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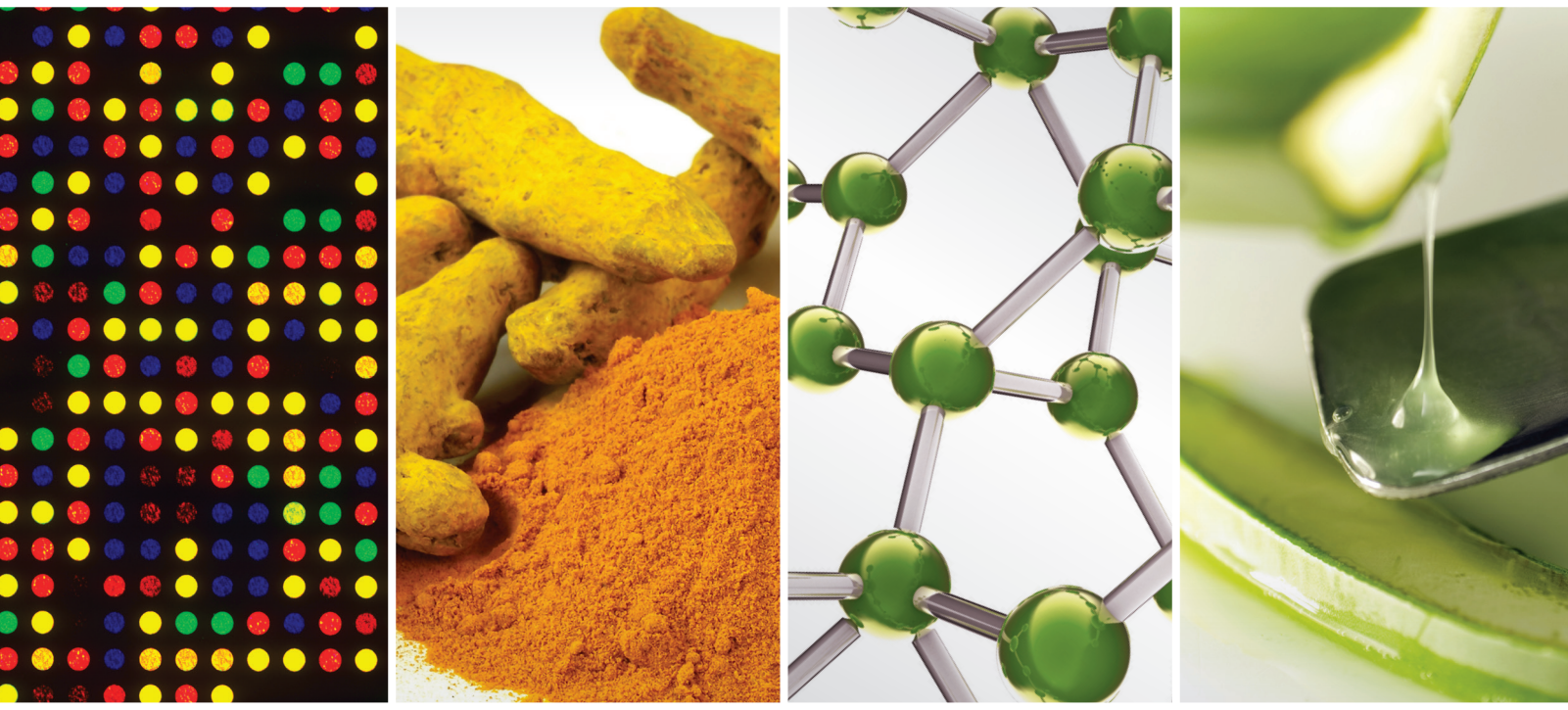
Scientific Evidence for Korean Medicine and Its Integrative Medical Research

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



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Editorial Board

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 967087, 2 pages
http://dx.doi.org/10.1155/2015/967087

Editorial

Scientific Evidence for Korean Medicine and Its Integrative Medical Research

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Received 13 October 2015; Accepted 13 October 2015

The Korean Medicine (KM) incorporates same clinical techniques as East Asian traditional medicine; however it also has typical characteristics such as Sasang typology, Saam acupuncture, Chuna therapy, pharmacopuncture, and Korean psychotherapy. Modernized KM currently utilizes cutting-edge techniques of orthodox medicine, though it is rooted on the *Donguibogam*, one of the best Korean medical classics written in 1610 and enlisted on the Memory of the World by UNESCO in 2009. In 2013, the National Health Insurance of Korea paid about 2 billion dollars for medical services provided by 18,000 KM doctors in 210 KM hospitals and 13,200 KM local clinics. The role of KM in health service is expected to expand since Korea is anticipated to be an aged society in 2018 and hyperaged society in 2028 which means increased chronic disease patients. Thus the evaluation of efficacy and safety of KM and improvement of its clinical skills have been emphasized recently.

Our special issue, which had opened for 6 months in the first half of 2015, focused on scientific evidence for KM and its integrative medical research.

An article by C. Na et al. described that Jakyak-Gamcho decoction extracted with 70% ethanol exhibits higher amounts of effective index components than that extracted with water; it may be worthwhile to investigate alternative extraction methods in terms of extraction efficiency and *in vivo* effectiveness for other herbal medicines besides Jakyak-Gamcho decoction. A study by D.-S. Hwang et al. evaluated that Kyung-Ok-Ko significantly protects against heat-induced damage to testicular function in male mice by inhibiting oxidative stress and apoptosis. B. Joh et al. described that *Morus alba* treatment of infertility, jaundice, cognitive disorder, and hyperpigmentation is found to be effective and diabetes with *Morus alba* is recognized to have clinical importance. An interesting study by S. J. Lee et al. elucidated the biopsychological mechanism underlying the Sasang typology (a traditional Korean personalized medicine) using Behavioral Inhibition System (BIS)/Behavioral Activation System (BAS) scale. S. J. Lee et al. reported significant differences in BIS and BAS scores between So-Yang and So-Eum Sasang types.

K. Lee and B.-J. Lee reported the exact plant origins, efficacies, uses, components, and toxicities of Polygoni Multiflori Radix, Cynanchi Wilfordii Radix, and Cynanchi Auriculati Radix so that they can be correctly understood and used. A study by D. R. Kim et al. reported that Trigonellae semen treatment could enhance sperm function by promoting spermatogenesis and the expression of cation channel of sperm proteins in mouse testes. J.-H. Hwang et al. reported that intratracheal Chung-pae administration effectively decreases the chronic inflammation and pathological changes in a porcine pancreatic elastase- and lipopolysaccharide-induced chronic obstructive pulmonary disease mouse model. An interesting study by H.-G. Kim et al. suggested that one can find the Bonghan systems under the skin as putative acupuncture points by tracing the intraexternal Bonghan systems, from which a new KM will be born.

T.-H. Kim et al. suggested a recommendation for reporting cases of acupuncture-related infections. The recommendation includes items on patient's condition and adverse events (or complications) in detail, which are necessary to establish the causality between acupuncture and the event as well as to provide information for judging appropriateness of acupuncture practice. A study by M.-H. So and Y.-K. Choi reported that the water extracts of Scutellariae radix and Liriopis Tube significantly suppressed the increased production of nitric oxide, interleukin-6, macrophage inflammatory protein-1α, macrophage inflammatory protein-1ß, macrophage inflammatory protein-2, and granulocyte colony-stimulating factor as well as the increase of the intracellular free calcium in mouse macrophages induced by lipopolysaccharide. A research article by J. S. Ha et al. reported that the ethyl acetate fraction from *Actinidia arguta* containing physiological phenolics might enhance drug-induced amnesia through acetylcholinesterase inhibition and neuroprotection.

Another study by W.-M. Jung et al. demonstrated that the indications of each acupoint were primarily associated with the corresponding meridian system, using data mining methods to analyze the characteristics of the indications of each acupoint and to visualize the relationships between the acupoints and disease sites in the classic KM text *Chimgoogyeongheombang*. A study by J. Y. Park et al. suggested that *Artemisia asiatica* extract and eupatilin could cure or prevent cisplatin-induced renal toxicity without any adverse effect; *Artemisia asiatica* extract can be used in combination with cisplatin to prevent nephrotoxicity. An interesting review article by M. Park and S. Kim reported that Sa-am acupuncture, which operates with five shu points as a main aspect of treatment, has the advantage of increasing parasympathetic nerve activity and adjusting the balance of the autonomic nervous system; to maximize this effect, inserting a needle into the skin layer and providing gentle and light stimulation while considering the respiratory phase may be desirable.

A research article by K. Kim et al. reported that KM combination therapy may be beneficial for decreasing pain and improving function in lumbar spinal stenosis patients and may produce comparatively few adverse events. Another study by J. W. Suh et al. reported that the Emotional Freedom Technique, a meridian-based psychological therapy that alleviates psychologic and psychosomatic conditions by applying tapping stimulations at certain meridian acupoints, is more effective in improving anger and anxiety in the Hwabyung patients compared to the conventional meditation technique of Progressive Muscle Relaxation. An interesting study by H. G. Kim et al. suggested that those with a Taeumin type (one of four Sasang types) may tolerate psychological or oxidative stress better than those with the other types in accordance with the differences in the serum levels of stress hormones and the oxidative stress markers.

In conclusion, we expect that this special issue updates scientific evidences in KM and makes useful progress on KM integrative research.

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 ECAM 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 that the special issue will bring readers useful academic reference in their research.

Wansu Park
Salih Mollahaliloglu
Vitaly Linnik
Han Chae

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 805819, 6 pages
http://dx.doi.org/10.1155/2015/805819

Research Article

Study on the Difference of BIS/BAS Scale between Sasang Types

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Received 26 June 2015; Accepted 17 September 2015

Academic Editor: Roberto Miniero

Introduction. The purpose of this study was to examine the differences in temperament between So-Yang (SY) and So-Eum (SE) Sasang types using Behavioral Inhibition System/Behavioral Activation System (BIS/BAS) scale to elucidate the biopsychological mechanism underlying the Sasang typology, a traditional Korean personalized medicine. *Methods*. 248 university students were categorized into three Sasang types, and series of t-tests were conducted, separately for male and female participants, to examine the difference of Behavioral Inhibition System (BIS), Behavioral Activation System (BAS), BAS-Drive (BAS-D), BAS-Fun Seeking (BAS-FS), and BAS-Reward Responsiveness (BAS-RR) scores between SY and SE Sasang types. *Results*. There were significant differences between Sasang types in the BIS/BAS subscales with consideration of gender. In male participants, BAS-total score of SY type (39.75 ± 4.56) was significantly ($t = 2.462$, $p = 0.016$) higher than that of SE type (36.68 ± 4.97). On the other hand, in female participants, BIS score of SY type (20.10 ± 4.01) was significantly ($t = -2.097$, $p = 0.039$) lower than that of SE type (21.83 ± 3.91). *Discussion*. The current study suggested relationship between Sasang typology and Behavior Inhibition and Activation Systems and showed significant differences in BIS/BAS scale between SY and SE Sasang types. Further studies on biological base of Sasang typology are needed.

1. Introduction

Sasang typology is a traditional Korean medical typology dividing people into four Sasang types based on their unique organ system [1], which determines type-specific temperaments [2, 3], pathophysiological characteristics [4], disease susceptibility [5, 6], and response to specific treatments [7]. Research has been reported that the psychobiological characteristics of Sasang typology might have biological basis [8–10]; however it was not satisfactory to indicate underlying mechanisms (Table 1).

The So-Yang Sasang type is an extroverted, active, inquisitive, outgoing, quick-tempered, excitable, dynamic, easy-going, and impulsive person with strong interest in the outside world; on the contrary, the So-Eum Sasang type is introverted, passive, negative, organized, reserved, static, meticulous, patient, cautious, and nervous person focusing on their inner world [8].

Previous studies on the temperament of each Sasang type presented that the characteristics of So-Yang (SY) and So-Eum (SE) Sasang types are opposing each other [2, 3, 8, 11]. It has been reported that the So-Yang and So-Eum types have contrasting characteristics with Eysenck's Neuroticism and Extraversion and Cloninger's Novelty-Seeking (NS) and Harm-Avoidance (HA) [2]. The SY type was high on Extraversion and NS whereas it was low on Neuroticism and HA. On the contrary, the SE type was high on Neuroticism and HA whereas it was low on Extraversion and NS [2, 3, 8].

Interestingly, Cloninger's NS and HA are known to have influence from Gray's Behavioral Activation and Inhibition System, which might have important meanings in Sasang typology, equivalently [10, 12]. Gray's Behavioral Activation and Inhibition System are two pivotal motivational systems, which are considered to be responsible for affective states, behavior, personality, and predispositions for various forms of psychopathology, and there have been considerable

Table 1: Characteristics of So-Yang and So-Eum Sasang types.

Sasang type (prevalence)	So-Yang (소양, 少陽) (30%)	So-Eum (소음, 少陰) (30%)
Origin of the nature	Anger (怒) by righteousness (義) They become angry when they are blocked. The anger can be regulated by fairness	Enjoyment (樂) by wisdom (智) Worries can be relieved with wisdom. They enjoy what they have now
Temperament or personality characteristics	Active, externally oriented, and talented for business. Unstable, easily getting bored, sacrificing, righteous, easily acceptable, quick tempered, active, and easy-going High Extraversion (NEO-PI). High Novelty-Seeking and low Harm-Avoidance (TCI). High SPQ	Still, internally oriented, and self-directed Neat, mild, negative, intelligent, organized, patient, jealous, perseverant, passive, static, and meticulous Low Extraversion. Low Novelty-Seeking and high Harm-Avoidance. Low SPQ. Low Positive Affect (PANAS). High in Trait Anxiety (STAI)
Pathophysiological characteristics	Strong intake and digestion and weak waste discharge	Strong waste discharge and weak intake and digestion
Concerns for the good health	Easy with defecation Avoid overactivation and overloads of bodily functions	Good digestion Maintain healthy digestive function, peristalsis, and body heat
Frequent symptoms or disease	Constipation, gastroesophageal (laryngopharyngeal) reflux disease, affective disorder, insomnia, and heat on chest	Indigestion or dyspepsia, upper respiratory infection, and neurotic symptoms
Type-specific medical herbs	Rehmanniae Radix, Corni Fructus, Hoeoen, Alismatis Rhizoma, Osterici Radix, and Angelicae Pubescentis Radix	Ginseng Radix, Atractylodis Rhizoma Alba, Glycyrrhizae Radix, Cinnamomi Cortex, Citri Pericarpium, Zingiberis Rhizoma Crudus
Type-specific acupuncture points	Diagnosis with HT3. Treatment with HT7(+)/SP3(−)	Diagnosis with HT7. Treatment with SP3(+)/LI4(−)

TCI: Temperament and Character Inventory; NEO-PI: NEO Personality Inventory; SPQ: Sasang Personality Questionnaire; PANAS: Positive and Negative Affect Schedule; STAI: State and Trait Anxiety Inventory.

number of studies for the measurement and understanding of its neuroanatomical and psychometric properties [13–15].

Considering the meanings of inhibition and activation from biological perspective, these two contrasting concepts can be translated into Yin and Yang of traditional medicine in dualistic perspective. Yin-Yang is a representative term for two opposite characteristics of nature as introvert-extrovert, passive-active, negative-positive, cold-hot, moon-sun, night-day, dark-bright, slow-fast, and so on [8].

As for a biological system, Yang is predisposed to respond actively and externally towards environmental stimuli, whereas Yin is predisposed to be withdrawn and passive. Comparing the concept of Yin-Yang in traditional medicine to Western biological concepts of Behavioral Inhibition and Activation is an interesting research topic, in that it attempts an integrated approach toward research on biopsychology across the East and the West.

Therefore, in the current study, it was aimed to test whether the Behavioral Activation and Inhibition systems reflect the characteristics of SY and SE Sasang types, which represents the biological characteristics of Yang and Yin [8], respectively. Carver and White's BIS/BAS scale was used for the measure of Behavioral Activation and Inhibition system [16, 17]. It measures Behavioral Inhibition System (BIS) and Behavioral Activation System (BAS). The BIS measures the activation from aversive stimuli such as anxiety, fear, and worry. The BAS is a sum of three subscales of BAS-Drive (BAS-D), BAS-Fun Seeking (BAS-FS), and BAS-Reward Responsiveness (BAS-RR). The BAS-D measures the degree to which individuals pursue appetitive goals. BAS-FS reflects tendency to seek new potentially rewarding experiences and to act on incentives of the moment. BAS-RR focuses on positive responses to an occurrence of reward.

The results in this study would be useful for analyzing biopsychological characteristics of Sasang typology and would provide new diagnostic tools for those who have interest in differentiating Sasang types in their clinics. Furthermore, we can find similarities and differences between traditional temperament theory of Yin and Yang and Western biopsychology of BAS and BIS, and it would be useful for providing foundations for integrative biopsychology across the East and the West [8, 12].

2. Methods

2.1. Participants and Measurement. A total of 270 individuals from School of Korean Medicine were asked to complete QSCCII for Sasang type classification and BIS/BAS scale

Table 2: Demographic features of the subjects in this study.

	So-Yang	Tae-Eum	So-Eum	
Sex*				Chi-square = 11.960, $p = 0.03$
(male/female)	20/38	42/22	67/59	
Age	28.53 ± 3.21	28.62 ± 3.64	29.00 ± 5.52	$F = 0.259$, $p = 0.772$
Education				Chi-square = 1.874, $p = 0.392$
Bachelor	41	52	95	
Master	17	12	31	

* $p < 0.05$.

for BAS-D, BAS-FS, BAS-RR, BAS, and BIS measurement. The following procedures were approved by the Institutional Review Board of Pusan National University. All participants were provided with a written consent form for this study.

The BIS/BAS scale was developed by Carver and White [16] and was tested for the psychometric structure [18, 19]. We used Korean version translated by K.-H. Kim and W.-S. Kim [17], which was reaffirmed by comparing the original and translated versions side-by-side. The BIS/BAS scale consists of 24 items including four filler items, and each item is scored using a 4-point Likert scale from "strongly disagree" [1] to "strongly agree" [4]. The BAS scale has 13 items including BAS-D (4 items), BAS-FS (4 items), and BAS-RR (5 items), whereas the BIS has 7 items.

The QSCCII, a Sasang typology-based diagnostic inventory, is composed of 121 items concerning type-specific body shapes, psychological characteristics, life style, and pathophysiological symptoms. It was developed in 1993 and was revised in 1996. It has been used as an objective measurement in Sasang typology studies. The correctly predicted percentage of QSCCII was reported as 70.08% [20]; and the internal consistency calculated with Cronbach's alpha for Tae-Yang (TY), So-Yang (SY), Tae-Eum (TE), and So-Eum (SE) Sasang types was 0.57, 0.58, 0.59, and 0.63, respectively [21].

2.2. Statistical Analysis. Descriptive statistics on gender, education level, and age of each Sasang type were analyzed. χ^2 test for gender and education level and Analysis of Variance (ANOVA) for age were used to find differences between Sasang types.

The internal consistency of BIS/BAS subscales was analyzed with Cronbach's alpha. Item number and mean and standard deviation for each subscale were calculated.

t-test was conducted to compare BAS-D, BAS-FS, BAS-RR, BAS, and BIS levels between SY and SE types. Statistical results were presented as frequency (%) or mean ± standard deviation, and statistical significance level was set at $p < 0.05$, $p < 0.01$, and $p < 0.001$. PASW Statistics 18.0 (IBM, Armonk, NY) was used for all statistical analysis.

3. Results

3.1. Demographic Characteristics of the Participants. Data from 248 participants were used in the final analysis. The numbers of So-Yang (SY), Tae-Eum (TE), and So-Eum (SE) types classified with QSCC II were 58, 64, and 126, respectively (Table 2). As a result of the analyses to find differences of Sasang types between groups, there was a significant difference of Sasang type between genders ($\chi^2 = 11.960$, $p = 0.03$). However, no significant difference was found in age or education level.

Table 3: Internal Consistency of the BIS/BAS scale.

	# of items	Mean	St. dev.	Cronbach's alpha
BAS	13	38.17	27.94	0.824
BAS-D	4	10.90	4.41	0.701
BAS-FS	4	11.25	5.26	0.705
BAS-RR	5	16.02	5.03	0.723
BIS	7	20.09	14.20	0.834

BAS: Behavior Activation System; BAS-D: BAS-Drive; BAS-FS: BAS-Fun Seeking; BAS-RR: BAS-Reward Responsiveness; BIS: Behavior Inhibition System.

3.2. Reliability of the BIS/BAS Scale. Internal consistencies of BIS and BAS items were calculated with Cronbach's alpha (Table 3). Data from 247 participants were used. The mean and standard deviation of BAS (13 items) were 38.17 ± 27.94, whereas the mean and standard deviation of BIS (7 items) were 20.09 ± 14.20. Cronbach's alpha levels were acceptable: 0.824 and 0.834 for BAS and BIS, respectively.

3.3. BIS/BAS Scale Profile of So-Yang and So-Eum Sasang Types in Male and Female Participants. In male participants, the results of t-test to evaluate the differences between SY and SE types are shown in Table 4. The differences of BAS-D and BAS between SY and SE types were significant. The BAS-D of SY type (11.70 ± 1.94) was significantly ($t = 2.216$, $p = 0.029$) higher than that of SE type (10.50 ± 2.15). The BAS of SY type (39.75 ± 4.56) was significantly ($t = 2.462$, $p = 0.016$) higher than that of SE type (36.68 ± 4.97).

In female participants, the results of t-test to find the differences between SY and SE types were shown in Table 5. The differences of BAS-FS and BIS between SY and SE types were significant. The BAS-FS of SY type (12.15 ± 1.96) was significantly ($t = 2.201$, $p = 0.030$) higher than that of SE type (11.11 ± 2.44). On the other hand, the BIS of SY type (20.10 ± 4.01) was significantly ($t = -2.097$, $p = 0.039$) lower than that of SE type (21.83 ± 3.91).

Table 4: Mean and SD of BIS/BAS subscales in male So-Yang (n = 20) and So-Eum (n = 67) Sasang type groups.

	So-Yang	So-Eum	
BAS*	39.75 ± 4.56	36.68 ± 4.97	$t = 2.462$, $p = 0.016$
BAS-D*	11.70 ± 1.94	10.50 ± 2.15	$t = 2.216$, $p = 0.029$
BAS-FS	11.70 ± 2.10	10.76 ± 2.22	$t = 1.672$, $p = 0.098$
BAS-RR	16.35 ± 1.98	15.41 ± 2.03	$t = 1.81$, $p = 0.074$
BIS	19.05 ± 3.11	19.86 ± 3.23	$t = -0.996$, $p = 0.322$

* $p < 0.05$.
BAS: Behavior Activation System; BAS-D: BAS-Drive; BAS-FS: BAS-Fun Seeking; BAS-RR: BAS-Reward Responsiveness; BIS: Behavior Inhibition System.

Table 5: Mean and SD of BIS/BAS subscales in female So-Yang (n = 38) and So-Eum (n = 59) Sasang type groups.

