authenticated by their morphological features as *Atractylodes japonica* Koidz. (Changchul and Baekchul), *Atractylodes chinensis* Koidz., and *Atractylodes macrocephala* Koidz., is confirmed as *Atractylodes japonica*, *Atractylodes chinensis*, and *Atractylodes macrocephala* by internal transcribed spacer sequencing; the results from chemometric analyses show that the chemical components of the crude drugs from *Atractylodes japonica* are significantly different from those from *Atractylodes macrocephala* but are similar to those from *Atractylodes chinensis* according to the results from chemometric analyses; the categorization by age of *Atractylodes japonica* as Changchul or Baekchul is not recommended. The results indicate that *Atractylodes japonica* should be categorized as "Changchul" and should not be further categorized by age.

A study by H.-Y. Cha et al. described that they investigated the preventive therapeutic effects of Hataedock (HTD) treatment on inflammatory regulation and skin protection in AD-induced NC/Nga mice under high-fat diet conditions;

HTD downregulates the levels of IL-4 and PKC but increases the levels of LXR; HTD also suppresses the mast cell degranulation and release of MMP-9, substance P; the levels of TNF-$\alpha$, p-I$\kappa$B, iNOS, and COX-2 are also decreased; the upregulation of inflammatory cell's apoptosis is confirmed as increase of apoptotic body and cleaved caspase-3 and decrease of Bcl-2; HTD also reduces edema, angiogenesis, and skin lesion inflammation; HTD suppresses various inflammatory response on AD-induced mice with obesity through the regulation of Th2 differentiation and the protection of lipid barrier.

W.-S. Jung et al. reported that Chunghyul-dan (CHD) is a herbal complex containing 80% ethanol extract and is composed of Scutellariae Radix, Coptidis Rhizoma, Phellodendri Cortex, Gardeniae Fructus, and Rhei Rhizoma; CHD has shown antilipidemic, antihypertensive, antiatherosclerotic, and inhibitory effects on ischemic stroke recurrence with clinical safety; the antilipidemic effect of CHD results from 3-hydroxy-3-methylglutaryl-coenzyme A reductase and pancreatic lipase-inhibitory activity; the antihypertensive effect likely results from the inhibitory effect on endogenous catecholamine(s) release and harmonization of all components showing the antihypertensive effects; anti-inflammatory and antioxidant effects on endothelial cells are implicated to dictate the antiatherosclerotic effects of CHD; CHD also shows neuroprotective effects on cerebrovascular and parkinsonian models; CHD could be helpful for the prevention of the recurrence of ischemic stroke; CHD could be a promising medication for treating and preventing cerebrovascular and cardiovascular diseases.

An interesting study by Y.-K. Song et al. evaluated that the serum C-peptide, insulin, leptin, lipocalin-2, and adipsin levels in the obese female Korean adults group are significantly higher than in the normal female Korean adults group; mean serum leptin of Eui-E-In-Tang group, which was randomized to receive Eui-E-In-Tang for 12 weeks, is significantly reduced at the end point of 12 weeks; Eui-E-In-Tang is composed of Coicis Semen, Angelicae Gigantis Radix, Atractylodis Rhizoma Alba, Ephedrae Herba, Cinnamomi Ramulus, Paeoniae Radix, and Glycyrrhizae Radix; Eui-E-In-Tang may exert immunomodulatory effect via reducing the circulating concentration of leptin in obese female Korean adults.

C. Yang et al. reported that they collected 166 injury cases from 94 Korean male and female national volleyball players; knee (25.9%), low back (13.3%), elbow, and ankle (8.4%) injuries are most common; joint (41.6%) and muscle (30.7%) are major injured tissues; KM team medical doctors utilize acupuncture (40.4%), chuna manual therapy (16.0%), physical therapy (15.2%), taping (9.0%), and cupping (7.8%) to treat volleyball injuries; any type of medications is used infrequently; additional physical and exercise therapies are preferred after receiving acupuncture (both 46.9%); injury and treatment parameters in this study could be useful to build advanced KM model in sport medicine.

In conclusion, we expect that this special issue updates innovative technologies in KM and makes useful progress for improving efficiency and accuracy in KM.

## Acknowledgments

We express our great appreciation to all authors for their excellent contributions and reviewers for their valuable help. We express our sincere thanks to the Editorial Board of this journal for their approval on this topic and continuous support in successful publication of this special issue. The lead guest editor would like to thank the three guest editors for their dedicated cooperation. We hope the special issue will give readers useful academic reference in their research.

*Wansu Park*
*Salih Mollahaliloglu*
*Vitaly Linnik*
*Han Chae*

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*Research Article*

# Management of Sport Injuries with Korean Medicine: A Survey of Korean National Volleyball Team

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The purpose of this study was to report the current state of Korean medicine (KM) treatment on sports injury by implementing survey with volleyball team medical doctors participating in 2013-2014 season. Six KM doctors completed a questionnaire that includes injury parameters: type, location, situation, and pain scores. We collected 166 injury cases from 94 Korean male and female national volleyball players. Knee (25.9%), low back (13.3%), elbow, and ankle (8.4%) injuries were most common. Joint (41.6%) and muscle (30.7%) were major injured tissues. KM team medical doctors utilized acupuncture (40.4%), chuna manual therapy (16.0%), physical therapy (15.2%), taping (9.0%), and cupping (7.8%) to treat volleyball injuries. Any types of medications were used infrequently. Additional physical and exercise therapy were preferred after receiving acupuncture (both 46.9%). This study presented the preliminary injury profile of Korean elite volleyball players. Injury and treatment parameters could be useful to build advanced KM model in sport medicine.

## 1. Introduction

The Korean medicine (KM) shares the same therapeutic techniques with East Asian traditional medicine. Some techniques such as acupuncture and cupping are used worldwide now. It also has distinctive features such as sasang typology, saam acupuncture, chuna manual therapy, pharmacopuncture, and pattern identification [1]. Research of KM in the field of sport medicine has begun in the early 1990s to integrate KM with sports injury care and to establish a firm foundation with a genuine system and developmental plan. KM therapies including acupuncture, moxibustion, chuna manual therapy, pharmacopuncture, and herbal medicine were expected to be useful to treat sports injuries [2, 3]. However, there is a paucity of well-designed clinical KM research regarding sports injury [4, 5]. To fully integrate KM into sports injury treatment, it is imperative to link KM with established sports related studies.

Globally, volleyball is one of the most popular sports. The Federation Internationale de Volleyball includes 220 member federations and presides over 5 continental confederations. Volleyball is a powerful sport that involve repeated forceful arm actions and jumps that can lead to shoulder and knee injuries. Several epidemiological surveys have described preventive strategies based on the injury mechanisms [6–8]. However, there was no study focusing on volleyball injuries managed with KM. The present study was conducted to report the current state of KM treatment with regard to sports injury based on the results of a survey of national male and female volleyball team medical doctors participating in the 2013-2014 season.

## 2. Materials and Methods

*2.1. Subjects.* The subjects were six licensed KM doctors who participated as team medical doctors in 2013-2014 international volleyball season. They reported 166 injury profiles and treatment models using structured questionnaire. Written consents were obtained from all subjects. Personal

information that can be used to identify an individual player was eliminated during data capturing.

*2.2. Questionnaire.* We developed a questionnaire to determine how KM volleyball team medical doctors treat sports injuries. It was developed in Korean language and queried the treatment backgrounds of the six doctors. Individual items were based on similar studies and modified to suit our subject matter [9].

The self-reported questionnaire included the following:

(1) diagnosis given by team medical doctor and number of visits;

(2) injury type and location and reason for visit;

(3) injury duration, pain scores, injury mechanism, type of activity during injury, and game or training status;

(4) previous treatment experience, reason for selecting particular treatment, and satisfaction;

(5) need for imaging diagnosis;

(6) treatment content and theoretical basis;

(7) treatment place, condition, and required treatment time;

(8) comanagement model.

*(I) Injury Questionnaire*

Diagnosis condition/reason for this consultation:___

(If there were more than two diagnoses, please fill out another questionnaire)

Number of treatment sessions:___

Injury type (please √ one box)

□ Bone
□ Joint (include ligament)
□ Muscle
□ Tendon
□ Contusion
□ Laceration
□ Central/peripheral nervous system
□ Other:___

Location of injury (please √ one box)

□ Head
□ Neck
□ Chest
□ Rib
□ Upper back
□ Lower back
□ Upper arm
□ Elbow
□ Forearm
□ Wrist
□ Palm
□ Thumb
□ Finger
□ Thigh
□ Knee
□ Shin
□ Ankle
□ Heel
□ Soles of the feet
□ Toe
□ Other:___

Reason for presentation (please √ one box)

□ New injury
□ Aggravation or exacerbation of an existing injury that had not fully resolved
□ Recurrence of a previous injury that had fully resolved
□ Maintenance/preventive/asymptomatic care
□ Illness
□ Other:___

How long has the player had this condition or pain for (please √ one box)

□ 0–7 days
□ 1–4 wks
□ 1–3 mths
□ 3–6 mths
□ 6–12 mths
□ 1-2 yrs
□ 2+ yrs

Please rate the degree of pain the player has for this condition (circle one number)

0 (No pain)
1
2
3
4
5
6
7
8
9
10 (Worst pain)

How did the injury occur? (please √ one box)

□ Contact/physical collision with another player or object [Specify:___
□ Non-contact/DID NOT involve physical contact [Specify:___

□ Uncertain/the injury gradually developed [Specify:___

Type of activity at the time of injury (please √ one box)

□ Competition [Specify period of game □ First □ Second □ Third □ Forth □ Fifth]
□ Training/Practice
□ Other:___

Did the player have to stop playing or training because of the injury? (please √ one box)

□ Yes
□ No

If no, was the player restricted or limited from full participation? (please √ one box)

□ Yes
□ No

What other practitioners has the player previously consulted for this condition? (please √)

□ None
□ Traditional Korean medical doctor
□ Medical doctor
□ Physical therapist
□ Trainer/Exercise therapist
□ Other:___

What is the reason the player stated for selecting a specific treatment for this condition? (please √ one box)

□ Nearby location/Convenience
□ Experience treated by oneself/Satisfied or improved following previous treatment
□ Suggestion of a coaching staff or surrounding people (family, team)
□ Renown of treatment group
□ Advertisement/Internet
□ Other:___
□ None

If player was previously treated for this condition, how did they feel about the outcome? (please √ one box)

□ Highly satisfied
□ Satisfied
□ Partially satisfied
□ Not satisfied
□ Not at all satisfied
□ None

*(II) Diagnosis and Treatment Questionnaire*

Was a referral for advanced imaging required? (please √ one box)

□ Yes
□ No
[Specify: □ X-ray □ CT/MRI □ Ultrasound □ Other___

What kind of treatment was provided? (please √)

□ Acupuncture treatment (include pharmacopuncture therapy)
□ Cupping therapy
□ Moxibustion therapy
□ Herbal medicine
□ Physical therapy
□ Chuna manual therapy
□ Exercise therapy
□ Taping
□ Over-the-counter medication
□ Prescription medication
□ Consultation
□ Other:___

Which treatment was provided with theory? (please √ one box)

□ Theory of Traditional Korean medicine
[Specify: □ Meridian system demonstration □ Organ demonstration □ Eight demonstration □ Other___
□ Western medicine
□ Combined Traditional Korean medicine and western medicine/used both
□ Other___
□ None

Where was the treatment administered? (please √ one box)

□ Training location
□ Match location
□ Other:___

When was treatment provided? (please √ one box)

□ Pre training
□ During training
□ Post training
□ Pre match
□ During match
□ Post match
□ Other:___

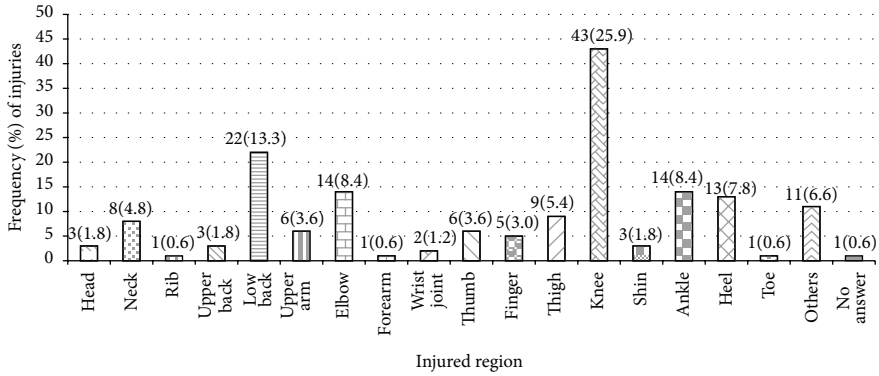


Figure 1: Location and frequency of injuries.

How many minutes did you spend treating this patient? (please √ one box)

□ <5
□ 6–10
□ 11–15
□ 16–20
□ 20–30
□ 31–45
□ 45–60
□ >60

Was co-management with another health care provider required? (please √ one box)

□ No
□ Yes
[Specify:___□ Medical doctor □ Ambulance □ Hospital □ Physiotherapist □ Trainer/Exercise therapist □ Other___

If applicable, was this provided at the event? (please √ one box)

□ Yes
□ No

*2.3. Ethical Review.* This study was approved by the institutional review board of the Korea Institute of Oriental Medicine.

## 3. Results

*3.1. General Characteristic.* Six KM team medical doctors reported 166 injury cases from Korean male and female national volleyball players. Total 94 players (age range of 17–33 years; male : female = 76 : 18) experienced one or more injuries during 2013-2014 season. Players visited KM team medical doctors with mean 2.18 sessions (maximum 10 sessions). Mean pain score at visit was 5.4 (0–10 numeric pain rating scale) and most severe pain was reported in new injuries of knee and head.

*3.2. Injury Location.* The most frequent injury site was the knee joint (43/166 cases, 25.9%) followed by the low back (22, 13.3%), elbow and ankle (each 14 cases, 8.4%), and heel (13 cases, 7.8%) (Figure 1). Gender differences were reported. Male players complained of knee (32 cases, 29.3%), low back (16 cases, 14.6%), and ankle (10 cases, 9.1%) injuries. Their knee injury was mainly recurrence or worsening of former damage (20 cases, 62.5%), while new damage was major cause (11 cases, 68.8%) in case of low back problem.

Female player complained of elbow (14 cases, 24.5%), knee (11 cases, 19.2%), and heel (8 cases, 14.0%) injuries. Female elbow and knee injuries were mainly caused by initial damage (12 cases, 85.7%, and 9 cases, 81.8%). Most frequent injured tissue was joint with 69 out of 166 cases (41.6%) followed by muscle with 51 cases (30.7%) and tendon with 14 cases (8.4%). Fracture was least frequent with 1 case (0.6%).

*3.3. Distribution of Treatment Frequency by Injured Tissue.* The most frequent treatment was acupuncture (151 cases, 40.4%) followed by chuna manual therapy (60 cases, 16.0%), physical therapy (57 cases, 15.2%), taping (34 cases, 9.0%), and cupping (29 cases, 7.8%). Korean over the counter (OTC) and prescribed medications were used infrequently. The injured tissue distribution revealed that acupuncture, chuna manual therapy, physical therapy, and taping were frequently used to treat joint injuries, while cupping was more used for treating muscle injury (Table 1).

*3.4. Comanagement Therapy.* No subjects required western medical assistant or emergency transfer after receiving treatment from KM team medical doctors. Two cases were reported to visit an external hospital after undergoing acupuncture, one case after physical therapy. Additional physical therapy and exercise were required after receiving acupuncture (23 cases each, 46.9%). No subject required additional physical therapy after receiving an initial session. After chuna manual therapy, 19 (54.3%) cases received physical therapy and 6 (17.1%) cases needed exercise based treatments from their trainers (Table 2).

## 4. Discussion

Beyond health benefits, the growing popularity of sports will result in increase of sport-induced injuries [10–13]. Advances

Table 1: Treatment type for injured tissue.

| Treatment | Category (total $n = 166$), $n$ (%) | | | |
|---|---|---|---|---|
| | Joint | Muscle | Tendon | Others |
| Acupuncture | 64 (38.1) | 45 (41.3) | 26 (44.8) | 16 (41.0) |
| Cupping | 12 (7.1) | 16 (14.7) | 0 (0.0) | 1 (2.6) |
| Moxibustion | 2 (1.2) | 0 (0.0) | 1 (1.7) | 0 (0.0) |
| Herbal medicine | 2 (1.2) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Physical therapy | 26 (15.5) | 22 (20.2) | 6 (10.3) | 3 (7.7) |
| Chuna manual therapy | 32 (19.0) | 9 (8.3) | 10 (17.2) | 9 (23.1) |
| Exercise treatment | 7 (4.2) | 4 (3.7) | 1 (1.7) | 1 (2.6) |
| Taping | 16 (9.5) | 7 (6.4) | 10 (17.2) | 1 (2.6) |
| Over-the-counter medication | 3 (1.8) | 2 (1.8) | 1 (1.7) | 4 (10.3) |
| Prescription medication | 2 (1.2) | 0 (0.0) | 1 (1.7) | 1 (2.6) |
| Consultation | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (2.6) |
| Others | 2 (1.2) | 4 (3.7) | 2 (3.4) | 2 (5.1) |
| Total | 168 (100.0) | 109 (100.0) | 58 (100.0) | 39 (100.0) |

Results are presented as number and percentage of cases.

Table 2: Comanagement after KM treatment.

| Comanagement | Acupuncture | Physical therapy | Chuna manual therapy |
|---|---|---|---|
| Medical doctor | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Ambulance | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Hospital | 2 (4.1) | 1 (10.0) | 0 (0.0) |
| Physiotherapist | 23 (46.9) | 0 (0.0) | 19 (54.3) |
| Trainer | 23 (46.9) | 9 (90.0) | 6 (17.1) |
| Not answered | 1 (2.0) | 0 (0.0) | 0 (0.0) |
| Total | 49 (100.0) | 10 (100.0) | 25 (100.0) |

Results are presented as number and percentage of cases.

in complementary and alternative medicine (CAM) techniques can have a major impact on modern sports medicine. In the field of professional and elite sports such as basketball, ice hockey, and wrestling, CAM treatment models are adopted to manage sport injuries [9, 14, 15].

The current KM system is being confronted by medical globalization and western-driven medical science development, making it difficult to maintain unique identity. Korean sports medicine is faced with a similar dilemma. There is lack of evidence reporting effectiveness of KM treating sport injury of elite sport player. Our survey of national male and female volleyball players would help clarify the role of Korean sports medicine.

This research was conducted to report the current state of KM treatment in sport injuries. It was carried out by using a questionnaire to assess the opinions of team medical doctors of national male and female volleyball players regarding various Korean sports medicine treatment methods and possible countermeasures. The questionnaire was designed in similar way as that employed in former sports injury studies [6, 9].

Volleyball involves repetitive movements, advanced technique, and excellent motor ability to spike, block, serve, receive, pass, and toss. Dynamic interplay involves various possible injuries. The most frequently injured location in this study was the knee joint (43/166 cases, 25.9%). Injuries of low back (22 cases, 13.3%), elbow and ankle (both 14 cases, 8.4%), and heel (13 cases, 7.8%) were reported more frequently than thumb (6 cases, 3.6%) and other fingers (5 cases, 3.0%) in this research. These results agree with former studies that described common injuries site including ankle, knee, and finger [6, 7, 16].

The most frequent treatment method was acupuncture (151 cases, 40.3%). As shown in Taiwanese wrestlers study, acupuncture has been used with sport injuries because it is rapid acting and easily applicable [15]. Acupuncture is effective for musculoskeletal disorders and there are positive reports on performance enhancement and postexercise recovery [14, 17–22].

Chuna manual therapy (60 cases, 16.0%) and physical therapy (57 cases, 15.2%) were also frequently used. Volleyball player repeatedly uses dominant shoulder and arm during serve or spike with extreme power. It affects muscle balance and range of motion of shoulder [23]. Chuna manual therapy is a KM therapy for pain control and repositioning of physical imbalance. Physical therapy is used less frequently because competitive provider such as western medical doctors and physiotherapist can use the same method. Taping therapy (34 cases) was the fourth most common treatment method; it was previously reported to have a beneficial effect on the knee joint [24]. However it was ranked fourth among

the treatment methods, likely because it can be done by players themselves and does not require doctor's assistance. Cupping and moxibustion generally occupied low frequency and was applied in limited conditions. Medication (herbal, OTC, and prescription drugs) was infrequently used, possibly because players and coaches were cautious about unintended antidoping rule violation. KM team medical doctor preferred physical and exercise therapies as comanagement models coupled with acupuncture or chuna manual therapy.

This study is the first survey report that presents the preliminary injury and KM treatment profile of Korean elite volleyball players. Injury and treatment parameters could be useful to build advanced KM model in sport medicine. More large studies including wide range of volleyball player are necessary to indicate generalized trends of sport injury and KM therapeutic modalities.

## 5. Conclusion

Our study findings revealed that the knee and the low back were major lesions of volleyball injury among Korean elite players. Acupuncture, chuna manual therapy, and physical therapy are widely used by KM team medical doctors whereas moxibustion, medication, and exercise treatment are used infrequently. Acupuncture was often combined with physical and exercise therapies. This study might be useful to understand circumstances of KM in the field of volleyball injury. Further outcome study that shows best practice model for each sport injuries would be required. In addition, KM multimodal strategy to cooperate with other health professionals for treat and prevent sport injury will be also needed to achieve advancement of Korean sports medicine.

## Competing Interests

The authors declare that they have no competing interests.

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*Research Article*

# Modulatory Effect of Eui-E-In-Tang on Serum Leptin Concentration in Obese Korean Female Adults: A Randomized Controlled Trial

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*Background.* Obesity is associated with chronic inflammation and cytokines. However, to date, the relationship between the serum levels of cytokines in obese individuals and taking herbal drugs remains largely unexplored. *Methods.* Serum cytokines were assessed by multiplex cytokine profiling assay. Serum samples of obese female Korean adults (obese group; $n = 20$) as well as normal female Korean adults (normal group; $n = 21$) were collected at the start and end of study period. Twenty obese female Korean adults were randomized to receive Eui-E-In-Tang (Eui-E-In-Tang group; $n = 9$) at a daily dose of 9 g or a matched placebo (placebo group; $n = 11$) for 12 weeks. *Results.* According to investigating serum cytokine levels at the start point of this study, the serum C-peptide, insulin, leptin, lipocalin-2, and adipsin levels in the obese group were found to be significantly higher than in the normal group. And the investigation of serum cytokine levels at the end point of this study demonstrated that mean serum leptin of Eui-E-In-Tang group was found to be significantly reduced ($P = 0.037$). *Conclusions.* This study provides preliminary evidence that Eui-E-In-Tang may exert immunomodulatory effect via altering the circulating concentration of leptin in Korean female adults.

## 1. Introduction

Obesity is a serious health problem worldwide. According to the World Health Organization (WHO) statistics, there were more than 1.9 billion overweight adults globally, of whom about 600 million were obese in 2014 [1].

Obesity is associated with an increased risk of life-threatening diseases including diabetes, cardiovascular diseases, metabolic syndrome, osteoarthritis, gout, and certain types of cancer [2–4]. Adya et al. have already reported that obesity is a major health burden with an increased risk of cardiovascular morbidity and mortality [5]. dos Santos et al. have reported that inflammation, the process aimed at restoring homeostasis after an insult, can be more damaging than the insult itself if uncontrolled, excessive, or prolonged [6]. Specifically, in mediators of chronic inflammation, cytokine (cell signaling protein) has been becoming the impressive target to deal with obesity. Alvehus et al. have reported that obesity can be considered as a low-grade inflammatory condition, strongly linked to adverse metabolic outcomes [7]. It has been suggested that adipocytokine (adipokine) such as leptin is secreted by adipose tissue and concerned with the pathogenesis of obesity-associated complications [8, 9].

Yen et al. have reported that inflammation has been found to be an important characteristic of adipose tissue in obese subjects and obesity is also associated with compromised immune responses to infections, which have not been fully understood [10]. Schulte et al. have reported that cytokines are important pleiotropic regulators of the immune response, which have a crucial role in the complex pathophysiology underlying sepsis [11]. Recently, anti-inflammatory effects of herbal drugs used in Korea, China, and Japan have been reported continuously. Eui-E-In-Tang is one of herbal drugs which are used for obese individuals in Korea.

However, to date, the relationship between the serum levels of cytokines in obese individuals and taking herbal drug such as Eui-E-In-Tang remains largely unexplored, especially in Korean women. In this study, we investigated

modulatory effects of Eui-E-In-Tang on serum concentrations of cytokines in obese female Korean adults.

## 2. Materials and Methods

*2.1. Study Design.* This was a randomized, double blind, placebo-controlled trial in which each patient received Eui-E-In-Tang (9 g/day) or a matched placebo for 12 weeks. All studies were approved by Bioethic Institutional Review Board at Gachon University. All subjects gave written informed consent prior to participation.

*2.2. Subjects.* Thirty-six apparently healthy adult Korean females were recruited from the local communities of Incheon and neighboring areas as normal subjects (the normal group). Obesity was defined by body mass index (BMI) $\geq$ 30.0 kg/m$^2$. Thirty-six obese adult Korean females were selected and recruited from the local communities of Incheon and neighboring areas as obese subjects (the obese group). Thirty-one individuals were excluded if they were taking any prescription or over-the-counter medication including oral contraceptives, hormone replacement therapy, or psychiatric drugs or had any acute or chronic medical illnesses during the study. Finally, 21 normal subjects and 20 obese subjects were selected for serum cytokine profiling assay in the study.

*2.3. Drugs.* Eui-E-In-Tang and placebo drug capsules were provided by the Hanpoong Pharmaceutical Co., Ltd. (Seoul, Korea). The components of Eui-E-In-Tang capsule are shown in Table 1. The placebo capsules were made of cornstarch and manufactured identical to Eui-E-In-Tang capsules in terms of color, size, and shape by the same manufacturer.

*2.4. Multiplex Cytokine Assay.* Multiplex cytokine profiling assay (MILLIPLEX MAP Human Metabolic Panel, Millipore, Billerica, MA, USA) was used to simultaneously measure serum levels of 21 cytokines such as C-peptide, ghrelin, glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1 (GLP-1), glucagon, interleukin- (IL-) 1$\beta$, IL-6, IL-8, insulin, leptin, monocyte chemotactic protein- (MCP-) 1, pancreatic polypeptide (PP), peptide YY (PYY), tumor necrosis factor- (TNF-) $\alpha$, nerve growth factor (NGF), hepatocyte growth factor (HGF), adiponectin, lipocalin-2, resistin, adipsin, and total plasminogen activator inhibitor type-1 (PAI-1). Samples were read on a Bio-Plex 200 suspension array system (Bio-Rad, Hercules, CA, USA) using Bio-Plex Manager 5.0 software system (Bio-Rad). The standard curve range for each cytokine was from 3.2 to 10,000 pg/mL.

*2.5. Statistical Analysis.* The results represent the mean ± SD. Descriptive analyses for the normal and obese groups are done using the independent sample $t$-test. The serum cytokine levels of the normal and obese groups were also compared via an independent sample $t$ test. Of them, C-peptide, insulin, leptin, HGF, adiponectin, lipocalin-2, adipsin, and total PAI-1—all of which showed significant differences between the two groups—were compared with a univariate analysis of covariance. Correlation coefficients were calculated using Spearman's test. A paired $t$-test was used to compare serum cytokine levels before and after Eui-E-In-Tang therapy. All tests were two-tailed at a probability level of 0.05. All analyses were performed using SPSS software 19.0 (SPSS Inc., Chicago, IL, USA).

Table 1: Composition of Eui-E-In-Tang.

| Herbal medicines | Ratio |
|---|---|
| Coicis Semen | 10 |
| Angelicae Gigantis Radix | 4 |
| Atractylodis Rhizoma Alba | 4 |
| Ephedrae Herba | 4 |
| Cinnamomi Ramulus | 3 |
| Paeoniae Radix | 3 |
| Glycyrrhizae Radix | 2 |

## 3. Results

Table 2 presents the demographic factors, height, weight, and body mass index of the individuals in the normal and obese groups. We also compared vascular risk factors between the normal and obese group. Significant differences in the serum glutamate-pyruvate transaminase (SGPT), alkaline phosphatase, gamma-glutamyl transpeptidase (GGTP), total cholesterol, high-density lipoprotein (HDL) cholesterol, triglyceride, uric acid, glucose, C-reactive protein (CRP), and white blood cell (WBC) levels were observed between the two groups.

Among the 21 cytokines, the number of samples with detectable levels and the range (pg/mL) varied according to the tested cytokines. Data of cytokines which were detected to be >80% of the total samples were used for further analysis and presented in Table 3. Data of cytokines included C-peptide, GIP, insulin, leptin, MCP-1, PP, IL-8, HGF, adiponectin, lipocalin-2, resistin, adipsin, and total PAI-1. Among the 13 cytokines analyzed, the serum C-peptide, insulin, leptin, HGF, lipocalin, adipsin, and total PAI-1 levels of the obese group were found to be significantly higher than those of the normal group ($t = -5.104$, $P < 0.001$; $t = -3.906$, $P = 0.001$; $t = -6.777$, $P < 0.001$; $t = -2.753$, $P = 0.01$; $t = -2.062$, $P = 0.046$; $t = -3.723$, $P = 0.001$; and $t = -3.441$, $P = 0.001$, resp.), whereas the serum adiponectin level of the obese group was found to be lower than that of the normal group ($t = 2.77$, $P = 0.009$) (Table 3). After adjusting for age, height, SGPT, alkaline phosphatase, GGTP, total cholesterol, HDL cholesterol, triglyceride, uric acid, glucose, CRP, and WBC, the serum C-peptide, insulin, leptin, lipocalin-2, and adipsin levels in the obese group were found to be significantly higher than in the normal group ($F = 4.673$, $P = 0.04$; $F = 4.358$, $P = 0.046$; $F = 28.469$, $P < 0.001$; $F = 7.236$, $P = 0.012$; $F = 5.37$, $P = 0.028$, resp.).

Table 4 presents correlations among serum cytokines in the overall population. C-peptide was significantly correlated with insulin, leptin, IL-8, HGF, adiponectin, adipsin, and total PAI-1. GIP was significantly correlated with insulin, adiponectin, and lipocalin-2. Insulin was significantly correlated with leptin, HGF, adipsin, and total PAI-1. Leptin was

Table 2: Demographic characteristics and obesity-related scale scores of participants.

| | Normal group ($n$ = 21, female) | Obese group ($n$ = 20, female) | $t$ | $P$ |
|---|---|---|---|---|
| Age (year) | 36.7 ± 11.1 | 41.4 ± 9.5 | −1.436 | 0.159 |
| Height (cm) | 161.5 ± 4.7 | 160.2 ± 6.2 | 0.796 | 0.431 |
| Weight (kg) | 53.8 ± 4.4 | 87.6 ± 12.2 | −11.729 | <0.001 |
| Body mass index (BMI) | 20.7 ± 1.5 | 34.0 ± 3.1 | −17.397 | <0.001 |
| Total protein (g/dL) | 7.5 ± 0.3 | 7.3 ± 0.5 | 1.018 | 0.315 |
| Albumin (g/dL) | 4.7 ± 0.2 | 4.6 ± 0.2 | 1.419 | 0.164 |
| Total bilirubin (g/dL) | 0.7 ± 0.3 | 0.6 ± 0.3 | 1.257 | 0.216 |
| SGOT (U/L) | 19.2 ± 6.8 | 24.4 ± 10.1 | −1.939 | 0.06 |
| SGPT (U/L) | 15.4 ± 8.9 | 25.0 ± 11.5 | −2.971 | 0.005 |
| Alkaline phosphatase (U/L) | 49.6 ± 14.3 | 67.4 ± 16.4 | −3.691 | 0.001 |
| GGTP (U/L) | 14.4 ± 6.8 | 33.0 ± 28.6 | −2.839 | 0.01 |
| Creatine phosphokinase (U/L) | 111.4 ± 123.8 | 98.8 ± 66.6 | 0.404 | 0.688 |
| Total cholesterol (mg/dL) | 174.1 ± 24.3 | 201.7 ± 46.7 | −2.359 | 0.025 |
| HDL cholesterol (mg/dL) | 62.1 ± 14.5 | 53.2 ± 13.0 | 2.061 | 0.046 |
| Triglyceride (mg/dL) | 74.8 ± 32.8 | 133.5 ± 60.7 | −3.822 | 0.001 |
| Uric acid (mg/dL) | 4.4 ± 0.8 | 5.6 ± 1.3 | −3.675 | 0.001 |
| Blood urea nitrogen (mg/dL) | 11.8 ± 2.4 | 12.8 ± 3.7 | −1.045 | 0.303 |
| Creatinine (mg/dL) | 0.7 ± 0.1 | 0.7 ± 0.1 | −0.737 | 0.465 |
| Glucose (mg/dL) | 85.8 ± 9.1 | 93.4 ± 10.2 | −2.512 | 0.016 |
| CRP (mg/dL) | 0.0 ± 0.1 | 0.3 ± 0.5 | −2.533 | 0.02 |
| Free T4 (ng/dL) | 1.2 ± 0.1 | 1.2 ± 0.2 | −0.573 | 0.57 |
| Thyroid-stimulating hormone ($\mu$IU/mL) | 1.6 ± 0.7 | 1.6 ± 0.9 | −0.141 | 0.888 |
| Hemoglobin (g/dL) | 13.3 ± 1.0 | 13.6 ± 0.9 | −1.111 | 0.273 |
| Hematocrit (%) | 40.3 ± 2.6 | 41.1 ± 2.1 | −1.069 | 0.291 |
| RBC count ($10^6/\mu$L) | 4.4 ± 0.4 | 4.5 ± 0.3 | −1.384 | 0.174 |
| WBC count ($10^3/\mu$L) | 5.7 ± 1.8 | 7.1 ± 1.8 | −2.408 | 0.021 |
| Platelet count ($10^3/\mu$L) | 268.1 ± 58.8 | 279.2 ± 46.7 | −0.668 | 0.508 |

Table 3: Comparison of serum cytokine levels using multiplex cytokine assays.

| Serum cytokine levels (pg/mL) | Normal ($n$ = 21, female) | Obese ($n$ = 20, female) | $t$ | $P$ |
|---|---|---|---|---|
| C-peptide | 1673.2 ± 787.3 | 3395.5 ± 1319.7 | −5.104 | <0.001 |
| GIP | 64.4 ± 54.3 | 102.5 ± 203.1 | −0.831 | 0.411 |
| Insulin | 27.7 ± 13.7 | 52.4 ± 24.9 | −3.906 | 0.001 |
| Leptin | 842.0 ± 706.2 | 3498.7 ± 1612.0 | −6.777 | <0.001 |
| MCP-1 | 1163.7 ± 493.1 | 1313.7 ± 430.1 | −1.036 | 0.307 |
| PP | 110.8 ± 61.8 | 119.2 ± 72.1 | −0.402 | 0.69 |
| IL-8 | 2098.1 ± 2500.2 | 1637.5 ± 3468.3 | 0.49 | 0.627 |
| HGF | 174.1 ± 84.0 | 285.3 ± 160.8 | −2.753 | 0.01 |
| Adiponectin | 10228.4 ± 1709.2 | 8732.5 ± 1748.4 | 2.77 | 0.009 |
| Lipocalin-2 | 3429.9 ± 1335.8 | 4552.5 ± 2086.3 | −2.062 | 0.046 |
| Resistin | 807.3 ± 530.7 | 1048.1 ± 669.4 | −1.28 | 0.208 |
| Adipsin | 16287.9 ± 1044.8 | 17351.4 ± 752.5 | −3.723 | 0.001 |
| Total PAI-1 | 1953.5 ± 647.3 | 2701.9 ± 743.7 | −3.441 | 0.001 |

significantly correlated with HGF, lipocalin-2, adipsin, and total PAI-1. PP was significantly correlated with adiponectin, resistin, and total PAI-1. IL-8 was significantly correlated with lipocalin-2. HGF was significantly correlated with lipocalin-2, resistin, adipsin, and total PAI-1. Lipocalin-2 was significantly correlated with resistin and adipsin. Resistin was significantly correlated with adipsin. However, MCP-1 was not significantly correlated with any other cytokines.

Table 5 presents that mean serum leptin of Eui-E-In-Tang group was found to be significantly reduced by Eui-E-In-Tang therapy for 12 weeks ($P$ = 0.037). In contrast, Eui-E-In-Tang did not alter serum levels of C-peptide, GIP, insulin, MCP-1,

Table 4: Spearman correlation analysis of cytokines.

| | C-peptide | GIP | Insulin | Leptin | MCP-1 | PP | IL-8 | HGF | Adiponectin | Lipocalin-2 | Resistin | Adipsin | Total PAI-1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C-peptide | 1 | 0.306 | 0.852** | 0.651** | −0.029 | −0.017 | −0.328* | 0.418** | −0.407** | 0.274 | 0.176 | 0.318* | 0.634** |
| GIP | | 1 | 0.409** | 0.261 | −0.232 | 0.033 | −0.153 | 0.084 | 0.312* | 0.364* | −0.017 | 0.179 | 0.273 |
| Insulin | | | 1 | 0.583** | −0.036 | −0.089 | −0.275 | 0.471** | −0.201 | 0.287 | 0.111 | 0.374* | 0.587** |
| Leptin | | | | 1 | 0.158 | 0.075 | −0.082 | 0.536** | −0.282 | 0.322* | 0.12 | 0.414** | 0.615** |
| MCP-1 | | | | | 1 | 0.115 | 0.052 | 0.083 | −0.007 | −0.15 | −0.12 | −0.078 | 0.06 |
| PP | | | | | | 1 | −0.027 | −0.063 | 0.35* | −0.04 | 0.409** | 0.093 | 0.383* |
| IL-8 | | | | | | | 1 | 0.276 | 0.155 | 0.422** | 0.289 | −0.085 | −0.272 |
| HGF | | | | | | | | 1 | −0.302 | 0.635** | 0.358* | 0.484** | 0.422** |
| Adiponectin | | | | | | | | | 1 | −0.032 | −0.033 | −0.119 | −0.041 |
| Lipocalin-2 | | | | | | | | | | 1 | 0.609** | 0.346* | 0.223 |
| Resistin | | | | | | | | | | | 1 | 0.407** | 0.296 |
| Adipsin | | | | | | | | | | | | 1 | 0.284 |
| Total PAI-1 | | | | | | | | | | | | | 1 |

*$P < 0.05$.
**$P < 0.01$.

Table 5: Effect of Eui-E-In-Tang on serum cytokine levels.

| Serum cytokine levels (pg/mL) | Study group | $N$ | Baseline | Endpoint | $P$ |
|---|---|---|---|---|---|
| C-peptide | Obese-Eui-E-In-Tang | 9 | 3290.7 ± 1194.0 | 3481.1 ± 1466.4 | 0.832 |
| | Obese-placebo | 11 | 3156.3 ± 1444.6 | 3129.7 ± 2203.9 | 0.664 |
| GIP | Obese-Eui-E-In-Tang | 9 | 76.3 ± 90.5 | 124.0 ± 265.9 | 0.703 |
| | Obese-placebo | 11 | 63.0 ± 48.9 | 58.6 ± 51.9 | 0.433 |
| Insulin | Obese-Eui-E-In-Tang | 9 | 57.9 ± 24.2 | 47.8 ± 25.6 | 0.262 |
| | Obese-placebo | 11 | 46.3 ± 17.7 | 91.9 ± 137.8 | 0.309 |
| Leptin | Obese-Eui-E-In-Tang | 9 | 3749.5 ± 1556.7 | 3293.5 ± 1701.6 | 0.037 |
| | Obese-placebo | 11 | 2281.4 ± 1148.6 | 2856.6 ± 1157.4 | 0.489 |
| MCP-1 | Obese-Eui-E-In-Tang | 9 | 1231.0 ± 381.9 | 1381.4 ± 473.0 | 0.471 |
| | Obese-placebo | 11 | 1409.2 ± 615.2 | 1462.4 ± 760.0 | 0.767 |
| PP | Obese-Eui-E-In-Tang | 9 | 131.0 ± 100.4 | 109.5 ± 39.8 | 0.365 |
| | Obese-placebo | 11 | 193.7 ± 175.0 | 161.4 ± 196.7 | 0.401 |
| IL-8 | Obese-Eui-E-In-Tang | 9 | 954.3 ± 1610.5 | 2196.5 ± 4474.1 | 0.231 |
| | Obese-placebo | 11 | 2473.8 ± 3287.8 | 3437.3 ± 3850.0 | 0.494 |
| HGF | Obese-Eui-E-In-Tang | 9 | 299.7 ± 162.5 | 273.5 ± 166.4 | 0.373 |
| | Obese-placebo | 11 | 240.3 ± 106.9 | 268.5 ± 127.4 | 0.938 |
| Adiponectin | Obese-Eui-E-In-Tang | 9 | 8954.3 ± 1676.9 | 8551.0 ± 1864.9 | 0.697 |
| | Obese-placebo | 11 | 9219.6 ± 1097.8 | 8470.6 ± 1669.0 | 0.916 |
| Lipocalin-2 | Obese-Eui-E-In-Tang | 9 | 4239.4 ± 1258.6 | 4808.5 ± 2615.8 | 0.323 |
| | Obese-placebo | 11 | 3465.1 ± 1897.7 | 4050.4 ± 1833.9 | 0.440 |
| Resistin | Obese-Eui-E-In-Tang | 9 | 1163.2 ± 763.1 | 953.9 ± 603.3 | 0.473 |
| | Obese-placebo | 11 | 940.7 ± 493.1 | 821.5 ± 415.1 | 0.556 |
| Adipsin | Obese-Eui-E-In-Tang | 9 | 17645.9 ± 591.6 | 17110.3 ± 808.6 | 0.751 |
| | Obese-placebo | 11 | 17553.3 ± 624.6 | 17067.4 ± 894.1 | 0.907 |
| Total PAI-1 | Obese-Eui-E-In-Tang | 9 | 2867.1 ± 866.5 | 2566.7 ± 636.9 | 0.438 |
| | Obese-placebo | 11 | 2556.1 ± 789.0 | 2858.0 ± 886.5 | 0.387 |

PP, IL-8, HGF, adiponectin, lipocalin-2, resistin, adipsin, and total PAI-1 ($P > 0.05$). Table 5 also presents that the serum cytokine levels of obese group with the placebo capsules for 12 weeks do not show any significant variation ($P > 0.05$).

## 4. Discussion

Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health by the WHO definition [1]. Obesity is a global health problem and is associated with chronic inflammation, which is linked to many diseases including diabetes, cardiovascular diseases, metabolic syndrome, and gout. According to the WHO statistics, about 13% of the world's adult population (11% of men and 15% of women) was obese in 2014 [1].

Ghayour-Mobarhan et al. have reported that obesity is associated with a strong inflammatory response and is often accompanied by increased levels of proinflammatory cytokines and impaired antioxidant status [12]. Spite et al. have reported that although inflammation is thought to be essential for reacting to pathologic infection, ungoverned inflammation is an underlying component of various diseases, such as sepsis, cardiovascular disease, diabetes, and other chronic inflammatory diseases [13]. Maskrey et al. have reported that chronic inflammation is a characteristic feature in virtually all inflammatory diseases, including cardiovascular disease such as atherosclerosis, and it is becoming increasingly clear that disturbance of the processes usually involved in resolution of inflammation is an underlying feature of chronic inflammatory conditions [14]. Skeldon et al. have reported that inflammation also is regarded as an important contributor to the development of metabolic disease and recent work has strongly implicated the inflammasome as having a pivotal role in the regulation of metabolism, obesity, insulin resistance, and cardiovascular disease [15].

It is well known that cytokine is an important mediator in acute or chronic inflammation. Burska et al. have reported that cytokines are small proteins which play important roles in cell signaling and they are secreted by a variety of cellular sources acting either on the cell producing them (autocrine) or on the surrounding cells (paracrine) [16]. Nakagawa et al. have reported that IL-6 is a multifunctional cytokine that is produced by many different cell types, and plays an important role in the regulation of inflammation, immune responses, the acute-phase response, and hematopoiesis [17]. Niebler et al. have reported that IL-1$\beta$ is also included in central key players in the immune surveillance interactome, which not only mediates inflammation but also links innate and adaptive immunity [18]. Tracey et al. have reported that TNF-$\alpha$ is a pleiotropic cytokine known to play a major role in host defense mechanisms, initiating a beneficial local inflammation which in excess, however, may cause tissue damage concerned with the pathogenesis of numerous autoimmune and/or inflammatory systemic diseases [19].

Cao has reported that obesity induces production of inflammatory cytokines (often referred to together with adipokines as adipocytokines) and infiltration of immune cells into adipose tissue, which creates a state of chronic low-grade inflammation [20]. Ganjali et al. have reported that the most important source of proinflammatory cytokines in obesity is macrophages that infiltrate adipose tissue as a response to the adipocyte growth, decreased blood supply, hypoxia, and tissue necrosis [21]. Blüher and Mantzoros have reported that the adipose tissue influences the regulation of several important physiological functions including but not limited to appetite, satiety, energy expenditure, activity, insulin sensitivity and secretion, glucose and lipid metabolism, fat distribution, endothelial function, hemostasis, blood pressure, neuroendocrine regulation, and function of the immune system through adipokines [9]. On the other hand, Park et al. have reported that Euiiin-tang (yiyiren-tang; Eui-E-In-Tang) granules exert anti-inflammation and antiobesity effect on high fat diet-induced obese C57 BL/6J mice [22].

These days, the number of reports for adipokines with obesity is increasing. Leptin is well known to be a forerunner of the adipokine superfamily. Park and Ahima have reported that leptin is secreted by adipose tissue and regulates energy homeostasis, neuroendocrine function, metabolism, immune function, and other systems through its effects on the central nervous system and peripheral tissues [8].

In order to examine the inflammatory mediators of obesity and determine a possible distinctive inflammatory blood marker, we measured the serum levels of 21 cytokines using multiplex cytokine assay. Unlike infectious inflammation diseases increase the serum levels of proinflammatory cytokines such as IL-1$\beta$, IL-6, TNF-$\alpha$, and MCP-1, our data represent that the serum C-peptide, insulin, leptin, HGF, lipocalin, adipsin, and total PAI-1 levels are higher in the obese group than the normal group; the serum adiponectin level is lower in the obese group than the normal group. After adjusting for age, height, SGPT, alkaline phosphatase, GGTP, total cholesterol, HDL cholesterol, triglyceride, uric acid, glucose, CRP, and WBC, the serum C-peptide, insulin, leptin, lipocalin-2, and adipsin levels in the obese group were found to be significantly higher than in the normal group. C-peptide was significantly correlated with insulin, leptin, IL-8, HGF, adiponectin, adipsin, and total PAI-1. Insulin was significantly correlated with C-peptide, GIP, leptin, HGF, adipsin, and total PAI-1. Leptin was significantly correlated with C-peptide, insulin, HGF, lipocalin-2, adipsin, and total PAI-1. Lipocalin-2 was significantly correlated with GIP, leptin, IL-8, HGF, resistin, and adipsin. Adipsin was significantly correlated with C-peptide, insulin, leptin, HGF, lipocalin-2, and resistin. How correlations of cytokines are varied and involved in obesity deserves further investigation in cells, animal models, and human populations.

And the investigation of serum cytokine levels at the end point of this study demonstrated that mean serum leptin of Eui-E-In-Tang group was found to be significantly reduced by Eui-E-In-Tang therapy for 12 weeks. In contrast, Eui-E-In-Tang therapy did not show any significant effect on the concentrations of C-peptide, insulin, lipocaline-2, adipsin, HGF, total PAI-1, GIP, MCP-1, PP, IL-8, adiponectin, and resistin. The findings on the modulatory effect of Eui-E-In-Tang on serum level of leptin deserve to be studied further to make a safe and effective treatment for obesity.

We acknowledge limitations in our study. The most important limitation relates to the small sample size. Another

limitation of the present study was the lack of gender diversity in the study group. It is recommended that further studies be designed for a large sample size including male individuals.

## Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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*Review Article*

# The Effects of *Chunghyul-Dan* (A Korean Medicine Herbal Complex) on Cardiovascular and Cerebrovascular Diseases: A Narrative Review

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*Chunghyul-dan* (CHD) is a herbal complex containing 80% ethanol extract and is composed of *Scutellariae Radix*, *Coptidis Rhizoma*, *Phellodendri Cortex*, *Gardeniae Fructus*, and *Rhei Rhizoma*. We have published several experimental and clinical research articles on CHD. It has shown antilipidemic, antihypertensive, antiatherosclerotic, and inhibitory effects on ischemic stroke recurrence with clinical safety in the previous studies. The antilipidemic effect of CHD results from 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and pancreatic lipase-inhibitory activity. The antihypertensive effect likely results from the inhibitory effect on endogenous catecholamine(s) release and harmonization of all components showing the antihypertensive effects. Furthermore, anti-inflammatory and antioxidant effects on endothelial cells are implicated to dictate the antiatherosclerotic effects of CHD. It also showed neuroprotective effects on cerebrovascular and parkinsonian models. These effects of CHD could be helpful for the prevention of the recurrence of ischemic stroke. Therefore, we suggest that CHD could be a promising medication for treating and preventing cerebrovascular and cardiovascular diseases. However, to validate and better understand these findings, well-designed clinical studies are required.

## 1. Introduction: Background, History, and Development

*Chunghyul-dan* (CHD) is a capsulated herbal complex, which contains an 80% ethanol extract (300 mg per capsule) composed of *Scutellariae Radix*, *Coptidis Rhizoma*, *Phellodendri Cortex*, *Gardeniae Fructus*, and *Rhei Rhizoma* (Table 1). It is also known as Daehwang-Hwang-Ryung-Haedok-Tang (Daio-orengedokuto in Japanese), which means Hwang-Ryung-Haedok-Tang (HRHT, Orengedokuto in Japanese) plus Daehwang (*Rhei Rhizoma*). In other words, CHD consists of HRHT and *Rhei Rhizoma*.