	So-Yang	So-Eum	
BAS	39.57 ± 5.05	38.57 ± 5.11	$t = 0.945$, $p = 0.347$
BAS-D	11.00 ± 2.18	10.94 ± 1.99	$t = 0.118$, $p = 0.906$
BAS-FS*	12.15 ± 1.96	11.11 ± 2.44	$t = 2.201$, $p = 0.030$
BAS-RR	16.42 ± 2.16	16.50 ± 2.20	$t = -0.191$, $p = 0.848$
BIS*	20.10 ± 4.01	21.83 ± 3.91	$t = -2.097$, $p = 0.039$

* $p < 0.05$.
BAS: Behavior Activation System; BAS-D: BAS-Drive; BAS-FS: BAS-Fun Seeking; BAS-RR: BAS-Reward Responsiveness; BIS: Behavior Inhibition System.

4. Discussion

The current study examined Carver and White's BIS/BAS scale profile of SY and SE Sasang types (Table 1) and showed that the BAS of male participants and the BIS of female participants were significantly different between SY and SE Sasang types (Tables 4 and 5).

The results in this study, in combination with previous studies with Cloninger's NS and HA, are partly confirmed as hypothesized, in that the mechanism of Sasang typology might be related to the neurobiological base of Gray's biological personality theory [10]. Gray's notion of Behavioral Activation and Inhibition Systems suggests two main brain systems regulating approach and withdrawal behaviors to environmental stimuli [8, 22].

The Yin and Yang (Eum and Yang) are two opposing and complementary sides of the nature and have been a theoretical basis of traditional medicine for thousands of years in East [8]. And this concept was shown to have phenotypic similarities with temperament (cold-warm) and humidity (wet-dry) in humoral theory of Hippocrates and Galen which is an archetype of Western personality theories [23].

It was reported in functional neuroscience literature that the differential activation patterns in hippocampus, parahippocampal cortex, and amygdala caused by fear-related stimuli are positively correlated with Carver and White's BIS [24], which was shown to play a role in modulating anxiety related behaviors and negative emotion [22]. And the mesolimbic and mesocortical dopamine projections including ventral striatum and orbital regions of the prefrontal cortex are shown to be related to Carver and White's BAS [25, 26], which is known to be crucial for approach behavior and reward-related motivation [22].

Considering existing research on the biological base of Sasang typology along with current study [2, 3, 8, 9], SY and SE Sasang type might have differences in Behavioral Activation and Inhibition system, which have neuroanatomical structural basis. Those with SE Sasang type might have anxiety-related and negative emotion-related serotonergic circuits in hippocampus, parahippocampal cortex, and amygdala that have innate tendency to show negative or avoidant reaction to the unknown or harmful stimulations. SY Sasang type might have predisposed development with approach and reward-related mesolimbic and mesocortical dopamine projections including ventral striatum and orbital regions of the prefrontal cortex which could result in active and explorative reaction to the outside environment [8, 10, 12, 22]. Further studies on the relationships of SY and SE Sasang types with neuronal structure and neurotransmitter would be needed.

As for the reason that there were gender differences in BIS and BAS scale, unlike the case with Cloninger's NS and HA, there would be some speculations. First, the psychometric structure of Carver and White's BIS/BAS scale might include gender differences, which was shown as pivotal for Sasang typology [8]. Carver and White previously reported that BIS and BAS-RR of female participants (21.09 and 17.90, resp.) were significantly higher than those of male participants (18.84 and 17.27, resp.) [16]. The gender-specific neuroanatomical basis of BIS/BAS scale [22] and the relationship between BIS/BAS scale and socioemotional functioning in children [27] were also reported.

Second, there is a possibility that some items of BIS/BAS scale are related to gender roles differentially applied in the East and the West [8]. Though the filler items are not included in the calculation, the following items might trigger gender roles in East Asian society: "a person's family is the most important thing in life," "how I dress is important to me," and "it is hard for me to find the time to do things such as getting a haircut."

Third, the BIS/BAS scale may measure different dimensions from those of Cloninger's HA and NS, which was reported to measure Gray's Behavioral Activation and Inhibition System [28, 29]. As an example, when correlation analysis was conducted between BIS/BAS and Tridimensional Personality Questionnaire (TPQ), Cloninger's previous biopsychological model of TCI, HA ($r = 0.59$, $p < 0.001$) was significantly correlated with BIS but NS ($r = -0.11$, *n.s.*) was not [16].

These results emphasize a need for additional correlation studies on the personality construct of BIS/BAS and TCI along with Sasang Personality Questionnaire (SPQ), which measures the temperamental dimension of Sasang typology. The SPQ is a Yin-Yang based objective dimensional measurement with proven clinical validity and reliable psychometric properties [8]. The SPQ-total score showed distinctive differences between SY and SE types with respect to age and gender [8].

Considering that the BIS/BAS scale can measure the differences between SY and SE Sasang types, the Sasang typology can be used in diverse clinical fields since its Korean BIS/BAS version [17] has been used for the studies on heart rate variability [30], eating behavior [31], internet game addiction [32], depression [33], problematic alcohol use [34], motivation and interest [35], response to affective stimuli [36], and subjective well-being [37].

The results of the current study supported our prediction that significant differences of BIS and BAS exist between SY and SE Sasang types when gender is taken into account. The understanding for biological basis of Sasang typology would become more profound and integral with further research incorporating more participants and cultural contexts [38].

Conflict of Interests

The authors have no conflict of interests to disclose.

Acknowledgment

This work was supported by a 2-Year Research Grant of Pusan National University.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 273409, 11 pages
http://dx.doi.org/10.1155/2015/273409

Review Article

The Reporting Quality of Acupuncture-Related Infections in Korean Literature: A Systematic Review of Case Studies

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Received 24 June 2015; Revised 29 August 2015; Accepted 8 October 2015

Academic Editor: Hongcai Shang

Objective. Acupuncture is generally accepted as a safe intervention when it is administered in appropriate clinical setting by well-educated and experienced practitioners. In this study, we reviewed observational studies on adverse events (AEs) or complications relevant to acupuncture practice in Korean literature for assessing their reporting quality and suggested recommendations for future ones on acupuncture-related infections. *Method*. Electronic databases including Medline, Embase, Cochrane library, Korean studies Information Service System, DBpia, National Digital Science Library, and Korean National Assembly Library were searched until May 2015. Combination of keywords including "acupuncture" and "infection" were used for searching databases. *Result*. A total of 23 studies from 2,739 literature articles were identified from electronic database searching until May 2015. From this review, we found that most case studies did not report enough information for judging causality between acupuncture and the AEs (or complications) as well as appropriateness of the acupuncture practice. In addition, acupuncture experts rarely participated in the reporting of these AEs (or complications). *Conclusion*. Based on these limitations, we suggest a tentative recommendation for future case studies on acupuncture-related infection. We hope that this recommendation would contribute to the improvement of the reporting quality of acupuncture-related AEs (or complications) in the future.

1. Introduction

Acupuncture is a considerably safe intervention when it is administered in an appropriate clinical setting by well-educated and experienced practitioners. According to a large-scaled prospective survey result, only self-limited minor adverse events (AEs) (e.g., bleeding and needling pain) occurred in few cases with an incidence rate of 671/10,000 consultations [1]. Moreover, most of the severe complications of acupuncture are mainly related to the improper practice settings and inexperience of half-fledged practitioners. In particular, acupuncture-related infections have been reported to be closely related to the poor usage of acupuncture and disinfection procedures [2].

By the way, acupuncture-related AEs have been reported continuously, and the number of AE cases has not decreased even until recently [3]. The most serious problem among these AE cases was that essential information for judging the appropriateness of the acupuncture practice was not reported clearly [3, 4]. In this sense, case studies on acupuncture-related AEs are not regarded to be adequately informative to reduce errors and to enhance safety in future acupuncture practices and only lead to exaggeration of acupuncture-related harm among the general public. For predicting future AEs and promoting prevention measures in avoidable AE cases, it is important to identify whether the AEs and complications in the past studies were caused by negligence in practice or adverse reaction to acupuncture, which is not possible to determine retrospectively.

Reporting guidelines have been developed for transparent and easy reporting of clinical trials [5] and cases studies [6]. Regarding AEs, the Consolidated Standards of Reporting Trials' (CONSORT) AE reporting guideline is available for clinical trials [7]. Guideline for acupuncture-related AE reporting was suggested earlier [8], but it has not been adopted frequently in the case studies since its publication. There

are both similarities and differences between AEs related to acupuncture practice and those of drug-related AEs in some aspects; therefore, the guideline for acupuncture-related AEs should include common components such as description of patient's demographic data, medical history, and risk factors for, detailed condition of, and clinical outcomes of the current AEs [9] as well as acupuncture-specific aspects such as details of acupuncture intervention (e.g., needling points, needling depth, practitioner's types and experience level, and acupuncture materials used) [8]. In addition, evaluation of the appropriateness of acupuncture practice and the correlation between acupuncture and AEs, which is a key for improving quality of future practice, need to be reported.

Acupuncture-related infection is one of the commonly reported AEs of acupuncture. Compared with other types of AEs, it shows comparatively low incidence [2], but it is considered to be one of the major issues for good acupuncture practice, because infection often introduces considerable harm to patient's safety [3, 4]. Considering this situation, transparent and rigorous reporting on acupuncture-related infection is necessary for acupuncture practitioners as well as medical consumers.

In this sense, the purpose of this study was to assess the status of the reporting quality of case studies on acupuncture-related AEs or complications, especially focusing on acupuncture-related infections in the Korean literature, and to suggest tentative reporting guidelines for future case reports.

2. Methods

This is a systematic review of the observational studies on acupuncture-related infections. In this review, we defined AEs and complications differently as follows: AEs and complications are common unexpected signs or symptoms related to acupuncture treatment, but AEs occur when acupuncture procedure is appropriate while complications are caused by the practitioner's negligence [10]. Distinction between these two concepts is crucial in the reporting of acupuncture-related infections, because complications are more relevant to individual practitioner's skill level and compliance with clinical guideline for safe practice including clean needle technique, whereas AEs are actually related to acupuncture itself.

Electronic databases including Medline, Embase, Cochrane library, Korean studies Information Service System (KISS), DBpia, National Digital Science Library (NDSL), and Korean National Assembly Library were searched until May 2015. Combination of keywords including "acupuncture" and "infection" was used for developing search strategies based on the characteristics and structures of the individual databases.

The search strategy for Medline was as follows: acupuncture AND (infection OR hepatitis OR HIV OR (auricular chondritis) OR endocarditis OR meningitis OR (spinal infection) OR septicaemia OR (necrotizing fasciitis and toxic shock) OR (septic arthritis) OR abscess OR skin OR herpes).

For this review, we only included case studies or series that reported infectious conditions related to acupuncture in Korean literature. Acupuncture covers a broad range of nondrug interventions including moxibustion in traditional East Asian medicine (TEAM) [11], but we only included the classic types of acupuncture (i.e., needle insertion into the skin) in this review. However, if other interventions including moxibustion, cupping, and blood-letting were added to classic acupuncture and it was impossible to define which intervention might be first cause of the AEs (or complications), we included them the study.

To assess the reporting quality of the individual studies, the following data were analyzed based on the literature: (1) data related to patient information: patient characteristics, disease or symptoms for which acupuncture treatment was performed, risk factors for AEs or complications, underlying diseases, detailed features of AEs or complications, final clinical outcomes and (2) data related to AE: occurrence time after acupuncture, laboratory or pathological findings, other possible causes, and spatial relationship between needling site and affected lesion. In addition, a description of acupuncture treatment including practitioner's type, needling site, depth of insertion, needle type, stimulation method, and acupuncture settings (i.e., where the acupuncture was practiced and disinfection procedure) was analyzed. Each item was judged as "well documented (WD)" when all the related information was reported appropriately in the literature, as "documented but not enough for the judgment (DE)" when information was suggested but not enough to clearly describe the patient's presentation and as "not documented (ND)" when no information was available in the literature.

Causality between the acupuncture and AEs (or complications) and appropriateness of acupuncture practice was evaluated subsequently. Causality was assessed according to the modified WHO-UMC causality assessment criteria: "Certain" when plausible time relationship between the event and acupuncture was observed in the literature without possible cause of other treatments or underlying diseases for AEs (or complications), "Probable" when reasonable time relationship was observed and AEs (or complications) are unlikely to be explained by other causes, "Possible" when reasonable time relationship was observed and AEs (or complications) were possibly explained by other causes, "Unlikely" when improbable time relationship was observed with other plausible causes, "Conditional" when more data from current undergoing examination was necessary for the evaluation, and "Unassessable" when information was insufficient for judgment [12]. The appropriateness of acupuncture was assessed based on the information of acupuncture practice in the literature: "Appropriate" when all the acupuncture procedure could not be a probable cause of the AEs (or complications), "Inappropriate" when any of the procedures might be the possible cause of the AEs (or complications), and "Unclear" when there was not enough information for the judgment of acupuncture procedure.

Data extraction and appraisal of causality and acupuncture appropriateness were conducted by two authors (Tae-Hun Kim and Jung Won Kang) independently. If there was any discordance between the two authors, which could not be solved by discussion, a third author (Wan-Soo Park) arbitrated them.

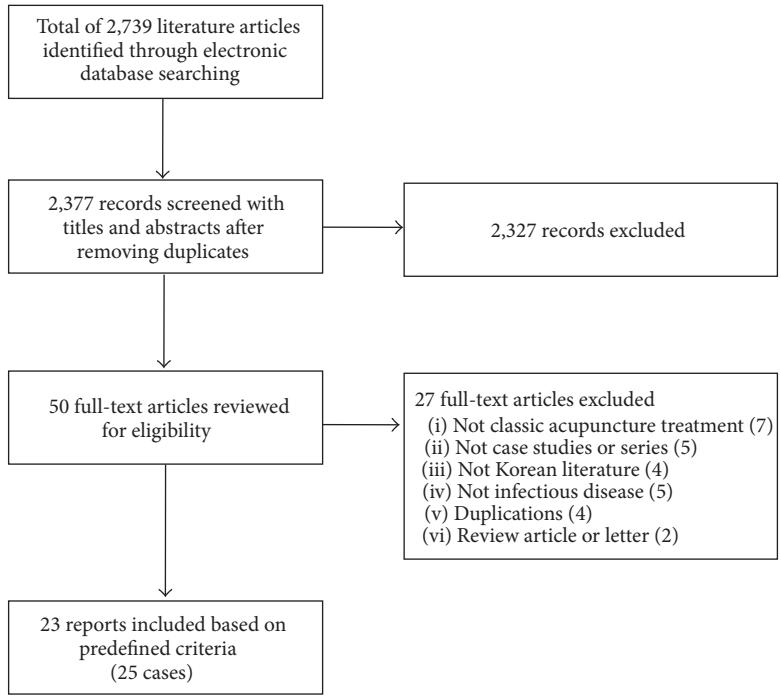

Figure 1: Study flow chart.

3. Results

From the electronic database search, a total of 2,739 records were identified and 50 hard copies were reviewed for eligibility. Among them, 23 reports (25 cases) were included in this review (Figure 1). Revealed infectious agents were *Streptococcus* species in four studies [13–16], *Staphylococcus* species in four studies [17–20], *Mycobacterium* species in four studies [21–24], *Escherichia coli* in two studies [19, 25], and unidentified ones in three studies [26–28]. In addition, other infection agents were *Actinomyces* [29], *Bifidobacterium longum* [30], *Gemella morbillorum* [31], Herpes simplex virus [32], *Klebsiella pneumonia* [33], *Vibrio cholera* [34], *Serratia liquefaciens* complex [17], and *Spirochaete* [35]. The infectious diseases were skin infection in five studies [21–24, 32], sepsis in four studies [18, 25, 30, 34], fasciitis in three studies [15–17], psoas abscess in three studies [14, 25, 27], epidural abscess [20, 28] or inflammatory granuloma [26] in three studies, and others including abdominal actinomycosis [29], liver abscess [13], mediastinitis [31], necrotizing aortitis [19], pericardial abscess [18], spondylitis [25], retroperitoneal abscess [33], and syphilis [35]. Among 23 studies, only one study [28] consulted an acupuncture specialist who could analyze the AE (or complication) cases and judge the appropriateness of acupuncture practice as well as causality between the acupuncture and AEs (or complications) [28] (Table 1).

3.1. The Reporting Quality of the Information on the Patient and AEs (or Complications) Related to Acupuncture Practice. In most studies, the information on the patient's general characteristics, detailed features of AEs (or complications), and final outcomes of the AEs (or complications) were described very well. However, important patient's information including preceding conditions or reasons for seeking acupuncture (percentage of inappropriately reported studies, 22%) [19–21, 23, 33] and predisposing risk factors to which relevant AEs (or complications) might be attributable (70%) [17–26, 28–33] were not reported appropriately in most studies. Essential items for judging causality including other possible causes of AEs (or complications) (65%) [15, 16, 18–23, 25–27, 30, 32–34] as well as explanation of the association between needling site and the affected lesion (87%) [13–27, 30, 31, 33–35] were not suggested at all or insufficiently in most studies (Table 1). In this sense, all the included studies insisted that strong association between acupuncture treatment and the event existed, but causality of the acupuncture-related AEs (or complications) could not be concluded with the information provided in most studies (Table 2).

3.2. Reporting Quality of the Information on Acupuncture Practice. Although all the AEs (or complications) cases were asserted to be related to acupuncture, information on acupuncture and acupuncture practice were not reported adequately in most studies. Information on acupuncture practice closely relevant to acupuncture-related complications including practitioner's type (87%) [14–22, 24–27, 29–35], settings for acupuncture practice (74%) [14, 17–27, 29, 30, 32–34], needle types (100%) [13–35], usage of disposable, sterile needles (100%) [13–35], and features of acupuncture practice including needling site (96%) [13–27, 29–35], depth of insertion (100%) [13–35], stimulation method for acupuncture (100%) [13–35], and whether disinfection procedure was implemented properly (100%) [13–35] and was not described in most studies. So, appropriateness of acupuncture practice

Table 1: Reporting status on the information of patients and adverse events (AEs) or complications related to acupuncture practice in the included studies.

				Patient's information				AEs (or complications) information				
Study ID	Infection agents (numbers of the cases)	Final diagnosis	Inclusion of acupuncture specialist among the authors*	Patient's characteristics	Preceding conditions or reasons for seeking acupuncture	Description on the risk factors for AEs (or complications)	Features of AEs (or complications)	Clinical outcome (follow-up)	Time relation between acupuncture and AEs (or complications)	Laboratory or pathological findings	Consideration of the other possible causes of AEs (or complications)	Explanation on the association between needling site and affected lesion
Bang and Lim 2006 [25]	*Escherichia coli* (1)	Psoas abscesses; epidural abscess; infectious spondylitis; sepsis	None	WD	WD	ND	WD	WD	WD	WD	ND	DE
Cho et al. 2003 [33]	*Klebsiella pneumonia* (1)	Retroperitoneal abscess	None	WD	DE	DE	WD	WD	WD	WD	DE	ND
Cho et al. 2010 [21]	*Mycobacterium abscessus* (1)	Mycobacterium abscessus cutaneous infection	None	WD	ND	DE	WD	WD	DE	WD	DE	ND
Cho et al. 2015 [31]	*Gemella morbillorum* (1)	Mediastinitis, osteomyelitis	None	WD	WD	DE	WD	WD	WD	WD	WD	ND
Choi et al. 2013 [13]	*Streptococcus intermedius* (1)	Pyogenic liver abscess	None	WD	WD	WD	WD	WD	DE	WD	WD	DE
Choi 2014 [17]	*Serratia liquefaciens* complex and *Staphylococcus intermedius* (1)	Cervical necrotizing fasciitis	None	WD	WD	ND	WD	WD	WD	WD	WD	WD
Ha et al. 1999 [30]	*Bifidobacterium longum* (1)	Sepsis	None	WD	WD	ND	WD	WD	WD	WD	DE	ND
Ha and Kim 2003 [26]	No organism growth (1)	Chronic inflammatory epidural granuloma	None	WD	WD	DE	WD	WD	DE	WD	ND	DE
Han et al. 2012 [18]	*Staphylococcus aureus* (1)	Pericardial abscess; sepsis	None	DE	WD	DE	WD	WD	WD	WD	DE	ND
Kang et al. 2012 [14]	*Streptococcus pneumonia* (1)	Psoas abscess; diabetic foot ulcer	None	WD	WD	WD	WD	WD	WD	WD	WD	DE
Jung et al. 2011 [32]	Herpes simplex virus (1)	Skin infection	None	WD	WD	DE	WD	ND	WD	WD	DE	WD
Jung et al. 2014 [22]	*Mycobacterium massiliense* (1)	Localized cutaneous infection	None	WD	WD	ND	WD	WD	DE	WD	DE	DE
Kang and Jeong 2006 [15]	*Streptococcus pyogenes* (1)	Necrotizing fasciitis	None	WD	WD	WD	WD	WD	WD	WD	DE	ND
Kim et al. 2003 [35]	*Spirochetes* (1)	Secondary syphilis	None	WD	WD	WD	WD	WD	DE	WD	WD	ND
Kim et al. 2010 [23]	*Mycobacterium tuberculosis* (3)	Primary inoculation tuberculosis	None	WD	DE	DE	WD	WD	WD	WD	ND	DE

Table 1: Continued.

Study ID	Infection agents (numbers of the cases)	Final diagnosis	Inclusion of acupuncture specialist among the authors*	Patient's information				AEs (or complications) information				
				Patient's characteristics	Preceding conditions or reasons for seeking acupuncture	Description on the risk factors for AEs (or complications)	Features of AEs (or complications)	Clinical outcome (follow-up)	Time relation between acupuncture and AEs (or complications)	Laboratory or pathological findings	Consideration of the other possible causes of AEs (or complications)	Explanation on the association between needling site and affected lesion
J. W. Kim and Y. S. Kim 2010 [27]	Unidentified (1)	Psoas abscess	None	WD	WD	WD	WD	WD	WD	WD	DE	ND
Kim et al. 2015 [29]	*Actinomyces* species (1)	Abdominal wall actinomycosis	None	WD	ND	ND	WD	WD	DE	WD	WD	WD
Lee et al. 1994 [24]	*Mycobacterium fortuitum* (1)	Cutaneous mycobacterial infection and abscesses	None	WD	WD	ND	WD	WD	DE	WD	WD	DE
Lee et al. 2008 [19]	*Escherichia coli*; MRSA (1)	Necrotizing aortitis	None	WD	ND	DE	WD	WD	DE	WD	ND	DE
Lee et al. 2012 [28]	Unidentified (1)	Cervical epidural abscess	Included	WD	WD	ND	WD	WD	WD	WD	WD	WD
Lim et al. 2013 [34]	Non-O1, Non-O139 *Vibrio cholera* (1)	Septicemia	None	WD	WD	WD	WD	WD	WD	WD	DE	ND
Song et al. 2006 [16]	*Streptococcus pyogenes* (1)	Necrotizing fasciitis	None	WD	WD	WD	WD	WD	DE	WD	DE	DE
Yu et al. 2013 [20]	*Staphylococcus aureus* (1)	Multiple epidural abscess	None	DE	ND	ND	WD	ND	WD	DE	ND	DE

AEs: adverse events, MRSA: methicillin resistant *Staphylococcus aureus*, WD: well documented, DE: documented but not enough for the judgment, ND: not documented, and NA: not applicable; *inclusion of acupuncture specialist among the authors was assessed based on the author's affiliation.

Table 2: Author's conclusion in the included studies and causality assessment based on the WHO-UMC criteria.

Study ID (author, yr)	Author's conclusion (Quotation from reports)	Causality assessment*
Bang and Lim 2006 [25]	*"Paraplegia might result from complications of an acupuncture therapy."*	Probable
Cho et al. 2003 [33]	*"We report a case of serious infectious complication caused by acupuncture."*	Conditional
Cho et al. 2010 [21]	*"We report a case of 59-year-old Korean woman who developed M. abscessus cutaneous infection after multiple acupunctures."*	Unlikely
Cho et al. 2015 [31]	*"It is plausible that the infection was caused by acupuncture therapy rather than a hematogenous infection."*	Unlikely
Choi et al. 2013 [13]	*"In this case, we assume that the patient had acupuncture needles and bacteremia after being treated with contaminated was maybe seeded in the liver."*	Unlikely
Choi 2014 [17]	*"In conclusion, acupuncture and herbal injection should be performed using clean care practices, and NF must be considered as a possible complication in high-risk patients and even also in healthy patients"*	Possible
Ha et al. 1999 [30]	*"Since there were no obvious predisposing conditions preceding anaerobic infection in the young male patient other than acupuncture therapy, it is speculated that the organism was introduced to the blood circulation either from improperly sterilized acupuncture needles or from the colon via minute perforations caused by those needles."*	Possible
Ha and Kim 2003 [26]	*"…we hypothesize that the epidural granuloma probably formed as the result of focal hemorrhage and low-grade infection by a microorganism after acupuncture…"*	Unassessable
Han et al. 2012 [18]	*"…based on the multifocal acupuncture therapy history of this patient and the absence of previous pericardial disease, the pericardial abscess may have been caused by hematogenous spread of Staphylococcus aureus from the soft tissue infection of the knees."*	Unlikely
Kang et al. 2012 [14]	*"Based on the aforementioned observations, it was likely that the abscess and septic arthritis were due to acupuncture and moxibustion."*	Conditional
Jung et al. 2011 [32]	*"We theorize that our patient acquired cutaneous herpes from direct viral inoculation via a contaminated acupuncture needle or reactivation of a cutaneous herpes viral infection due to mechanical trauma."*	Probable
Jung et al. 2014 [22]	*"We present a case of a localized cutaneous infection due to M. massiliense of the sole associated with acupuncture."*	Conditional
Kang and Jeong 2006 [15]	*"We present this extremely unusual case of a patient after taking acupuncture who survived severe necrotizing fasciitis of the chest wall following wide debridement of the necrotic tissue and broad-spectrum antibiotic therapy."*	Unassessable
Kim et al. 2003 [35]	*"We herein report a rare case of secondary syphilis with clinical features of annular pustular psoriasis following the repeated acupuncture and venous drainage by a her doctor."*	Possible
Kim et al. 2010 [23]	*"Herein, we report 3 cases of primary inoculation tuberculosis resulting from illegal acupuncture in the same nursing home on the same day by a person with no medical training or license."*	Probable
J. W. Kim and Y. S. Kim 2010 [27]	*"Here, we report the first documented case of psoas abscess caused by acupuncture procedure in a hemodialysis patient."*	Possible
Kim et al. 2015 [29]	*"Herein, we report an unusual case of abdominal wall actinomycosis which developed in a patient after acupuncture and presented as abdominal wall mass that was first mistaken for abdominal wall invasion of diverticulum perforation."*	Possible
Lee et al. 1994 [24]	*"In our case, the contaminated acupuncture needle could be an infection source of cutaneous lesions."*	Probable
Lee et al. 2008 [19]	*"In the present case, a long acupuncture needle penetrated from the patient's back is the most suspicious cause for the aortic infection."*	Unlikely
Lee et al. 2012 [28]	*"In this case, we suspect that wet cupping and/or acupuncture in poorly controlled hygiene might have led to the cervical epidural abscess."*	Certain
Lim et al. 2013 [34]	*"We report a 56-year-old cirrhotic patient of non-O1, non-O139 septicemia caused by cellulitis of both lower extremities after acupuncture."*	Unlikely
Song et al. 2006 [16]	*"We report a rare case of necrotizing fasciitis on the face of a 62-year-old man, who had uncontrolled diabetes mellitus following acupuncture treatment."*	Unassessable
Yu et al. 2013 [20]	*"Multiple epidural abscess after acupuncture."*	Possible

*Causality was assessed according to the WHO-UMC criteria based on the information from the reports on AEs (or complications).

Table 3: Reporting status on the specific features of acupuncture treatments in the included studies.

Study ID (author, yr)	Details of acupuncture practice								
	Practitioner's type	Needling site (acupuncture points)	Usage of disposable, sterile needles	Depth of insertion	Needle type	Stimulation method	Acupuncture settings	Disinfection procedure	Appraisal for the appropriateness of acupuncture*
Bang and Lim 2006 [25]	ND	DE	ND	DE	DE	ND	ND	ND	Unclear
Cho et al. 2003 [33]	ND	ND	ND	ND	ND	ND	ND	ND	Unclear
Cho et al. 2010 [21]	ND	ND	ND	ND	ND	ND	ND	ND	Unclear
Cho et al. 2015 [31]	DE	ND	ND	ND	ND	ND	WD	ND	Unclear
Choi et al. 2013 [13]	WD	DE	ND	ND	DE	ND	WD	ND	Unclear
Choi 2014 [17]	ND	DE	ND	ND	ND	ND	ND	ND	Unclear
Ha et al. 1999 [30]	ND	DE	ND	ND	DE	ND	DE	ND	Unclear
Ha and Kim 2003 [26]	ND	DE	ND	ND	ND	ND	DE	ND	Unclear
Han et al. 2012 [18]	ND	DE	ND	ND	ND	ND	ND	ND	Unclear
Kang et al. 2012 [14]	ND	DE	ND	ND	ND	ND	ND	ND	Unclear
Jung et al. 2011 [32]	ND	ND	ND	ND	ND	ND	ND	ND	Unclear
Jung et al. 2014 [22]	ND	DE	ND	ND	ND	ND	ND	ND	Unclear
Kang and Jeong 2006 [15]	ND	DE	ND	ND	ND	ND	WD	DE	Unclear
Kim et al. 2003 [35]	ND	DE	ND	ND	ND	ND	WD	ND	Unclear
Kim et al. 2010 [23]	WD	DE	ND	ND	ND	ND	DE	ND	Inappropriate
J. W. Kim and Y. S. Kim 2010 [27]	ND	DE	ND	ND	ND	ND	ND	ND	Unclear
Kim et al. 2015 [29]	ND	DE	ND	ND	ND	ND	ND	ND	Unclear
Lee et al. 1994 [24]	ND	DE	ND	ND	ND	ND	ND	ND	Unclear
Lee et al. 2008 [19]	ND	DE	ND	ND	DE	ND	ND	ND	Unclear
Lee et al. 2012 [28]	WD	WD	ND	WD	ND	ND	WD	DE	Unclear
Lim et al. 2013 [34]	ND	DE	ND	ND	ND	ND	ND	ND	Unclear
Song et al. 2006 [16]	ND	DE	ND	ND	ND	ND	WD	ND	Unclear
Yu et al. 2013 [20]	ND	DE	ND	ND	ND	ND	ND	ND	Unclear

WD: well documented, DE: documented but not enough, ND: not documented, and NA: not applicable; *acupuncture treatment was appraised to be Appropriate when all the acupuncture procedures could not be a probable cause of the adverse events or complications, Inappropriate when any of procedures might be the possible cause of the adverse events or complications, and Unclear when there is not enough information for deciding the appropriateness of acupuncture treatment.

Table 4: Recommendation for reporting cases of acupuncture-related infections.

Items	Content
Title	Types of acupuncture practice and AEs (or complications) should be included in the title.
Authors	Acupuncture specialists need to be included among the authors.
Description for the patient	
Demographic data	Sex, age, ethnicity, and residence need to be described.
Preceding conditions or reasons for seeking acupuncture	The patient's diseases or symptoms for seeking acupuncture treatment should be described for assessing appropriateness of acupuncture.
Description on the risk factors for AEs (or complications)	Patient's underlying conditions or cointerventions which might be related to AEs (or complications) need to be declared.
Details of acupuncture intervention [37]	
Acupuncture practitioner's type	Certification, education status, and clinical experience level need to be declared.
Needling sites (acupuncture points)	Location and number of points for acupuncture or needling need to be described in detail using WHO standard acupuncture point locations guideline [38].
Usage of disposable, sterile needles	Usage of disposable, sterile needles should be assessed and reported.
Depth of insertion	Depth and direction of needle insertion should be suggested.
Needle type	Length, diameter, material, and manufacturer of acupuncture needles should be declared.
Stimulation method	Stimulation method for acupuncture including manual, electric stimulation, or other stimulating methods needs to be reported.
Acupuncture settings	Medical institutions or conditions of the physician's office need to be suggested.
Disinfection procedure	Detailed disinfection measure before and after acupuncture should be reported in detail.
Description for the AEs (or complications)	
Time relation between acupuncture and AEs (or complications)	Time line of acupuncture treatment and the occurrence of AE (or complication) symptoms should be suggested clearly.
Explanation on the association between needling site and affected lesion	Relationship between needling site and affected lesion should be evaluated appropriately.
Features of AEs (or complications)	Information on the clinical presentation of AEs (or complications) needs to be suggested sufficiently to assess the causality between acupuncture and the event.
Laboratory or pathological findings	Laboratory or pathological findings related to the AEs (or complications) should be suggested.
Consideration of the other possible causes of AEs (or complications)	Based on the preceding risk factors, other treatments, assessment of acupuncture appropriateness, and other possible causes of AEs (complications) should be evaluated fairly and scientifically.
Appraisal for the appropriateness of acupuncture	Appropriateness of acupuncture practice appraised based on the information about acupuncture intervention, procedure, settings, and disinfection method should be reported.
Causality assessment	Causality category according to the WHO-UMC criteria needs to be suggested based on the clear reason for the decision [39].
Discussion and conclusion	
Previous evidence on the AEs (or complications) related to acupuncture	Previous case reports or literature with rigorous evidence on the current AEs (or complications) needs to be reported.
Conclusion	Conclusion should be written based on the results of the appraisal for the appropriateness of acupuncture practice and causality between acupuncture and the event in a neutral position.
Clinical implication	Preventive measures against current acupuncture-related infection need to be suggested based on the analysis of appropriateness of acupuncture for future safe practice.

could not be appraised in all studies except for one [23] (Table 3).

4. Discussion

From this systematic review on the acupuncture-related infection case reports in Korean literature, we found that essential information for judging causality and appropriateness of acupuncture practice was not reported adequately. In particular, preceding risk factors, other possible causes, and spatial association between acupuncture needling and the affected lesion, which are necessary information when deciding causality, were not reported sufficiently. In this sense, we could not find concrete evidence in each report for the judgment of the causality between acupuncture practice and occurrence of the AEs (or complications) except for the time-order relationship. Description of the acupuncture procedure could not confirm the appropriateness of the acupuncture practice because it was insufficient and inadequate. Acupuncture specialists with expertise in these cases were not involved in the reporting process. These factors negatively affect the reporting quality of acupuncture-related AEs (or complications).

This review has several specific points. First, we assessed individual components in the case reports to identify possible errors related to the practice in these studies. When assessing the reporting quality of case reports on acupuncture-related infections, appropriateness of acupuncture practice should be evaluated. In this review, only one case had a complication that was definitely introduced by a wrong practice of acupuncture [23] and in other cases it could not be conclusively established whether the events were AEs or complications because the description was improper and deficient. However, previous systematic reviews on acupuncture-related infections only focused on the type and frequency of AEs but did not pay much attention to the appropriateness of acupuncture [4, 36]. Misconduct or errors during acupuncture can affect clinical outcomes; therefore, the assessment of appropriateness can give insight into future safe practice for acupuncture. Second, we evaluated causality rigorously based on the original case reports. To ensure transparency, two different authors whose specialty is acupuncture assessed causality individually and discussed the results. From this review, we found that most studies did not assess causality between acupuncture and AEs (or complications) appropriately, so case reports, although abundant, have limitations in that they do not help warn practitioners against the risks of acupuncture and are not informative enough to improve acupuncture practice. Causality of acupuncture-related AEs (or complications) should be assessed transparently and fairly based on the time association between the practice and the event, pathological mechanism of AEs (or complications), and exclusion of other potential causes [12]. In addition, acupuncture-related AEs (or complications) are different from drug-related AEs in that discontinuation and readministration of acupuncture hardly affect the clinical outcomes unlike with drugs [12]. Judging the causality between acupuncture and AEs (or complications), these factors need to be analyzed appropriately.

This study has limitations as well. First, we only assessed Korean literature, which reflects the reality of clinical practice in Korean context. Two different expert occupations in health-care sector, conventional medical doctors and Korean Medicine doctors, are in charge of national health with equal legal position, and competition among them is intensifying. Considering this conflicting situation, reporting of AEs (or complications) related to acupuncture might be overexaggerated (even with malicious intention) and not based on sound scientific evidence. Another explanation for this status could be that acupuncture specialists hardly participated in the case reporting of the included studies. The purpose of this study was to evaluate the quality of Korean literature, so this limitation is inevitable. Second, we only assessed acupuncture-related infections in this study. Apart from infections, other frequently reported acupuncture-related conditions such as traumatic injuries including pneumothorax and nerve injury need to be assessed in similar manner. We only assessed a small portion of acupuncture-related AEs (or complications), and the same issues on the reporting quality can be adapted to other types of AEs (or complications). These two limitations will be covered through further successive reviews in the future.

Based on the current review results, we now suggest a recommendation for reporting cases of acupuncture-related infections (Table 4). This recommendation includes items on patient's condition and AEs (or complications) in detail, which are necessary to establish the causality between acupuncture and the event and to provide information for judging appropriateness of acupuncture practice. Compared with a previously published reporting guideline for acupuncture-related AEs [8], this recommendation includes detailed information which is necessary for the judgment of causality between acupuncture and AEs (or complications) such as time and spatial relationship between acupuncture and AEs as well as the assessment of the other possible causes of AEs and appropriateness of acupuncture practice. In addition to this, we recommend that acupuncture specialists join as one of the authors for the AE reporting which is important for scientific and transparent reporting and lessons for future safe practice of acupuncture practice from the AE accident, which can be suggested as a clinical implication of the report. We hope that this tentative recommendation can be useful for reporting future cases of acupuncture-related infections in a clear and informative way as well as developing more advanced recommendation based on the future multidisciplined, mixed-method researches.

Conflict of Interests

The authors declare that there is no competing interest.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 203965, 8 pages
http://dx.doi.org/10.1155/2015/203965

Research Article

Anti-Inflammatory Effect of Combination of Scutellariae Radix and Liriopis Tuber Water Extract

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Received 22 June 2015; Revised 10 September 2015; Accepted 4 October 2015

Academic Editor: Salih Mollahaliloglu

Scutellariae Radix and Liriopis Tuber have been used to treat the inflammatory diseases in traditional Korean medicine and anti-inflammatory effect of each herb has been shown partially in several articles. However, the combined extract of these medicinal herbs (SL) has not been reported for its anti-inflammatory effects. In this study, we investigated the effects of SL on the creation of several proinflammatory mediators in RAW 264.7 cell mouse macrophages induced by Lipopolysaccharide (LPS). SL inhibited significantly the increase of NO, the release of intracellular calcium, the increase of interleukin-6 (IL-6), macrophage inflammatory proteins (MIP-1α, MIP-1β, and MIP-2), and granulocyte colony-stimulating factor (G-CSF) in LPS-induced RAW 264.7 cell at the concentrations of 25, 50, and 100 μg/mL, and SL inhibited significantly the increase of macrophage colony-stimulating factor (M-CSF) at the concentrations of 25 and 50 μg/mL, and tumor necrosis factor (TNF) at the concentration of 25 μg/mL. These results implicate that SL has anti-inflammatory effects by suppressing the production of various inflammatory mediators in macrophages. But SL did not inhibit significantly the increase of granulocyte macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), and Regulated on Activation, Normal T cell Expressed and Secreted (RANTES); therefore, further study is demanded for the follow-up research to find out the possibility of SL as a preventive and therapeutic medicine for various inflammatory diseases.

1. Introduction

Inflammation, a defense mechanism against various stimuli inducing injury, accompanies symptoms of flare, fever, swelling, pain, and various functional disorders. Many cytokines and proteins as well as prostaglandin E_2 (PGE_2), lysosomal enzymes, and free radicals are known to be involved in inflammation [1]. Macrophages, involving innate immunity via phagocytic process as well as acquired immunity via antigen-presenting function, are representative immune cells activated by pathogens, cellular injury, and cytokines secreted by different types of immune cells. The activated macrophages trigger inflammatory reactions by secreting cytokines, such as TNF-α, IL-1, and other various inflammatory mediators, that is, nitric oxide (NO), reactive oxygen species (ROS), and prostaglandins [2]. Lipopolysaccharide (LPS), a major outer membrane component of Gram-negative bacteria [3], acting as an external immunostimulator, known to generate various immune cells and to promote the secretion of proinflammatory cytokines, is an established model molecule for inflammation research [4]. Inflammation not just affects simple inflammatory reactions but also acts on inflammatory mediators, which mediate many chronic diseases and inflammation-related disorders, hence getting more attention. Anti-inflammatory medicines show their effectiveness by suppressing the metabolism of these inflammatory mediators; therefore, a lot of researches to discover novel candidates to control inflammatory reactions by regulating various chemical inflammatory mediators have been actively pursued [5].

According to a report, while many experiments employing the extracts of a single medicinal herb focused on in vitro effect, combined extracts have been rather tested for either animal or clinical researches [6]. Scutellariae Radix (SR)

and Liriopis Tuber (LT) have shown their anti-inflammatory effects with their extract alone in several research articles [7–11], yet the combined extract of these medicinal herbs has not been reported for its anti-inflammatory effect.

In this study, anti-inflammatory effect of the mixture of Scutellariae Radix (SR) and Liriopis Tuber (LT) was evaluated. Each plant was extracted alone in hot water and then combined mixture called sample "SL" was tested in RAW 264.7, a mouse macrophage cell line, for cell viability rate and the effect of NO production. The levels of NO production, intracellular free calcium, and production of several cytokines, such as interleukin (IL), macrophage inflammatory protein (MIP), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF), leukemia inhibitory factor (LIF), and Regulated on Activation, Normal T cell Expressed and Secreted (RANTES), were significantly affected by the treatment of the sample "SL" in RAW 264.7 cells stimulated by LPS.

2. Materials and Methods

2.1. Preparation of Sample (SL). 20 g of Scutellariae Radix and 20 g of Liriopis Tuber were extracted in 2,000 mL of distilled water each using a reflux extractor. The decoction was boiled for 2 hrs after temperature reached boiling point. Each extract was vacuum-filtered through filter papers (Advantec No. 2, Japan) and then concentrated in a rotary vacuum evaporator. Each concentrate was freeze-dried, resulting in 6.9 g of Scutellariae Radix (extraction yield, 34.5%) and 4.5 g of Liriopis Tuber (extraction yield, 22.5%). The mixed extracts (1 : 1 ratio) yielded the testing material (SL), which was used throughout in this study.

2.2. Cell Culture. RAW 264.7 cells were cultured in DMEM medium with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C under 5% CO_2 in a CO_2 culture incubator. The cells were sufficiently expanded in 75 cm^2 flask (Falcon, USA), washed with phosphate buffered saline (PBS) every 3 days. The cells were then treated with 0.25% trypsin-EDTA (1 mL/50 mL flask) for 1 min and incubated at 37°C for additional 5 min to detach from the culture flask after trypsin solution was removed. The resulting detached cells suspended in 10 mL DMEM 10% FBS were split 1 : 2 ratio and divided into new culture flasks and incubated in the CO_2 incubator (37°C, 5% CO_2) [7].

2.3. Cell Viability Assay. MTT assay was carried out as follows [12, 13]. 100 μL of the cells (1×10^5 cells/well) was added into a 96-well plate and incubated at 37°C, 5% CO_2 incubator for 24 hrs. After the plate was washed with phosphate buffered saline (1x PBS), various concentrations of the sample in PBS of the same volume were treated in the designated wells and incubated. At the end of incubation, 100 μL of 1 mg/mL MTT (Sigma, USA) in PBS was added to each well. The plate was protected from light with aluminum foil wrapping and incubated at the same condition for 2 hrs. After removing the culture medium, 100 μL DMSO was added to the wells and left at 37°C for 2 hrs. Cell viability was assessed by measuring the absorption in a microplate reader (Molecular Devices, USA) at 490 nm.

2.4. Measurement of NO Production. L-Arginine, the substrate of NO, is converted to L-citrulline and nitric oxide (NO), which are quickly stabilized to NO_2, nitrite, and nitrate. Griess reagent (0.5% sulfanilamide, 2.5% phosphoric acid, and 0.5% naphthylethylenediamine) chemically reacts with nitrite, forming azo dye in purple color, of which concentration is identical to that of NO. The nitrite concentration was estimated from azo dye concentration; therefore, the microplate reader was set to measure the absorption at 540 nm to determine the production of NO. To investigate the effect of the testing sample (SL) on the production of NO in RAW 264.7 cells, the following experiments were carried out. LPS (1 mg/mL) alone or with various concentrations of the testing sample (SL) was treated to each well and incubated at 37°C, 5% CO_2 incubator for 24 hrs. The supernatant (100 μL) added to a 96-well plate was mixed with 100 μL Griess reagent and reacted for 15 min. The NO production was estimated by measuring the absorption at 540 nm with a microplate reader (Bio-Rad, USA) [14].

2.5. Intracellular Free Calcium Measurement. To investigate the effect of the testing sample (SL) on the intracellular free calcium release in RAW 264.7 cells, fluo-4 calcium assay was carried out as follows. 100 μL of the cells (1×10^5 cells/well) was added into a 96-well plate and incubated at 37°C, 5% CO_2 incubator for 24 hrs. LPS (1 μg/mL) alone or with various concentrations of the testing sample (SL) was treated to each well and incubated at 37°C, 5% CO_2 incubator for 18 hrs. After the plate was washed with phosphate buffered saline (1x PBS), various concentrations of the sample in PBS of the same volume were treated in the designated wells and incubated. After the incubation, the medium in the wells was removed and 100 μL fluo-4 dye solution was added to each well and incubated at 37°C, 5% CO_2 incubator for 30 min. The fluorescence intensity of each well was measured by spectrofluorometer (485 nm for excitation filter; 535 nm for emission filter) [14].