Various herbal medicines have been used to treat cardiovascular and cerebrovascular diseases such as atherosclerosis, angina, and stroke in Korean medicine clinics. HRHT, which consists of *Scutellariae Radix*, *Coptidis Rhizoma*, *Phellodendri Cortex*, and *Gardeniae Fructus*, is one of the most famous herbal medicines for treating cardiovascular and cerebrovascular diseases. The first record of the medicinal properties of the HRHT was documented in the Chinese medicine classic, "Oe-Dae-Bi-Yo (published in 752)." Traditionally, it has been used to treat pathological inflammation in gastrointestinal and cardiovascular and cerebrovascular diseases.

The modern clinical and experimental studies have also shown various evidences for the effect of HRHT on cardiovascular and cerebrovascular diseases. For instance, HRHT enhanced cerebral blood flow, decreased blood pressure, and exerted anti-inflammatory and vasodilatory effects [1]. Clinically, HRHT showed effects on abdominal obesity [2] and on the accessory symptoms of hypertension [3]. It could be suggested that these clinical effects are based on anti-inflammatory [4], antilipidemic [5], and antiplatelet effects [6] of HRHT. Furthermore, each herb in the HRHT is known to have neuroprotective, antioxidant, and antihypertensive

Table 1: Composition of *Chunghyul-dan*.

| Constituent herbs | Scientific name (country of origin) | Weight (g) |
|---|---|---|
| *Scutellariae Radix* | *Scutellaria baicalensis* Georgi (Korea) | 0.28 |
| *Coptidis Rhizoma* | *Coptis japonica* Makino (Korea) | 0.28 |
| *Phellodendri Cortex* | *Phellodendron amurense* Ruprecht (Korea) | 0.28 |
| *Gardeniae Fructus* | *Gardenia jasminoides* Ellis (Korea) | 0.28 |
| *Rhei Rhizoma* | *Rheum palmatum* L. (China) | 0.07 |
| *Total* | | 1.2 |

effects [7–11]. Based on this, it was suggested that HRHT could have effects on cardiovascular disease and cerebrovascular disease.

To enhance the above-mentioned effects of HRHT, we combined HRHT and *Rhei Rhizoma*. The resulting combination medication was named as *Chunghyul-dan* (CHD), which means "purification of blood." A previous meta-analysis [12] suggested that *Rhei Rhizoma*-based herbal preparations have significant effects on the improvement of the clinical efficacy rates, the Barthel Index, National Institutes of Health Stroke Scale, Glasgow Coma Scale, and neurological deficit scores when compared with controls using only western medicine. *Rhei Rhizoma* has been used to remove blood stasis (oehyul in Korean and oketsu in Japanese). Clinically, *Rhei Rhizoma* has been used to treat severe gastrointestinal disorders including constipation or ileus, severe inflammation such as appendicitis or pneumonia, hypertension, and cerebrovascular diseases. Furthermore, *Rhei Rhizoma* has shown neuroprotective effects on experimental ischemic stroke [13] and antilipidemic effects [14].

The process of CHD development is as follows. Herbs were extracted with 80% ethanol in boiling water for 2 hours. The extracts were filtered, evaporated with a rotary vacuum evaporator, and freeze-dried. To standardize the quality of CHD, berberine in *Coptidis Rhizoma* and *Phellodendri Cortex*, baicalin in *Scutellariae Radix*, geniposide in *Gardeniae Fructus*, and sennoside A in *Rhei Rhizoma* were quantitatively assayed according to the standardized methods [15]. To enhance dry weight yields (%) of extract, CHD was extracted with 80% ethanol instead of water. In previous studies, extracted yield of each herb with 80% ethanol was higher than extracted yields with water [16, 17]. Furthermore, we used capsulated form to improve drug compliance.

Since 2002, we have reported extensive experimental and clinical research articles related to CHD. It has shown antilipidemic, antihypertensive, antiatherosclerotic, and enhancing effects on vascular endothelial cells. Furthermore, CHD shows inhibitory effect on recurrence of ischemic stroke (small vessel occlusion type). From here onwards, this review introduces and provides the effects of CHD on cardiovascular and cerebrovascular diseases.

## 2. CHD in Dyslipidemia

Dyslipidemia is defined as an abnormal amount of cholesterol and fat in the blood. It is known as a moderate risk factor for cardiovascular and cerebrovascular diseases such as hypertension [18], coronary artery disease [19], and ischemic stroke [20]. The conventional therapies for dyslipidemia are statins (3-hydroxy-3-methylglutaryl-coenzyme A [HMG-CoA] reductase inhibitors), fibric acid derivatives, and bile acid sequestrants. Among these, statins are the most preferred therapeutic option. Although statins are an effective medication for dyslipidemia, they have severe adverse effects such as myopathy, rhabdomyolysis, and hepatic dysfunction [21, 22]. Therefore, new types of effective and safer antilipidemic agents need to be explored.

Chung et al. [23] investigated the effects of CHD on serum lipids in patients with hyperlipidemia (see Table 2). CHD (1800 mg/day for 8 weeks) was administered to 34 patients (10 males and 24 females) with serum levels of total cholesterol, LDL cholesterol, and triglycerides higher than 200 mg/dL, 130 mg/dL, and 200 mg/dL, respectively. Follow-up lipid profile check was performed after 4 weeks (34 patients) and 8 weeks (15 patients). Four weeks later, total cholesterol and LDL cholesterol levels showed significant decrease (−8.3% ($p < 0.05$) and −7.4% ($p < 0.05$), resp.). Eight weeks later, total cholesterol and triglyceride levels showed significant decrease (−7.7% ($p < 0.05$) and −21.1% ($p < 0.05$), resp.). No serious adverse effect was observed during the follow-up. Kim et al. [24] investigated the antilipidemic effect of CHD and compared it with atorvastatin. Study design was a case-control, open-label study. The subjects were divided into 2 groups, CHD group (further subdivided into two groups based on dosage; i.e., CHD 1 group received 600 mg/day CHD and CHD 2 group received 1200 mg/day CHD) and atorvastatin group (receiving 10 mg/day atorvastatin), to investigate and identify the dose-dependent effect of CHD on hyperlipidemia. Although atorvastatin was more powerful than 600 mg or 1200 mg CHD in lowering lipid levels, both CHD 1 and CHD 2 groups showed a statistically significant lipid-lowering effect (total cholesterol ($p < 0.05$), from 268.1 ± 30.2 mg/dL to 248.6 ± 29.2 mg/dL). There was no adverse effect such as hepatic or renal toxicity during CHD treatment. However, there was no significant difference between CHD 1 and CHD 2 groups in lowering lipids. Cho et al. [25] conducted a case-control, open-label study for evaluating the therapeutic effects of CHD on hypercholesterolemia. The subjects of this study were hyperlipidemia patients whose total serum cholesterol was more than 240 mg/dL. Subjects were divided into two groups, namely, CHD group and atorvastatin group that were treated (8 weeks) with CHD (600 mg/day, $n = 21$) and atorvastatin (10 mg/day, $n = 12$), respectively. After 8 weeks, CHD showed significant

Table 2: The efficacy of *Chunghyul-dan* in cardiovascular and cerebrovascular diseases in clinical studies [23–25, 32, 44, 56–58].

| Disease | Author (year) | Subjects and Design | Intervention | Results |
|---|---|---|---|---|
| Dyslipidemia | Chung et al. [23] | 34 hyperlipidemia patients Before and after study | 1800 mg/day CHD for 8 weeks | After 4 weeks, total cholesterol: −8.3% ($p < 0.05$), LDL cholesterol: −7.4% ($p < 0.05$After 8 weeks, total cholesterol: −7.7% ($p < 0.05$), triglyceride: −21.1% ($p < 0.05$) |
| | Kim et al. [24] | 62 hyperlipidemia patients Case-control, open-label study | CHD1: 600 mg/day CHD2: 1200 mg/day Atorvastatin: 10 mg/day atorvastatin for 8 weeks | After 8 weeks, in CHD 1 and 2, total cholesterol: 268.1 ± 30.2 mg/dL → 248.6 ± 29.2 mg/dL ($p < 0.05$) There was no significant difference between CHD 1 and 2 group Atorvastatin was superior to 600 mg or 1200 mg CHD |
| | Cho et al. [56] | 33 hyperlipidemia patients Case-control, open-label study | CHD: 600 mg/day CHD Atorvastatin: 10 mg/day atorvastatin for 8 weeks | After 8 weeks, in CHD group, total cholesterol: 269.5 ± 21.3 mg/dL → 246.9 ± 23.7 mg/dL ($p < 0.01$), LDL cholesterol: 171.2 ± 29.8 mg/dL → 155.4 ± 26.5 mg/dL ($p < 0.05$) CHD was superior to historical controls used diet therapy or placebo Atorvastatin was superior to 600 mg CHD |
| Hypertension | Yun et al. [32] | 28 stroke patients with stage 1 hypertension Randomized controlled, open-label study | CHD: 1200 mg/day CHD Control: No treatment for 2 weeks | After 2 weeks, in CHD group SBP: 141.37 ± 8.96 mmHg → 132.28 ± 9.46 mmHg ($p = 0.03$, vs control, $p = 0.036$) In control group, SBP: 138.71 ± 11.36 mmHg → 132.27 ± 8.93 mmHg ($p > 0.05$) |
| Atherosclerosis (Arterial stiffness) | Park et al. [44] | 35 subjects with increased baPWV (>1400 cm/sec) Randomized controlled, open-label study | CHD: 1800 mg/day CHD Control: No treatment for 8 weeks | After 8 weeks, in CHD group baPWV: 1736.0 ± 271.1 cm/sec → 1599.0 ± 301.9 cm/sec ($p = 0.032$) In control group, baPWV: 1668.3 ± 116.2 cm/sec → 1653.3 ± 184.1 cm/sec ($p = 0.774$) |
| Stroke prevention (SVO type) | Cho et al. [25] | 31 asymptomatic ischemic stroke patients Observational study | 600 mg/day CHD for 1 year | Complete follow-up patients ($n = 21$): no stroke recurrence Lost follow-up/dropped-out patients ($n = 10$): 2 patients suffered recurrence |
| | Cho et al. [57] | 158 ischemic stroke patients Observational study | 600 mg/day CHD for 1 year | Complete follow-up patients ($n = 73$): 3 patients (4.1%) experienced stroke recurrence Lost follow-up/dropped-out patients ($n = 85$): Among 85, 54 patients included in the final analysis → 8 patients (9.4%) had stroke recurrence. OR of CHD for stroke recurrence (vs lost to follow up): 0.12 times |
| | Cho et al. [58] | 356 ischemic stroke patients Case-control, open-label study | CHD: 600 mg/day CHD Antiplatelet: Antiplatelet agent therapy for 2 years | In CHD group, recurrence occurred in 3 subjects (2.0%). In Antiplatelet group, recurrence occurred in 17 subjects (8.2%) OR of CHD for stroke recurrence(vs Antiplatelet group): 0.208 times |

CHD: *Chunghyul-dan*; LDL: low-density lipoprotein; SBP: systolic blood pressure; baPWV: brachial-ankle pulse wave velocity; SVO: small vessel occlusion.

lipid-lowering effect (total cholesterol ($p < 0.01$), from 269.5 ± 21.3 mg/dL to 246.9 ± 23.7 mg/dL; LDL cholesterol ($p < 0.05$), from 171.2 ± 29.8 mg/dL to 155.4 ± 26.5 mg/dL). Although the antilipidemic effect of CHD was less than atorvastatin, it was higher than historical controls from previous studies using diet therapy or placebo. During CHD treatment, there was no adverse effect on hepatic or renal toxicity.

CHD has also shown antilipidemic effect in previous experimental studies. It inhibits HMG-CoA reductase and pancreatic lipase. Kim et al. [1] assessed the HMG-CoA reductase and pancreatic lipase-inhibitory effects of CHD in hyperlipidemic model rats treated with Triton WR-1339. They showed that CHD decreased total serum cholesterol and LDL cholesterol levels in the hyperlipidemic model rats. It potently inhibited HMG-CoA reductase and pancreatic lipase, simultaneously. Therefore, they suggested that the antilipidemic effect of CHD could originate from the inhibition of pancreatic lipase and HMG-CoA reductase. Another experimental study [17] also showed that *Rhei Rhizoma,* which is a component of CHD, exerted inhibitory effects on pancreatic lipase. Flavonoid extracts from *Scutellariae Radix* such as wogonin showed antilipidemic and body weight reducing effect in mice [26, 27]. Furthermore, alkaloids from *Coptidis Rhizoma* [28, 29] and crocin isolated from *Gardeniae Fructus* [30] also revealed antihyperglycemia and antihyperlipidemia effect in experimental studies.

Therefore, we suggest that CHD could be a safe-effective medication for controlling dyslipidemia. Although the effect of CHD on dyslipidemia is lower than that of statins, it did not show any adverse effects such as myopathy or hepatic dysfunction. The mechanism of the effect of CHD on dyslipidemia can be implicated to results from its pancreatic lipase-inhibitory effects. CHD can be an alternative medication for controlling dyslipidemia in the patients with adverse effects of statins. Further studies, such as examining whether statin with CHD therapy is superior to statin only therapy, are needed to ascertain the effect of CHD on dyslipidemia and clinical use.

## 3. CHD in Hypertension

Hypertension is one of the risk factors for atherosclerotic diseases such as stroke and coronary artery disease. In a previous clinical study, HRHT (a component of CHD) exhibited therapeutic effects on abdominal obesity [2] and the accessory symptoms of hypertension [3].

We conducted a preliminary study to determine an optimal dose for antihypertensive effect of CHD [31]. In this study, 1200 mg/day CHD (twice a day, p.o., 600 mg/each time) showed short-term antihypertensive effect on stroke patients with stage 1 hypertension (systolic blood pressure 140–159 mmHg and diastolic blood pressure 90–99 mmHg).

Based on the preliminary study, Yun et al. [32] evaluated the antihypertensive efficacy of CHD on stroke in patients with stage 1 hypertension using 24-hour ambulatory blood pressure monitoring (24ABPM). Forty stroke patients with stage 1 hypertension were enrolled for the study. They were randomly assigned into two groups: CHD group and control group. Subjects in CHD group ($n = 15$) were treated with CHD (1200 mg/day) for 2 weeks, whereas control group ($n = 13$) did not receive CHD. Systolic blood pressure (SBP) of CHD group decreased from 141.37 ± 8.96 mmHg to 132.28 ± 9.46 mmHg after 2-week CHD administration ($p = 0.03$). However, SBP of control group did not show statistically significant decrease (from 138.71 ± 11.36 mmHg to 132.27 ± 8.93 mmHg). After 2 weeks of treatment, there was a significant difference in SBP between the CHD and the control groups ($p = 0.036$, Mann-Whitney $U$ test). However, diastolic blood pressure and pulse rate in both groups had no significant change after treatment.

The antihypertensive effect of CHD can be explained by the findings of the following studies. HRHT and SamHwang-SaShim-Tang (SHSST, Sanoushasin-to in Japanese) exert an inhibitory effect on releasing endogenous catecholamines in experimental studies [33, 34]. Another study suggested that SHSST attenuated the increase in systemic and pulmonary arterial blood pressure induced by U46619 in rats. It downregulated the expression of phosphodiesterase type 5 (PDE5), Rho-kinase (ROCK) II, and cyclooxygenase-2 (COX-2) and upregulated the expression of soluble guanylyl cyclase (sGC) alpha(1) and sGCbeta(1) in U46619 treated primary pulmonary smooth muscle cells [35]. Berberine, a main compound of *Coptidis Rhizoma*, has shown inhibitory effects on endoplasmic reticulum stress in the carotid arteries of spontaneously hypertensive rats [36]. Furthermore, *Scutellariae Radix* showed inhibitory effect on adenylate cyclase activity in experimental studies [33, 34]. In addition, crocetin (a carotenoid from *Gardeniae Fructus*) also showed protective effect against hypertension and cerebral thrombogenesis in stroke-prone spontaneously hypertensive rats [9]. Furthermore, *Rhei Rhizoma* has a depressive effect on noradrenergic and dopaminergic nerve activities [33, 34] that also may be a mechanism of antihypertensive effects of CHD.

Based on these findings, we suggest that CHD can be used as an antihypertensive agent for stage I hypertension. However, further evaluation with a larger sample size and long-term follow-up is warranted.

## 4. CHD in Endothelial Dysfunction and Atherosclerosis

The effect of CHD on atherosclerosis, especially in endothelial cell dysfunction, has been reported. Endothelial cell dysfunctions are responsible for cardiovascular diseases such as the focal localization of atherosclerotic plaque [37]. Deregulation of endothelial cell function is closely associated with the incidence of atherosclerosis. Therefore, we investigated the effect of CHD on vascular endothelial cell dysfunction. CHD exhibited antiapoptotic effects and acted as a cell-cycle-progression and cell-migration-promoting agent in a previous study [38]. Molecular studies showed that CHD activates nitric oxide synthase (NOS) mRNA, which plays an important role in the protection against atherosclerosis. It suppresses vascular cell adhesion molecule-1 (VCAM-1) mRNA, which is expressed in human endothelial cells on sites predisposed to atherosclerotic lesions [39]. Another study [40] implicated CHD in controlling a variety of inflammation

related activities by regulating MCP-1 and VCAM-1 gene expression in endothelial cell. Based on this, we suggest that antiatherosclerotic effect of CHD stems from antiapoptotic, anti-inflammatory, and antioxidant effects in human vascular endothelial cells.

The effects of components of CHD on atherosclerosis have also been reported. Wogonin, an active component of *Scutellariae Radix*, revealed inhibitory effect on monocyte chemotactic protein-1 gene expression in human endothelial cells [41]. Berberine, a natural extract from *Coptidis Rhizoma*, also revealed antiatherosclerotic effect via suppression of adhesion molecule expression including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) [42] and activation of AMP-activated protein kinase (AMPK) [43].

To ascertain the clinical antiatherosclerotic effects of CHD, we investigated the effect of CHD on increased arterial stiffness using brachial-ankle pulse wave velocity (baPWV) [44]. Arterial stiffness is a contributor to the progression of atherosclerosis [45], as the increased cycle stress on the arterial walls can affect the progression. Pulse wave velocity (PWV) is a surrogate marker for atherosclerosis and is a valuable index of arterial stiffness [46]. Subjects (35) with increased baPWV (>1400 cm/sec) were enrolled for this study. All subjects were randomized and divided into 2 groups; the CHD group ($n = 20$) received 1800 mg CHD for 8 weeks and the control group ($n = 15$) was without CHD medication. After 8 weeks, baPWV was significantly decreased in the CHD group (from 1736.0 ± 271.1 cm/sec to 1599.0 ± 301.9 cm/sec, $p = 0.032$), while there was no significant change in the control (from 1668.3 ± 116.2 cm/sec to 1653.3 ± 184.1 cm/sec, $p = 0.774$). There was no clinical adverse effect.

Arterial stiffness is closely associated with atherosclerosis [46] and there is a correlation of PWV and intima-media thickness (IMT) [47]. Based on the above findings, it is suggested that CHD may prevent the progression of atherosclerosis. The mechanism of CHD mediated antiatherosclerotic effects could be the antiapoptotic and NOS activation effects on endothelial cells.

## 5. The Neuroprotective Effect of CHD in Brain Ischemia and Cerebrovascular and Neurodegenerative Diseases

Traditionally, HRHT and *Rhei Rhizoma* have been the most famous herbal preparations for stroke and ischemic brain pathology in East Asia. As mentioned above, HRHT enhances cerebral blood flow, decreases blood pressure, and exerts anti-inflammatory and vasodilatory effects [1]. Furthermore, *Rhei Rhizoma*-based herbal medicines have significant effects on the improvement of stroke as compared with controls using only western medicine in a meta-analysis [12]. Therefore, we hypothesized that CHD could also exert neuroprotective effect on brain ischemia and neurodegenerative disease.

Previous experimental studies suggested that components of CHD such as *Rhei Rhizoma* [48] and *Scutellariae Radix* [49] revealed neuroprotective and prophylactic effects on the brain ischemia of rats. Berberine, the major pharmacological active constituent of *Coptidis Rhizoma*, is also suggested to regulate neuronal apoptosis in cerebral ischemia [8]. Geniposide, a pharmacologically active component purified from *Gardeniae Fructus*, is also suggested as a suppressor of neuroinflammation through inhibiting receptor for advanced glycation end products- (RAGE-) dependent signaling pathway in Alzheimer model [50, 51].

In an experimental study [52], CHD decreased the lipopolysaccharide- (LPS-) induced expression of mRNAs encoding inducible NO synthase, tumor necrosis factor- (TNF-) alpha, interleukin-1beta, cyclooxygenase-2, and prostaglandin E2 in rat brain microglia. Furthermore, CHD significantly decreased LPS-induced phosphorylation of the ERK1/2 and p38 signaling proteins. These results suggest that CHD exerts neuroprotective effect by reducing the release of various proinflammatory molecules from activated microglia.

An experimental study using rat model of focal ischemia-reperfusion investigated the effect of CHD on ischemic brain damage [53]. CHD was administered just before reperfusion and then 2 hours after reperfusion to evaluate its neuroprotective effect. After CHD treatment, cerebral infarct volume indicated significant reduction (100, 200, and 400 mg/kg; $p < 0.05$). It also lowered microglial activation and neutrophil infiltration ($p < 0.05$). Brain-derived neurotrophic factor- (BDNF-) positive cells were significantly increased after CHD treatment ($p < 0.05$). It is thus likely that the neuroprotective mechanisms of CHD result from inhibition of microglial activation, reduction of neutrophil infiltration, and enhancement of BDNF expression. Subsequently, another study [54] showed that CHD treatment markedly decreased the cytotoxicity in 42-hour hypoxia condition and H/R condition ($p < 0.01$ and $p < 0.05$, resp.). It also significantly decreased Bax expression ($p < 0.01$) and slightly decreased Bcl-2 expression. Based on these findings, it is likely that CHD shows neuroprotective effect in N2a cells subjected to H/R by increasing the expression of the proapoptotic protein Bax.

As CHD inhibited microglial activation, the neuroprotective effects of CHD on Parkinson's disease (PD) models were also investigated [55]. In an *in vivo* study, CHD (50 mg/kg, 5 days) reduced dopaminergic neuronal damage in the substantia nigra pars compacta (SNpc) and striatum and ameliorated bradykinesia. In an *in vitro* study, CHD exhibited significant protective effects in PC12 cells by inhibiting intracellular reactive oxygen species (ROS) generation and by regulatory effects on the heme oxygenase-1 and gp91 phagocytic oxidase. Furthermore, CHD protected dopaminergic neurons in a primary mesencephalic culture against 1-methyl-4-phenylpyridinium (MPP+) neurotoxicity. These results indicated that CHD could protect neuronal cell death in PD model by inhibition of ROS generation and associated mitochondrial dysfunction.

Based on these findings, it is likely that the anti-inflammatory and antioxidant properties of CHD on brain provide the neuroprotective effect. Therefore, CHD may be used as an alternative agent for brain ischemia as well as neurodegenerative diseases in the near future. However,

further clinical studies using perfusion CT to evaluate the effect of CHD in clinical set-up are required.

## 6. CHD in Prevention of Small Vessel Occlusion (SVO) Type Ischemic Stroke

As mentioned earlier, CHD has antilipidemic [17, 23–25], antihypertensive [31, 32], anti-inflammatory [52, 53], and antioxidative [54] effects and can improve endothelial cells [38–41]. It was predicted that CHD could improve and prevent the progress of microangiopathy, which is strongly associated with small vessel disease type ischemic stroke. Clinical studies were conducted to evaluate the effectiveness of CHD on the prevention of small vessel occlusion type ischemic stroke.

First, the inhibitory effect of CHD on stroke occurrence in subjects with asymptomatic SVO type ischemic stroke was investigated through observational study. For this study [56], patients who had spotty lesions (3 mm in diameter or larger) in area supplied by deep perforating artery, showing high intensity in T2 weighted (TR = 3000, TE = 80) and FLAIR images and low intensity in the T1 weighted (TR = 450, TE = 10) image were included. According to the inclusion criteria, 31 patients were recruited. 600 mg/day CHD was administered to all subjects for 1 year. Stroke occurrence and adverse effects were monitored for 1 year. Follow-up brain MRI was performed to detect new ischemic lesions 1 year after the treatment. Ten subjects dropped out and only 21 subjects completed the follow-up. Among the 21 subjects who completed follow-up, no subject experienced clinical symptoms characterized by typical stroke and no new lesions were detected in the follow-up MRI. However, among the 10 drop-out patients, 2 patients experienced ischemic stroke. The CHD administration period for drop-out patients was 2.3 ± 1.8 months.

To further understand these results, the inhibitory effect of CHD on stroke recurrence in subjects with SVO type ischemic stroke (including asymptomatic and symptomatic stroke) was investigated [57]. Seventy-three patients with SVO type ischemic stroke were treated with 600 mg/day CHD for 1 year. Among them, three patients (4.1%) experienced new ischemic stroke (symptomatic stroke = 2; asymptomatic stroke = 1).

An expansion study with a 2-year follow-up was conducted in multicenters [58]. There were 2 groups in this study: the CHD group ($n$ = 148) with 600 mg/day CHD for 2 years and the control group ($n$ = 208) with antiplatelet agents. New brain lesions occurred in only 3 subjects (2.0%) of the CHD group, whereas 17 subjects (8.2%) experienced stroke recurrence in the control. The OR of the CHD group for stroke recurrence was 0.232 times that of the control. Furthermore, the OR of the CHD group decreased to 0.208 when adjusted for other relevant risk factors (age, sex, antiplatelet gage medication, smoking, previous stroke, hypertension, diabetes mellitus, and hyperlipidemia). Although it was not a randomized controlled study, we suggest that the inhibitory rate of CHD on stroke recurrence is much higher than that of antiplatelet agents.

A conclusion about the effect of CHD on prevention of SVO type stroke recurrence cannot be reached due to lack of randomized controlled studies. However, from the limited data available, recurrence rate of SVO stroke in the CHD therapy group (2.0%) was lower than conventional antiplatelet therapy (6.1~12.8%) using aspirin, clopidogrel, cilostazol, triflusal, or dipyridamole [59–65]. The most common adverse effect of antiplatelet agent therapy is bleeding, which can be fatal. For instance, the overall incidence rate of hemorrhagic events was 5.58 per 1000 person-years for aspirin users and 3.60 per 1000 person-years for those without aspirin use, and the incidence rate ratio (IRR) was 1.55 (95% CI, 1.48–1.63) [66]. However, there were no serious adverse events such as bleeding during CHD treatment. Based on this, we suggest that CHD may be a safe and effective agent for prevention of SVO type ischemic stroke. We are now conducting long-term follow-up study for the effect of CHD on SVO type stroke patients to improve our understanding.

## 7. Safety and Adverse Effect

To examine the safety of CHD, a retrospective cohort review study was performed [67]. Among 656 subjects with CHD treatment, there were clinical adverse effects in 13 subjects (2.0%), 8 with gastrointestinal symptoms such as indigestion, headache, insomnia, chest discomforts, general fatigue, and thirst appearing in 1 subject, respectively. This apparent frequency of adverse effects was much lower than the safety of previous medications such as statins [68, 69]. There were no serious adverse effects such as hepatic or renal dysfunction. Furthermore, other studies on effects of CHD on dyslipidemia [23–25], hypertension [32], and arterial stiffness [44] did not show any other adverse effects. Therefore, it may be suggested that CHD is a safe medication.

## 8. Summary and Future Considerations

CHD has antilipidemic, antihypertensive, antiatherosclerotic, antioxidant, and neuroprotective effects, which could exert an inhibitory effect on microangiopathy resulting in prevention of ischemic stroke. Furthermore, several clinical trials have proven its status as a safe alternative medication. To confirm the effect of CHD, well-designed and large sized clinical studies are required to assess the potential of CHD and validate these findings.

## Competing Interests

The authors declare that they have no competing interests.

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*Research Article*

# Hataedock Treatment Has Preventive Therapeutic Effects in Atopic Dermatitis-Induced NC/Nga Mice under High-Fat Diet Conditions

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This study investigated the preventive therapeutic effects of Hataedock (HTD) treatment on inflammatory regulation and skin protection in AD-induced NC/Nga mice under high-fat diet conditions. Before inducing AD, the extract of *Coptidis Rhizoma* and *Glycyrrhiza uralensis* was administered orally to the 3-week-old mice. After that, AD-like skin lesions were induced by applying DNFB. All groups except the control group were fed a high-fat diet freely. We identified the effects of HTD on morphological changes, cytokine release and the induction of apoptosis through histochemistry, immunohistochemistry, and TUNEL assay. HTD downregulated the levels of IL-4 and PKC but increased the levels of LXR. HTD also suppressed the mast cell degranulation and release of MMP-9, Substance P. The levels of TNF-$\alpha$, p-I$\kappa$B, iNOS, and COX-2 were also decreased. The upregulation of inflammatory cell's apoptosis is confirmed by our results as increase of apoptotic body and cleaved caspase-3 and decrease of Bcl-2. HTD also reduced edema, angiogenesis, and skin lesion inflammation. Our results indicate HTD suppresses various inflammatory response on AD-induced mice with obesity through the regulation of Th2 differentiation and the protection of lipid barrier. Therefore, HTD could be used as an alternative and preventive therapeutic approach in the management of AD.

## 1. Introduction

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease with a typical distribution of pruritic skin lesions. As with any other inflammatory skin disease, it greatly affects the quality of life of patients [1, 2]. Many studies have supported the definition of AD as a complex trait, in that interactions between genes and environmental factors and the interplay between multiple genes contribute to disease manifestation [3].

Traditionally, it was thought that the primary pathogenic mechanism of AD was initiated largely due to immune dysfunction, with the key roles played by Th1/Th2 cell dysregulation, IgE production, dendritic cell signaling, and mast cell hyperactivity, leading to pruritic, inflammatory dermatosis, and the secondary disruption of the epidermal barrier [4–6].

Recently, the importance of environmental factors during infancy and early childhood in the expression of AD has become well-recognized. It was suggested that the immune and respiratory systems are relatively immature during infancy and that they continue to develop during early childhood [7]. In addition, it was suggested that allergic diseases have their origins in early life and that the priming

of the immune system starts in utero [8]. Previous studies have found that obesity is associated with defective skin barrier function [9, 10]. In addition, obesity results in a chronic low-grade inflammatory condition that may directly contribute to inflammatory pathways in AD [11]. Although the precise link between obesity and AD has not been well established, adiposity is believed to induce systemic inflammation, which may negatively influence the immature immune system and atopic disorders [12]. Based on these studies, we can conclude that early life is a crucial period of development for the immune system and that excess fat may influence the pathogenesis or expression of allergic diseases among susceptible infants and children.

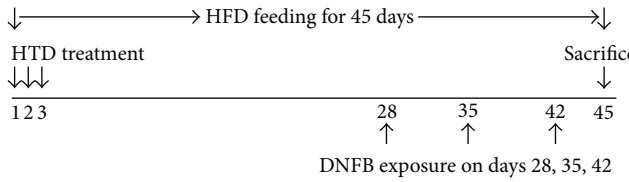
Within traditional Korean Medicine, AD is thought to be caused by children being influenced by "heat" during fetal development, which is referred to as "fetal heat" [13]. This "fetal heat" is influenced by maternal diet or mentality during pregnancy. Therefore, traditional Korean Medicine treats AD with "heat-clearing" medicines to reduce accumulated heat in the skin [14]. Hataedock (HTD) treatment is a "heat-clearing" treatment that dispels "fetal heat" and the meconium collected by the fetus via orally administering herbal extracts to a newborn baby. In our previous study, HTD treatment alleviated inflammatory skin damages in NC/Nga mice through regulating of inflammation and downregulation of protein kinase C (PKC) and Th2 cytokines, which are involved in the initial steps of AD development [15, 16].

An extract of *Coptidis Rhizoma* and *Glycyrrhiza uralensis* is traditionally used in HTD treatment. Recent studies have indicated that *Coptidis Rhizoma*, a kind of classical heat-clearing and detoxifying herb according to traditional Korean pharmacology, has antihyperglycemia, antihyperlipidemia, antihypertension, anti-inflammatory, and antioxidant effects [17]. It is also known that *Coptidis Rhizoma* and its main active compound, berberine, improve glucose and lipid metabolism disorders and have antiobesity activities [18, 19]. *Glycyrrhiza uralensis* is used to treat several inflammatory disorders, enhance the activity of other ingredients, reduce toxicity, and improve flavor [20]. The antioxidant and anti-inflammatory activities of flavonoids separated from *Glycyrrhiza uralensis* have been reported in recent years [21, 22]. The combination of these effects may lead to enhanced lipid metabolism and reduced skin inflammation in AD.

Based on this background, we evaluated the preventive therapeutic effects of HTD treatment on inflammatory regulation and skin protection in AD-induced NC/Nga mice fed a high-fat diet.

## 2. Materials and Methods

*2.1. Preparation of HTD Herb Extract.* The procedure used to manufacture the herb extract for HTD treatment was as follows: 100 g of *Coptidis Rhizoma* and 100 g of *Glycyrrhiza uralensis* were decocted in 1,000 mL of distilled water for 3 hours and then filtered; after concentrating this mixture to 50 mL under reduced pressure using a rotary evaporator, the filtrate was freeze-dried. We obtained 31 g of the extract (yield: 15.5%) for use.




| Group | HFD feeding | HTD treatment | Dermatitis elicitation |
|---|---|---|---|
| Ctrl | – | – | PBS |
| HF | + | – | PBS |
| HDE | + | – | DNFB |
| HTT | + | + | DNFB |

Figure 1: Experimental design. Before inducing AD, the extract of *Coptidis Rhizoma* and *Glycyrrhiza uralensis*, which is traditionally used in HTD treatment, was administered orally to the HTT group on days 1, 2, and 3. Mice were challenged by DNFB on days 28, 35, and 42. During the experimental period, all groups except the Ctrl group were fed a high-fat diet freely. Ctrl: normal feeding, HF: high-fat diet, HDE: high-fat diet and untreated AD-induced, HTT: high fat diet and Hataedock treated AD-induced, HFD: high-fat diet, HTD: Hataedock, PBS: phosphate-buffered saline, and DNFB: dinitrofluorobenzene.

*2.2. Animal and AD Induction.* Male 3-week-old NC/Nga mice (13–15 g each) were obtained from Central Lab Animal Inc. (Seoul, Korea). The mice were divided into four groups ($n = 10$ per group) as follows: the normal feeding group (Ctrl group), high-fat diet group (HF group), high-fat diet and AD-induced with no treatment group (HDE group), and high-fat diet and AD-induced with HTD treatment group (HTT group). In the HTT group, 3-week-old mice were given HTD treatment; they were given the extract of *Coptidis Rhizoma* and *Glycyrrhiza uralensis* orally (20 mg/kg) on days 1, 2, and 3. To induce AD-like skin lesions, the back regions of the mice were stripped, and 1 mL of 5% sodium dodecyl sulfate (SDS) (Sigma-Aldrich, St. Louis, MO, USA) was rubbed on the back of each mouse 20 times using a cotton swab to remove the lipid lamella of the stratum corneum. On day 28, the mice were sensitized via exposure to 100 $\mu$L of 1% dinitrofluorobenzene (DNFB) in acetone/olive oil (4 : 1). On days 35 and 42, the mice were challenged with 2% DNFB 100 $\mu$L. On day 45, the mice were deeply anesthetized with sodium pentobarbital and killed. Mice in the HF, HDE, and HTT groups except the control group were fed a high-fat diet (fat, 60%; carbohydrate, 20%; protein, 20%; DIO DIET, USA) freely for the experimental period.

All animal experiments were approved by the Institutional Animal Care and Use Committee of Pusan National University (IACUC number: PNU-2014-0732). We followed the NIH Guide for the Care and Use of Laboratory Animals throughout this study. The experimental design is summarized in Figure 1.

*2.3. Fingerprinting Analysis.* High-performance liquid chromatography- (HPLC-) based fingerprinting was performed with an Agilent 1200 Series HPLC System (Agilent Technologies, Santa Clara, CA, USA), binary solvent delivery pump (G1312A), autosampler (G1329A), column oven

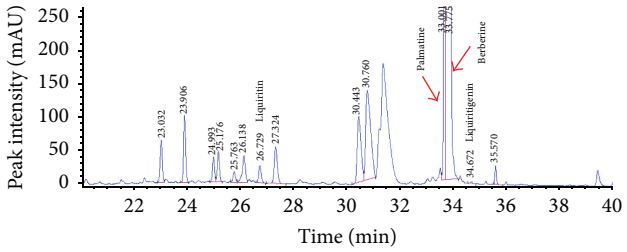



FIGURE 2: The HPLC analysis of the extract of *Coptidis Rhizoma* and *Glycyrrhiza uralensis*. Palmatine was detected at approximately 33.001 minutes, Berberine was detected at approximately 33.775 minutes, Liquritin was detected at approximately 26.729 minutes, and Liquiritigenin was detected at approximately 34.672 minutes. HPLC: High-performance liquid chromatography.

(G1316A), diode array detector (DAD; G1315D), vacuum degasser (G1322A), and Capcell PAKMGII C18 column (3.0 × 150 mm, 3.0 $\mu$m; Shiseido, Tokyo, Japan). The flow rate of the column was set at 0.6 mL/min, the temperature was maintained at 35°C, and the injection volume was set at 15 $\mu$L. The mobile phase consisted of 0.5% formic acid in water (v/v; A) and acetonitrile (B) with the following linear gradient profile: initiation 5 min, 2% B, 12 min, 10% B, 20 min, 25% B, 27 min, 25% B, 25 min, 80% B, 37 min, 80% B, 40 min, 30% B, and 45 min, 2% B. A standard solution containing Palmatine, Berberine (ChemFaces, Wuhan, China), Liquritin, and Liquiritigenin (Sigma-Aldrich, USA) was prepared by dissolving these compounds in distilled water (10 mg/100 mL). The solution was filtered through a 0.45 $\mu$m syringe filter, after which HPLC was performed.

To identify the constituents of the herb extract used for HTD treatment (the extract of *Coptidis Rhizoma* and *Glycyrrhiza uralensis*) in the study, we conducted HPLC fingerprinting. The standard constituents of our component analysis of the extract were Palmatine, Berberine, Liquritin, and Liquiritigenin. The HPLC analysis is shown in Figure 2.

*2.4. Tissue Process and Histochemistry.* After the mice were sacrificed, dorsal skins were obtained and fixed in 10% NBF at room temperature for 24 h and embedded in paraffin for serial sectioning (5 $\mu$m).

To investigate histological changes such as epithelial hyperplasia, capillary distribution, and collagen fiber distribution, we performed Masson's trichrome staining, which is used to detect collagen fibers and collagen deposition. The samples were fixed using Bouin's fluid (50–60°C) for 1 hr. The picric acid was then removed with 70% ethanol. The samples were incubated in Weigert's iron hematoxylin working solution for 10 min to stain the nuclei, and then, the collagen fibers were stained blue with Biebrich scarlet-acid fuchsin solution and phosphomolybdic-phosphotungstic acid for 15 min each and aniline blue solution for 5 min.

To investigate the distribution and morphological changes of the mast cells that were activated by neuropeptide, we performed histochemical staining with Luna's stain. We stained the mast cell granules using an aldehyde fuchsin solution for 30 minutes and Weigert's iron hematoxylin working solution for 10 minutes and then counterstained in methyl orange solution for 5 minutes.

To investigate changes in the lipid lamella in the stratum corneum, we used the oil red O staining method. To create frozen sections for lipid staining, the samples were fixed with 10% NBF and 10% formol-calcium. We immersed the sample in a cryoprotection solution of 30% sucrose and made the frozen sections 10 mm in width by freezing microtome (Microm, Germany). After that, we placed the slices in oil red O for 10 minutes to drain off the water, counterstained with Harris hematoxylin for 2 minutes, rinsed in distilled water, then mounted the sample with glycerin jelly, and observed the slices.

*2.5. Immunohistochemistry.* The skin slices were steeped in proteinase K solution (20 $\mu$g/mL) to undergo proteolysis for 5 minutes. The proteolysed slices were incubated in blocking serum (10% normal goat serum) for 4 hours. Then, the slices were incubated with goat anti-LXR (1 : 200, Santa Cruz Biotec, USA), goat anti-PKC (1 : 100, Santa Cruz Biotec, USA), goat anti-IL-4 (1 : 100, Santa Cruz Biotec, USA), goat anti-Substance P (1 : 100, Santa Cruz Biotec, USA), goat anti-MMP-9 (1 : 200, Santa Cruz Biotec, USA), goat anti-TNF-$\alpha$ (1 : 100, Santa Cruz Biotec, USA), goat anti-p-I$\kappa$B (1 : 500, Santa Cruz Biotec, USA), goat anti-iNOS (1 : 200, Santa Cruz Biotec, USA), goat anti-COX-2 (1 : 200, Santa Cruz Biotec, USA), goat anti-Bcl-2 (1 : 100, Santa Cruz Biotec, USA), and goat anti-cleaved caspase-3 (1 : 100, Santa Cruz Biotec, USA), all of which are primary antibodies, for 72 hours in a 4°C humidified chamber. Next, the slices were linked with biotinylated rabbit anti-goat IgG (1 : 100, Santa Cruz Biotec), which is a secondary antibody, for 24 hours at room temperature. After the slices were exposed to the secondary antibody, an avidin biotin complex kit (Vector Lab, USA) was applied for 1 hour at room temperature. Finally, the slices were developed with 0.05 M tris-HCl buffer solution (pH 7.4), which consisted of 0.05% 3, 3′-diaminobenzidine and 0.01% HCl, and then counterstained with hematoxylin.

*2.6. TUNEL Assay.* To investigate apoptosis, a TUNEL assay was performed using an *in situ* apoptosis detection kit (Apoptag, Intergen, USA). We carried out proteolysis using proteinase K for 5 minutes and then applied equilibration buffer for 5 seconds. The proteolysed slices were added strength TdT enzyme (36 $\mu$L TdT enzyme : 72 $\mu$L reaction buffer). Then, the slices were incubated in a humidified chamber at 37°C for 1 hour and then agitated for 10 minutes in strength stop/wash buffer. Next, the slices were treated with anti-digoxigenin peroxidase and DAB for 1 hour. Finally, we observed the sections counterstained with eosin using an optical microscope.

*2.7. Image Analysis and Statistical Analysis.* To produce numerical data from our immunohistochemistry, an image analysis was performed using image Pro Plus (Media cybernetics, USA). In image analysis of our 400x magnification exposure photography, the positive reacted particle as pixel cells (80–100 intensity range) was counted in 10 randomly

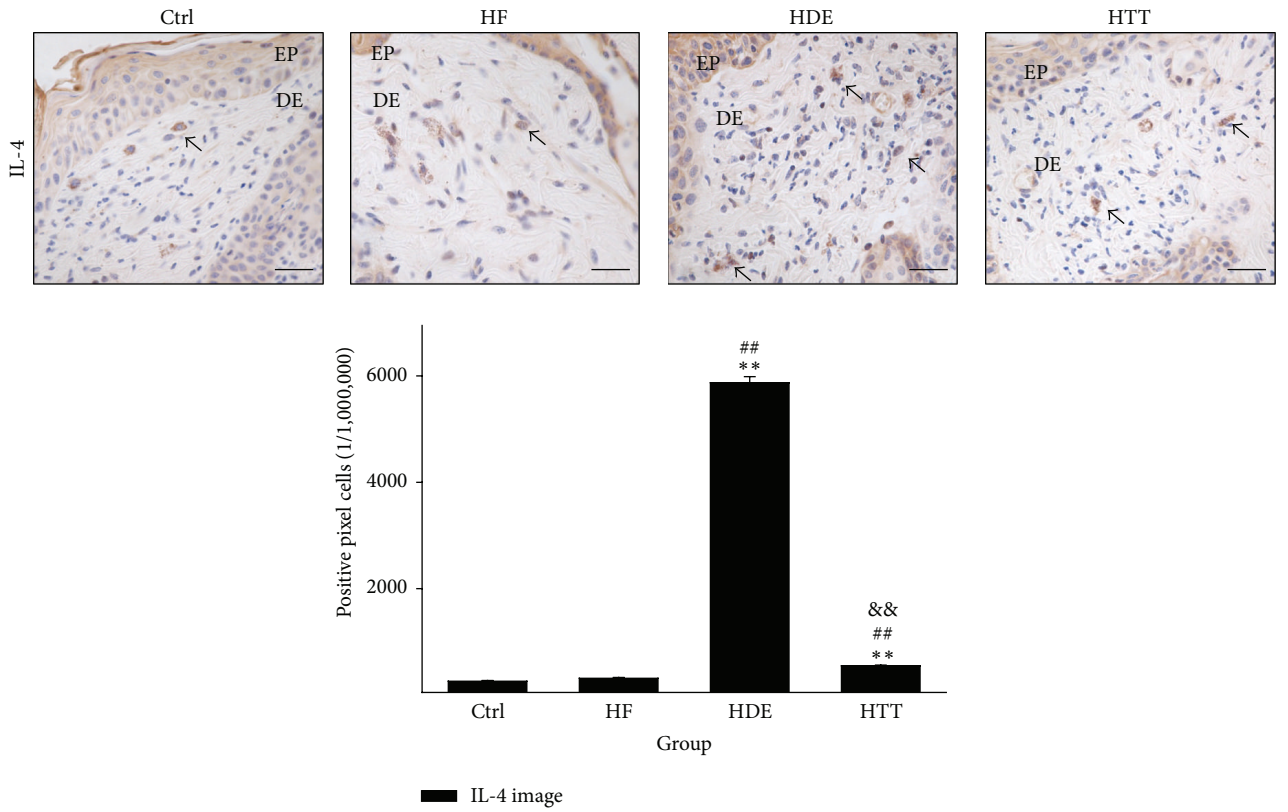



Figure 3: The regulation of Th2 differentiation. IL-4-positive reaction (arrow indicates dark brown) decreased in the HTT group compared with the HDE group (IL-4 immunohistochemistry; bar size, 50 $\mu$m). Data of IL-4 image analysis was also showing the same result ($p < 0.01$). Ctrl: normal feeding, HF: high-fat diet, HDE: high-fat diet and untreated AD-induced, HTT: high fat diet and Hataedock treated AD-induced, EP: epidermis, and DE: dermis. $^{**}p < 0.01$, compared with the Ctrl group; $^{\#\#}p < 0.01$, compared with the HF group; $^{\&\&}p < 0.01$, compared with the HDE group.

selected fields of each group (total pixel cells 100,000,000 or 1,000,000 by various results of immunohistochemistry condition such as nonspecific structure and artificiality). The data were presented as the means ± standard error. The statistical significances of the differences were analyzed with SPSS software (SPSS 23, SPSS Inc., USA), using a one-way ANOVA and Levene's (LSD) test with a significance level of $p < 0.01$.

## 3. Results

*3.1. The Regulation of Th2 Differentiation.* The regulation of Th2 differentiation was estimated by measuring the IL-4-positive reaction. The IL-4-positive reaction was seen in the cytoplasm of dermal papilla cells. The levels of IL-4 in the HTT group were shown to be decreased by 54% ($p < 0.01$) as compared with the HDE group (Figure 3).

*3.2. The Maintenance of Lipid Barrier in Epidermis.* The protective effects of the lipid barrier were estimated by measuring the liver X receptor- (LXR-) and PKC-positive reactions. The levels of LXR-positive reaction that were seen diffusely in the cytoplasm of cells in the stratum corneum and the stratum granulosum were remarkably decreased in the HDE group, but the levels of the HTT group were increased by 148% ($p < 0.01$) as compared with the HDE group (Figure 4).

An increase in the levels of PKC-positive reaction appearing in damaged keratinocytes and in the intercellular space was observed in the HDE group as compared with the Ctrl and HF group. This elevation was significantly decreased by HTD treatment. The levels of PKC in the HTT group were shown to be decreased by 54% ($p < 0.01$) as compared with the HDE group (Figure 4).

Moreover, we observed that skin damage, such as the elimination of the intercellular lipid lamellae in the stratum corneum, was remarkably reduced in the HTT group as compared with HDE group (Figure 4).