2.6. Measurement of Cytokine Level. The following experiment by referencing Ryu et al. [10, 14, 15] was carried out to find whether the testing sample (SL) affects the secretion of immunoproteins. 100 μL of the cells (1×10^5 cells/well) was added into a 96-well plate and incubated at 37°C, 5% CO_2 incubator for 24 hrs. After the plate was washed with phosphate buffered saline (1x PBS), various concentrations of the sample in PBS in the same volume of the medium were treated in the designated wells and incubated. LPS (1 μg/mL) alone or with various concentrations of the testing sample (SL) was treated to each well and incubated at 37°C, 5% CO_2 incubator. At the end of incubation, cell culture supernatant from each well was reacted with the antibody-conjugated capture beads put in the wells in advance. The reacted capture beads in the wells of a filter plate were washed in 150 μL per well of the washing buffer. After reacting with added detection antibody, the plate was incubated for 30 min. After

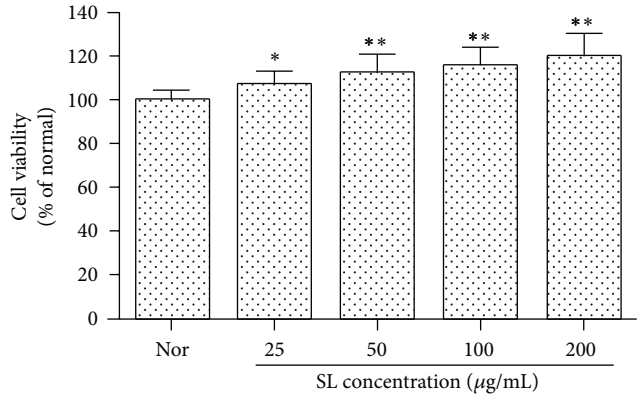

Figure 1: Effect of SL on cell viability in RAW 264.7 cells. Normal (Nor): treated with media only. SL: Scutellariae Radix and Liriopis Tuber water extract. Each RAW 264.7 cell group was incubated with SL at the concentrations of 25, 50, 100, and 200 μg/mL for 24 hrs. The test material at the concentrations of 25, 50, 100, and 200 μg/mL significantly increased the cell viability. Results are represented as mean ± SD. $*$ represents $P < 0.05$ compared to the normal. $**$ represents $P < 0.01$ compared to the normal.

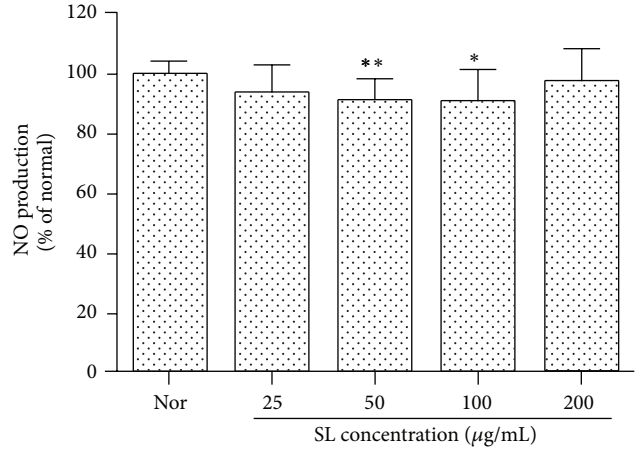

Figure 2: Effect of SL on NO production in RAW 264.7 cells. Normal (Nor): treated with media only. SL: Scutellariae Radix and Liriopis Tuber water extract. Each RAW 264.7 cell group was incubated with SL at the concentrations of 25, 50, 100, and 200 μg/mL for 24 hrs. SL at the concentrations of 50 and 100 μg/mL inhibited the production of NO significantly. Results are represented as mean ± SD. $*$ represents $P < 0.05$ compared to the normal. $**$ represents $P < 0.01$ compared to the normal.

three times of washing in the washing buffer, 120 μL reading buffer was added into each well, and 5 min of shaker culture (300~500 rpm) at the room temperature was followed. Using Bio-Plex array reader (Bio-Plex 200), the target cytokine levels were determined.

2.7. Statistical Analysis. All the results in this study are expressed as mean ± SD, and the difference between control value and that of the test material was analyzed by Student's t-test. Less than 0.05 of P value was considered as statistically significant.

3. Results

3.1. The Effect to RAW 264.7 on the Cell Viability. The test material at the concentrations of 25, 50, 100, and 200 μg/mL significantly increased the cell viability (Figure 1).

3.2. The Effect on the NO Production

3.2.1. In the Simple RAW 264.7 Cells. The result of 24-hour treatment of SL to RAW 264.7 cells showed that SL at the concentrations of 50 and 100 μg/mL inhibited the production of NO significantly (Figure 2).

3.2.2. In the LPS-Stimulated RAW 264.7 Cells. When RAW 264.7 cells were cotreated with LPS (1 μg/mL) and various concentrations of SL for 24 hrs, the production of NO in the LPS-stimulated RAW 264.7 cells was decreased significantly at all the concentrations of SL (25, 50, 100, and 200 μg/mL) (Figure 3).

3.3. The Effect on the Intracellular Free Calcium in the LPS-Stimulated RAW 264.7 Cells. When RAW 264.7 cells were cotreated with LPS (1 μg/mL) and various concentrations of SL for 18 hrs, the increase of intracellular free calcium in the LPS-stimulated RAW 264.7 cells was inhibited significantly by the treatment of SL at all the concentrations (25, 50, 100, and 200 μg/mL) (Figure 4).

3.4. The Effects on Cytokines Production

3.4.1. IL-6 Production in RAW 264.7 Cells. When RAW 264.7 cells were cotreated with LPS (1 μg/mL) and various concentrations of SL for 24 hrs, the increase of the production of IL-6 in the LPS-stimulated RAW 264.7 cells was significantly inhibited by the treatment of SL at all the concentrations (25, 50, and 100 μg/mL) (Table 1).

3.4.2. MIP-1a Production in RAW 264.7 Cells. When RAW 264.7 cells were cotreated with LPS (1 μg/mL) and various concentrations of SL for 24 hrs, the increase of the production of MIP-1a in the LPS-stimulated RAW 264.7 cells was significantly inhibited by the treatment of SL at all the concentrations (25, 50, and 100 μg/mL) (Table 1).

3.4.3. MIP-1β Production in RAW 264.7 Cells. When RAW 264.7 cells were cotreated with LPS (1 μg/mL) and various concentrations of SL for 24 hrs, the increase of the production of MIP-1β in the LPS-stimulated RAW 264.7 cells was significantly inhibited by the treatment of SL at all the concentrations (25, 50, and 100 μg/mL) (Table 1).

3.4.4. MIP-2 Production in RAW 264.7 Cells. When RAW 264.7 cells were cotreated with LPS (1 μg/mL) and various concentrations of SL for 24 hrs, the increase of the production of MIP-2 in the LPS-stimulated RAW 264.7 cells was significantly inhibited by the treatment of SL at all the concentrations (25, 50, and 100 μg/mL) (Table 1).

Table 1: Effects of SL on various cytokines production in LPS-treated RAW 264.7 cells.

Cytokines (pg/mL)	Normal	Control	SL25	SL50	SL100
IL-6	58.17 ± 4.54	25515.17 ± 96.13$^{\#}$	24079.17 ± 632.48*	23759.67 ± 243.89**	24204.33 ± 614.87*
MIP-1α	7259.00 ± 614.07	27791.33 ± 33.50$^{\#}$	25886.33 ± 247.03**	25939.67 ± 58.29**	26115.17 ± 381.14**
MIP-1β	7369.17 ± 2454.09	2588.00 ± 352.36$^{\#}$	24379.50 ± 287.76**	23904.33 ± 386.25**	24090.33 ± 276.86*
MIP-2	64.33 ± 17.04	25599.83 ± 68.31$^{\#}$	24114.50 ± 494.84**	23695.67 ± 163.49**	24334.00 ± 132.53*
G-CSF	84.33 ± 32.13	27370.00 ± 7.00$^{\#}$	25154.17 ± 341.76**	24702.50 ± 503.57**	25769.00 ± 132.32**
M-CSF	40.67 ± 4.62	77.00 ± 6.08$^{\#}$	68.67 ± 7.37	69.00 ± 5.29	64.17 ± 6.75
GM-CSF	57.33 ± 3.79	6185.17 ± 463.36$^{\#}$	4947.83 ± 706.00	4781.00 ± 547.75	6210.67 ± 1406.94
TNF-α	169.33 ± 17.50	7013.00 ± 245.35$^{\#}$	6393.67 ± 269.24*	6561.17 ± 656.97	6685.50 ± 1020.25
LIF	38.50 ± 4.92	7757.33 ± 376.11$^{\#}$	6878.83 ± 644.82	6626.33 ± 615.58	7765.50 ± 794.14
RANTES	134.67 ± 19.01	13871.83 ± 257.82$^{\#}$	13535.33 ± 1275.87	13082.50 ± 907.42	13789.67 ± 420.85

Normal: cytokine production in the RAW 264.7 cells treated with media only.
Control: cytokine production in the RAW 264.7 cells treated with LPS (1 μg/mL).
SL25: cytokine production in the LPS-stimulated RAW 264.7 cells incubated with Scutellariae Radix and Liriopis Tuber water extract at the concentration of 25 μg/mL.
SL50: the production of cytokines in the LPS-stimulated RAW 264.7 cells incubated with Scutellariae Radix and Liriopis Tuber water extract at the concentration of 50 μg/mL.
SL100: the production of cytokines in the LPS-stimulated RAW 264.7 cells incubated with Scutellariae Radix and Liriopis Tuber water extract at the concentration of 100 μg/mL.
Results are represented as mean ± SD.
represents $P < 0.05$ compared to the normal.
$*$ represents $P < 0.05$ compared to the control.
$**$ represents $P < 0.01$ compared to the control.

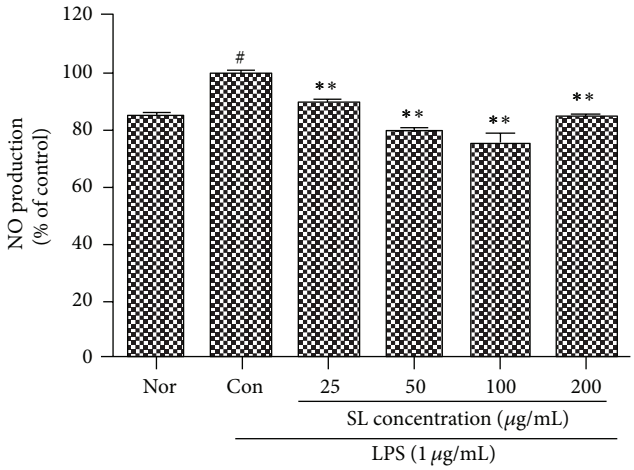

Figure 3: Effect of SL on NO production in LPS-treated RAW 264.7 cells. Normal (Nor): treated with media only. Control (Con): treated with LPS (1 μg/mL). SL: Scutellariae Radix and Liriopis Tuber water extract. Each RAW 264.7 cell group was incubated with SL at the concentrations of 25, 50, 100, and 200 μg/mL with LPS for 24 hrs. The production of NO in the LPS-stimulated RAW 264.7 cells was decreased significantly at all the concentrations of SL (25, 50, 100, and 200 μg/mL). Results are represented as mean ± SD. # represents $P < 0.05$ compared to the normal. $**$ represents $P < 0.01$ compared to the control.

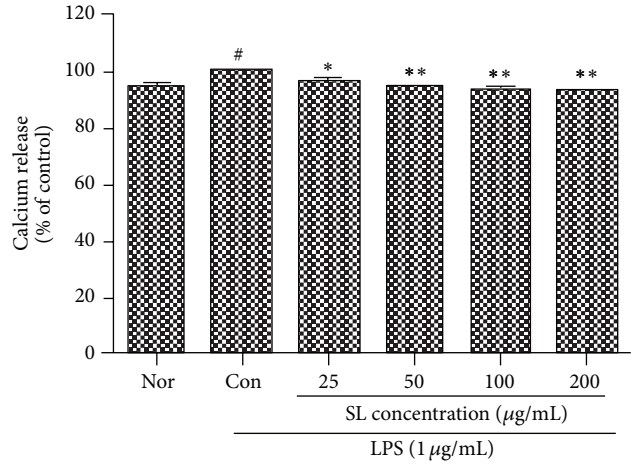

Figure 4: Effect of SL on calcium release in LPS-treated RAW 264.7 cells. Control (Con): treated with LPS (1 μg/mL). SL: Scutellariae Radix and Liriopis Tuber water extract. Each RAW 264.7 cell group was incubated with SL at the concentrations of 25, 50, 100, and 200 μg/mL with LPS for 18 hrs. The increase of intracellular free calcium in the LPS-stimulated RAW 264.7 cells was inhibited significantly by the treatment of SL at all the concentrations (25, 50, 100, and 200 μg/mL). Results are represented as mean ± SD. # represents $P < 0.05$ compared to the normal. $*$ represents $P < 0.05$ compared to the control. $**$ represents $P < 0.01$ compared to the control.

3.4.5. G-CSF Production in RAW 264.7 Cells. When RAW 264.7 cells were cotreated with LPS (1 μg/mL) and various concentrations of SL for 24 hrs, the increase of the synthesis of G-CSF in the LPS-stimulated RAW 264.7 cells was significantly inhibited by the treatment of SL at all the concentrations (25, 50, and 100 μg/mL) (Table 1).

3.4.6. The Effect on M-CSF Production in RAW 264.7 Cells. When RAW 264.7 cells were cotreated with LPS (1 μg/mL) and various concentrations of SL for 24 hrs, the increase of the synthesis of M-CSF in the LPS-stimulated RAW 264.7

cells was inhibited by the treatment of SL but not significantly (Table 1).

3.4.7. The Effect on GM-CSF Production in RAW 264.7 Cells. When RAW 264.7 cells were cotreated with LPS (1 μg/mL) and various concentrations of SL for 24 hrs, the increase of the synthesis of GM-CSF in the LPS-stimulated RAW 264.7 cells was inhibited by all the concentrations of treated SL but not significantly (Table 1).

3.4.8. TNF-α Production in RAW 264.7 Cells. When RAW 264.7 cells were cotreated with LPS (1 μg/mL) and various concentrations of SL for 24 hrs, the increase of the production of TNF-α in the LPS-stimulated RAW 264.7 cells was significantly inhibited by the treatment of SL at the concentration of 25 μg/mL (Table 1).

3.4.9. The Effect on LIF Production in RAW 264.7 Cells. When RAW 264.7 cells were cotreated with LPS (1 μg/mL) and various concentrations of SL for 24 hrs, the increase of the synthesis of LIF in the LPS-stimulated RAW 264.7 cells was inhibited by the treatment of SL but not significantly (Table 1).

3.4.10. The Effect on RANTES Production in RAW 264.7 Cells. When RAW 264.7 cells were cotreated with LPS (1 μg/mL) and various concentrations of SL for 24 hrs, the increase of the synthesis of RANTES in the LPS-stimulated RAW 264.7 cells was inhibited by the treatment of SL but not significantly (Table 1).

4. Discussion

Inflammation is fundamentally an immune reaction occurring in most of human body and can be divided by chronic and acute types. The latter reacting to physical stimulations or foreign body infections induces tissue injury instantly and, on the other hand, the former takes longer to occur and lasts longer, involving characteristic infiltration of monocytes, macrophages, lymphocytes, and other plasma cells, inducing fibrosis or vascularization via tissue destruction and healing process [16]. Macrophages, spreading out all the body tissues, are immune cells taking charge of the innate immune responses, preying on and phagocytizing foreign bodies, bacteria, virus, and aged cells [17]. And macrophages carry out important roles in the process of inflammation by producing many inflammatory mediators, that is, proinflammatory cytokines, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), nitric oxide (NO), or prostaglandins (PG) [18]. The activated macrophages, for example, secrete a large quantity of inflammatory mediators in the primary inflammatory reaction, inducing various inflammatory diseases such as bronchitis, arthritis, multiple sclerosis, atherosclerosis, stroke, degenerative brain diseases, and viral infection, often leading to aggravation of the diseases [19, 20]. Therefore, researches to discover anti-inflammation agents effectively reducing the diverse anti-inflammatory mediators in the midst of inflammation have flourished [16]. So, the in vitro researches showing anti-inflammatory effects, using a single medicinal herb or combined extracts of multiple medicinal herbs, have been reported [6].

Examining the existing view of the medical action of Scutellariae Radix, the extract of Scutellariae Radix, or the components of Scutellariae Radix, especially the medical action of baicalein and wogonin, flavonoids, has been actively researched [8] on the subjects of antitumor and anti-inflammatory effects [5, 7–10, 21–23]. Particularly, Yoon et al. reported that water extract of Scutellariae Radix inhibits the production of NO and synthesis of IL-6 and IL-10 in the LPS-stimulated macrophages [7]; Ha and Kim reported that baicalein, a primary component of Scutellariae Radix, regulates the production of inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, and the expression of COX-2 mRNA, and it represses the microglial activity, suggesting that the flavonoid could be a good candidate for the therapy of central nervous system inflammation [8]. Besides, Park also reported that the water extract of Scutellariae Radix keeps increasing or maintaining the hydrogen peroxide production in mouse macrophages, implicating immunopotentiating effect of the extract [9]. However, most of these reports are subjected to a single extract of Scutellariae Radix. The research of Kim et al. employed combined extracts including Scutellariae Radix, finding that Huanggeumjakyak-tang is effective as an anti-inflammation since the agent treated in macrophages repressed the production of NO, PGE_2, and IL-6 and the expression of iNOS and COX-2 mRNA [5]. In this context, since Scutellariae Radix has shown to be preventive as well as having therapeutic effects in various inflammation-related diseases, the authors of this study investigated the effect of Scutellariae Radix combined with other agents on the anti-inflammatory effect.

Liriopis Tube is reported to have diverse medical actions, such as antidiabetic effect, anti-inflammation, and suppressing Ig M antibody production [11, 24–26]. Rhee et al. reported that anti-inflammatory action, in particular, of the extract of Liriopis Tube is effective in pulmonary fibrosis by inflammation adjustment via increasing macrophages ratio and decreasing lymphocytes and neutrophils ratios [11]. In addition, Lee et al. reported the effects of Liriopis Tube combined with schizandra on the levels of IL-4, IL-5, and IL-6 in an asthma animal model [25].

As yet, experiment showing the effect of anti-inflammation of the combined extracts of Scutellariae Radix and Liriopis Tube has not been reported. The water extracts of Scutellariae Radix and Liriopis Tube were combined, yielding the testing sample (SL), which was applied to RAW 264.7 cells, a mouse macrophage cell line, to investigate the effect of the combined extracts on the anti-inflammation effect by measuring cell viability, NO production, intracellular free calcium level, and production of various cytokines.

In this study, SL treatment at 25, 50, 100, and 200 μg/mL did not reduce significantly the cell viability; rather it increased the rate significantly compared with those of control, implying that SL does not induce cellular toxicity on the macrophage cell line (Figure 1).

NO is known to be an inflammation modulator by involving various physiological functions such as vasodilation, neurotransmitter system, antibacteria, and immunomodulation. NO, produced as radical by NOS activity on L-arginine, plays an important intracellular secondary signal transducer. NOS

can be divided into two classes, constitutive NOS (cNOS) and inducible NOS (iNOS). cNOS largely works on vasodilation and neurotransmission and NO production by cNOS has an important role in body homeostasis [27]. iNOS involves immune toxicity and is activated by a variety of stimuli, such as LPS, interferon-γ (IFN-γ), IL-1, and TNF-α. The enzyme generates NO for a long period of time in macrophages, vascular smooth muscle cells, endothelial cells, liver cells, and cardiac muscle cells, by which high level of NO production in vivo results in destruction of host cells, vasodilation by shock, and tissue destruction by the induced inflammation reaction [28, 29]. In this study, SL at 50 and 100 μg/mL decreased the production of NO significantly. When the sample was applied to RAW 264.7 cell stimulated by LPS, SL at all the concentrations treated (25, 50, 100, and 200 μg/mL) decreased significantly the production of NO (Figures 2 and 3).

Calcium is known to play a very important role in inflammation. The activated signal transduction by Toll-like receptor increases intracellular free calcium, which subsequently mediates the increase of diverse inflammatory mediators [6]. SL treatment at 25, 50, 100, and 200 μg/mL suppressed significantly the intracellular free calcium production in RAW 264.7 cells stimulated by LPS (Figure 4).

Cytokines are water-soluble proteins produced in human body cells, regulating immunity and inflammation by affecting growth, differentiation, expansion, and activation of immune cells. IL-6 has an important role in the host defense mechanism and immune response [30]. SL treatment at 25, 50, and 100 μg/mL suppressed significantly the IL-6 production in RAW 264.7 cells stimulated by LPS (Table 1).

MIP is a member of the chemokine subfamily that was originally purified from the conditioned media of an LPS-stimulated macrophage cell line. Two major forms of MIP, MIP-1α and MIP-1β, are highly related in immune and inflammatory response. They activate granulocytes which can lead to acute neutrophilic inflammation. And they also induce the synthesis and the release of other proinflammatory cytokines such as IL-1, IL-6, and TNF-α from fibroblasts and macrophages. MIP-1 is best known for its chemotactic and proinflammatory effects but can also promote hematopoiesis. Particularly, MIP-1β is expressed by T cells, B cells, and monocytes after antigen or mitogen stimulation [31, 32]. SL treatment at 25, 50, and 100 μg/mL suppressed significantly the MIP-1α, the MIP-1β, and the MIP-2 production in RAW 264.7 cells stimulated by LPS (Table 1).

CSF, facilitating growth and differentiation of bone marrow stem cells as a hematopoietic growth factor, has gotten much attention since the factor has been reported to facilitate the differentiation of granulocytes and macrophages. Granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), and granulocyte macrophage colony-stimulating factor (GM-CSF) belong to CSF and multi-colony-stimulating factor is also known as IL-3 [5, 33–35].

G-CSF facilitates granulopoiesis and its level is increased in respiratory disease such as asthma [30, 31]. SL treatment at 25, 50, and 100 μg/mL suppressed significantly the G-CSF production in RAW 264.7 cells stimulated by LPS (Table 1).

M-CSF produced in osteoblasts or mesenchymal stem cells involves the differentiation of osteoclasts and big mononuclear cells and has an important role in the recombination of monocyte-macrophage cell lines [6]. SL treatment suppressed M-CSF production in RAW 264.7 cells stimulated by LPS but not significantly (Table 1).

GM-CSF facilitates growth and activation of granulocytes and macrophages as a protective tool against infection and inflammation [35]. SL treatment suppressed GM-CSF production in RAW 264.7 cells stimulated by LPS but not significantly (Table 1).

The role of TNF-α is in the regulation of immune cells. TNF-α can induce the release of chemokines, prostaglandins, protease, and growth factors by activating endothelial cell, neutrophil, B cell, and so forth [16]. SL, just treatment at 25 μg/mL, suppressed significantly the TNF-α production in RAW 264.7 cells stimulated by LPS (Table 1).

LIF is an IL-6 class cytokine and RANTES are known for kinds of chemokine. SL treatment suppressed LIF and RANTES production in RAW 264.7 cells stimulated by LPS but both not significant (Table 1).

These results suggest that SL treatment does not harm the viability of cells and SL could be used to alleviate the symptoms of acute and chronic inflammatory diseases which are induced by overproduction of various inflammatory mediators in macrophages, for example, by infectious agents such as LPS, or inflammatory autoimmune diseases [36, 37]. Further study, for example, the effect of SL on more diverse inflammatory mediators, is demanded for the follow-up research to find out the possibility of SL as a preventive and therapeutic medicine for various inflammatory diseases.