*3.3. The Regulation of Mast Cells Activation.* The regulation of mast cells activation was estimated by measuring the Substance P and matrix metallopeptidase 9- (MMP-9-) positive reaction in dermal papilla. Marked increases of Substance P and MMP-9-positive reactions, which were seen in the cytoplasm, were observed in the HDE group. Treatment with Hataedock suppressed the production of Substance P and MMP-9 significantly. The levels of Substance P in the HTT

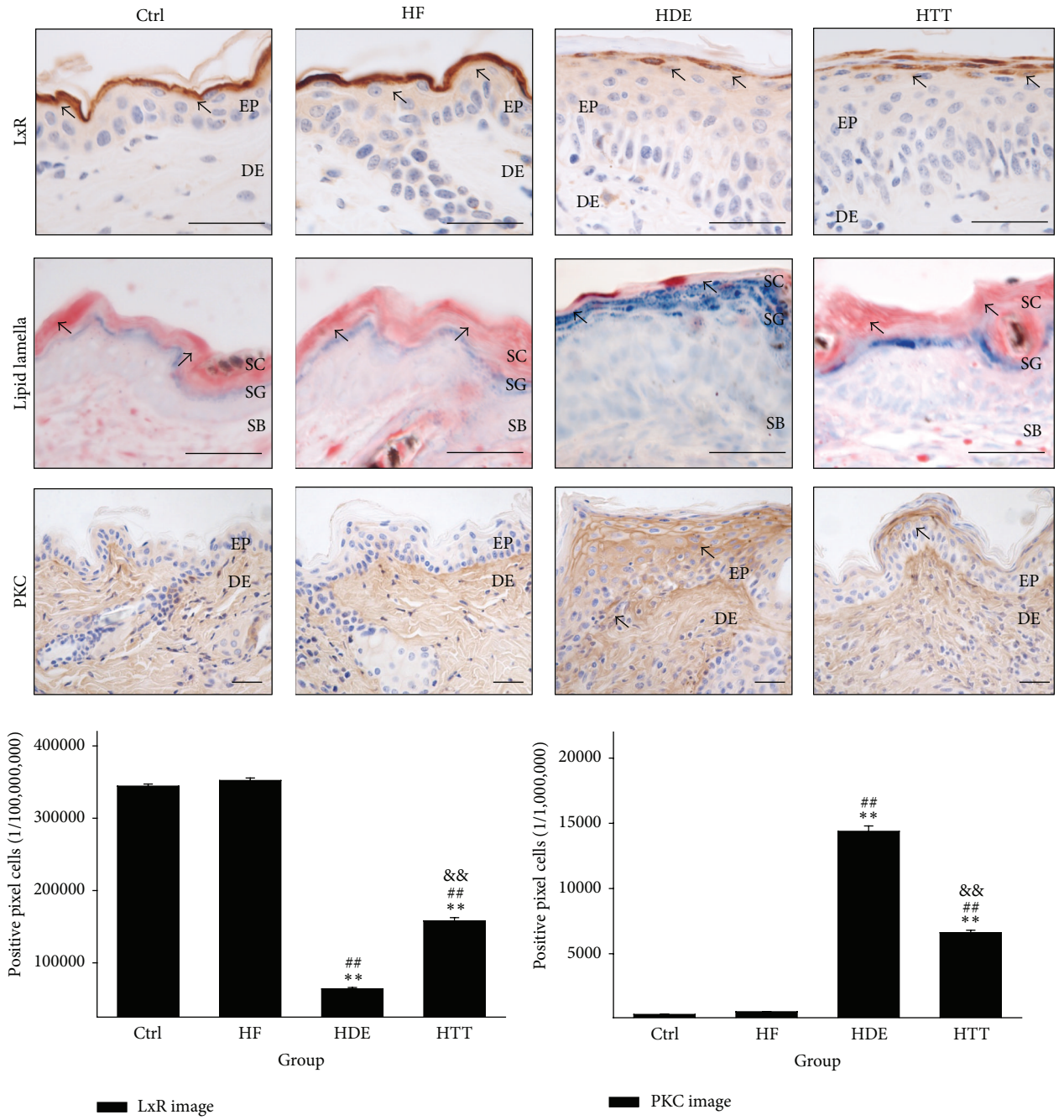



FIGURE 4: The maintenance of lipid barrier in epidermis. The LXR-positive reaction (arrow indicates dark brown) in HDE remarkably decreased but was maintained in HTT (LXR immunohistochemistry; bar size, 50 $\mu$m). The intercellular lipid lamellae (arrow indicates reddish line) of the stratum corneum disappeared in HDE but appeared in HTT (oil red O; bar size, 50 $\mu$m). The PKC-positive reaction (arrow indicates dark brown) in HTT remarkably decreased (PKC immunohistochemistry; bar size, 50 $\mu$m). Data of LXR and PKC image analysis was also showing the same result ($p < 0.01$). Ctrl: normal feeding, HF: high-fat diet, HDE: high-fat diet and untreated AD-induced, HTT: high fat diet and Hataedock treated AD-induced, EP: epidermis, DE: dermis, SC: stratum corneum, SG: stratum granulosum, SB: stratum basale, LXR: liver X receptor, and PKC: protein kinase C. $^{**}p < 0.01$, compared with the Ctrl group; $^{\#\#}p < 0.01$, compared with the HF group; $^{\&\&}p < 0.01$, compared with the HDE group.

group were shown to be decreased by 54% ($p < 0.01$) as compared with the HDE group. The levels of MMP-9 in the HTT group were also shown to be decreased by 48% ($p < 0.01$) as compared with the HDE group (Figure 5).

Furthermore, the results of Luna's staining showed that many degranulated mast cells from the dermal papilla to the area around the subcutaneous layer were observed in the HDE group. On the other hand, decreases in degranulated

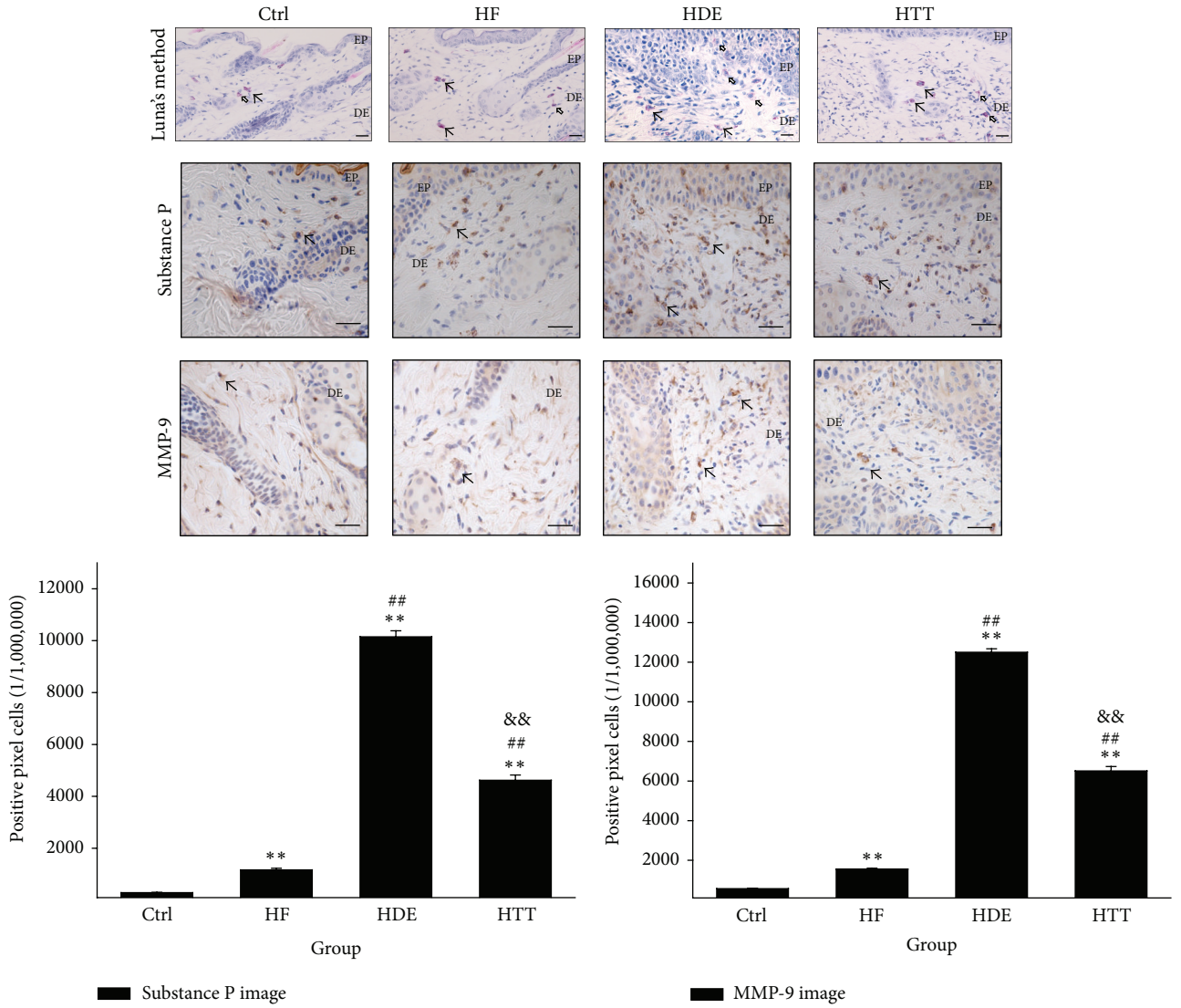



Figure 5: The regulation of mast cells activation. The distribution of degranulated mast cell (vacant arrow) in dermal papillae was increased in the HDE group but decreased in the HTT group (Luna's method; bar size, 50 $\mu$m). The Substance P positive reaction (arrow indicates dark brown) in HTT significantly decreased (Substance P immunohistochemistry; bar size, 50 $\mu$m). The MMP-9 positive reaction (arrow indicates dark brown) in HTT remarkably decreased (MMP-9 immunohistochemistry; bar size, 50 $\mu$m). Data of Substance P and MMP-9 image analysis was also showing the same result ($p < 0.01$). Ctrl: normal feeding, HF: high-fat diet, HDE: high-fat diet and untreated AD-induced, HTT: high fat diet and Hataedock treated AD-induced, EP: epidermis, DE: dermis, and MMP-9: matrix metalloproteinases-9. $^{**}p < 0.01$, compared with the Ctrl group; $^{\#\#}p < 0.01$, compared with the HF group; $^{\&\&}p < 0.01$, compared with the HDE group.

mast cells were observed in the HTT group as compared with the HDE group (Figure 5).

*3.4. Downregulation of Inflammation.* To estimate the anti-inflammatory effects of HTD, we measured the levels of TNF-$\alpha$-, p-I$\kappa$B-, iNOS-, and COX-2-positive reactions in stratum basale and dermal papilla. The results of the immunohistochemical staining showed the appearance of TNF-$\alpha$-, p-I$\kappa$B-, iNOS-, and COX-2-positive reactions in the cytoplasm. Compared with the HDE group, HTD treatment significantly decreased the levels of TNF-$\alpha$-, p-I$\kappa$B-, iNOS-, and COX-2-positive reactions. The HTT group showed a 32% ($p < 0.01$) decrease in TNF-$\alpha$ as compared with the HDE group. The HTT group showed a 61% ($p < 0.01$) decrease in p-I$\kappa$B as compared with the HDE group. The levels of iNOS in the HTT group were also shown to be decreased by 63% ($p < 0.01$) as compared with the HDE group. In addition, COX-2-positive reaction levels were decreased by 51% ($p < 0.01$) in the HTT group (Figure 6).

*3.5. Upregulation of Apoptosis.* The results of the TUNEL assay indicated the upregulation of apoptosis in the dermal papilla cells via the HTD treatment. In the HTT group, the apoptotic body as synonymous with gathering of DNA fragmentation on nucleus was remarkably increased by 373% ($p < 0.01$) as compared to the HDE group (Figure 7).

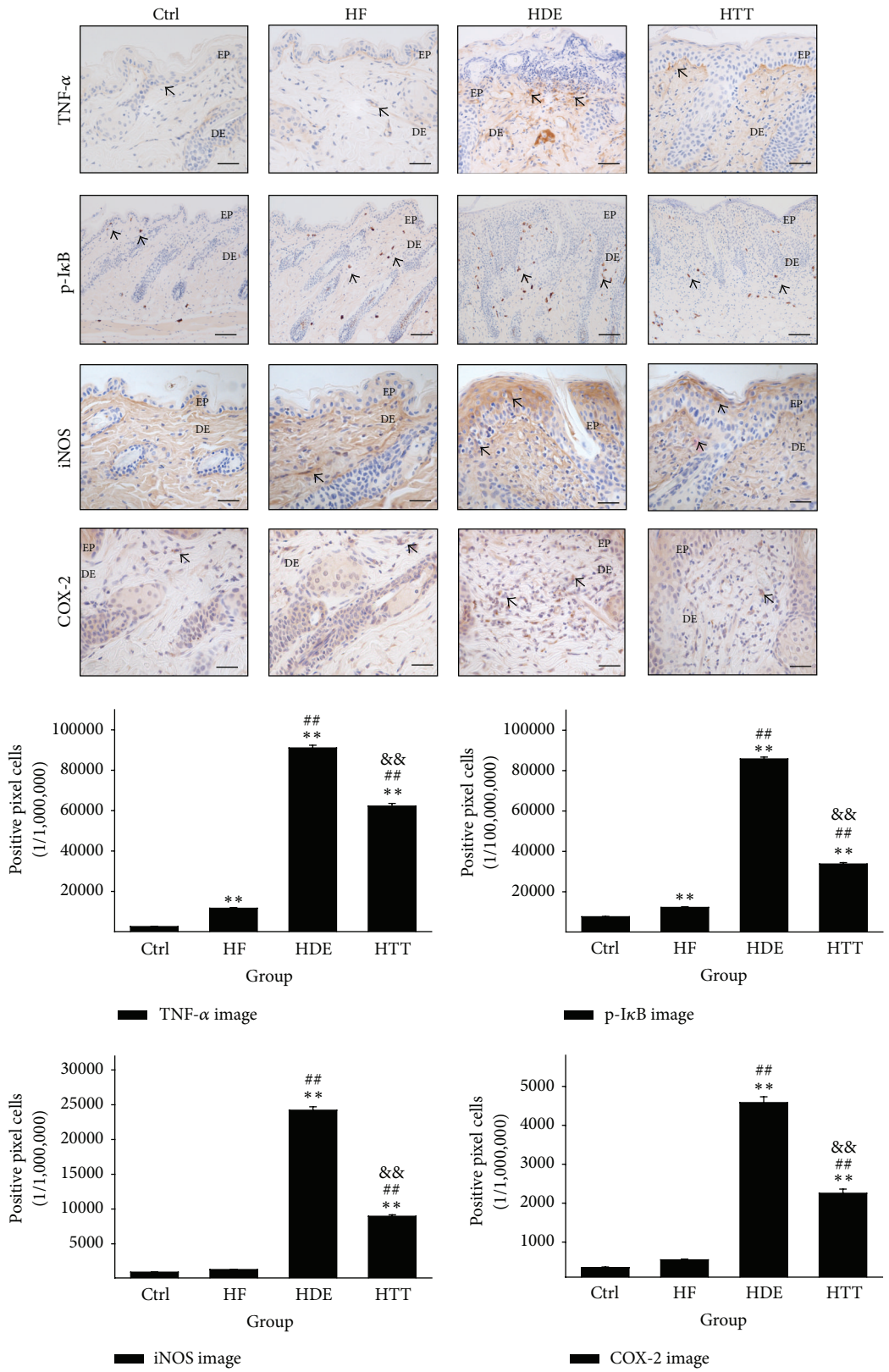



Figure 6: Downregulation of inflammation. In HTT group, the excessive inflammation condition such as increase of TNF-$\alpha$, p-I$\kappa$B, iNOS, and COX-2 was ameliorated by HTD treatment. These positive reactions (arrow indicates dark brown) were remarkably decreased compared with those of the HDE group (immunohistochemistry; bar size, 50 $\mu$m, only p-I$\kappa$B bar size 100 $\mu$m). Data of TNF-$\alpha$, p-I$\kappa$B, iNOS, and COX-2 image analysis was also showing the same result ($p < 0.01$). Ctrl: normal feeding, HF: high-fat diet, HDE: high-fat diet and untreated AD-induced, HTT: high fat diet and Hataedock treated AD-induced, EP: epidermis, DE: dermis. $^{**}p < 0.01$, compared with the Ctrl group; $^{\#\#}p < 0.01$, compared with the HF group; $^{\&\&}p < 0.01$, compared with the HDE group.

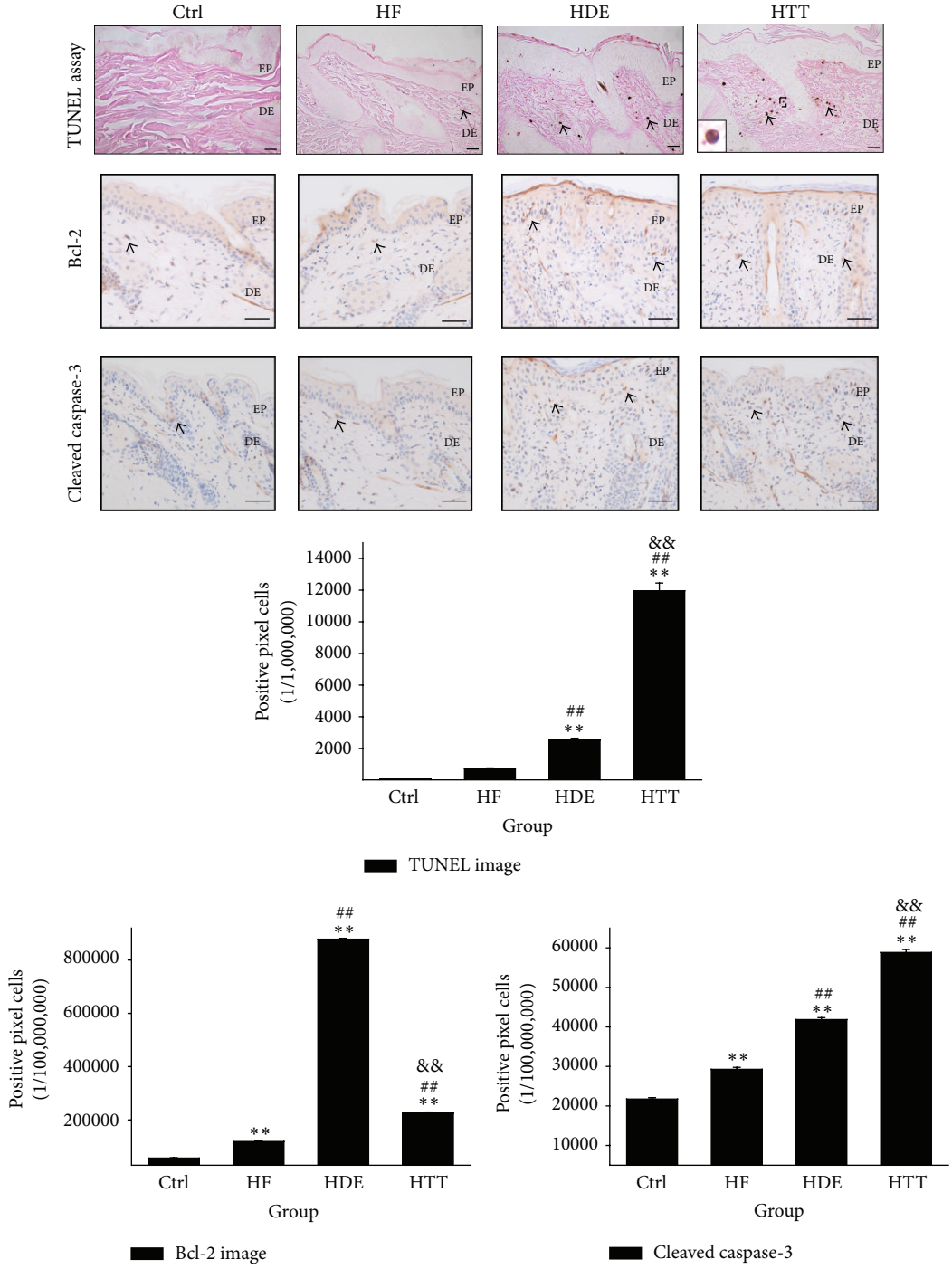



Figure 7: Upregulation of apoptosis. Upregulation of apoptosis in dermatitis induced by HTD treatment. The apoptotic body (arrow indicates dark brown) in the HTT group was remarkably increased compared to the HDE group (TUNEL assay; square box, enlarged DNA fragmentation of nucleus with TUNEL positive reaction; bar size, 50 $\mu$m). The Bcl-2 positive reaction (arrow indicates dark brown) in HTT group was remarkably decreased compared with those of the HDE group (immunohistochemistry; bar size, 50 $\mu$m). The cleaved caspase-3 positive reaction (arrow indicates dark brown) in HTT group was increased compared with those of the HDE group (immunohistochemistry; bar size, 50 $\mu$m). Data of TUNEL, Bcl-2, and cleaved caspase-3 image analysis was also showing the same result ($p < 0.01$). Ctrl: normal feeding, HF: high-fat diet, HDE: high-fat diet and untreated AD-induced, HTT: high fat diet and Hataedock treated AD-induced, EP: epidermis, and DE: dermis. $^{**}p < 0.01$, compared with the Ctrl group; $^{\#\#}p < 0.01$, compared with the HF group; $^{\&\&}p < 0.01$, compared with the HDE group.

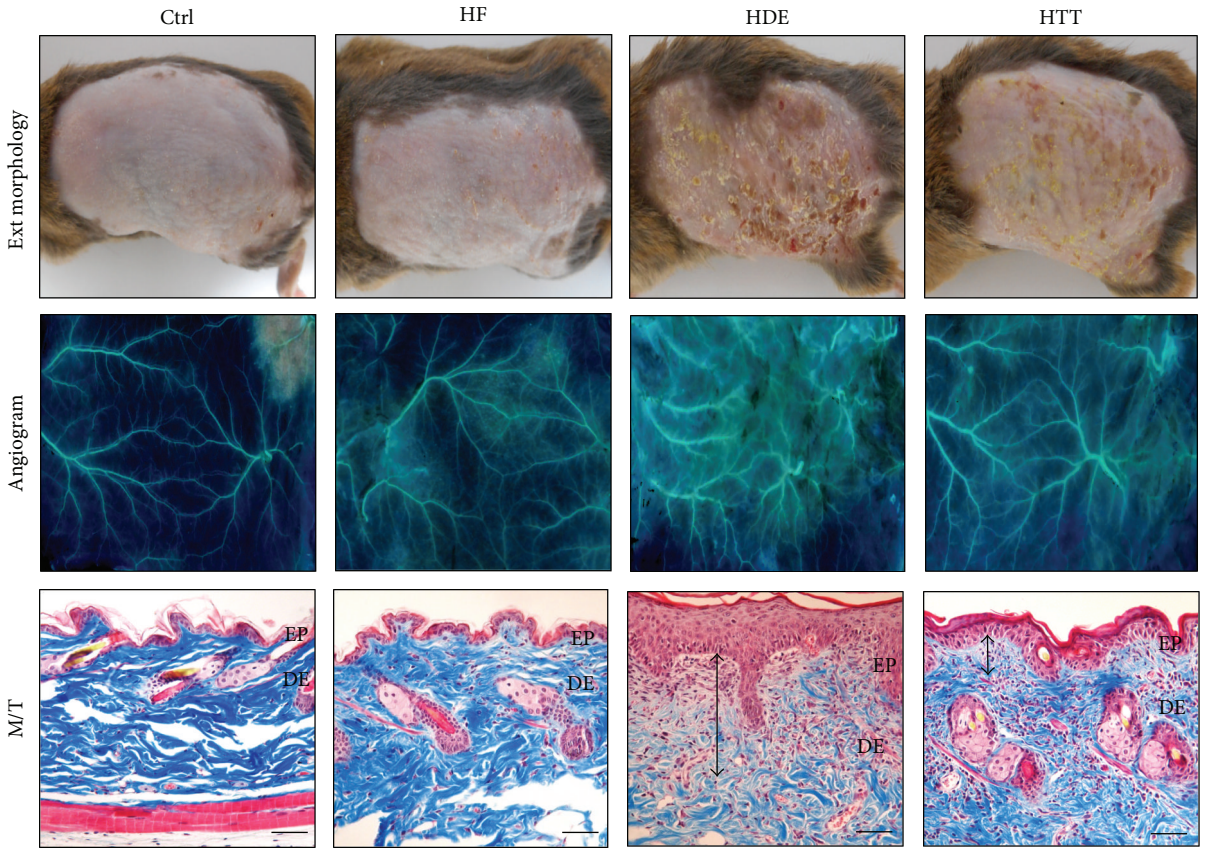



Figure 8: The mitigative effect of HTD treatment for dermatitis. The skin damage as eczema was mitigated in HTT group. The angiogenesis was increased in HDE group but decreased in HTT group (×4). The histological features of AD such as vacuolation of keratinocytes, hyperplasia, edema (up-down arrow), infiltration of inflammatory cells, and increase of capillary were increased in HDE group but decreased in HTT group (bar size, 100 $\mu$m). Ctrl: normal feeding, HF: high-fat diet, HDE: high-fat diet and untreated AD-induced, HTT: high fat diet and Hataedock treated AD-induced, EP: epidermis, DE: dermis, and M/T: Masson trichrome method.

Compared with the HDE group, HTD treatment significantly changes the apoptosis signal. The HTT group showed a 74% ($p < 0.01$) decrease in Bcl-2, an antiapoptotic protein, as compared with the HDE group. The HTT group showed a 41% ($p < 0.01$) increase in cleaved caspase-3, proapoptotic protein, as compared with the HDE group (Figure 7).

*3.6. The Mitigative Effect of HTD Treatment on Dermatosis.* The therapeutic effectiveness of HTD (administering the extract of *Coptidis Rhizoma* and *Glycyrrhiza uralensis*) was explored by examining its effect on dermatosis severity. AD-induced mice that were fed a high-fat diet and did not receive any treatment (the HDE group) showed the highest level of dermatosis severity. The obtained image of the HDE group showed various pathological features, such as severe erythema, blood clot, edema, superficial erosion, deep excoriation, and dry skin. In contrast, the high-fat-diet-fed, AD-induced mice treated with HTD (the HTT group) exhibited better control of AD symptoms (Figure 8).

A comparison of angiogenesis was conducted between the HDE and HTT groups. Our angiogram reveals that the HTT group experienced much better angiogenesis reduction than the HDE group (Figure 8).

For the results obtained from Masson's trichrome stains, the atopic mice of the HDE group also exhibited significant damage such as vacuolation of keratinocytes, hyperplasia in epidermis, disappearance of collagen fibers, the infiltration of inflammatory cells, and an increase in dermal capillary density.

On the other hand, when mice were treated with HTD, the amelioration of epidermal hyperplasia, the improvement of collagen fiber density, and fewer inflammatory cell infiltrates in the dermis were remarkably observed (Figure 8).