5. Conclusions

The water extracts of Scutellariae Radix and Liriopis Tube were combined, yielding the testing sample (SL), which was applied to RAW 264.7 cells to investigate the effect of SL on the cell viability, NO production, intracellular free calcium level, and production of various cytokines. As described in this study, SL treatment at all the concentrations of 25, 50, 100, and 200 μg/mL did not show any particular toxicity in the mouse macrophages; rather it increased cell viability and at 50 and 100 μg/mL NO production was decreased significantly. In addition, SL at all the concentrations of treatment significantly suppressed the production of NO and the increase of the intracellular free calcium in the mouse macrophages stimulated by LPS. The increases of IL-6, MIP-1α, MIP-1β, MIP-2, and G-CSF induced by LPS treatment in mouse macrophages were significantly suppressed by the treatment of SL at the concentrations of 25, 50, and 100 μg/mL.

These results implicate that SL has anti-inflammatory effect by suppressing the production of various inflammatory mediators in macrophages.

Conflict of Interests

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

Acknowledgment

This work was supported by a grant from Gachon University.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 876484, 13 pages
http://dx.doi.org/10.1155/2015/876484

Research Article

Antiamnesic Effect of *Actinidia arguta* Extract Intake in a Mouse Model of TMT-Induced Learning and Memory Dysfunction

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Received 9 June 2015; Revised 11 September 2015; Accepted 17 September 2015

Academic Editor: Tetsuya Konishi

The antiamnesic effects of ethyl acetate fraction from *Actinidia arguta* (EFAA) on trimethyltin- (TMT-) induced memory impairment were investigated to find the possibility of functional food substances. EFAA showed a potent AChE inhibitory effect (IC_{50} = 53 μg/mL) and efficient neuroprotection against H_2O_2-induced oxidative stress. The administration of EFAA significantly decreased TMT-induced cognitive deficit in Y-maze, passive avoidance, and Morris water maze (MWM) tests. After the behavioral tests, the antioxidant activities were confirmed using mice brain tissues. EFAA not only showed the inhibition of AChE activity and the decline of malondialdehyde (MDA) level as a sign of lipid peroxidation but also presented the increase of the superoxide dismutase (SOD) level and the decrease of the oxidized glutathione (GSSG)/total glutathione (GSH + GSSG) ratio. Finally, the phenolics in EFAA were identified using liquid chromatography coupled with hybrid triple quadrupole-linear ion trap mass spectrometry, and four main phenolics, such as quinic acid, chlorogenic acid, caffeoyl hexose, and quercetin-3-glucoside, were identified. These results suggest that EFAA containing physiological phenolics might enhance drug-induced amnesia through AChE inhibition and neuroprotection.

1. Introduction

Alzheimer's disease (AD), which is one of the most serious diseases in the aged societies of developed countries, is a neurodegenerative disorder characterized by loss of learning and memory. Cholinergic hypothesis was proposed as an AD pathogenesis, owing to its relation to the extensive loss of neurons in the nucleus basalis of Meynert [1]. A decrease of choline acetyltransferase (ChAT) levels and an increase of acetylcholinesterase (AChE) levels were related to cognitive dysfunction through the decrease of acetylcholine (ACh) levels, which is a neurotransmitter [2, 3].

Reactive oxygen species (ROS), the products of oxygen metabolic processes, such as the superoxide anion radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$), can cause neuronal apoptosis and impair cellular function and membrane integrity [4]. Many researchers have not only demonstrated an increase of oxidative stress in the brains of AD patients but also reported that the increase of antioxidant uptake is inversely related to the risk of AD incidence [5]. Some phenolic compounds through the intake of foods such as fruits or vegetables may reduce the risk of AD owing to their antioxidant properties, which protect the neuronal cell from oxidative stress caused by ROS, which may be related to neurodegenerative disease [6]. Recently, phenolic compounds have been studied as a source of natural antioxidants [7, 8].

Actinidia arguta (*A. arguta*), which belongs to the family Actinidiaceae, is a high-value food resource, globally 2–5 genera, 280–560 species, and native *Actinidia* spp. in Korea

include *A. arguta*, *A. polygama*, *A. rufa*, and *A. kolomikta* [9]. *Actinidia* spp. in Korea have strong cold resistance and pest resistance, and the whole of the fruit can be eaten without peeling it, owing to its thin bark, hairless, and mouthful-sized properties. Although its size is relatively small compared with the kiwi, it can be utilized in a variety of foods owing to its high sugar content and various nutrients, including vitamin C. *A. arguta* is known to alleviate fever and thirst as well as dyspepsia in Korean folk medicine [10]. The root of *A. arguta* is also used for the treatment of vomiting and arthralgia, and its rich vitamin C content can prevent fatigue and scurvy.

Trimethyltin (TMT) is an organometal neurotoxic compound. TMT exposure in rats has been reported to induce extensive hippocampal damage as well as abnormal behavior, such as hyperactivity [11]. Additionally, behavioral tests using TMT-induced animals are useful for the study of memory dysfunction, such as neurodegenerative disease [12].

The physiological activities of *A. arguta*, including the antiallergic, anti-inflammatory, antidiabetic, and antioxidant effects, have been reported by several recent studies [13, 14]. However, research on *A. arguta* related to cognitive function is insufficient, and most of all, physiological and cognitive improvement effects have not yet been reported. Consequently, the aim of the present study is to evaluate ameliorating effect of *A. arguta* on TMT-induced learning and memory deficits in ICR mice and is to identify main phenolic compounds.

2. Materials and Methods

2.1. Materials. Vitamin C, thiobarbituric acid, acetylthiocholine, H_2O_2, TMT, dimethyl sulfoxide (DMSO), 2′,7′-dichlorofluorescein diacetate (DCF-DA), 2′,3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kit, lactate dehydrogenase (LDH) assay kit, 9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate (tacrine), superoxide dismutase (SOD) assay kit, and solvents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), and glutathione (GSH) detection kit was also purchased from Enzo Life Science Inc. (Enzo Diagnostics, NY, USA).

2.2. Extraction of A. arguta. The fruit of *A. arguta* (cultivar: Autumn Sense) was received from the Korea Forest Research Institute in September 2013 and was authenticated by the Korea Forest Research Institute. A voucher specimen was deposited at the Herbarium of the Department of Special Purpose Trees, Korea Forest Research Institute. After *A. arguta* was washed with running tap water, it was ground. Mixture (200 g) was suspended and extracted with 80% ethanol (4 L) at 60°C for 2 h. The extracts were filtered through Whatman number 2 filter paper (Whatman International Limited, Kent, UK) and evaporated. The evaporated materials were redissolved until 300 mL of distilled water. These redissolved solutions were consecutively partitioned in a separatory funnel with the equivalent amount of three solvents (*n*-hexane, chloroform, and ethyl acetate). The fractions were concentrated in a vacuum evaporator (N-N series, EYELA Co., Tokyo, Japan) at 60°C and were lyophilized. The lyophilized ethyl acetate fraction from *A. arguta* (EFAA) was stored at −20°C until used.

2.3. AChE Inhibitory Assay. The AChE inhibitory activity was carried out by the Ellman method using acetylthiocholine iodide as a substrate [15]. Cultured fluid of PC 12 cells was homogenized with 5 mL lysis buffer (pH 7.4), containing 10 mM Tris-HCl, 1 M NaCl, 50 mM $MgCl_2$, and 1% Triton X-100 using the Glas-Col homogenizer, and supernatant was obtained by centrifugation at 14,000 g for 30 min. The supernatant was used as an enzyme, and all processing was performed at 4°C. Protein level in the supernatant was measured using the Quant-iT protein assay kit (Invitrogen, Carlsbad, CA, USA). After adding each 10 μL EFAA and 10 μL enzymes, preincubation was at 37°C for 15 min. Then after adding an Ellman reaction mixture in a 50 mM sodium phosphate buffer (pH 8.0) to the above reaction mixture absorbance was measured at 405 nm.

2.4. Neuronal Cell Culture and Measurement of Intracellular Oxidative Stress. PC 12 cells (KCLB 21721; Korea Cell Line Bank, Seoul, Korea) were reproduced in an RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum, 25 mM HEPES, 25 mM sodium bicarbonate, 50 units/mL penicillin, and 100 μg/mL streptomycin. The cells were cultured under the conditions (5% CO_2 and 37°C).

Levels of intracellular ROS were measured using the DCF-DA assay [4]. DCF-DA as a nonfluorescent compound, upon entering into a cell, is deesterified, and then it forms substrate (fluorescence DCF) by intracellular ROS such as H_2O_2. Cells (10^4 cells/well on 96-well plate) were treated with EFAA or vitamin C (positive control). After 48 h, cells were treated with or without 200 μM H_2O_2, and cells were incubated for 2 h. Finally, cells were treated by the 50 μM DCF-DA dissolved in phosphate buffered saline (PBS). Fluorescence was measured by fluorescence microplate reader (Infinite 200, Tecan Co., San Jose, CA, USA) with 485 nm excitation and 530 nm emission filters.

2.5. Determination of Cell Viability. Neuroprotective effect on H_2O_2-induced oxidative stress was measured by the MTT reduction assay. PC 12 cells (10^4 cells/well on 96-well) were treated with EFAA or vitamin C (positive control); then they were preincubated for 48 h. The cells were treated with or without 200 μM H_2O_2 for 3 h. The amount of formed MTT formazan by ability to return of mitochondria in living cells was measured using a microplate reader (Bio-Rad, Tokyo, Japan) at a test wavelength of 570 nm and a reference wavelength of 690 nm [4].

Protective effect of neuronal cell membrane was also confirmed using the LDH assay kit. In brief, cells were settled by centrifugation at 250 g for 4 min, and 100 μL of supernatants was transferred into new 96-well. Damage of neuronal cell membrane was evaluated by measuring the amount of the intracellular enzyme, LDH released into the medium [4].

2.6. Animals. All experimental procedures were approved by the guidelines established by the Animal Care and Use Committee of Gyeongsang National University (certificate: GNU-131105-M0067). The Institute of Cancer Research (ICR)

mice (4 weeks old, male) were purchased from Samtako (Osan, Korea), and mice were housed two per cage in a room maintained with a 12 h light-dark cycle, 55% humidity, and 23–25°C temperature. The EFAA was dissolved in drinking water at concentrations of 5, 10, and 20 mg/kg body weight, and once a day was oral administration through stomach tube. The mice were allowed free access to feed and water for 3 weeks. After 3 weeks, the TMT (7.1 μg/kg of body weight (2.5 mg/kg)) was dissolved in 0.85% sodium chloride solution (w/v), and mice were intraperitoneally treated with a single injection (100 μL). The control group was injected (100 μL) with only sodium chloride solution without TMT [16].

2.7. Behavioral Tests in TMT-Induced Amnesic Mouse Model. Recording spontaneous alternation behavior in a Y-maze test was used to evaluate the immediate spatial working memory performance with the SMART video-tracking system (SMART v3.0, Panlab SL, Barcelona, Spain). The Y-maze test was performed after 3 days on the TMT injection. The maze was made of black-painted plastic, and each arm of the maze was 33 cm long, 15 cm high, and 10 cm wide and was positioned at equal angle. Each mouse was placed at the end of one arm and allowed to move freely through the maze for 8 min. The arm entry of mouse was considered to have been completed only when the hind paws of the mouse were placed completely in the arm of the maze. Alternation behavior is defined as successive entries into the three arms in an overlapping triplet set. The percentage of alternation behavior was calculated by the following formula using the total arm entry with a score [17]:

$$\begin{aligned}&\text{Alternation behavior (\%)}\\&\quad=\frac{\text{Actual alternation}}{\text{Maximum alternation}}\times 100,\\&\text{Maximum alternation}\\&\quad=\text{total number of arm entries}-2.\end{aligned}\tag{1}$$

The passive avoidance test was performed to investigate the short-term memory ability. Test box was divided into two parts, one illuminated and one dark, and a wire mesh floor. The mice were allowed to move freely through a tunnel between the two parts. The training trial was carried out after 4 days of the TMT injection. Mouse was placed in the light part and inescapable electric shock was provided (0.5 mA, 3 s) when the hind paws of the mouse were completely placed in the dark part. After single training trial, the passive avoidance test (5 days after TMT injection) was conducted. The mouse was again placed in the light part, and the time latency was measured and when consumed the mouse was reentered intothe dark part and step-through latency time into the dark part was evaluated (the step-through latency maximum testing limit was 300 s) [17].

The Morris water maze (MWM) test was carried out by referring to the Morris study with some modification [18]. The equipment consisted of a stainless steel circular pool (90 cm in diameter and 60 cm in height) that was randomly divided into quadrants (E, W, S, and N zones) with visual cues on the walls for navigation. The circular pool was filled up to 30 cm (high) using squid ink (Cebesa, Valencia, Spain) in water (22 ± 2°C). A platform (6 cm in diameter) was installed in the middle of the W zone, the position of which was unchanged during the training session. The mice were allowed to swim and the latency time until they escaped from the water onto the submerged platform up to a maximum of 60 s was recorded, and they were allowed to stay on the platform for 15 s. In the training session (6 days after TMT injection), the mice were subjected to swim for escape during four trials per day. The probe test (10 days after TMT injection) was conducted to evaluate the spatial memory and long-term memory without the platform for 60 s, and the time spent in the W zone was recorded using a SMART 3.0 video-tracking system.

2.8. Biochemical Analysis of the Mice Brains. For biochemical analysis, preparation for SOD activity involves homogenizing small pieces of whole brain with 40 volumes of ice-cold PBS. To get the pellets, the homogenates were directly centrifuged at 400 g for 10 min at 4°C. The pellets in 5–10 volumes of ice-cold 1x Cell Extraction Buffer (10% SOD buffer, 200 μM phenylmethane sulfonylfluoride, and 0.4% (v/v) Triton X-100 in distilled water) were incubated on ice for 30 min and centrifuged at 10,000 g for 10 min at 4°C to get the supernatant. The protein concentration was determined using the Quant-iT protein assay kit (Invitrogen, Carlsbad, CA, USA).

The preparation for the determination of GSH and oxidized glutathione dimer (GSSG) level involves homogenizing small pieces of whole brain with 20 volumes of 5% metaphosphoric acid and direct centrifugation at 14,000 g for 15 min at 4°C to get the supernatant. To determine GSSG, the supernatant was treated with 2 M 4-vinylpyridine and incubated for 1 h at room temperature. The measurements of SOD and GSH were carried out using commercial kits. The concentration of protein was determined using the Quant-iT protein assay kit (Invitrogen, Carlsbad, CA, USA).

Brains of mice were dissected and homogenized with PBS corresponding to the 10 volumes of whole brain tissues. To get the supernatant, the homogenates were centrifuged at 10,000 g for 60 s at 4°C. 160 μL of each supernatant was mixed with 960 μL of 1% (v/v) phosphoric acid followed by addition of 320 μL 0.67% (v/v) thiobarbituric acid solution. The mixture was incubated at 95°C in water bath for 1 h. The reactant (colored complex) was centrifuged at 1,600 g for 10 min, and absorbance of supernatant was measured at 532 nm using tetramethoxypropane as a standard. Malondialdehyde (MDA) levels as a token of lipid peroxidation were expressed as nmole/mg protein [8].

2.9. Phenolic Compounds Analysis. Analysis for physiological phenolics in EFAA was performed using the 3200 QTRAP with a hybrid triple quadrupole-linear ion mass spectrometer (Applied Biosystems, Foster City, CA, USA), and C_{18} column (250 × 4.6 mm, 5.0 μm, ProntoSIL, BISCHOFF Chromatography, Germany) was used. The eluent solvents were used to A (0.1% formic acid in distilled water) and B (0.1% formic acid in acetonitrile), and a gradient condition was applied

as follows (min, %B): (0, 20), (20, 60), (30, 90). The flow rate was 0.5 mL/min with a 20 μL injection volume, column oven temperature of 30°C, and all the analyses were carried out using a TurboIonSpray ionization source, and ESI-MS conditions were as follows: negative-ion mode, curtain gas (N_2) 20 (arbitrary units), drying gas (N_2) heated to 650°C, and a variety of collision energies.

Analysis for contents of phenolics in EFAA was performed using the high performance liquid chromatography (HPLC) with a photodiode array UV-Vis detector system (Shimadzu Corporation, Kyoto, Japan), and C_{18} column (250 × 4.6 mm, 5.0 μm, ProntoSIL, BISCHOFF Chromatography, Germany) was used. The mobile phase was used to acetonitrile: 10 mM KH_2PO_4 (10 : 90, v/v), isocratic, and monitored for 30 min (wavelength 205 nm). The flow rate was 1.0 mL/min with a 10 μL injection volume and column oven temperature of 30°C.

2.10. Statistical Analysis. All data were expressed as mean ± SD. Verification of each average value was subjected to analysis of variance (ANOVA) using the SAS software (version 9.1, SAS Institute, Cary, NC, USA). Duncan's new multiple range test was used to determine the difference of means, and $p < 0.05$ was considered to be statistically significant.

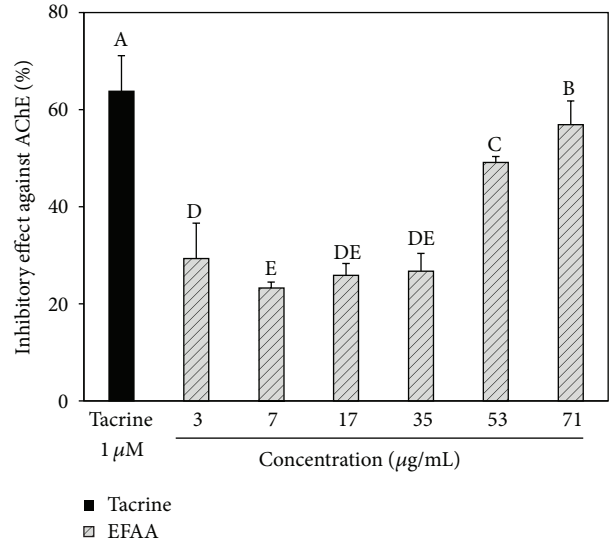

Figure 1: Inhibitory effect of ethyl acetate fraction from *Actinidia arguta* (EFAA) on cellular AChE. Inhibition was expressed as a percentage of enzyme activity inhibited with the control value (100%). Results shown are mean ± SD ($n = 3$). Data were statistically considered at $p < 0.05$, and different letters in graph represent statistical difference.

3. Results and Discussion

3.1. Cellular AChE Inhibitory Effect of EFAA. Neurodegenerative disease is related to damage or to the death of the neuronal cells that generate ACh as a neurotransmitter, and it can be decreased by AChE [1]. Drugs for neurodegenerative diseases have been used to maintain high ACh levels, but reported side effects include gastrointestinal disturbances [19]. Hence, an AChE inhibitor has been demanded that is a safe natural product without side effects, and our study also examined the AChE inhibitory effect of EFAA as a natural plant source.

EFAA showed a significant AChE inhibitory effect similar to 1 μM of tacrine (positive control) (Figure 1). Tacrine showed the highest inhibitory effect against AChE (63.79%), and most of the EFAA groups significantly inhibited the AChE activity and showed an IC_{50} value of 53 μg/mL. Neurodegenerative diseases are related to reduced ACh levels as well as relatively high AChE levels resulting from the loss of cholinergic neurons [1]. The phenolics of a natural plant were reported to have an AChE inhibitory effect [3], and kiwifruit belonging to *Actinidia* spp. showed a high AChE inhibitory effect in *in vitro* analysis [20]. Therefore, the EFAA might be helpful in improving cognitive dysfunction through inhibition of AChE.

3.2. Inhibitory Effect of EFAA on Intracellular Oxidative Stress and Neuronal Cell Protective Effect of EFAA. Oxidative stress caused by excessive accumulation of ROS may impair neuronal cells, and this increased oxidative stress has been implicated in most neurodegenerative diseases [5]. Neuronal cells are particularly vulnerable to ROS, such as H_2O_2, and excessive exposure to ROS can lead to neurodegenerative diseases resulting from neuronal cell death [4]. Because cellular oxidative stress is an important factor in neurodegenerative diseases, such as AD, the effect of EFAA was measured by DCF-DA assay.

The H_2O_2 group intracellular oxidative stress level was increased (approximately 114.93%) compared with that of the control group (100.00%) (Figure 2(a)). In contrast, the EFAA groups' intracellular oxidative stress levels dose-dependently decreased compared with that of the H_2O_2 group. In particular, EFAA (1000 μg/mL) showed a potent inhibitory effect (approximately 41%) on intracellular oxidative stress compared with the vitamin C group (92.16%), and all EFAA groups showed a significantly low oxidative stress level compared with that of the control group. These results indicated that EFAA protected the neuronal cells against H_2O_2-induced oxidative stress. A previous study reported that the *A. arguta* sprout showed a significant reducing effect on the intracellular ROS level caused by H_2O_2-induced oxidative stress [14]. In addition, natural antioxidants, such as phenolics, have a superior protective effect on neuronal cell damage caused by oxidative stress [6]. Therefore, phenolics in EFAA may protect neuronal cells by reducing increased oxidative stress.