## 4. Discussion

The results of the studies suggest that HTD treatment may be an effective preventive treatment for AD. HTD treatment was effective in attenuating inflammation and maintaining the skin barrier in AD-induced NC/Nga mice under high-fat-diet conditions. Much progress has been made in understanding the genetic background and pathophysiology of AD, thus allowing more specific therapeutic interventions to be introduced [23]. Among these interventions, we paid attention to those that intervene very early in the lives of infants and young children by controlling skin inflammation

at the earliest time point [24]. Moreover, there is now plenty of evidence indicating close ties between the metabolic and immune systems. It is now clear that obesity is associated with a state of chronic low-level inflammation [25]. In this respect, an unbalanced maternal diet during breastfeeding may be a risk factor underlying the later development of atopic sensitization in the infant, regardless of maternal atopic disease [26]. It was also reported that neonatal adiposity is a predictor of AD [12].

We hypothesized that both immune system dysregulation and a high-fat diet may affect inflammatory responses in the infant. Further, we hypothesized that these responses may amplify the development of AD. Based on these hypotheses, we investigated the preventive therapeutic effects of HTD treatment on inflammatory regulation and skin protection in AD-induced NC/Nga mice under high-fat diet conditions. This study differs from previous studies with regard to the timing of treatment administration. Previous studies administered the herbal extracts after AD symptom onset [27–30]. On the other hand, this study administered the herbal extracts before clinical symptom onset. We also established a high-fat-diet-induced model and so attempted to identify relevant neonatal adiposity and the development of AD.

*4.1. The Regulation of Th2 Differentiation.* It was known that the Th2-biased immune responses that characterize neonates may influence the later onset of allergic disease [31]. Th2 cells mediate these functions by producing various cytokines. Particularly, IL-4 is a key Th2 cytokine that is critical for Th2 cell differentiation, IgE production, and eosinophil recruitment, among other functions [32]. It was reported that high levels of IL-4-producing T cells and low levels of IFN-$\gamma$ at birth may enhance the risk of the subsequent development of AD [33].

To estimate the regulation of Th2 differentiation, we measured the level of IL-4-positive reaction in the dermal papilla cells. We show that the levels of IL-4 positive reaction were remarkably increased in the HDE group but that the levels of the HTT group were decreased (Figure 3). This finding suggests that HTD treatment reduced the level of IL-4. This decrease of IL-4 may contribute to the regulation of Th2 differentiation and these results may also contribute to the improvement of AD.

*4.2. The Maintenance of Lipid Barrier in Epidermis.* We show that the levels of LXR-positive reaction were remarkably decreased in the HDE group but that the level of the HTT group was maintained. Moreover, the lipid barrier in the intercellular space was also maintained in the HTT group. On the other hand, an increase in the levels of PKC was observed in the HDE group as compared with the Ctrl group. This elevation was significantly decreased in the HTT group (Figure 4).

It is known that dysfunctional ceramides in the SC barrier may contribute to the disruption of the epidermal barrier, resulting in mechanisms that operate in the pathophysiology of AD [34]. This condition is induced by PKC activation, which plays a role in the initiation of epidermal barrier dysfunction [35]. The PKC signaling pathway is activated by a broad spectrum of extracellular stimuli that promote lipid hydrolysis and play a fundamental role in numerous biological facets, such as differentiation, proliferation, apoptosis, and neuronal transmitter release [36]. It is known that transactivation by LXR was decreased by the activation of the PKC signaling pathway [37].

LXRs play a critical role in the control of lipid metabolism, acting as regulators of cholesterol and fatty acid metabolism [38]. Previous studies have demonstrated that LXR activators stimulate epidermal differentiation, improve permeability barrier homeostasis, and inhibit epidermal proliferation [39, 40]. Additionally, it is known that LXR activators exhibit potent antihyperplastic and anti-inflammatory activity in irritant-contact dermatitis and acute allergic-contact dermatitis [41]. LXR activation also accelerates permeability barrier recovery following acute barrier disruption [42].

Therefore, the protective effects for the lipid barrier may be caused by the activation of LXRs and the inhibition of PKC. Such effects may influence the maintenance of the lipid barrier in the HTT group.

*4.3. The Regulation of Mast Cells Activation.* Mast cells play a central role in both acute and chronic allergic reactions through the release of a number of mediators and cytokines [43]. Because most studies have shown increased numbers of mast cells in skin lesions in AD models, it is generally assumed that mast cells contribute to skin inflammation [2]. Upon activation, mast cells release their membrane-bound cytosolic granules, leading to the release of several molecules that are important in the pathogenesis of AD and host defense [44].

Mast cell associated nerves in the skin are predominantly Substance P-positive [45]. Substance P, an established neurotransmitter, evokes an immune inflammatory response involving the degranulation of mast cells [46]. Interestingly, it has also been pointed out that stress and anxiety worsen dermatitis via Substance P-dependent neurogenic inflammation in mice [47]. Thus, Substance P is currently considered to be one of the key pruritogenic factors [48].

Matrix metalloproteinase- (MMP-) 9 has been recognized in the process of inflammation and tissue remodeling and repair. Also, mast cells can produce MMP-9, which may contribute to extracellular matrix degradation and absorption in the process of allergic and nonallergic responses [49]. Thus, it is assumed that MMP-9 could be important in the pathogenesis of AD [50].

In the present study, many degranulated mast cells were observed in the HDE group. On the other hand, primarily granular mast cells appeared in HTT group. Moreover, the levels of Substance P and MMP-9 were significantly decreased in the HTT group (Figure 5). Therefore, these results imply that HTD reduces the infiltration of degranulated mast cells and can prevent the release of Substance P and MMP-9.

*4.4. Downregulation of Inflammation.* TNF-$\alpha$ is one inflammatory mediator that has been implicated in AD, due to its participation in lipid and protein synthesis in the epidermis

and as a consequence of effect of skin barrier lipid composition and organization [51]. It is known that facilitated translocation of NF-$\kappa$B may worsen the allergic inflammation including AD by enhancing the production of inflammatory cytokines and chemokines [52]. And the phosphorylated I$\kappa$B (p-I$\kappa$B) allow the NF-$\kappa$B subunit to translocate to the nucleus [53]. For this reason, deregulation of NF-$\kappa$B and p-I$\kappa$B is a hallmark of chronic inflammatory diseases [54]. iNOS and COX-2 are also known to play important roles in the regulation of inflammatory reactions [55, 56]. iNOS produces high amounts of NO induced by cytokines such as interferon gamma and TNF-$\alpha$ [57]. NO derived from iNOS also plays important roles in the modulation of symptoms in patients with inflammatory diseases, including AD [58]. It is also known that COX products are increased in the skin of patients with AD [59].

To elucidate the role of the anti-inflammatory effects of HTD, we analyzed the levels of TNF-$\alpha$-, p-I$\kappa$B-, iNOS-, and COX-2-positive reactions. Marked increases in TNF-$\alpha$, p-I$\kappa$B, iNOS, and COX-2 production were observed in the HDE group. These increases were effectively lowered by HTD treatment in the HTT group (Figure 6). While the inflammatory response plays important roles in protecting the host and repairing tissues, it can also damage normal skin tissues. Therefore, these results of the decrease in inflammatory cytokines imply that HTD may contribute to the reduction of inflammation and improvement of AD.

*4.5. Upregulation of Apoptosis.* Apoptosis is highly important in the renewal of cells and formation of epidermal structure [60]. Apoptotic cells may not stimulate inflammation if they are ingested by phagocytes before they release their intracellular substances. Moreover, during this process apoptotic cells can stimulate phagocytes to induce anti-inflammatory cytokines [61]. It has also been reported that dysregulated apoptosis may contribute to the development and persistence of AD [62].

Overexpression of the Bcl-2 has been demonstrated to prevent apoptosis either by sequestering proforms of death-driving caspases or by preventing the release of cytochrome c into the cytoplasm [63, 64]. On the other hand, sequential activation of caspases plays an important role in the execution-phase of cell apoptosis. Among them, caspase-3 is a commonly activated death protease, catalyzing the specific cleavage of many key cellular proteins [65]. The caspase-3 is known to act downstream on bax/bcl-2 control [66]. The cleavage products of bcl-2 are located to the mitochondria resulting in the release of cytochrome c and leading to more caspase-3 activation as a positive feedback effect, strengthening the apoptotic effect [67].

In the present study, apoptotic cells were determined via TUNEL assay. The apoptotic bodies in the HTT group were remarkably increased in number as compared to the HDE group. The cleaved caspase-3 positive reaction was also increased in the HTT group. In contrast, the Bcl-2 positive reaction in HTT group was remarkably decreased (Figure 7). This finding suggests that HTD treatment may contribute to stimulate the apoptotic cascade activation that ultimately leads to improvement of AD.

*4.6. The Mitigative Effect of HTD Treatment on Dermatosis.* In this study, we demonstrated the skin barrier maintenance and anti-inflammatory effects of HTD treatment under high-fat-diet conditions in AD. In our animal model, the repeated application of DNFB caused skin damage and increased angiogenesis and spongiosis in the HDE group. However, in the HTT group, damage to the intercellular space, hyperplasia, edema, the infiltration of inflammatory cell, and increased capillaries were decreased (Figure 8).

Therefore, these findings suggest that HTD was more effective in maintaining skin integrity during the course of dermatosis and treatment under high-fat-diet conditions. These results indicate that HTD may alleviate the underlying inflammatory reactions and effectively attenuate these skin damages.

## 5. Conclusions

In summary, we demonstrated that HTD treatment was effective in attenuating inflammation and maintaining the skin barrier in AD-induced NC/Nga mice under high-fat-diet conditions. These results imply that HTD may alleviate the symptom of AD through the regulation of Th2 differentiation, the maintenance of lipid barrier in epidermis, the regulation of mast cells activation, downregulation of inflammation, and upregulation of apoptosis. In conclusion, HTD could be used as an alternative and preventive therapeutic approach in the management of AD. Further studies about detailed mechanism of these protective immune responses are needed.

## Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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*Research Article*

# Evaluation of Medicinal Categorization of *Atractylodes japonica* Koidz. by Using Internal Transcribed Spacer Sequencing Analysis and HPLC Fingerprinting Combined with Statistical Tools

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*Atractylodes* rhizomes have been used as the herbal medicine "Changchul" or "Baekchul," according to their clinical purpose, in Korea, China, and Japan. Among the *Atractylodes* species, the medicinal use of *Atractylodes japonica* has been controversial, as it is categorized as both Changchul and Baekchul in those countries, and, moreover, parts of the rhizome have been differently used, depending on age of the plant, in Korea. Chromatographic fingerprinting by using HPLC combined with chemometric analyses and internal transcribed spacer (ITS) sequencing analysis were conducted to classify and identify 34 crude drugs derived from *Atractylodes* rhizomes. The identification of the samples, authenticated by their morphological features as *A. japonica* Koidz. (Changchul and Baekchul), *A. chinensis* Koidz., and *A. macrocephala* Koidz., was confirmed as *A. japonica*, *A. chinensis*, and *A. macrocephala* by ITS sequencing. The results from chemometric analyses showed that the chemical components of the crude drugs from *A. japonica* were significantly different from those from *A. macrocephala* but were similar to those from *A. chinensis*. The analyses also suggested that the categorization by age of *A. japonica* as Changchul or Baekchul is not recommended. The results indicate that *A. japonica* should be categorized as "Changchul" and should not be further categorized by age.

## 1. Introduction

The genus *Atractylodes* (Asteraceae) are perennial herbs distributed in Korea, China, and Japan. Their dried rhizomes have been classified into two kinds of herbal medicines according to their clinical purpose, "Baekchul" (Baizhu in Chinese, Byakujutsu in Japanese) and "Changchul" (Cangzhu in Chinese, Soujutsu in Japanese) [1]. In medicinal applications in Korea, Japan, and China, the rhizomes of *A. lancea* DC. and *A. chinensis* Koidz. have been classified as Changchul, while that of *A. macrocephala* Koidz. has been classified as Baekchul in the pharmacopeias of Korea, China, and Japan [2–4].

However, there has been disagreement between countries in classifying the rhizome of *A. japonica* Koidz.: Korean and Japanese pharmacopeias, as well as some studies from Korea and Japan, have classified the rhizome of *A. japonica* as Baekchul, whereas Chinese studies have classified it as "Gwan-Changchul" (Guan-Cangzhu in Chinese), a type of Changchul, which is not even listed in the Chinese pharmacopeia [5, 6]. Moreover, *A. japonica* is recorded as a synonym of *A. lancea* in the *Flora of China* [7]. Confusion also occurs in local herbal markets in Korea, where the rhizomes of *A. japonica* have been used as Changchul when the fibrous substance has formed after it has grown over two years [8]. The dried rhizomes of *Atractylodes* species can be identified by their morphological features; however, it is difficult to discriminate them by macroscopic observation, a subjective method, due to their morphological similarity. Consequently, the misuse of *Atractylodes* rhizomes may occur

when identification is based only on their morphological features. Therefore, strict classification is required for the exact use of *Atractylodes* rhizomes for medicinal purposes.

Genetic identification achieved by hybridization, polymerase chain reaction (PCR), and sequencing techniques is considered more objective, precise, and reliable method for identifying and authenticating herbal species [9, 10]. Internal transcribed spacer (ITS) regions, which are rapidly evolving regions of nuclear ribosomal DNA, have been widely used for the identification of plant materials, and phylogenetic analysis using ITS regions has been conducted to investigate the genetic variability of complicated herbal species [11–13]. It has been reported that the four species of *Atractylodes* (*A. japonica*, *A. macrocephala*, *A. lancea*, and *A. chinensis*) can be distinguished by their different genotypes on ITS sequences [14]. In contrast, one study conducted by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the ITS region on nucleotide ribosome DNA (nrDNA) reported that *A. japonica* is not different from *A. macrocephala* [15]. Other studies have demonstrated that the geographical distributions, morphological features, and genetic differentiation between *A. lancea* and *A. chinensis* were not consistent, and *A. japonica* was most closely related to *A. lancea*, according to the results from ITS and *trnL-F* sequences [16, 17]. The results of these studies demonstrate that the classification of *Atractylodes* rhizomes, particularly that of *A. japonica*, remains controversial.

Chemical fingerprinting by using HPLC is an effective and reliable method for the investigation of chemical components in herbal medicines, owing to its high separation efficiency and high detection sensitivity [18]. HPLC fingerprinting also enables a systematic and comprehensive approach to the identification and quantification of the components in herbal medicines [19]. HPLC fingerprinting, combined with chemometric statistical analysis, has been widely used for the quality control of herbal medicines and related herbal products [20, 21]. Moreover, such techniques have also been used to discriminate interspecies differences in herbal medicines, by incorporating principal components analysis (PCA) [22–24]. In previous studies, *A. lancea* was chemically differentiated from *A. chinensis* by HPLC fingerprinting combined with PCA or orthogonal partial least squares-discriminant analysis [25, 26]. However, the chemical discrimination of *Atractylodes* species, using HPLC fingerprinting and chemometric analysis, has not been conducted.

In the present study, chromatographic fingerprinting and chemometric statistical analyses, including PCA, hierarchical clustering analysis (HCA) analysis, and Pearson's correlation coefficient analysis were conducted to classify the crude drugs derived from *Atractylodes* rhizomes. The ITS sequences from nrDNA were also examined to identify the *Atractylodes* species.

## 2. Materials and Methods

*2.1. Plant Materials and Reagents.* Thirty-four samples of crude drugs from *Atractylodes* rhizomes were collected or purchased from the wild, agricultural fields, or local markets in Korea and China. The samples were authenticated by their morphological features through identification criteria by authors. Fifteen rhizomes were identified as *A. chinensis* (coded as "AC"); seven samples of Baekchul and five samples of Changchul were "young" and "aged" dried rhizomes of *A. japonica* (coded as "AJB" and "AJC," resp.); and seven rhizomes were identified as *A. macrocephala* (coded as "AM") (Table 1). The voucher specimens have been deposited in the herbarium of the College of Korean Medicine, Woosuk University (Jeonju, Jeonbuk, Korea).

Nine specimens (coded as Arabic number) of *Atractylodes* plants were collected as species reference samples for identification of *Atractylodes* rhizomes in Table 1 and those samples were deposited in the Korea Institute of Oriental Medicine as voucher specimens. The classification result of nine dried-voucher specimens is shown in Table 2.

*2.2. Preparation of Genomic DNA.* The genomic DNA was extracted from the crude drugs of *Atractylodes* rhizomes according to the manuals of NucleoSpin® Plant II kit (Macherey-Nagel, Dueren, Germany). For some samples, 10% cetyl trimethyl ammonium bromide (CTAB) and 0.7 M NaCl were used to remove the phenolic compounds and polysaccharides.

*2.3. PCR Amplification.* For ITS amplification, PCR was performed using T-personal cycler (Biometra, Germany). In brief, 600 nM of primer set of ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [27], 1x BluePreMIX-HF (Macrogen, Korea), and 50 ng of genomic DNA were used for PCR amplification. PCR cycling conditions which were followed by predenaturation process (95°C, 5 min) were as follows: denaturation process (95°C, 30 s); annealing process (52°C, 30 s); extension process (72°C, 40 s) × 36 cycles; and final extension process (72°C, 5 min). The amplified PCR product was separated from other gradients using 1.5% agarose gel electrophoresis after the staining by the addition of Safe-white™ (abm, Canada). Amplified products were analyzed using MyImage (Seoulin Biotechnology, Korea).

*2.4. Determination of DNA Sequence of PCR Product.* PCR product separated from agarose gel was cloned using MG™ TOPcloner TA kit (Macrogen, Korea) and DNA sequence of cloned PCR product was determined through the interpretation performed by Macrogen (Korea).

*2.5. Analysis of DNA Sequence and Preparation of Dendrogram.* DNA sequence was analyzed using ClustalW multiple sequence alignment (Bioedit, v7.0.9; available at http://www.mbio.ncsu.edu/BioEdit/page2.html) and the phylogenetic tree was created by using DNADist (Bioedit). To study the relationship of *Atractylodes*, the nucleotide sequences of the genera *Atractylis* and *Carlina* deposited in NCBI GenBank were used. The genera *Brachylaena*, *Cardopatium*, *Cirsium*, *Echinops*, *Phonus*, and *Tarchonanthus* were also used as reference for phylogenetic relationship. *Magnolia heptapeta*,

Table 1: Morphological identification of the crude drugs of *Atractylodes* rhizomes.

| Code | Species | Origin | Code | Species | Origin |
|---|---|---|---|---|---|
| AC-1 | *A. chinensis* Koidz. | China | AJB-3 | *A. japonica* Koidz. | China |
| AC-2 | *A. chinensis* Koidz. | China | AJB-4 | *A. japonica* Koidz. | Unclear |
| AC-3 | *A. chinensis* Koidz. | China | AJB-5 | *A. japonica* Koidz. | Unclear |
| AC-4 | *A. chinensis* Koidz. | China | AJB-6 | *A. japonica* Koidz. | Unclear |
| AC-5 | *A. chinensis* Koidz. | China | AJB-7 | *A. japonica* Koidz. | Korea |
| AC-6 | *A. chinensis* Koidz. | Korea | AJC-1 | *A. japonica* Koidz. | Korea |
| AC-7 | *A. chinensis* Koidz. | Korea | AJC-2 | *A. japonica* Koidz. | Unclear |
| AC-8 | *A. chinensis* Koidz. | China | AJC-3 | *A. japonica* Koidz. | Unclear |
| AC-9 | *A. chinensis* Koidz. | China | AJC-4 | *A. japonica* Koidz. | Korea |
| AC-10 | *A. chinensis* Koidz. | China | AJC-5 | *A. japonica* Koidz. | Unclear |
| AC-11 | *A. chinensis* Koidz. | China | AM-1 | *A. macrocephala* Koidz. | China |
| AC-12 | *A. chinensis* Koidz. | China | AM-2 | *A. macrocephala* Koidz. | China |
| AC-13 | *A. chinensis* Koidz. | China | AM-3 | *A. macrocephala* Koidz. | China |
| AC-14 | *A. chinensis* Koidz. | China | AM-4 | *A. macrocephala* Koidz. | China |
| AC-15 | *A. chinensis* Koidz. | China | AM-5 | *A. macrocephala* Koidz. | China |
| AJB-1 | *A. japonica* Koidz. | Korea | AM-6 | *A. macrocephala* Koidz. | China |
| AJB-2 | *A. japonica* Koidz. | Korea | AM-7 | *A. macrocephala* Koidz. | China |

Table 2: Identification of dried-voucher specimens coded as Arabic numbers.

| Number | Identification |
|---|---|
| 1 | *A. japonica* Koidzumi |
| 2 | *A. lancea* de Candolle |
| 3 | *A. lancea* de Candolle |
| 4 | *A. macrocephala* Koidzumi |
| 5 | *A. japonica* Koidzumi |
| 6 | *A. japonica* Koidzumi |
| 8 | *A. lancea* de Candolle |
| 9 | *A. japonica* Koidzumi |
| 17 | *A. japonica* Koidzumi |

*Zanthoxylum rhoifolium*, and *Lilium cernuum* were used as out-groups in the phylogenetic analyses, based on previous studies [28, 29]. ITS sequences of these taxa were collected from GeneBank in NCBI (the accession numbers were shown in Table 3).

*2.6. Preparation of Samples for HPLC Analysis.* The dried powder of the rhizomes (100 mg) was weighed and then extracted by sonication with 2 mL of ethanol (HPLC grade; Phillipsburg, NJ, USA) for 40 min. The extract was filtered through a 0.45 $\mu$m membrane filter (Adventec, Tokyo, Japan) and concentrated in vacuum oven at 45°C. Concentrated extract was dissolved with methanol at the concentration of 5000 $\mu$g/mL and filtered through a 0.45 $\mu$m membrane filter, prior to HPLC injection.

*2.7. HPLC Conditions for Chromatographic Fingerprinting.* An Agilent 1260 liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA) equipped with an autosampler, degasser, quaternary solvent pump, and diode array detector (DAD) was used for chromatographic fingerprinting. The data were processed by using Chemstation software (Agilent Technologies Inc., USA). The separation of compounds was carried out on a Capcell Pak Mg II $C_{18}$ column (4.6 mm × 250 mm, 5 $\mu$m; Shiseido, Tokyo, Japan) with Mg II $C_{18}$ guard cartridge (4.0 mm × 10 mm, S-5; Shiseido, Tokyo, Japan) at 35°C. The flow rate was 1 mL/min and the injection volume was 10 $\mu$L. The mobile phase consisted of solvent A (HPLC-grade water; Phillipsburg, NJ, USA) and solvent B (HPLC-grade acetonitrile; Phillipsburg, NJ, USA), with the following gradient elution, 20% (B) over 0–2 min, 20–55% (B) over 2–10 min, 55% (B) over 10–13 min, 55–60%

Table 3: The GenBank accession number of species that are used in phylogenetic tree analysis.

| Species | GenBank accession number |
|---|---|
| *Atractylis cancellata* | AY826231.1 |
| *Atractylis arabica* | KF850563.1 |
| *Atractylis carduus* | AY826232.1 |
| *Atractylis arbuscula* | KF301215.1 |
| *Carlina falcata* | AY826243.1 |
| *Carlina vulgaris* | KF301217.1 |
| *Carlina acanthifolia* | KF301216.1 |
| *Echinops viscosus* | AY826283.1 |
| *Phonus riphaeus* | AY826310.1 |
| *Cirsium palustre* | EU143268.1 |
| *Cardopatium corymbosum* | AY826238.1 |
| *Tarchonanthus camphoratus* | AY826340.1 |
| *Brachylaena discolor* | AY826236.1 |
| *Magnolia heptapeta* | AY858638.1 |
| *Zanthoxylum rhoifolium* | KC502933.1 |
| *Lilium cernuum* | HQ686064.1 |

(B) over 13–35 min, 60% (B) over 35–38 min, and 60–75% (B) over 38–50 min, held for 2 min, and then reequilibrated to 20% until the end of the analysis. Detection was performed using a UV detector at the wavelengths of 255, 275, 295, 315, and 340 nm.

*2.8. Chemometric Statistical Analysis.* The 34 samples that were genetically identified and recoded were used for PCA, HCA, and Pearson's correlation analysis. Total 27 peaks were selected as "reference peaks," and their absolute area was calculated by peak area integration. The 27 reference peaks for the chromatographic fingerprinting, which were representative and >1.0% of total peak area, were chosen at their optimal UV absorption. The absolute area of chosen peak was calculated for chromatographic fingerprinting. A matrix composed of the rows (*Atractylodes* sample) and columns (absolute area of each reference peak) was used for construction of PCA plot, HCA dendrogram, and Pearson's correlation analysis, which were conducted using open-source software R (v.3.1.1).

## 3. Results

*3.1. ITS Genotype and Genetic Identification of Atractylodes Rhizomes.* The amplification of internal transcribed spacer (ITS) region produced overall 733 bp of nucleotide sequences from 34 samples listed in Table 1 and nine dried-voucher specimens (Figure 1). The determined ITS nucleotide sequence of samples were confirmed by using DNA sequence registered in NCBI GenBank as well as previous paper [14] with comparison of the accession numbers: *A. japonica* (AB219405), *A. macrocephala* (AB219406), *A. lancea* (AB219407), *A. chinensis* (AB219408), and *A. koreana* (AB219409). As presented in Table 4, there was nucleotide substitutions observed on 37 sites on the ITS regions of *Atractylodes* samples. Type 1, ITS sequence of *A. japonica*, showed multiple sequences comparing to other species, while types 2 and 3 were the genotypes of *A. macrocephala* and *A. lancea*, respectively. Type 4, the genotype of *A. chinensis*, was identical to type 5, the genotype of *A. koreana*.

All 7 samples labelled as "AM" were determined as *A. macrocephala* and the difference of DNA sequence between samples was not observed. Among 15 samples labelled as "AC," AC-1, AC-2, AC-4, AC-5, AC-6, and AC-11 were determined as *A. chinensis*, whereas the rest were *A. japonica*. The sequence of AC-1 was different from that of type 4 by 1 bp (A → G) at nucleotide position, 128 bp, which indicates intraspecific variation. All 7 samples labelled as "AJB" as well as 5 samples labelled as "AJC" were determined as *A. japonica*. AC-13 and AJB-5 were different form that of type 1 by 1 bp (G → A), at nucleotide position, 80 bp. AJC-2, AJC-3, and AJC-5 have 2 different nucleotide positions at 80 bp (G → A) and 481 bp (C → T), showing intraspecific variation.

*3.2. Genetic Relationship of the Atractylodes.* Nine dried-voucher specimens were confirmed as *A. japonica, A. lancea*, and *A. macrocephala*. Thirty-four samples of *Atractylodes* rhizomes were identified as *A. chinensis*, *A. japonica*, and *A. macrocephala*. Phylogenetic classification based on ITS region was constructed and the inferred evolutionary relationships among *Atractylodes* rhizomes were represented on phylogenetic tree. The genus *Atractylodes* is well separated from other close genera and out-groups. The samples of *A. japonica*, *A. lancea*, and *A. chinensis* formed *A. lancea* complex, whereas those of *A. macrocephala* formed their own complex, namely, *A. macrocephala* complex. Five AC samples, AC-2, AC-4, AC-5, AC-6, and AC-11, were involved in *A. chinensis* group. AJB, AJC samples, and the rest AC samples were contained in *A. japonica* group (Figure 2).

Comparing the genotypes listed in Figures 1 and 2 and Table 4, the species of 34 samples in Table 1 of *Atractylodes* rhizomes were recoded as shown in Table 5.

*3.3. HPLC Fingerprinting of Atractylodes Samples.* Through macroscopic observation of the results of HPLC fingerprinting, the chromatograms of *A. japonica* samples (AJBs + AJCs + AJs) and *A. chinensis* samples (ACs) showed peak patterns similar to each other, whereas the chromatograms of *A. macrocephala* samples (AMs) represented different peak patterns compared with those of *A. japonica* and *A. chinensis* samples. Moreover, chromatographic difference between Baekchul samples (AJBs) and Changchul samples (AJCs) was not apparently distinguished (Figures 3 and 4).

*3.4. Chemometric Statistical Analysis of Atractylodes Samples.* The results of the chromatographic fingerprinting were further analyzed using principle component analysis (PCA), hierarchical clustering analysis (HCA), and Pearson's correlation analysis to evaluate the correlations between the samples whose code names were redetermined by genetic identification as listed in Table 5.

Principle component 1 (PC1) score divided the samples of "*A. japonica* + *A. chinensis*" group in the positive plot with exception of AC-6, AJ-9, and AJC-5, while "*A. macrocephala*" group was in the negative plot. PC2 score further differentiated the samples having positive PC1 scores into positive PC2 plot (AC-1, AC-3, AC-5, AJ-1, AJ-2, AJ-6, AJ-7, AJ-8, AJB-2, AJB-3, AJB-7, AJC-1, and AJC-2) and negative PC2 plot (AC-2, AC-4, AJ-3, AJ-4, AJ-5, AJB-1, AJB-4, AJB-5, AJB-6, AJC-3, and AJC-4) and samples having negative PC1 scores into positive PC2 (AM-1, AM-5, AM-6, and AM-7) and negative PC2 (AM-2, AM-3, and AM-4) (Figure 5).

HCA results also showed similar result obtained from PCA. Below the height of 30000, while the samples of *A. macrocephala* formed their own clustering, those of *A. japonica* and *A. chinensis* were gathered together, where *A. japonica* samples and *A. chinensis* samples were mixed. Therefore, apparent discrimination between *A. japonica* samples and *A. chinensis* samples was not observed. Moreover, as seen in PCA score plot, AJB and AJC samples were not clearly distinguished in HCA dendrogram, as they were grouped undistinguishably within the same levels (Figure 6).

Box plot of average Pearson's correlation coefficient ($r$) showed two distinct sample groups, the group of *A. macrocephala* samples and the group of *A. japonica* and *A. chinensis* samples. The average coefficients of *A. macrocephala* samples

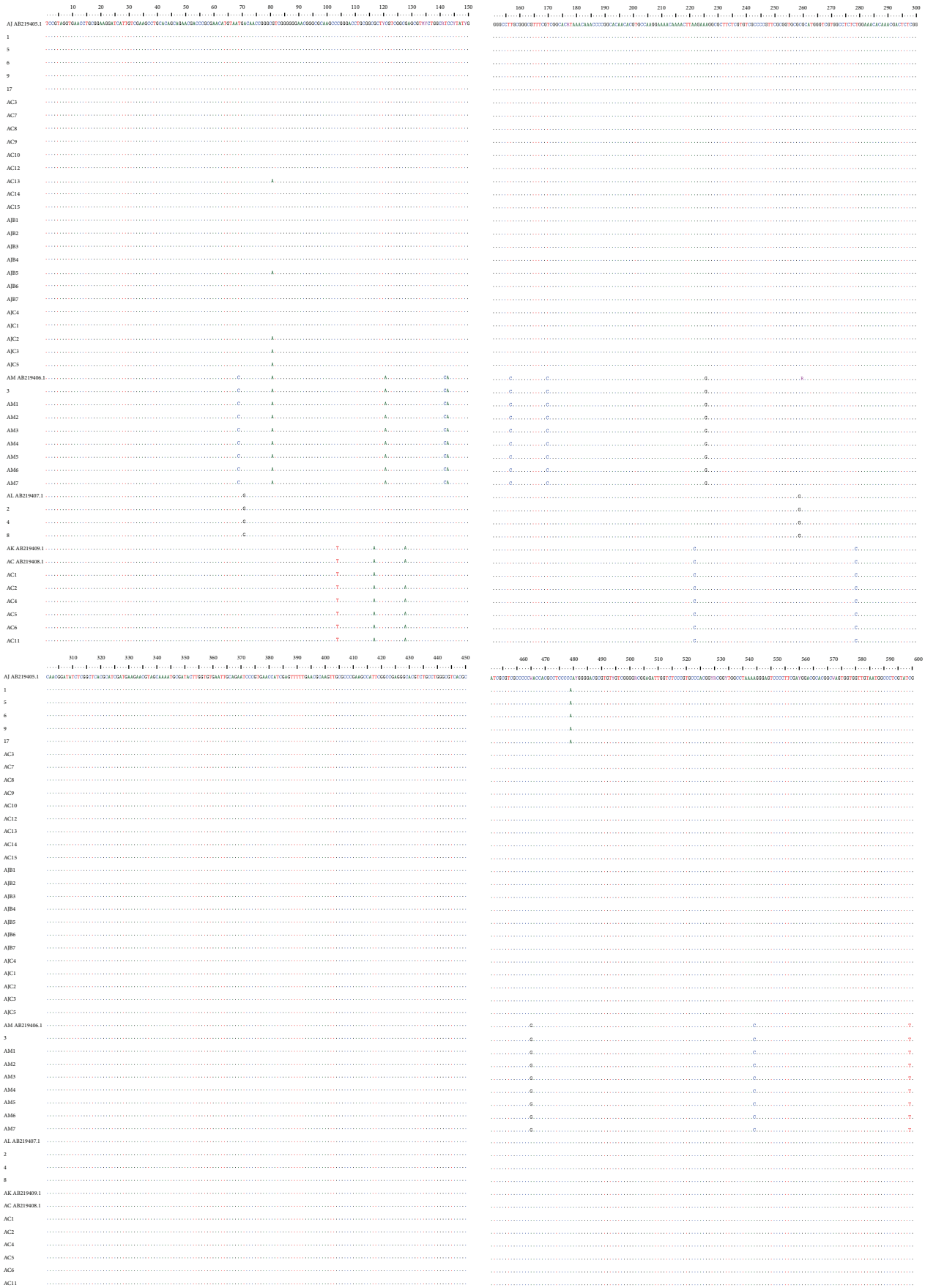

Figure 1: Continued.

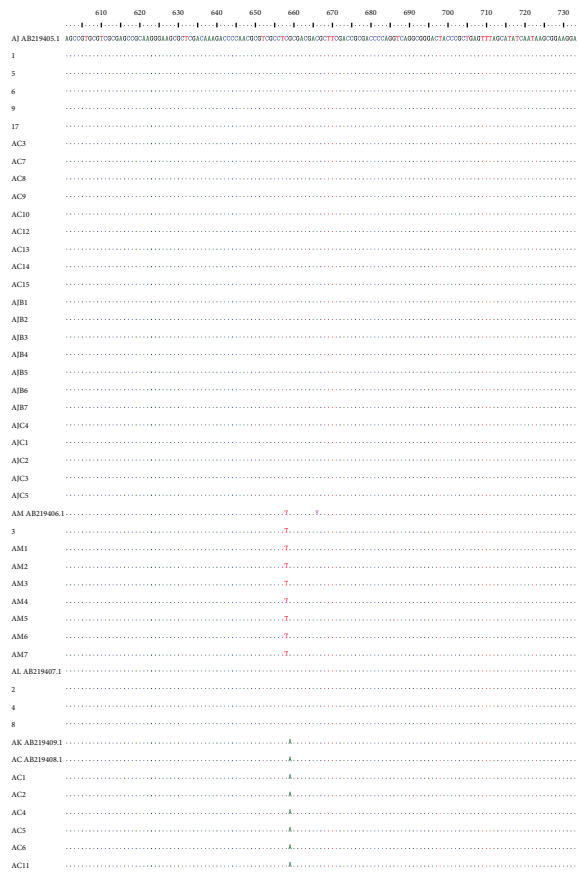


Figure 1: Multiple alignments of ITS nucleotide sequence among the sample listed in Table 1. The dots indicate the consensus nucleotide, and the dashes represent the gaps. AB219405.1 accession numbers of NCBI GenBank for the nucleotide sequences of the ITS for *Atractylodes japonica*; AB219409.1 and AB210407.1 accession numbers of NCBI GenBank for the nucleotide sequences of the ITS for *A. koreana* and *A. lancea*, respectively; AB219406.1 accession number of NCBI GenBank for the nucleotide sequence of ITS for *A. macrocephala*; and AB219408.1 accession number of NCBI GenBank for the nucleotide sequence of ITS for *A. chinensis*. The samples with Arabic number were dried-voucher specimens deposited in the Korea Institute of Oriental Medicine.

ranged from −0.2 to 0.0, whereas those of *A. japonica* and *A. chinensis* samples ranged from 0.5 to 1.0, except for AJ-9 showing the average coefficient between 0.0 and 0.2. Furthermore, AJB and AJC samples showed similar values of average Pearson's coefficients (Figure 7).

## 4. Discussion

Discrimination of herbal medicines according to their therapeutic effect is most important in using herbal medicines as therapeutic agents. To achieve such purpose, correct classification of herbal medicines should be preceded at their species levels. The rhizome of *A. japonica* has been at the center of controversy as it is differently classified in many countries; Korea and Japan have used that as "Baekchul," whereas China has used that as "Changchul." Furthermore, the rhizome of *A. japonica* is further divided into "Baekchul" and "Changchul" by the age; tuberous part grown for less than a year is used as Baekchul, while fibrous part grown for more than two years is used as Changchul. Hence, we classified *Atractylodes* samples which were genetically identified by ITS genotypes using analytical tools combined with chemometric statistics, in order to propose correct categorization of *Atractylodes* rhizomes, especially *A. japonica* rhizomes.

*4.1. Genetic Identification of Atractylodes Samples.* *Atractylodes*, *Atractylis,* and *Carlina* belong to subtribe Carlininae [28]. ITS sequence of *A. japonica* showing multiple sequences indicates that there are diverse intraspecific variations in *A. japonica* samples. The genotype of *A. chinensis* was identical to that of *A. koreana*. As the distribution of *A. koreana* is limited in Shandong and Liaoning area, *A. koreana* has been possibly derived from *A. chinensis* [14, 17]. The samples of *A. japonica*, *A. lancea*, and *A. chinensis* formed *A. lancea* complex, in accordance with the previous research [17]. *A. japonica* is most closely related to *A. lancea* and next related to *A. chinensis*, as suggested in previous researches [17, 30, 31]. The samples identified as *A. japonica* were obviously separated from those identified as *A. macrocephala* by DNA sequencing analysis [14, 16, 32]. According to the results,

Table 4: Genotypes of ITS nucleotide sequences deposited in the NCBI GenBank for recognition in the *Atractylodes* species.

| Nucleotide position | 69 | 71 | 81 | 104 | 117 | 120 | 121 | 128 | 134 | 135 | 141 | 142 | 143 | 145 | 149 | 157 | 170 | 177 | 222 | 226 | 259 | 260 | 279 | 465 | 481 | 494 | 502 | 529 | 530 | 534 | 544 | 560 | 572 | 599 | 658 | 659 | 666 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotype | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Type 1 | T | A | G | C | G | **Y** | C | G | **M** | **Y** | **R** | T | C | Y | Y | T | T | R | A | A | C | G | T | **W** | **Y** | **Y** | **R** | **Y** | **R** | **Y** | A | **Y** | **W** | C | C | G | C |
| Type 2 | C | A | A | C | G | C | A | G | C | C | G | C | A | C | C | C | C | G | A | G | C | **R** | T | G | C | C | G | C | G | C | C | C | T | T | T | G | **Y** |
| Type 3 | T | G | G | C | G | C | C | G | C | C | G | T | C | C | C | T | T | A | A | A | G | G | T | A | C | C | G | T | G | C | A | C | A | C | C | G | C |
| Type 4 | T | A | G | T | A | C | C | A | C | C | G | T | C | C | T | T | T | G | C | A | C | G | C | T | C | C | G | C | G | C | A | C | T | C | C | A | C |
| Type 5 | T | A | G | T | A | C | C | A | C | C | G | T | C | C | T | T | T | G | C | A | C | G | C | T | C | C | G | C | G | C | A | C | T | C | C | A | C |

*Bold characters indicate nucleotide additives, M = A and C; R = A and G; W = A and T; Y = C and T. Type 1 genotype resulted from deposited ITS nucleotide sequences in NCBI Genbank of *Atractylodes japonica*. Type 2 and type 3 represent *A. macrocephala* and *A. lancea*. Type 4 and type 5 represent *A. chinensis* and *A. koreana*.

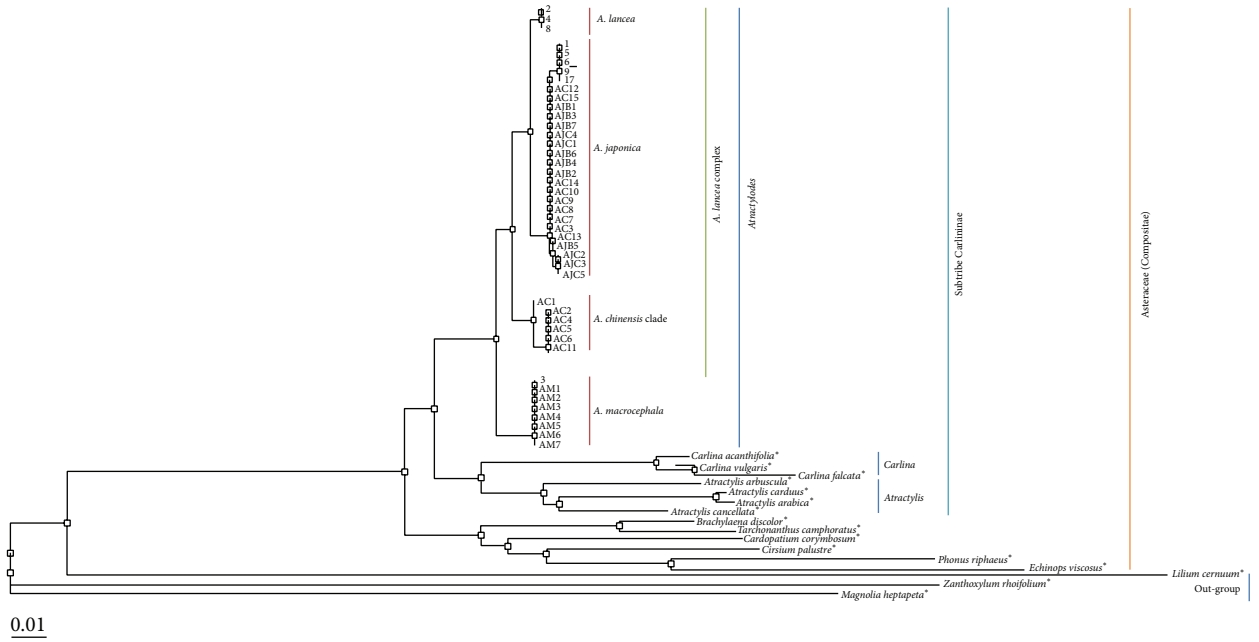


Figure 2: Phylogenic tree from DNADist (Neighbor phylogenetic tree) analysis of the ITS nucleotide sequences. The ITS sequences of taxa with "∗" such as *Atactylis*, *Carlina*, and out-group were downloaded from Genbank in NCBI. The samples with Arabic number were dried-voucher specimens deposited in the Korea Institute of Oriental Medicine.

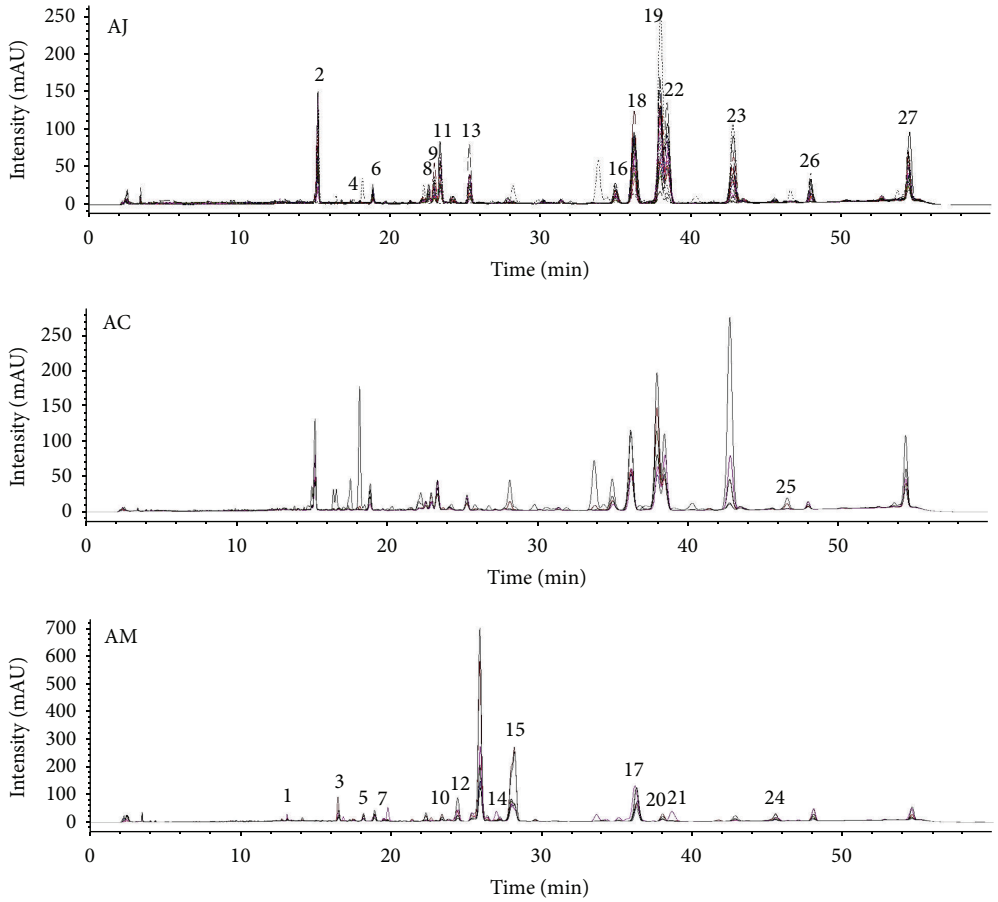


Figure 3: Representative chromatograms of the methanol extracts of AC, AJ, and AM at 255 nm. AC: *A. chinensis* Koidz.; AJ: *A. japonica* Koidz.; AM: *A. macrocephala* Koidz.

Table 5: Genetic identification of original species of *Atractylodes* rhizomes listed in Table 1.

| Code | Genetically identified species | Recode | Code | Genetically identified species | Recode |
|---|---|---|---|---|---|
| AC-1 | *A. chinensis* | AC-1 | AJB-3 | *A. japonica* | AJB-3 |
| AC-2 | *A. chinensis* | AC-2 | AJB-4 | *A. japonica* | AJB-4 |
| AC-3 | *A. japonica* | AJ-1 | AJB-5 | *A. japonica* | AJB-5 |
| AC-4 | *A. chinensis* | AC-3 | AJB-6 | *A. japonica* | AJB-6 |
| AC-5 | *A. chinensis* | AC-4 | AJB-7 | *A. japonica* | AJB-7 |
| AC-6 | *A. chinensis* | AC-5 | AJC-1 | *A. japonica* | AJC-1 |
| AC-7 | *A. japonica* | AJ-2 | AJC-2 | *A. japonica* | AJC-2 |
| AC-8 | *A. japonica* | AJ-3 | AJC-3 | *A. japonica* | AJC-3 |
| AC-9 | *A. japonica* | AJ-4 | AJC-4 | *A. japonica* | AJC-4 |
| AC-10 | *A. japonica* | AJ-5 | AJC-5 | *A. japonica* | AJC-5 |
| AC-11 | *A. chinensis* | AC-6 | AM-1 | *A. macrocephala* | AM-1 |
| AC-12 | *A. japonica* | AJ-6 | AM-2 | *A. macrocephala* | AM-2 |
| AC-13 | *A. japonica* | AJ-7 | AM-3 | *A. macrocephala* | AM-3 |
| AC-14 | *A. japonica* | AJ-8 | AM-4 | *A. macrocephala* | AM-4 |
| AC-15 | *A. japonica* | AJ-9 | AM-5 | *A. macrocephala* | AM-5 |
| AJB-1 | *A. japonica* | AJB-1 | AM-6 | *A. macrocephala* | AM-6 |
| AJB-2 | *A. japonica* | AJB-2 | AM-7 | *A. macrocephala* | AM-7 |

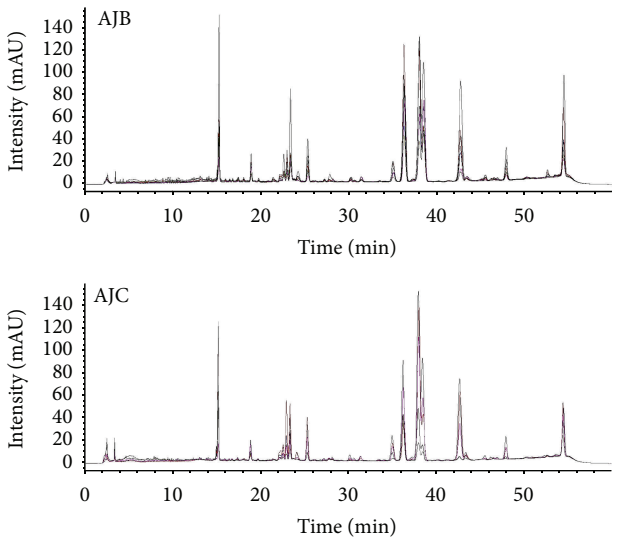


Figure 4: Representative chromatograms of the methanol extracts of AJB and AJC at 255 nm. AJB and AJC: *A. japonica* Koidz.

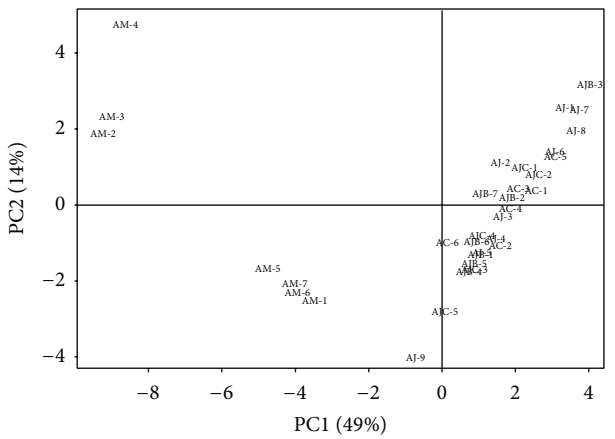


Figure 5: Score plot of principal components (PC1 versus PC2) on the variables (absolute area of reference peaks) with *Atractylodes* samples. PC1 and PC2 represent 49% and 14% of the total variance, respectively. AC: *A. chinensis* Koidz.; AJ, AJB, and AJC: *A. japonica* Koidz.; AM: *A. macrocephala* Koidz.

there was difference in classification between morphological identification and genetic identification which might be caused by morphological similarity.