Mitochondria might be regarded as one of the major targets that could be easily damaged by oxidative stress, causing neuronal cell death, because the induction of the mitochondrial permeability transition (MPT) pore can lead to mitochondrial cell death, which is related to the release of cytochrome C [21]. The cell viability of *A. arguta* on H_2O_2-induced neurotoxicity was examined by MTT assay, and the results are shown in Figure 2(b). The H_2O_2 group showed low

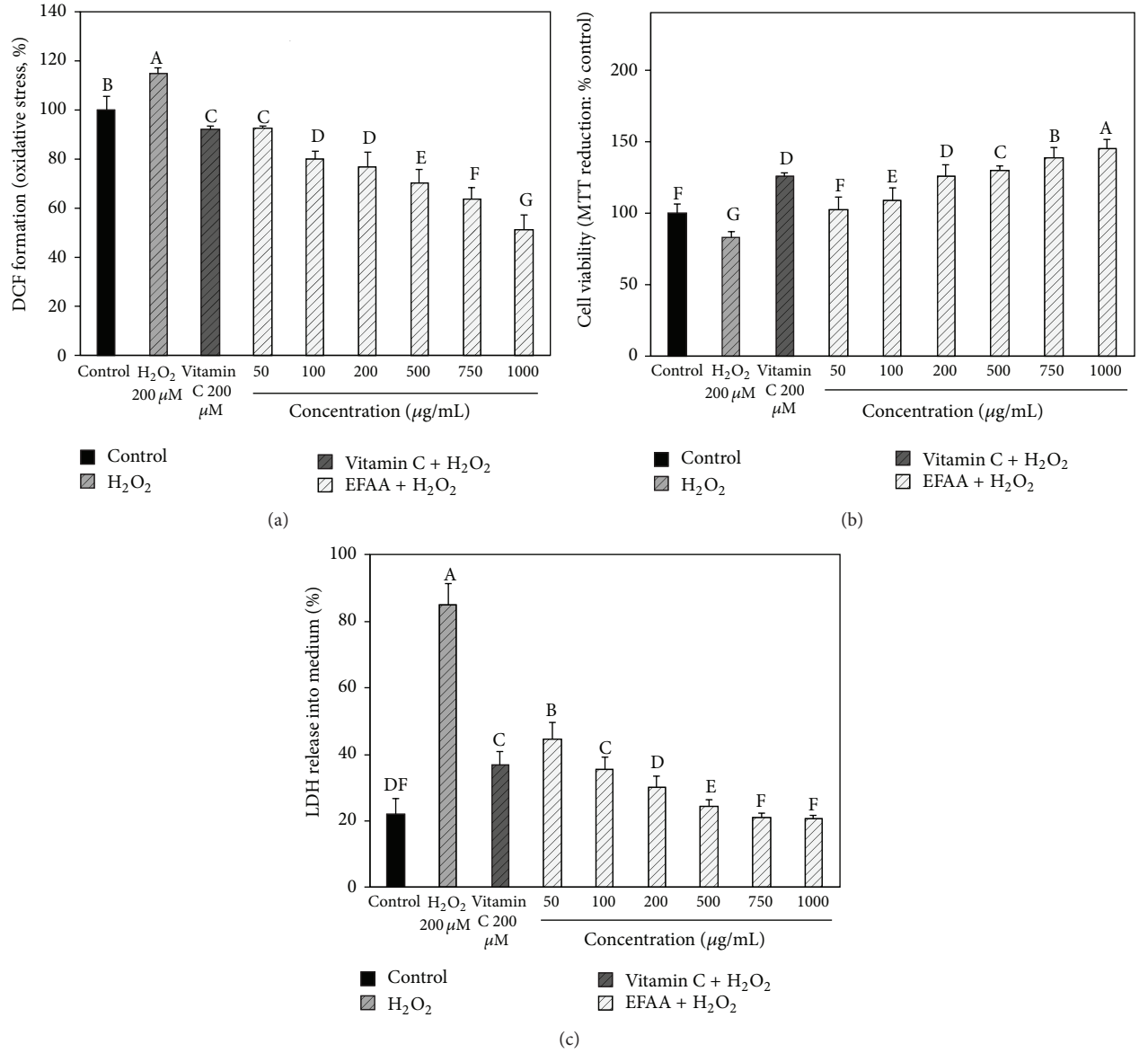

Figure 2: Protective effect of ethyl acetate fraction from *Actinidia arguta* (EFAA) on ROS production by the H_2O_2-induced cellular oxidative stress in PC 12 cells (a), neuronal cell protective effect on H_2O_2-induced cytotoxicity (b), and LDH release inhibitory effect on H_2O_2-induced membrane damage (c). Results shown are mean ± SD ($n = 3$). Data were statistically considered at $p < 0.05$, and different letters in graph represent statistical difference.

cell viability (an approximately 17% decrease) compared with the control group (100.00%), and the neuronal cell protective effect (125.82%) of the vitamin C group was higher than in the control group. EFAA increased the cell viability compared with the H_2O_2 group and showed slightly higher cell viability than the vitamin C group in 500–1000 μg/mL.

LDH is released into the medium by various ROS, which can lead to changes in the integrity and fluidity of the cell membrane, because neuronal cells have vulnerable structural characteristics against oxidative stress owing to a relatively high amount of lipid ingredients [4]. LDH was measured as a marker of neurodegenerative disease. The protective effect of EFAA against H_2O_2-induced cell membrane damage was examined by LDH assay, and the results are shown in Figure 2(c). The H_2O_2 group increased the LDH release quantity (approximately 60%) compared with the control group (22.07%), whereas the vitamin C group decreased the LDH release quantity (approximately 48%) compared with the H_2O_2 group, protecting the neuronal cell membranes. EFAA groups (≥200 μg/mL) showed an excellent inhibitory effect on LDH release into the medium compared with the vitamin C group as a positive control. In particular, the EFAA groups (≥750 μg/mL) showed a LDH release quantity similar to that of the control group.

Polyunsaturated fatty acids, such as linoleic acid and arachidonic acid, in the neuronal cells of the brain are weak to attacks by ROS [22]. Some studies have demonstrated significantly increased lipid peroxidation products (e.g.,

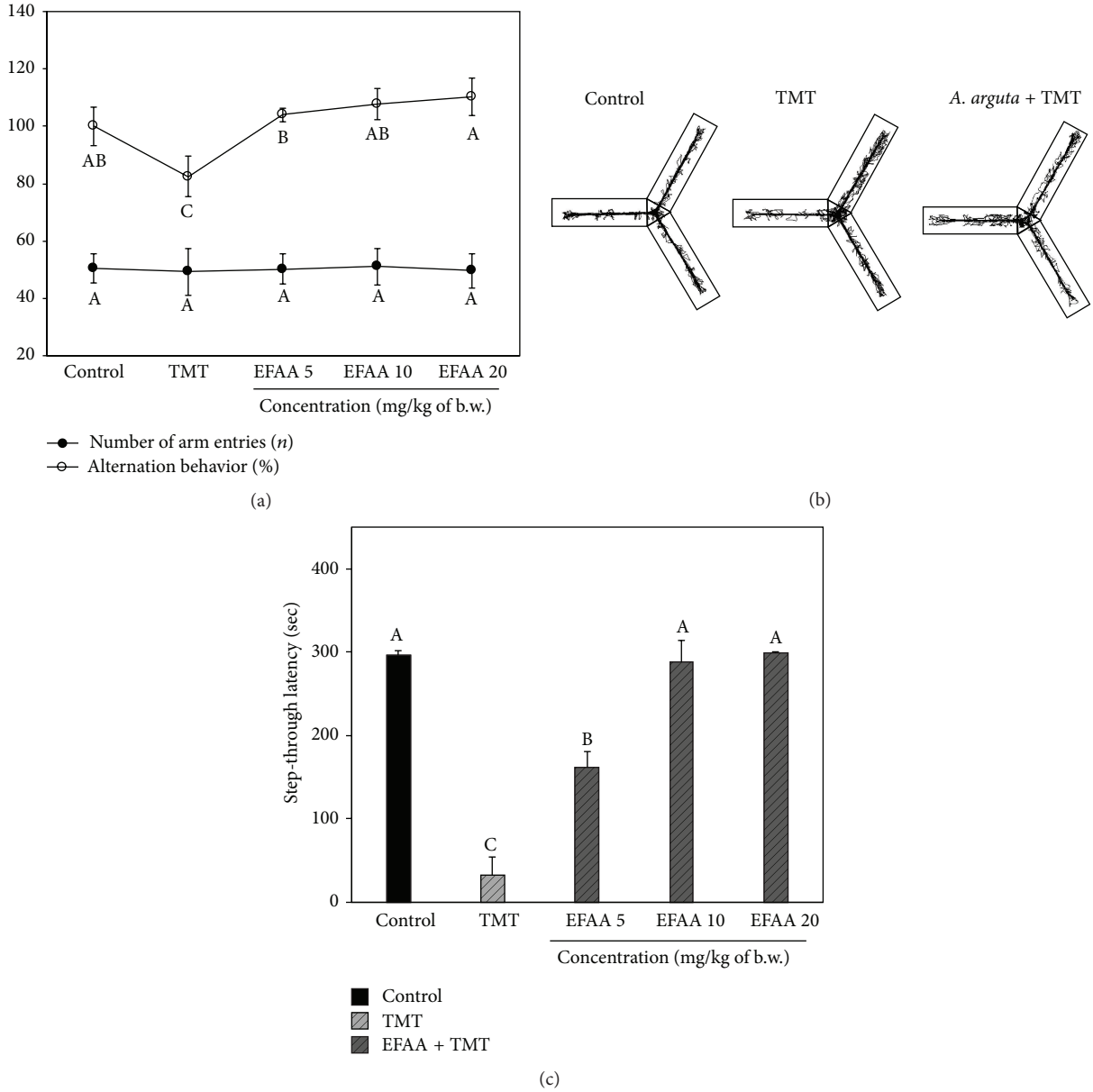

Figure 3: Protective effect of ethyl acetate fraction from *Actinidia arguta* (EFAA) against Y-maze and passive avoidance tests in TMT-induced amnesia. The spontaneous alternation behavior and number of arm entries (a) and path tracing of each group (b) were measured. Passive avoidance test was conducted 3 days after the TMT injection (c), and step-through latency (300 s) in the retention trial test was measured. Results shown are mean ± SD ($n = 3$). Data were statistically considered at $p < 0.05$, and different letters in graph represent statistical difference.

MDA, 4-hydroxynonenal, and acrolein) in the brains of AD patients [23]. If lipid peroxidation by ROS is inhibited, this may protect neuronal cells from acrolein, which expresses toxicity for the mitochondria [22]. The above results suggest that EFAA displayed protective effects on neuronal cells by inhibiting mitochondrial injury and cell membrane damage against H_2O_2-induced neurotoxicity.

3.3. Effect of EFAA on Behavioral Tests. Learning and memory impairments as the primary symptoms of AD have been related to the cholinergic system. TMT is known to cause various types of damage in terms of behavioral and biochemical deficits by causing pyramidal cell loss in the hippocampus as a potent neurotoxicant [11]. Therefore, our studies were carried out to confirm the beneficial effect of EFAA on TMT-induced cognitive dysfunction using Y-maze, passive avoidance, and MWM tests.

The Y-maze test was carried out using the innate tendencies of mice, which prefer to explore new environments in the maze rather than previously visited environments. In Figure 3(a), the TMT group showed impaired spatial working memories (118%, 18% decrease in alternation behavior) compared with those of the control group (100%). The EFAA groups showed increased alternation behavior (EFAA

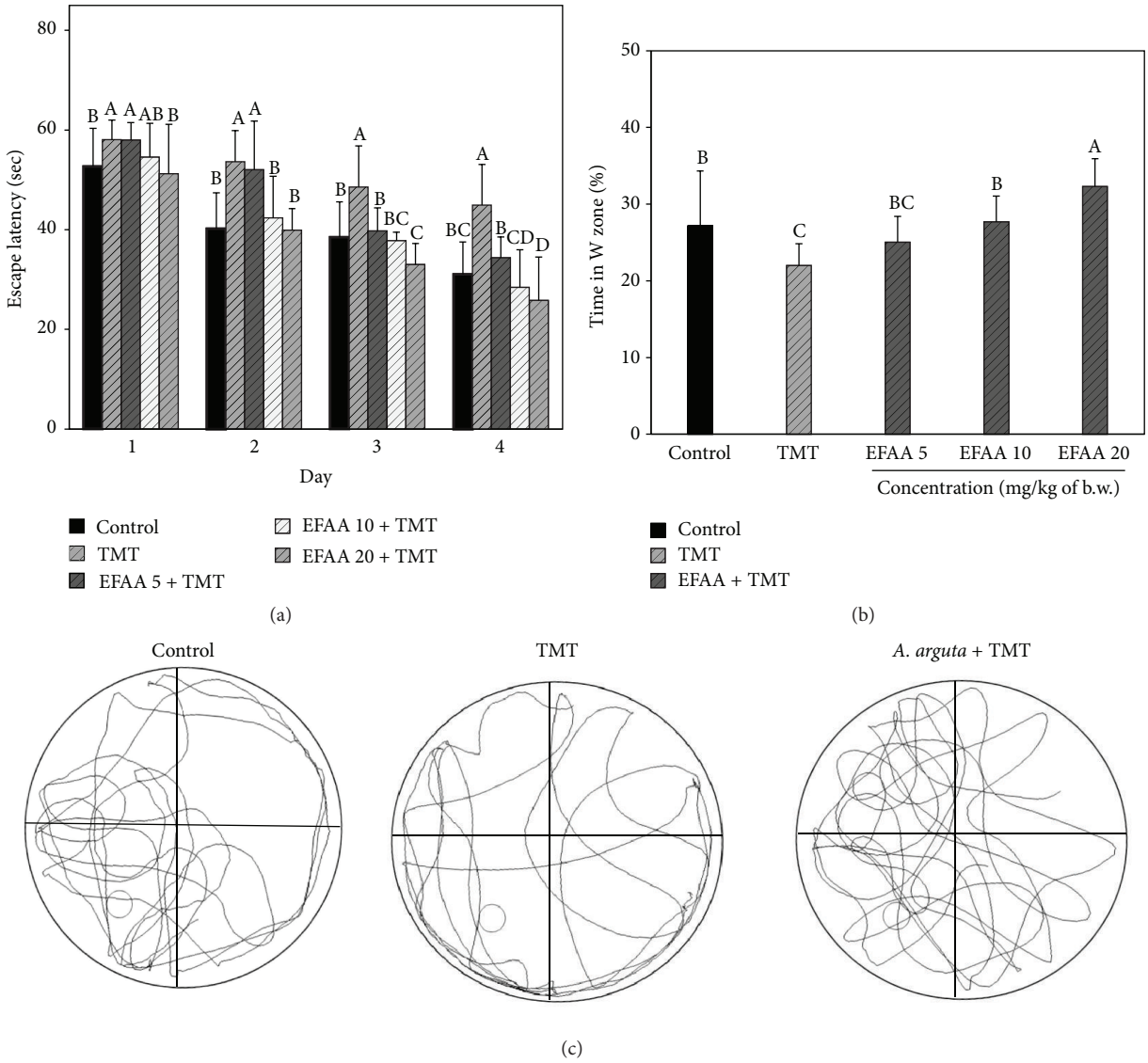

Figure 4: Protective effect of ethyl acetate fraction from *Actinidia arguta* (EFAA) against Morris water maze test in TMT-induced amnesia. The test was performed 5 days after the TMT injection, and escape latency in the training trial (a), platform crossings of probe trial sessions (b), and movement routes of each group in the probe trial (c) were measured during the 5 days. Results shown are mean ± SD ($n = 3$). Data were statistically considered at $p < 0.05$, and different letters in graph represent statistical difference.

5: 103.99%, EFAA 10: 107.65%, and EFAA 20: 110.21%). In contrast, the basic motor ability of the mice was not affected by TMT, because the number of arm entries showed no statistical difference between all experimental groups. Furthermore, the TMT group indicated that abnormal behavior, such as hyperactivity, was induced, because the movement routes of the TMT group were imbalanced compared with those of the control group. However, the EFAA group showed a similar shape to that of the control group (Figure 3(b)).

A passive avoidance test was performed to examine learning and short-term memory abilities in mice, which were given an unavoidable electronic shock when entering a dark place (Figure 3(c)). The TMT group showed a significantly low latency time (32.83 s, an 88.95% decrease in step-through latency) compared with that of the control group (297.20 ± 4.76 s). In contrast, the EFAA groups showed effectively attenuated step-through latency against TMT-induced impairment (EFAA 10: 288.20 s and EFAA 20: 298.75 s).

Based on these results, a long-term learning and spatial memory test was performed via the MWM test, and the results are shown in Figure 4(a). The TMT groups showed relatively high escape latency times on days 1, 2, 3, and 4 during the training trials compared with the control group. However, the EFAA groups showed decreased escape latency time during the training trials compared with the TMT group. In particular, the EFAA 20 group had lower escape latency times on days 3 and 4 in the training trials than the control group. After the training trials, long-term learning and spatial memory were examined in the probe trial without the platform. The TMT group spent relatively less time

(22.00%) in the W zone compared with the control group (27.15%); however, the EFAA groups showed a relatively higher retention time (EFAA 5: 25.05%, EFAA 10: 27.69%, and EFAA 20: 32.31%) in the W zone (Figure 4(b)). In addition, Figure 4(c) shows the movement routes of the mice in each group. The EFAA groups showed a relatively large amount of movement routes in the platform area compared with the other areas, whereas the TMT group showed random moving patterns. The mice of the EFAA group spent more time in the platform area compared with the TMT group.

The brains of rodents, including the hippocampus and prefrontal cortex, are involved in tasks such as learning and memory [18]. Previous research has reported that TMT exposure may lead to neurodegenerative disease, including neurobehavioral alteration, behavioral abnormality, aggression, and learning impairment caused by hippocampal damage [11]. Therefore, the present *in vivo* results demonstrated TMT-induced hippocampal damage via behavioral tests (Y-maze, passive avoidance, and MWM tests) of mice, and the ameliorating effect of EFAA was confirmed. According to previous studies, ferulic acids as phenolics showed an ameliorating effect on cognitive function against TMT-induced amnesia in *in vivo* tests (Y-maze and passive avoidance tests) and phenolics protected neuronal cells from damage by oxidative stress and increased ChAT activity [2]. In our results, the TMT-exposed mice showed low cognitive ability in each behavioral test compared with the control group, and this cognitive dysfunction caused by TMT was consistent with the findings of a previous study [12]. The EFAA groups showed improved learning and memory functions against TMT-induced cognitive deficit, and these beneficial effects may be considered as affected by phenolics in EFAA. Additionally, hippocampus lesions are related to spatial memory impairment [18]. The TMT group may have experienced significant hippocampal damage caused by TMT, because the TMT-exposed mice showed decreased escape ability in the training trials and probe tests in the MWM test. In addition, the EFAA group showed improved long-term and spatial memory, owing to relatively much more time spent in the platform area than that of the TMT-exposure mice. These results strongly suggest that phenolics in EFAA may have a significant effect on improving cognitive function. Therefore, phenolics in EFAA not only might be helpful for improving cognitive function by protecting neuronal cells or inhibiting AChE but also could enhance spatial memory and long-term memory against TMT-induced amnesia.

3.4. AChE Activity in Mice Brain Tissues. Regarding the mechanisms of TMT-induced amnesia, it has been speculated that damage to the cholinergic system in the hippocampus is related to the change of neurotransmitters, as well as neuronal cell loss. ACh level plays an important role in the modulation of cognitive performance and signal transfer in the synapses, and it may correlate with neurodegenerative disease [24]. Therefore, TMT-induced AChE activation in mice brains and the inhibitory effect of EFAA were investigated. In Figure 5, the TMT group shows increased AChE activity (approximately 39%) compared with the control group (100.00%),

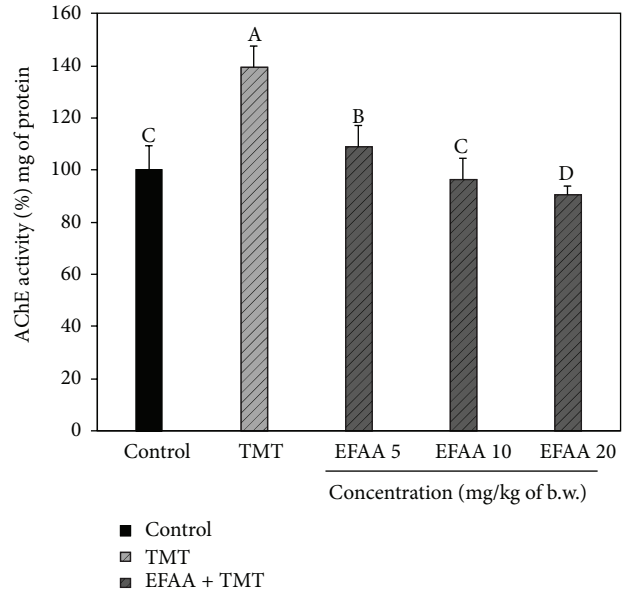

Figure 5: Inhibitory effect of ethyl acetate fraction from *Actinidia arguta* (EFAA) on AChE from TMT-induced defective mice brain homogenates. Results shown are mean ± SD ($n = 3$). Data were statistically considered at $p < 0.05$, and different letters in graph represent statistical difference.

whereas the AChE activity of the EFAA groups is significantly decreased. In particular, the EFAA 20 group showed decreased AChE activity (approximately 10%) compared with the control group. According to a previous study, the brain tissues of TMT-induced ICR mice showed high AChE activity and *Poncirus trifoliata* extract significantly inhibited AChE activity [12]. This was consistent with our results, which show increased AChE activity by TMT, and EFAA displayed inhibitory activity against TMT-induced AChE in the brain tissues. Excessive release of AChE, which is well known as a biomarker for memory malfunction, might be a factor in reducing cognitive function owing to the acceleration of the hydrolysis of ACh [3]. The present study suggests that EFAA is an effective natural source against AChE inhibition, because EFAA has a strong effect on ameliorating cognitive impairment caused by AChE overactivation.

3.5. Biochemical Analysis of Mice Brain Tissues. SOD, which is one of the antioxidant enzymes, is an enzyme that catalyzes the conversion of superoxide radical into molecular oxygen (O_2) and H_2O_2. H_2O_2 is sequentially neutralized through scavenging by catalase, peroxidase, and variable antioxidants. SOD has a role in maintaining physiological redox balance or reducing oxidative stress [25]. A previous report showed that SOD activity in brain tissue was diminished by TMT exposure in mice [26]. In Figure 6(a), the TMT group shows SOD activity (2.19 U/mg protein) and the EFAA 20 group shows statistically increased SOD activity (2.72 U/mg protein) compared with the TMT group.

The GSH of the brain tissues is also involved in the detoxification process of intracellular ROS, including free radicals, lipid peroxides, and electrophilic substances [25].

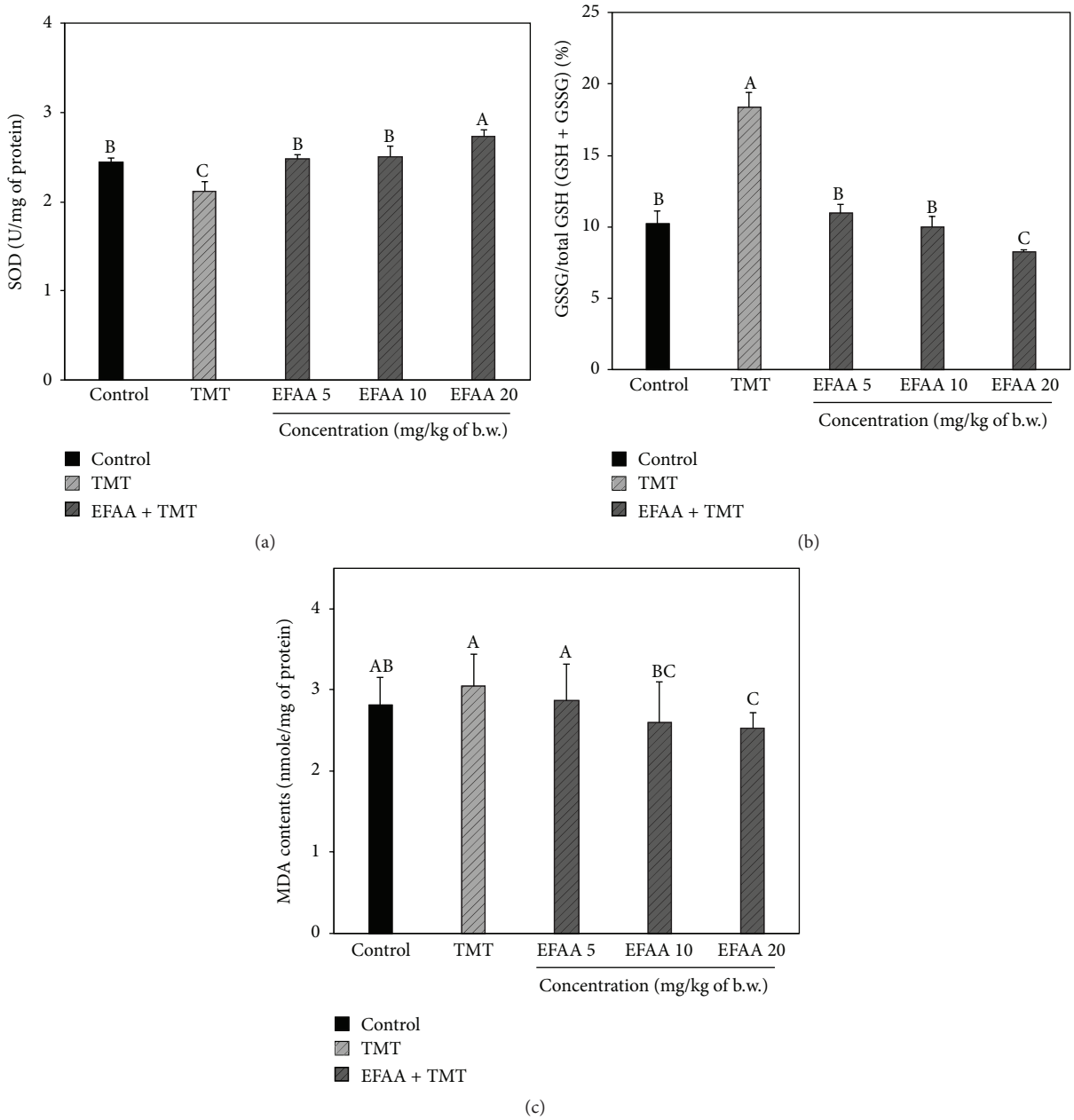

Figure 6: Effect of ethyl acetate fraction from *Actinidia arguta* (EFAA) on SOD contents (a), GSSG/total GSH (GSH + GSSG) ratio (b), and MDA contents (c) from TMT-induced defective mice brain homogenates. Results shown are mean ± SD ($n = 3$). Data were statistically considered at $p < 0.05$, and different letters in graph represent statistical difference.