*4.2. Classification of Atractylodes Samples Using Chemometric Analysis.* Principal components analysis (PCA) was performed for the clustering of the samples and for investigating the relationships among the samples using principal components on the PCA plot. Principal component 1 (PC1) explains most of the variance, and PC2, which is orthogonal to PC1, represents most of the variance not explained by PC1 [33]. HCA is another clustering method which classifies similar objects mathematically into the same group and the classifications were represented as tree diagram called dendrogram [34]. Pearson's correlation coefficient of each sample was calculated to evaluate the correlations between samples ($-1 \leq r \leq +1$; the closer the $r$ is to 1, the more correlated the two fingerprints are) [35].

From the results of PCA, HCA, and Pearson's correlation analysis, the *A. japonica* samples were obviously distinct from *A. macrocephala* samples but distributed close to the *A. chinensis* samples without apparent separation of AJBs and AJCs samples. These results indicate that *A. japonica* is chemically different from *A. macrocephala*; however, the crude drugs derived from *A. japonica* and *A. chinensis* were not clearly distinguishable by their chemical components. These results indicate that the therapeutic effects of *A. japonica* and

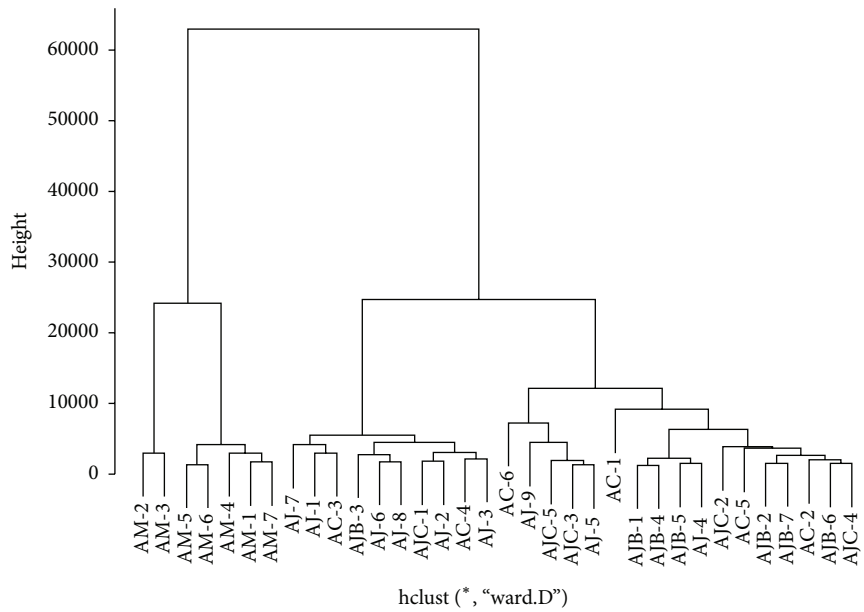


Figure 6: Hierarchical clustering analysis of *Atractylodes* samples. AC: *A. chinensis* Koidz.; AJ, AJB, and AJC: *A. japonica* Koidz.; AM: *A. macrocephala* Koidz.

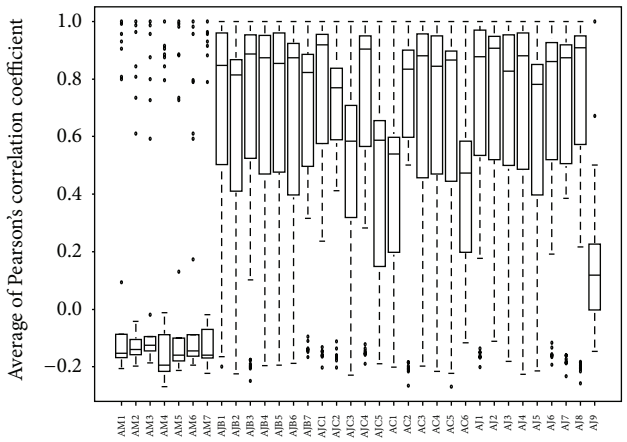


Figure 7: Average coefficients of Pearson's correlation of 34 *Atractylodes* samples. AC: *A. chinensis* Koidz.; AJ, AJB, and AJC: *A. japonica* Koidz.; AM: *A. macrocephala* Koidz.

*A. chinensis* whose effects can be exerted by the chemical components are thought to be presumably analogous [36], although they are genetically different. Moreover, the separation of *A. japonica* rhizomes as "Baekchul" and "Changchul" is not recommended because they had the closest PC scores, equal levels of hierarchies, and similar Pearson's coefficients [37]; therefore, AJBs and AJCs can be assumed to be the same medicinal parts.

*4.3. Assured Medicinal Categorization of Atractylodes japonica Rhizomes.* The categorization of *A. japonica* as Baekchul in Korea and Japan, therefore, should be removed, because its relationship to *A. macrocephala*, the only species regarded as Baekchul in China, is not close, which supports the results from previous studies [14, 17, 32, 38]. Moreover, we do not recommend the segregation of *A. japonica* into Baekchul and Changchul, because the segregated crude drugs of *A. japonica* are not distinguishable by their chemical and genetic characteristics. This suggests that there is the lack of chemical or genetic reason to categorize the rhizome of *A. japonica* as Changchul and Baekchul based on plant age.

Therefore, we propose that *A. japonica* should be separated from *A. macrocephala* and should be used as "Changchul" as the Chinese literatures define it, regardless of its age. Further biological and clinical experiments are necessary to confirm the results of the present study.

In conclusion, the 34 crude drugs derived from *Atractylodes* rhizomes were identified using ITS sequencing analysis and classified using HPLC fingerprinting and statistical tools such as PCA, HCA, and Pearson's correlation analysis. The macroscopically authenticated *Atractylodes* samples were genetically identified as *A. japonica*, *A. chinensis*, and *A. macrocephala* by ITS DNA sequencing. The hyphenated HPLC fingerprinting and statistical analyses showed that the chemical components of *A. japonica* were not related to those of *A. macrocephala* but related to those of *A. chinensis*. Moreover, the rhizome of *A. japonica* could not be segregated as Baekchul and Changchul by its age. The results from these chemical and genetic analyses demonstrate that *A. japonica* should be classified as "Changchul" and should not be classified by plant age.

## Disclosure

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

## Competing Interests

The authors declare that they have no competing interests.

## Authors' Contributions

J.-H. Kim and E.-J. Doh contributed equally to this work.

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*Research Article*

# Ultrastructure of a Mobile Threadlike Tissue Floating in a Lymph Vessel

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Observations of the primo vascular system (PVS) floating in lymph ducts were reported by various groups. There have been, however, no studies on the ultrastructure of the entire cross section of a primo vessel (PV) inside a lymph vessel with a transmission electron microscope (TEM). In the current study we took the TEM images of a cross section of the PV inside a lymph vessel. We used the Alcian blue staining method for the finding of the target PV in a lymphatic vessel by injecting the dye into the inguinal lymph nodes. The stained PV was harvested together with the lymph vessel and some parts of the specimens were used for studying with optical microscopes. Some other parts were treated according to a standard protocol for TEM. As the results the TEM study revealed the loosely distributed collagen fibers with plenty of empty spaces and the lumens with the endothelial nuclei. It turned out to be very similar to the ultrastructure of the PVs observed on the surfaces of internal organs. It also showed how compactly the PV is surrounded with lymphocytes. In conclusion, the detailed morphological features like the distribution of fibers in the PV were revealed and shown to be similar to another kind of the PV on the surfaces of internal organs.

## 1. Introduction

Korea has practiced acupuncture for thousands of years and made many original contributions. For instance, Saam acupuncture was developed by Saam several hundred years ago [1], and the "Constitutional Medicine" by JM Lee was introduced more than a hundred years ago [2]. More recently, in the early 1960s, Kim claimed that his team discovered the anatomical structure of acupuncture meridians as a circulating system which governs the regeneration of damaged and aged tissues and organs [3]. This system has been revived since 2002 and renamed as the primo vascular system (PVS) [4] and its relation to acupuncture is to be established even though relevant data are being accumulated [5].

The medical significance of the PVS besides the relation to acupuncture is steadily increasing. For example, the abundance of mast cells and other innate immune cells in the PVS [6] suggests a major role of the PVS in the innate immune functions. The regeneration function of the PVS that Kim claimed and emphasized [3] was also strongly supported by the discovery of the abundance of embryonic-like stem cells in the PVS [7].

The PVS in which stem cells were found were harvested from the blood vessels and lymph vessels. The primo vessel (PV) afloat in the lymph flow as a mobile threadlike structure is hard to observe without a suitable staining. Most frequently used staining dye was Alcian blue [8], and recently hollow gold nanoparticles were found to be more effective for

visualizing purpose [9]. Although numerous articles on the observation of the lymphatic PVS were reported, there has not been a thorough study of the ultrastructure of the PV with transmission electron microscope (TEM). A previous TEM study of a lymphatic PV showed only part of the PV cross section in comparison with a lymph vessel [10]. Therefore the detailed histological characterization of the PV has not been given in comparison with the lymphocytes that surround the PV. In this paper we present the whole view of the cross section of the PV compactly surrounded by lymphocytes so that the characteristic histological difference of the PV from the lymphocytes can be seen despite their similar sizes. Consequently, the current work firmly establishes the existence of the PV by providing firm data showing its characteristic ultrastructure.

## 2. Materials and Methods

*2.1. Animals.* Rats (Sprague-Dawley, male, 7 weeks old, 210~230 g) were obtained from DooYeol Biotech (Seoul, Republic of Korea) and housed in a temperature-controlled environment (23°C). All animals were exposed to a 12-hour light-dark cycle and were provided food and water *ad libitum*. The procedures involving the animals and their care were in full compliance with current international laws and policies (*Guide for the Care and Use of Laboratory Animals*, National Academy Press, 1996) and were approved by the Institutional Ethics Committee of the Advanced Institute of Convergence Technology (Approval number: WJIACUC20150804-3-04). The rats were anesthetized by intramuscular injection of a regimen consisting of 1.5 g/kg of urethane and 20 mg/kg of xylazine.

*2.2. Visualization and Observation of the Primo Vascular System.* Rats were injected with Alcian blue, and the 1.0% Alcian blue (A5268, Sigma-Aldrich, St. Louis, MO, USA) solution in boiled phosphate-buffered saline (PBS, pH 7.4) was filtered by using a 0.22 $\mu$m membrane filter (Merck Millipore, Darmstadt, Germany). After the inguinal node had been exposed, the prepared 1.0% Alcian blue dye was injected into the node. The lymph duct from the inguinal node to the axillary node was exposed to observe the PV in it. After the rats were sacrificed by using an intracardiac injection of urethane (1 mL), the lymph duct, including the PV, was harvested for histological study.

To confirm the distribution of rod-shaped nuclei with 4′,6-diamidino-2-phenylindole (DAPI), the specimen was stained with 300 nM DAPI (D1306, Invitrogen, MO, USA) solution for 20 minutes. The shape and distribution of nuclei were examined under a phase contrast microscope (Olympus, U-LH100HG, Japan) and a confocal laser scanning microscope (CLSM; C1 plus, Nikon, Japan).

For the observations with TEM, the specimens of the PV within a lymph vessel were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer for overnight. After washing in 0.1 M phosphate buffer, the specimens were postfixed with 1% osmium tetroxide in the same buffer for 1 hr. The specimens were dehydrated with a series of the graded ethyl alcohol, and pure acetone. The specimen was embedded in Epon 812 and the polymerization was performed at 60°C for 3 days. The cross sections of the PV inside a lymph vessel were first studied with optical microscope images of the semithin sections (1 $\mu$m) of the specimen before taking ultrathin sections (60–70 nm) for TEM. These semithin section images helped us to find the appropriate location for TEM. Ultrathin sections (60–70 nm) were obtained by ultramicrotome (Leica Ultracut UCT, Germany). Ultrathin sections collected on grids (200 mesh) were examined in TEM (JEM 1010, Japan) operating at 60 kV and images in the TEM were recorded by CCD camera (SC1000, Gatan, USA). Length on the electron micrograph was measured using GMS software (Gatan, USA).

## 3. Results

The lymph vessel we studied was the one starting from the inguinal node, running along the epigastric blood vessel in the skin and entering the axillary node (Figure 1(a)). The PV in this lymph vessel was observed *in vivo in situ* as a mobile floating threadlike structure stained blue with Alcian blue (Figure 1(b)).

The lymph vessel with the PV inside was harvested as shown in Figure 2(a). A piece of PV was extracted from the lymph vessel (Figure 2(b)) and stained with DAPI for studying nuclei distribution in the PV. It was examined with a confocal microscope. As shown in the cross section view below the PV was not pure but rather thickly shrouded with lymphocytes; consequently the nuclei seen in the surface of the apparent threadlike structure had round shapes. The optical sections of five $\mu$m thickness revealed the characteristic rod-shaped nuclei at the 15 $\mu$m depth from the surface of the extracted specimen whose thickness was 23 $\mu$m. It means the PV was located somewhat off-centered in the harvested specimen of the stained threadlike structure. The lengths of the rod-shaped nuclei were about 10 $\mu$m (Figure 2(c)).

A piece of the harvested lymph vessel was embedded in OCT and its micro section was examined after defreezing (Figure 3(a)). The Alcian blue stained threadlike structure containing the PV is clearly isolated from the lymph vessel which looked nearly void of lymphocytes. But another section we took for the TEM study was full of lymphocytes as shown in the toluidine blue stained semithin section (Figure 3(c)). Among the deep dark blue stained lymphocytes in the lymph vessel laid the light blue stained PV. Thanks to the precise positioning of the PV in the semithin section we were able to identify the PV in the TEM images (Figure 3(c)) which is a mosaic of several photographs to cover the whole lymph vessel. The PV is compactly surrounded by lymphocytes and is hard to recognize it unless its location is given by other means. The confounding factors are the small size of the PV and the thinness and softness of the surrounding membrane of the PV which makes the distinction of the PV from the adhered materials. Figure 3(d) shows the PV with its two endothelial nuclei and loosely scattered distribution of collagen fibers with many empty spaces.

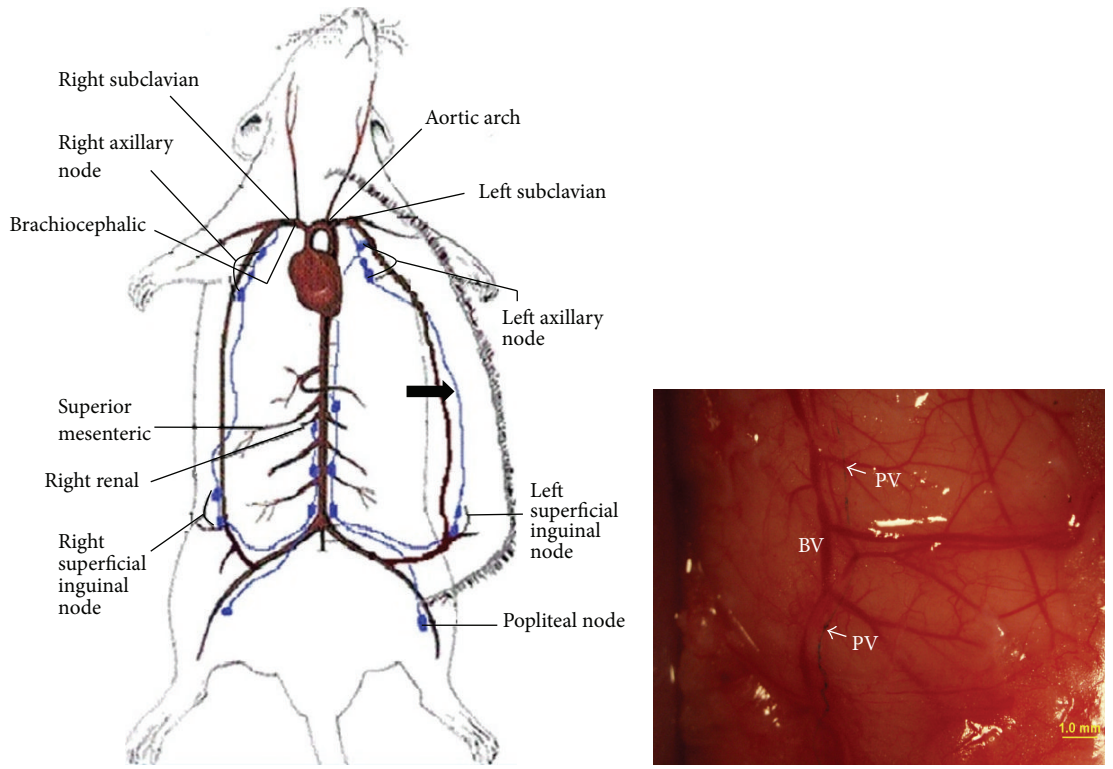


Figure 1: Stereomicroscopic images of lymph ducts in which a PVS was stained with Alcian blue. (a) Illustration of the locations of the lymph nodes and ducts along the epigastric blood vessels (thick arrow) in skin. (b) The blue stained primo vessel (PV) in the lymph duct along the blood vessel (BV) is indicated with arrows. The lymph vessel is hardly visible in a stereomicroscopic image.

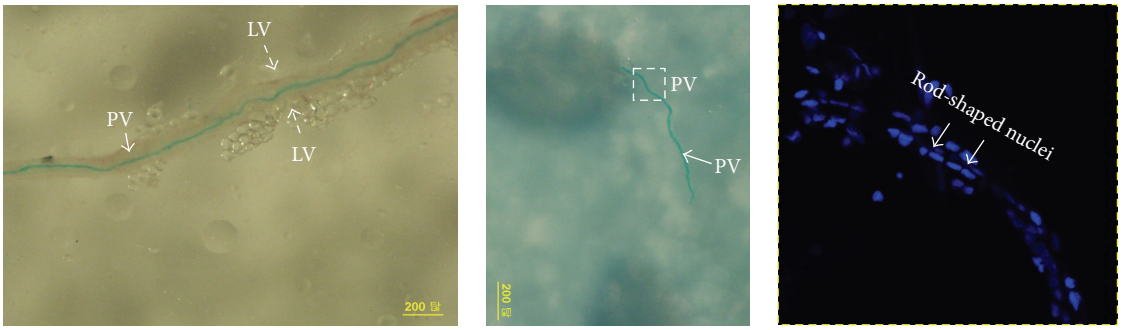


Figure 2: Images of a harvested lymph duct in which a PV was stained with Alcian blue. (a) A piece of the lymph vessel (LV) was harvested and image was taken with a phase contrast microscope. The PV is clearly seen due to the Alcian blue staining. (b) The stained PV was extracted from the lymph vessel with a forceps. It was treated with DAPI to stain its nuclei. (c) The boxed region of the PV in (b) was examined with a confocal microscope. The rod-shaped nuclei of the endothelial cells of the PV were observed at the 15 $\mu$m depth from the surface of the stained threadlike structure whose thickness was 23 $\mu$m. The PV became thicker because it was covered with lymphocytes.

## 4. Discussion

The PV inside lymph vessel has been identified through several stages of examinations: The PV stained blue with Alcian blue *in vivo in situ* is to stay afloat unbroken and not crushed to pieces when the lymph vessel is pushed and moved by a forceps. When the lymph vessel is harvested the PV should be strong enough to be extracted through a hole by pulling one end of the PV with forceps. Most critically, the PV after staining with DAPI should show the presence of the rod-shaped nuclei distributed in broken lines along the direction of the lymph vessel, which can be seen with a confocal microscope. Figures 1(b), 2(a), 2(b), and 2(c) show that the specimen we studied passed these identification criteria.

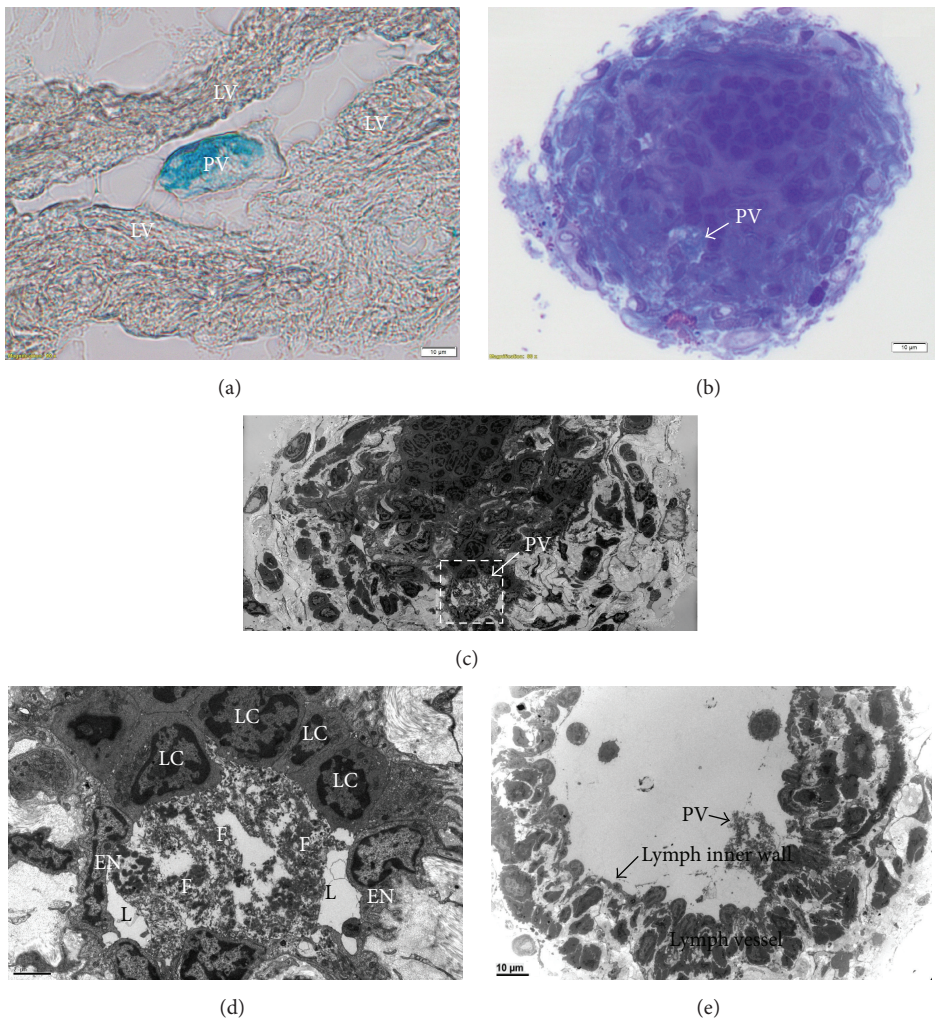


Figure 3: Cross-sectional images of a PV in the harvested lymph duct. (a) A cross section of the lymph duct (LV) with a blue stained PV in it. This specimen was frozen in OCT and the image was taken with a phase contrast microscope. This part of the lymph duct contained the PV without surrounding lymphocytes. (b) The semithin section of the toluidine blue stained lymph duct which were full of lymphocytes. The PV was located below from the center of the lymph duct and it was somewhat light blue colored because of the Alcian blue. This image helped us to find the PV among the lymphocytes. This semithin image is necessary for pointing the precise location of the PV, which is in turn helpful to apply TEM study. (c) The mosaic of the TEM images of the lymph duct which was full of lymphocytes. The location of the PV was identified with the aid of the above toluidine image. The PV showed the loose distribution of collagen fibers. (d) The boxed region of (c) is magnified to show the details of the PV. Two endothelial cell nuclei (EN) are seen along the lumens (L) which are different from the nuclei of the surrounding lymphocytes (LC). The collagen fibers (F) are extracellular material filling most of the PV. (e) A lymph vessel in which a PV was found without surrounding lymphocytes. The PV has similar distribution of fibers and lumens as (d).

The main contribution of the present work was to show the ultrastructure of the PV in the lymph vessel with TEM images. Although the TEM images of the lymphatic PV were previously taken they were only small parts of the PV and lymph vessel [10]. This time we took the whole cross-sectional view of the containing lymph vessel and the PV. It showed the characteristics loose distribution of the collagen fiber with many empty spaces, which was in good agreement with the previous work of the lymphatic PV [10] and the PV on the organ surfaces [11]. The thin surrounding membranes of the PV are another agreeing feature which is very thin as Kim described [3]. In addition, it revealed the endothelial nuclei and the lumens as shown in Figures 3(c) and 3(d). This is the most important histological property that could be examined with TEM. This detailed ultrastructural characteristic can only be seen with TEM and is necessary to identify the PV in a complicated situation incurred by the accumulation of lymphocytes. Without the help of the semithin section stained with toluidine blue in Figure 3(b) it would very difficult to locate the position of the PV.

The medical significance of the PVS is still to be investigated but data have already been accumulated showing its possible roles in innate immunity suggested by the abundant immune cells like mast cells in the PVS [6, 11]. There are also data showing the role as a source of very small stem cells [7], a path for metastasis [12], and a haven for cancer stem cells [13].

In conclusion, the study of the ultrastructural morphology of the lymphatic PV showed its detailed structure enough to identify the PV among the complicated situation when compactly surrounded with lymphocytes. This also explains how difficult it is to find the PV without careful and intentional search for it with TEM.

## Conflict of Interests

The authors listed above declare no conflict of interests.

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