Irreversible damage occurs when the neuronal cells are not able to maintain GSH homeostasis, and the deficiency of GSH accelerates the signal processes leading to TMT-induced neuronal cell death [27]. In contrast, phenolics may help to maintain the homeostasis of GSSG/GSH owing to their antioxidant activity [28]. The ratio of GSSG/GSH is often used to indicate the oxidative stress level in the cell. In Figure 6(b), the TMT groups show an increased GSSG/total GSH (GSH + GSSG) ratio (approximately 18%) compared with the control group (10.22%). However, the EFAA groups show a decrease in the GSSG/total GSH (GSH + GSSG) ratio. The EFAA 20 group presents a lower GSSG/total GSH (GSH + GSSG) ratio (8.20%) than the control group.

MDA, the final product of lipid peroxidation generated in damaged tissues by ROS, such as free radicals, is considered an indicator of oxidative stress [26]. Since the brain tissue has more plentiful unsaturated fatty acids than other tissues, oxidative stress occurring in the brain tissue continuously leads to memory loss and cognitive disorders [23]. TMT exposure has been found to increase the production of MDA through an excitotoxic effect on neuronal cells in the hippocampus of the brain [26]. Therefore, the lipid

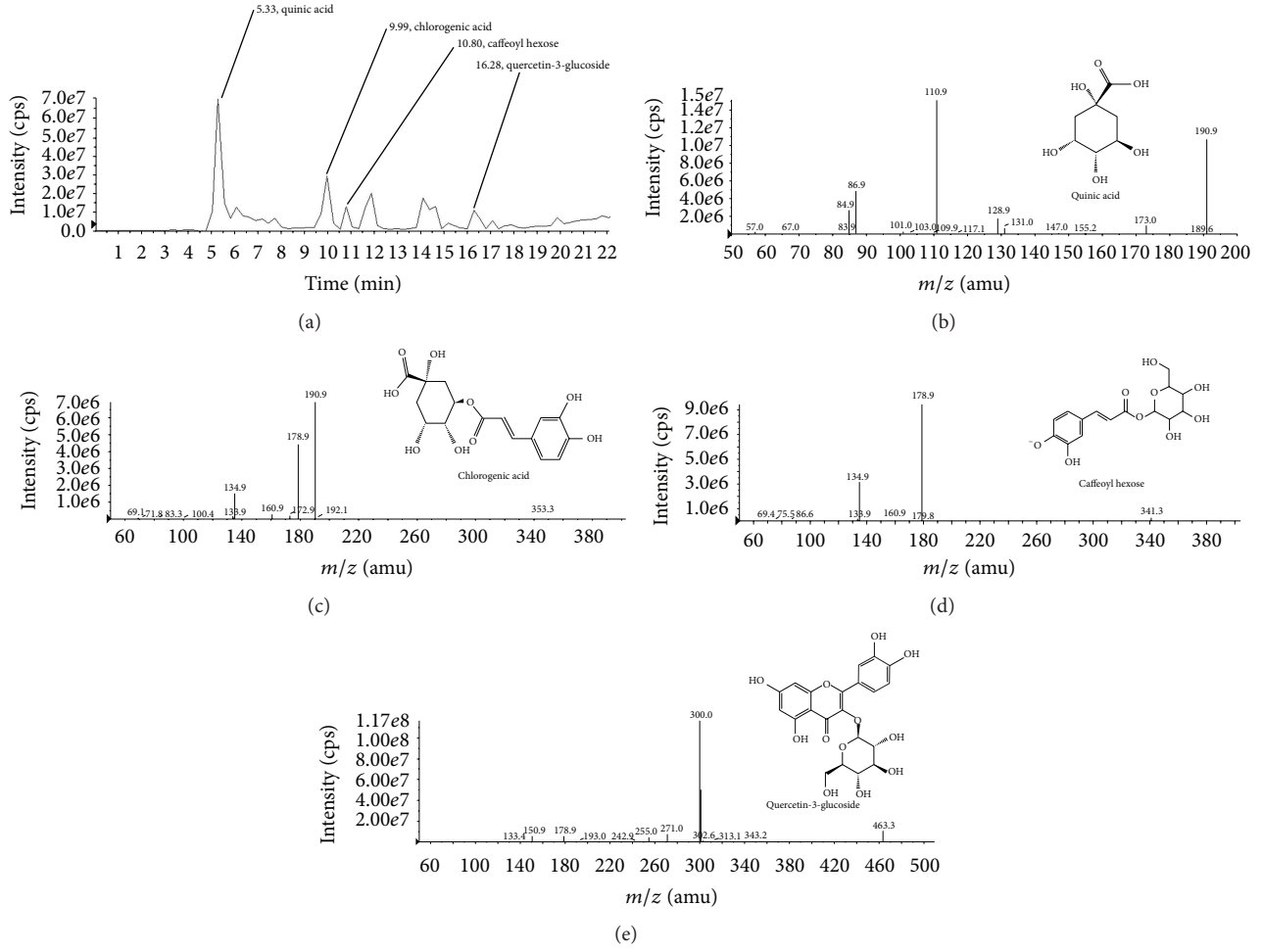

Figure 7: Liquid chromatography coupled with 3200 QTRAP mass spectrometer in negative-ion mode. Base peak chromatogram of mass scan (a), MS^2 scan data for quinic acid (b), chlorogenic acid (c), caffeoyl hexose (d), and quercetin-3-glucoside (e). MS^2 data patterns indicated a variety of collision energies (CE) −20 eV (b), −29 eV (c), −28 eV (d), and −35 eV (e), respectively.

peroxidation levels of TMT-induced amnesia were estimated by the amount of MDA in brain tissues. The TMT group showed slightly higher MDA contents (3.04 nmole/mg protein) than the control group (2.82 nmole/mg protein), while EFAA was shown to inhibit lipid peroxidation in mice brain tissues (Figure 6(c)). The EFAA 20 group showed effectively inhibited lipid peroxidation (approximately 18%) compared with the TMT group.

In our results, EFAA was shown to inhibit the biochemical change against TMT-induced oxidative stress in the mice brain tissues. Previous research reported that *A. arguta* has various phenolics, including chlorogenic acid, catechin, rutin, and quercetin [7]. Chlorogenic acid has also been reported to increase the GSH level by scavenging the streptozotocin-induced oxidative stress in diabetes [29]. SOD and GSH are the most important enzymes in the cell antioxidant defense system, and their quantitative balance could inhibit the MDA production resulting from lipid peroxidation. Therefore, EFAA could assist the improvement of TMT-induced amnesia by increasing SOD activity and GSH activity and inhibiting the production of MDA.

3.6. Identification of Main Phenolics in EFAA. The phenolics of EFAA were identified by 3200 QTRAP with a hybrid triple quadrupole-linear ion trap mass spectrometer for retention time, UV-Vis spectrum, a full scan of mass data, and an MS^2 scan for mass fragmentation. Four phenolics in EFAA were identified as two major peaks (retention time at 5.33 and 9.99 min) and two minor peaks (retention time at 10.80 and 16.28 min) by the base peak chromatogram of mass scan (Figure 7(a)). The main four peaks presented in the ESI-MS spectra were identified by the molecular ions $[M\text{-}H]^-$ at *m/z* values of 190.9, 341.3, 353.5, and 463.3, respectively. These four molecular ions were tentatively identified by a detailed analysis of their negative MS^2 scan data. The product ion at *m/z* 190.9 was observed to have fragment ions at *m/z* 173.0, 128.9, 110.9, 101.0, 86.9, and 84.9 (Figure 7(b)); these data were consistent with the fragment ions of quinic acid [30]. The product ion at *m/z* 353.5 (Figure 7(c)) was tentatively identified as a 3-*O*-caffeoylquinic acid (chlorogenic acid) that has fragment ions at *m/z* 190.9, 178.9, 172.9, and 134.9 via LC/MS^3 data analysis for monoacylchlorogenic acid [30, 31]. In Figure 7(d), caffeoyl hexose as a deprotonated

molecular ion at *m/z* 341.3, which coupled with a dehydrated hexose at *m/z* 178.9 and decarboxylated caffeoyl at *m/z* 134.9, was also identified [30]. Finally, the product ion at *m/z* 463.3 (Figure 7(e)) was identified as a quercetin-3-glucoside through comparison with the MS^2 fragment ions reported previously, which showed the loss of a dehydrated glucoside at *m/z* 300.0, and the fragment ions were confirmed as follows: at *m/z* 300.0, 271.0, 255.0, 178.9, and 150.9 [32].

A. arguta is widely known as a food with antioxidant properties, and it is steadily consumed. Due to the rich phenolics in *A. arguta*, it has been studied in terms of its antioxidant, anti-inflammation, antidiabetic, antiallergic, and other effects [13, 14]. A previous study investigated the polyphenol contents of kiwifruit (*A. deliciosa*), and various phenolic compounds were found, including caffeic acid, ferulic acid, syringic acid, ellagic acid, quercetin, catechol, pyrogallol, vanillin, and gallic acid [33]. Although the main phenolics in EFAA were confirmed as different compounds to *A. deliciosa*, these phenolics may be considered as having an ameliorating effect on cognitive function. Chlorogenic acid is one of the caffeoylquinic acid derivatives, including chlorogenic acid, 1,3-di-*O*-caffeoylquinic acid, and 1,5-di-*O*-caffeoylquinic acid. It is an ester formed between quinic acid and caffeic acid, and it is a polyphenol widely present in the leaves and fruits. It has also been reported to have good antioxidant activity, as evidenced by the decrease in oxidative stress [29]. Another report stated that although the antioxidant mechanisms of chlorogenic acid are still unclear, the antioxidant ability of chlorogenic acid is expected because of its redox-regulated transcription factors [28]. In addition, the effects of chlorogenic acid were also reported to ameliorate scopolamine-induced amnesia by inhibiting AChE, oxidative stress, and lipid peroxidation [34]. Caffeoyl hexose, which belongs to the hydroxycinnamic acid family, is collectively known as chlorogenic acid. Additionally, quinic acid is a colorless crystalline acid obtained from plant products or made synthetically by the hydrolysis of chlorogenic acid. Caffeoyl hexose and quinic acid have not yet been researched in many studies, but they may be helpful as antioxidants by scavenging the free radical. Finally, quercetin is one of the flavonoids that has potent antioxidant properties compared with vitamin C and vitamin E, and it was reported to protect the neuronal cell against $A\beta_{1-42}$-induced neurotoxicity [35]. Since recent research reported that *A. arguta* contains the quercetin-3-glucoside compound, our study also tentatively identified quercetin-3-glucoside among various quercetin derivatives [13]. In addition, the free radical scavenging activity of quercetin-3-glucoside was reported to show higher activity than phloridzin, 3-hydroxyphloridzin, chlorogenic acid, epicatechin, epicatechin dimer (procyanidin B2), trimer, and quercetin-3-glucoside [36]. Quinic acid, as a main phenolic compound in EFAA, was analyzed as 32.10 μg/mg of *A. arguta*.

Consequently, EFAA, including four phenolics, showed a significant antiamnesic effect on TMT-induced cognitive defects through AChE inhibition and antioxidant activity in a mouse model. Therefore, in this assay, the identified phenolics, including chlorogenic acid and quercetin-3-glucoside, were found to potentially contribute to the enhancement of cognitive function. Therefore, it could be considered that the learning and memory effect of EFAA may be due in part to the presence of chlorogenic acid, quercetin-3-glucoside, quinic acid, and caffeoyl hexose.

4. Conclusion

EFAA showed significant cellular AChE inhibitory effect and neuronal cell protective effect based on the cellular antioxidant activity caused by the H_2O_2. TMT-induced amnesia in the ICR mouse model was effectively improved by EFAA treatment. After *in vivo* behavioral tests, mice brain tissues were collected for examining AChE activity and several antioxidant systems. EFAA showed AChE inhibitory effect in brain and excellent antioxidant activity in SOD, GSSG/total GSH (GSH + GSSG), and MDA assay. The ameliorating effect of EFAA on TMT-induced amnesia may be affected by its main phenolics identified as a quinic acid, chlorogenic acid, caffeoyl hexose, and quercetin-3-glucoside. Consequently, our results suggest that *A. arguta* as natural food resources might be considered possible substance to prevent neurodegeneration through AChE inhibition and strong antioxidant activity.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

Ho Jin Heo participated in the design of the study. Jeong Su Ha, Dong Eun Jin, Chang Hyeon Park, and Tae Wan Seung conducted the experiments. Jeong Su Ha, Dong Eun Jin, and Seon Kyeong Park analyzed the data and drafted the paper. Jeong Su Ha, Seon Kyeong Park, Dae-Ok Kim, and Dong-Won Bae identified and confirmed main phenolics. All authors read and approved the final version of the paper.

Acknowledgments

This work was supported by the Development Program for Forest Science and Technology of Korea Forest Service (2013-Free10), Republic of Korea. This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2015R1D1A3A01015931). J. S. Ha, S. K. Park, C. H. Park, and T. W. Seung were supported by the BK21 Plus Program (2013), Ministry of Education, Republic of Korea.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 263898, 5 pages
http://dx.doi.org/10.1155/2015/263898

Research Article

Nonoperative Korean Medicine Combination Therapy for Lumbar Spinal Stenosis: A Retrospective Case-Series Study

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Received 10 March 2015; Revised 9 April 2015; Accepted 29 April 2015

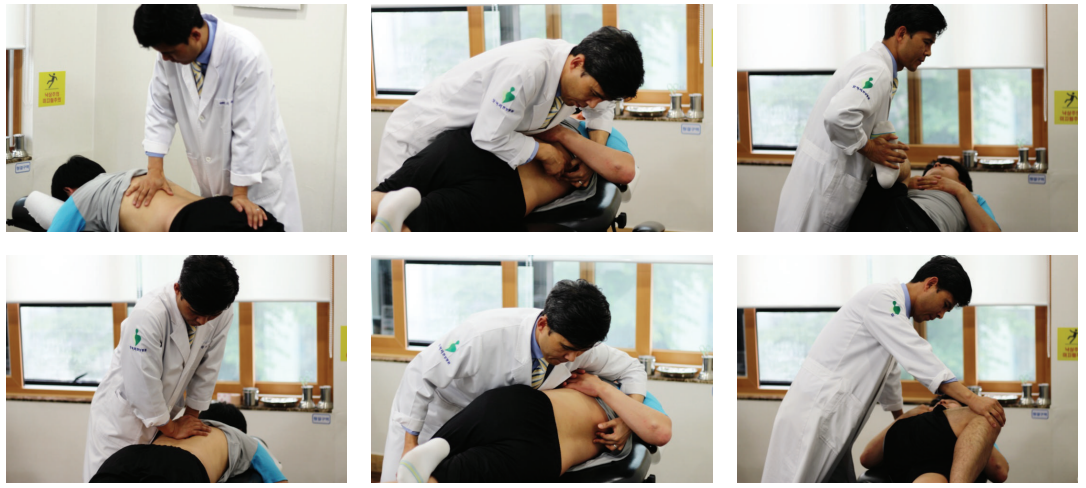
Academic Editor: Salih Mollahaliloglu

This is a retrospective case series exploring the therapeutic benefits and harm of nonoperative Korean medicine combination therapy for lumbar spinal stenosis (LSS). The medical records of a total of 33 LSS patients, who were treated as inpatients at Mokhuri Neck and Back Hospital, Republic of Korea, from November 2010 to January 2012, were reviewed first and telephone survey on these patients was conducted after one year. Body acupuncture, pharmacoacupuncture, Chuna, and oral administration of herbal medicines were offered to all patients. A Visual analogue scale (VAS) of pain and the walking duration without pain were used to assess the patients during the approximately 1-month treatment period. The average VAS score of pain and the walking duration improved significantly; the VAS score decreased from 9 (SD, 1.15) to 2.75 (2.22) ($p < 0.01$), and the walking duration increased from 5.5 (6.66) to 16.75 (13.00) minutes ($p < 0.01$). No adverse event was reported during the treatment. In addition, the decreased pain level and improved function continued for over one year. Although we did not find definitive evidence, the study results suggest that KM combination therapy may be beneficial for decreasing pain and improving function in LSS patients and may produce comparatively few adverse events.

1. Introduction

Lumbar spinal stenosis (LSS) refers to a narrowed spinal canal that commonly arises from degenerative changes in the bony structures or ligaments of the lumbar vertebrae. The characteristic symptoms of LSS include low back and leg pain in one or both sides and neurological claudication, which deteriorates in a lumbar extension posture [1]. Surgery to decompress the entrapped spinal cord is usually recommended, but complications occur quite often, and over 40% of patients reported complications after surgery in a previous report [2]. However, nonsurgical treatment has recently been identified as an important treatment strategy for decreasing pain and improving function. It is therefore commonly recommended as an initial therapy for LSS when no neurological contraindications or symptoms are evident [3].

Although a current Cochrane review on the effect of nonoperative treatment modalities for LSS only suggests a low quality of evidence [4], many procedures are currently used in clinical practice, for example, oral administration of analgesics including gabapentin or methylcobalamin, epidural steroid injections, home or intensive inpatient physical therapies, manual therapies, and multimodal nonsurgical therapies [5]. In Korean medicine (KM), several different nonsurgical interventions are currently used for the treatment of LSS. According to the clinical guidelines of the Korean Acupuncture and Moxibustion Medicine Society, acupuncture is recommended for the treatment of LSS. Korean pharmacoacupuncture, a comparatively novel intervention that consists of acupuncture-point injection of distillates of medicinal herbs, is frequently used for low back pain [6]. Various herbal decoctions are used for low back pain as single interventions or combined with acupuncture or other nondrug treatments [7, 8]. Because low back pain is the leading cause for visits to KM hospitals [9], clinical evidence regarding the various interventions of KM for spinal stenosis



(a) (b) (c)

Figure 1: The Chuna procedure (Korean-style manual therapy). (a) Extension-mobilization technique of lumbar vertebrae. (b) Manipulation technique of lumbar vertebrae in the lateral recumbent position. (c) Relaxation technique for lumbar vertebrae and the hip joints.

should be established. Observational studies including case studies and case series cannot provide conclusive evidence, but they play a complementary role to randomized controlled trials (RCTs). If no RCTs are available, case series can provide the best information with which practitioners and policy makers can make health care decisions [10]. The objective of this retrospective case series was to summarize the results of KM treatment of LSS in a local Korean hospital and provide basic evidence supporting its use and a clear outline of the proper procedures.

2. Methods

This was a retrospective case-series study. The study consisted of two parts; the medical records during the admission treatment period from all of the eligible patients were reviewed to assess the short-term effect of KM treatment for LSS first, and then a telephone follow-up survey of the available patients was conducted after one year to assess the midterm effects. The medical records of all patients who were diagnosed with LSS and treated as inpatients at the Mokhuri Neck and Back Hospital in the Republic of Korea from November 2010 to January 2012 were reviewed. Criteria for eligibility included patients less than 80 years of age diagnosed with LSS with at least a 3-month history of LSS symptoms and neurological claudication. All patients were required to meet the radiological diagnostic criteria, including an anterior-posterior (AP) diameter of the spinal canal less than 12-mm in MRI axial images [1].

All patients received the same treatment interventions during the admission period; the treatments consisted of body acupuncture, pharmacoacupuncture, Chuna (Korean-style manual therapy), and the oral administration of herbal medicines. Semi-individualized acupuncture points were selected for body acupuncture. GV3, GV4, BL23, BL25, and GB30 were treated in all patients, and BL56, BL57, GB34, GB39, SP9, and SP6 were selectively treated based on the symptoms of the individual patients. A 0.25 ∗ 40-mm disposable stainless steel acupuncture needle (Dong Bang Co., Korea) was used for needling, and acupuncture treatment was conducted once a day. The needle retention time was approximately 15 minutes. Hwangryunhaedoktang pharmacoacupuncture solution (1-2 cc; Korean Pharmacoacupuncture Institute, Korea), which consisted of *Coptidis rhizoma, Scutellariae radix, Phellodendri cortex*, and *Gardeniae fructus* extracts, was injected subcutaneously in the same locations as the acupuncture needles using a 26-gauge insulin syringe once daily. Chuna is a Korean manipulation technique, which is comprised of mobilization within the limit of the passive range of joint motion and muscle relaxation according to the patient's respiration (Figure 1) [11]. The patients attended Chuna manual therapy five times per week. Herbal decoctions mainly consisting of *Eucommiae cortex, Achyranthis radix, Cibotii rhizoma, Sorbus commixta, Geranium thunbergii, Saposhnikoviae radix*, *and Acanthopanacis cortex* were administered three times a day. In addition to these core treatments, information for daily activities and walking exercise was offered to patients by consultation with a KM doctor five times a week. Only low back and lower limb stretching exercises at the bed side and flatland walking were allowed. It was recommended that patients should not walk past the pain threshold and that they increase walking distance in a stepwise manner. A KM doctor evaluated the condition and exercise status of the patients at every consultation. All treatments were continued for the admission period of approximately 1 month for each patient.

A 0 (no pain) to 10 (worst possible pain) visual analogue scale (VAS) for the daily average of low back pain and the walking duration without pain were used to assess patients before and after admission treatment. The telephone survey of each patient was conducted one year after treatment by the same KM doctor who assessed the VAS score and walking duration without pain. Statistical analyses were conducted with a t-test or Wilcoxon signed-rank test based on

the normality of data with PASW Statistics 18 software (Polar Engineering and Consulting). The patients were evaluated for adverse events at every treatment. All of the study personnel complied with the ethical guidelines of the Mokhuri Neck and Back Hospital regarding all treatment procedures and patient medical record assessments.

3. Results

A total of 39 patients were admitted for treatment of symptoms related to LSS, and 4 patients were excluded from the study because they did not meet the diagnostic criteria for LSS; that is, the AP diameter of the spinal canal was over 12 mm on an L-spine MRI. In addition, 2 patients were excluded from the study during the treatment course; 1 refused admission for treatment, and the other developed an elevated serum aspartate aminotransferase (AST) level. As a result, the medical records of 33 patients were reviewed. Among the 33 LSS patients, 24 were contacted by telephone and finished the secondary interviews.

A total of 5 male and 28 female patients received the treatments. The median age was 66 years old (25% and 75% quartile ranges of 62 and 73 years, resp.), and the average disease duration was 9 (SD: 9.42) months. The average admission period was 28.75 (4.11) days. Seven patients were advised to undergo decompression surgery, and 13 had previously undergone a nerve block treatment. The L4 to L5 intervertebral space was the most frequent location where stenosis occurred (26 patients) followed by the L3 to L4 space (10 patients), the L2 to L3 space (8 patients), and the L5 to S1 space (4 patients); 13 patients had multilevel lesions. According to the radiological diagnostic criteria, absolute LSS (an AP diameter of the spinal canal of <10 mm) was observed in 14 patients, and the remaining patients showed relative LSS (an AP diameter 10 to 12 mm). Four patients were diagnosed with spondylolysis, and 9 were diagnosed with spondylolisthesis.

Not all of the outcome data were normally distributed according to the Shapiro-Wilk test for normality. Therefore, the Wilcoxon signed-rank test was used for the statistical analysis on the difference between before and after treatments. The average VAS score for pain was significantly decreased from 9 (1.15) to 2.75 (2.22) ($p < 0.01$, Figure 2). The average walking duration without pain was also significantly improved from 5.5 (6.66) to 16.75 (13.00) minutes ($p < 0.01$, Figure 3). No adverse events were reported during the treatment period.

To assess the midterm effects of KM treatment for LSS, data from the 24 patients who finished the one-year follow-up telephone interview were used for analysis. Among the 24 patients, no patient underwent spinal surgery during the follow-up period. Statistical analyses were conducted with a t-test based on the normality of data. The pain VAS score decreased significantly during the treatment from 7.88 (1.48) to 2.33 (1.37, $p < 0.001$), and it continued to improve over the one-year follow-up period (2.10 (1.98), $p < 0.001$, Figure 2). The walking duration without pain also showed significant improvement during the treatment (4.21 minutes (5.48) at the pretreatment and 16.00 (7.30) minutes at the posttreatment assessments), and it continued to improve after one year (20.25 (10.56), $p < 0.001$, Figure 3).

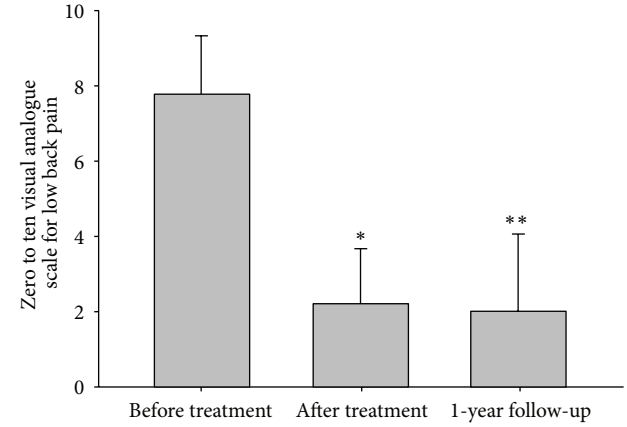


Figure 2: Visual analogue scale of pain. ∗: Wilcoxon signed-rank test of the before and after treatment values, $p < 0.05$; ∗∗: t-test of the before treatment and after one year of follow-up values, $p < 0.05$.

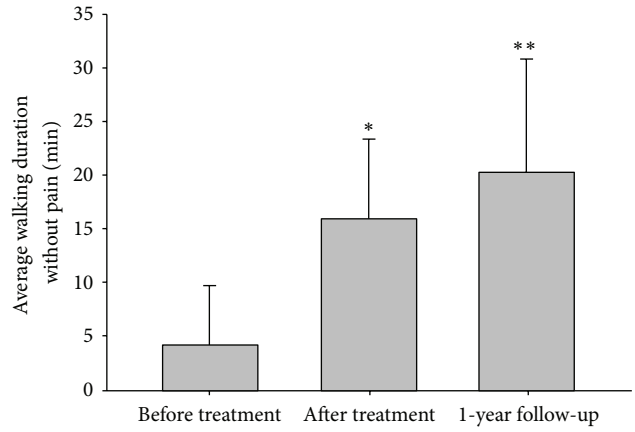


Figure 3: Walking duration without pain. ∗: Wilcoxon signed-rank test of the before and after treatment values, $p < 0.05$; ∗∗: t-test of the before treatment and after one year of follow-up values, $p < 0.05$.

4. Discussion

The results of this study demonstrate that approximately one month of nonoperative KM combination therapy may result in decreased pain and improved function with a comparatively low adverse event rate for LSS patients. The patients had extremely severe pain (average VAS: 9) at the beginning of the study, but only mild pain remained shortly after the treatment (average VAS: 2.75). Considering that the minimum clinically important difference in low back pain after spinal fusion surgery is approximately 2.2 points based on the recent literature [12], a 6.25-point decrease for a 0 to 10 VAS for back pain indicates considerable clinical significance. Neurological claudication was also significantly improved. Patients could walk without leg pain for more than three times as long after treatment (from 5.5 to 16.75 minutes).

No participant complained of adverse events related to the treatments. In addition, the study also showed that the decreased pain level and improved function continued for one year. Among the 24 patients who participated in the telephone survey, the severity of low back pain and walking duration without pain continued to improve after one year of follow-up.

The possible mechanism of KM combination interventions for the treatment of LSS has not been previously well established. However, a mechanism can be hypothesized based on the effects of separate individual interventions, which have been assessed in previous studies. Evidence of the effectiveness of acupuncture for low back pain has been shown, and practitioners generally agree with its clinical efficacy [13]. A previous animal experimental study suggested that acupuncture at the lumbar region may enhance sciatic nerve blood flow as well as the peripheral vasa nervorum through regulation of blood pressure and vasodilator nerve activity, which comprises a possible mechanism for the improvement of claudication [14]. The herbs used in this study might regulate inflammatory mediators and affect the chronic inflammatory conditions of the spinal lesion [15–17]. Although Chuna manual therapy has not been shown to be as effective on LSS as chiropractic or other types of manipulation techniques [18], it might relax the shortened muscles and correct the deformity related to long-term changes in the spinal bony structures and their arrangement, contributing to the functional improvement of LSS. Hwangryunhaedoktang pharmacoacupuncture is a novel Korean acupuncture technique that is believed to simultaneously deliver the effects of herbal medicine and acupuncture-point stimulation. Currently, clinical evidence has not been established for this treatment, but previous experimental studies suggest that it might have anti-inflammatory and analgesic effects in animal models [19]. The individual effects of each intervention may combine and contribute to the positive clinical result as a whole.

This study has several limitations. First, as we mentioned earlier, conclusive evidence for the use of combination therapy to treat LSS cannot be established from this study because case series have only limited value and occupy a low grade in the hierarchy of levels of evidence. Second, only short-term effects were evaluated, and well-validated, standardized, LSS-specific outcome assessment tools were not used. In particular, walking capacity was needed to be evaluated using condition-specific tools or questionnaires with ensured validity and credibility to support the clinical evidence [20]. The evaluation of walking duration without pain is a well-tolerated and easy clinical outcome for walking capacity in LSS patients, but the validity and credibility were not tested. In future clinical trials, appropriate outcomes for pain and function of LSS patients should be adopted. Third, in the follow-up telephone survey, we could not contact all of the patients who were included in the medical record review. This might exaggerate treatment effects and emphasize the positive results after one year. Fourth, due to the methodological limitations of this case series, we cannot confirm whether each treatment was effective individually or if a combination of the various treatments was a key factor for the clinical improvements seen in patients. Future clinical trials comparing the clinical effectiveness of individual interventions will be necessary for the assessment of individual and combined effects. Finally, the relevance of the study results cannot be ensured because the study results only reflect our specific experience with Korean patients with respect to LSS treatment. Various interventions are usually applied to treat low back pain in Korean medicine, which are not commonly used in biomedical clinics. A recent survey of Korean medicine (KM) suggests that general Korean medicine hospitals offer combination treatments with herbal medication, acupuncture, pharmacoacupuncture, physiotherapy, and Chuna to most patients with low back pain simultaneously [21]. Additionally, comparatively long-term nonsurgical, inpatient, and alternative therapies are quite common in Korea. In this sense, the clinical outcomes should be expected to be more conservative in a different cultural context.

Despite these limitations, this study is, to our knowledge, the first English language report of KM treatment for LSS. We hope the clinical efficacy and effectiveness of KM combination treatment for LSS will be established through future rigorous RCTs with larger sample sizes. A standardized treatment protocol for KM treatment using the appropriate assessment tools will be necessary for future studies.

Conflict of Interests

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

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 703439, 8 pages
http://dx.doi.org/10.1155/2015/703439

Review Article

A Modern Clinical Approach of the Traditional Korean Saam Acupuncture

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Received 19 March 2015; Accepted 28 April 2015

Academic Editor: Salih Mollahaliloglu

Saam acupuncture is one of the original therapeutic modalities representing traditional Korean medicine. It was originally described in a manuscript that is estimated to be published at some point between 1644 and 1742, in the middle of the Cho Sun dynasty, by a Korean Buddhist monk whose name is unknown. The principle of combining five shu points is based on the theory of Nanjing. The treatment and diagnosis concepts in Saam acupuncture were mainly influenced by Dongeuibogam and Chimgoogyeongheombang. The basic characteristic of combining five shu points in Saam acupuncture is the selection of the tonification and sedation points along the self-meridian and other meridians based on creation and governor relationships. Saam acupuncture clinical studies have mainly focused on musculoskeletal pain and autonomic nervous system regulation. From a neurophysiological perspective, Saam acupuncture, which involves five shu points as the main treatment aspect, has the advantage of increasing parasympathetic nerve activation and adjusting the balance of the autonomic nervous system. Inserting a needle into the skin layer while considering the respiratory phase and stimulating the needle gently and lightly could maximize the effect of Saam acupuncture. The specific Saam acupuncture prescribed should be identified on the basis of the neurobiological perspective.

1. Introduction

Saam acupuncture, which is one of the original therapeutic modalities representing traditional Korean medicine, is a unique treatment method that has a different origin than the modalities from China and Japan. The basic characteristic of combining five shu points in Saam acupuncture is the selection of the tonification and sedation points along the self-meridian and other meridians based on creation and governor relationships. In China, five element acupuncture, tonification, and sedation points along only the self-meridian are selected. Japanese meridian therapy added source point, connecting point, cleft point, alarm point, and transport point on the basis of Korea Saam acupuncture conception of the combined five shu points [1].

Saam acupuncture is based on the traditional concepts of yin-yang, five elements, ZangFu (viscera and bowels), qi, and meridians. Saam acupuncture treatment cannot be separated from these viewpoints. In particular, it involves the application of five shu points according to the creation and control cycles of the five-element theory. Therefore, the combination of acupoints in Saam is easier to understand from the perspective of traditional medicine.

The meridian is divided into three parts: the arm or foot, three yin and yang, and six ZangFu parts. A total of 24 deficiency and excess symptoms, with 24 coldness and fire symptoms, exist across the 12 meridians, but the diagnostic criteria related to these symptoms are too ambiguous for selecting a correct meridian. Except for the regular 48-treatment protocol, the treatment strategies are largely variable. Efforts were made to produce Saam treatments that are more effective by including other acupoints, with the main points firmly based on the regular pattern [2]. However, because the explanation regarding acupoint selection is very brief or elided, applying Saam acupuncture in the clinic is difficult.

Various Korean scholars have suggested different methods for applying Saam acupuncture in the clinic. Lee [3] proposed a diagnostic system by comparing pulse examination, whereas Kim [4] proposed a symptom-based diagnosis.

Cho [5] established visceral pattern identification for providing easy access to Saam acupuncture by analyzing Neijing (Internal Classic), Nan-jing (Classic of Difficult Issues), and Yixuerumen (Introduction to Medicine). Moreover, Kwon [6] studied constitutional acupuncture, and Kim [7] reviewed the mind-based aspect of Saam acupuncture.

However, a description of the mechanism of acupuncture to both clinicians and patients in mainstream medicine by using the contemporary concepts of neurobiology would be helpful for incorporating Saam acupuncture into mainstream medicine [8]. An important difference between traditional Korean medicine and the Western medical approach is the diagnostic process. In traditional Korean medicine, the diagnosis is made by using the principles of syndrome differentiation, which could be in accordance with the state of the ZangFu or qi or with the doctrine of the meridians [9]. This diagnostic process does not match the patterns in Western medicine.

In this paper, several neurobiological mechanisms of Saam acupuncture treatment are presented from the perspective of the clinician. Saam acupuncture clinical trials are also discussed. Further, the historical background of Saam acupuncture and the basic principle of combining acupoints are briefly reviewed.

2. The Historical Background of Saam Acupuncture

The original manuscript that described Saam acupuncture is estimated to have been published between 1644 and 1742, in the middle of the Cho Sun dynasty, by a Korean Buddhist monk whose name is unknown. Because Chimgoogyeong-heombang (Experiential Prescriptions of Acupuncture and Moxibustion), which was published in 1644, is quoted in the manuscript of Saam acupuncture, the publication period of the Saam acupuncture manuscript can be estimated to be after 1644 [10]. After 1742, Gy-san's clinical experience was added to the original manuscript of Saam acupuncture, that is, Gyeongjeyogyeol (Essential Rhymes on Acupuncture and Moxibustion by Master Saam). This is the oldest existing manuscript associated with Saam acupuncture. The physiology, pathology, and disease identification sections of Saam were handed down to its disciples, but the clinical experience section of Gy-san coexists in the currently published Saam acupuncture-related books [11].

Viscera/bowel-based acupuncture was developed in Chimgoogyeong-heombang (Experiential Prescriptions of Acupuncture and Moxibustion), with integration of the meridian/exterior theory with the viscera manifestation theory, which in turn provided various methods for viscera diagnosis. Viscera identification became the basis of Saam acupuncture treatment [12].

The Dongeuibogam (Treasured Mirror of Eastern Medicine) contains the following phrase: "One acupuncture needle in all the diseases, up to within four acupuncture needles, acupuncture on the whole body is not a good idea." This phrase represents the characteristics of the traditional Korean acupuncture method. In this context, Saam acupuncture treated the disease within four acupuncture points. Further, an acupuncture method based on the five element principles is presented in the acupuncture section of the Dongeuibogam. The idea of treating a disease derived from viscera/bowel dysfunction by using five shu points influenced Saam acupuncture [1].

3. The Basic Theory of Combining Five Shu Points in Saam Acupuncture

The following phrase is taken from the 69th chapter of Nan-jing: "In the case of depletion, fill the respective mother, in the case of repletion, drain the respective child, one must fill the first and then drain afterward." Gao-Wu, of the Ming Dynasty of China (1519 A.D.), was the first acupuncturist to describe the use of tonification and sedation points along the self-meridian by using the five shu points according to the 69th issue of Nan-Jing. Zhang Shi Xian advocated the use of five shu points in other meridians in Jiao Zheng Tu Zhu Nan Jing (Illustrated note of Classic of Difficult Issues) [11]. On the basis of Gao-Wu and Zhang Shi Xian treatment, Saam added the role of the governor. This notion originated from the 50th and 75th chapters of Nan-Jing.

In Saam acupuncture, the relationship with the governor is important, as is the relationship between the mother and son. The governor is sedated under the condition of deficiency and is tonified under the condition of excess [13].

The basic rules are those of the creation and governor relationships. In the case of insufficiency of any meridian, the mother points of its mother and its own meridians should be tonified and the governor points of its governor and its own meridians should be sedated. For example, the following is applied if the lung meridian is diagnosed as deficient: earth tonification, lung meridian-earth point LU9 and spleen meridian-earth point SP3 and fire sedation, lung meridian-fire point LU10, and heart meridian-fire point HT8 (Table 1). The other meridians follow the same rule, as described above.

In the case of the excess of any meridians, the governor points of its governor and its own meridians should be tonified and the son points of its son and its own meridians should be sedated. For example, the following is applied if the lung meridian is diagnosed to be excessive: fire tonification, lung meridian-fire point LU10, and heart meridian-fire point HT8 and water sedation, lung meridian-water point LU5, and kidney meridian-water point KI10 (Table 2).

Another simple but rarely used one is coldness-fire acupuncture treatment derived from the deficiency-excess acupuncture treatment. For cold symptoms, the fire points of its own and the fire meridians are tonified and the Water points of its own and the water meridians are sedated. For heat symptoms, the water points of its own and the water meridians are tonified and the fire points of its own and the fire meridians are sedated (Table 2).

Among the clinical case studies of Saam, 85% used either a tonification or a sedation formula, whereas 15% used variations of the tonification and the sedation formulae [13]. Saam treatment primarily focuses on the viewpoints of deficiency-excess symptoms rather than cold-heat symptoms [1]. The 240 acupuncture treatments described in the Gyeongjeyogyeol consist of 100 regular forms on the basis of

Table 1: Saam's combination of five shu points for deficiency and excess of the meridians.

Meridian	Deficiency				Excess			
	Tonify		Sedate		Tonify		Sedate	
Lung	SP3	LU9	HT8	LU10	HT8	LU19	KI10	LU5
Large intestine	ST36	LI11	SI5	LI5	SI5	LI5	BL66	LI2
Stomach	SI5	ST41	GB41	ST43	GB41	ST43	LI1	ST45
Spleen	HT8	SP2	LR1	SP1	LR1	SP1	LU8	SP5
Heart	LR1	HT9	KI19	HT3	KI19	HT3	SP3	HT7
Small intestine	GB41	SI3	BL66	SI2	BL66	SI2	ST36	SI8
Bladder	LI1	BL67	ST36	BL54	ST36	BL54	GB41	BL65
Kidney	LU8	KI7	SP3	KI3	SP3	KI3	LR1	KI1
Pericardium	LR1	PC9	KI10	PC3	KI10	PC3	SP3	PC7
Triple energizer	GB41	TE3	BL66	TE2	BL66	TE2	ST36	TE10
Gall bladder	BL66	GB43	LI1	GB44	LI1	GB44	SI5	GB38
Liver	KI10	LR8	LU8	LR4	LU8	LR4	HT8	LR2

Table 2: Saam's combination of five shu points for cold and heat symptoms of the meridians.

Meridians	Cold				Fire			
	Tonify		Sedate		Tonify		Sedate	
Lung	HT8	LU10	LU5	KI10	LU5	KI10	SP3	LU9
Large intestine	SI5	ST41	LI2	BL66	LI2	BL66	SI5	ST41
Stomach	ST41	SI5	ST44	BL66	ST44	BL66	ST36	BL54
Spleen	SP2	HT8	SP9	KI10	SP9	KI10	SP3	KI3
Heart	HT8	KI2	HT3	KI10	HT3	KI10	HT8	KI2
Small intestine	SI5	BL60	SI2	BL66	SI2	BL66	SI8	ST36
Bladder	SI5	BL60	SI2	BL66	SI2	BL66	ST36	BL54
Kidney	HT8	KI2	KI10	HT3	KI10	HT3	SP3	KI3
Pericardium	HT8	PC8	PC3	HT3	PC3	HT3	SP3	PC7
Triple energizer	TE6	BL60	TE2	BL66	TE2	BL66	TE6	BL60
Gall bladder	GB38	SI5	GB43	BL66	GB43	BL66	BL54	GB34
Liver	LR2	HT8	KI10	LR8	KI10	LR8	LR3	SP3

the 69th issue of Nan-Jing, 140 irregular forms on the basis of the 73rd and 75th issues, and various special acupuncture points, which include the source, connecting, accumulation, alarm, and back-transporting points [14].

4. Saam Acupuncture Clinical Research

To review clinical articles on Saam acupuncture, we used the following six databases: "Korean Studies Information Service system (KISS)", "National Discovery for Science Leaders (NDSL)," "Research Information Sharing Service (RISS)," "Oriental Medicine Advanced Searching Integrated System (OASIS)," PUBMED, and Google Scholar. The key words were "Saam" and "Saam acupuncture." The retrieved papers were screened so that only articles related to clinical research were retained. We selected 28 case studies and 17 clinical studies.

4.1. Saam Acupuncture Case Studies. Saam acupuncture has been applied for several diseases, and these are summarized in Table 3. However, the number of cases is low, and various therapeutic modalities were combined with Saam acupuncture except for some cases. Therefore, recognizing the effect that can be attributed to Saam acupuncture is difficult. Notably, treatments other than Saam acupuncture were well controlled in five cases.

In the case of a sleep disorder caused by a traffic accident, tonification of gall bladder improved sleep quality [15]. In traditional Chinese medicine (TCM), the gall bladder serves to buffer psychological anxiety. Because the cause of insomnia was determined to be a gallbladder deficiency, gallbladder tonification was selected as the solution.

Modified Saam acupuncture was used to treat 17 patients with refractory, sudden sensory-neuronal hearing loss of more than 3 weeks after a failed trial of conventional treatment including corticosteroids. The total improvement rate at 70.4 days after the initial visit was 47.1%. Thus, Saam acupuncture might be effective for refractory sensory-neuronal hearing loss in which conventional therapy has failed [16].

A 30-year-old woman with a right adnexal mass was treated with Saam acupuncture for 14 weeks. After treatment, transvaginal sonography revealed disappearance of the right adnexal mass. This effect may have been evoked by modification of autonomic nerve activity as a result, for

Table 3: Saam acupuncture clinical case reports.

Year	Disease	*N*	Period
1964	Eyelid edema	1	7 days
1975	Indigestion, HNP of lumbar	3	2–5 days
1981	Neurosis, duodenal ulcer	2	14 days
1998	Hyperemesis gravidarum	1	15 days
2002	Hwa-byung	2	5 months
2002	Wei symptom	1	2 months
2002	Oral dyskinesia	1	1 month
2003	Hemichorea-hemiballism	1	8 days
2003	Sequelae of CVA	1	10 months
2003	Hemorrhoid	2	10 days
2003	Fracture	1	1 month
2004	Lumbar compression fracture	1	14 days
2004	Insomnia	20	3 days
2006	Otitis media	3	1 month
2007	Tic disorder	1	3 months
2008	Knee strain	1	1 week
2008	Inflammatory acne	1	2 months
2009	Cancer pain	1	2 months
2010	Hearing loss	17	10 weeks
2011	Foot coldness	1	1 week
2012	ALS	1	5 days
2012	Meniere's disease	1	3 weeks
2013	Adnexal mass	1	14 weeks
2013	Calcific tendinitis of shoulder	1	7 days
2013	ALS	18	5 days
2014	Cancer	10	14 days
2014	Chronic post-stroke hemiparesis	7	1 month

N: number, ALS: amyotrophic lateral sclerosis, CVA: cerebrovascular accident, and HNP: herniated nucleus pulposus.

example, of reflex alteration of ovarian sympathetic nerve activity [17].

Modified Saam acupuncture (LU8, BL66, SI5, TE4, and CV12) was used to treat 10 cancer patients for 2 weeks with 4 sessions. CD3+, CD8+, and T-cell subsets were significantly increased and the fatigue severity scale score was significantly decreased. Therefore, Saam acupuncture may improve the immune system [18].

Finally, peripheral capillary oxygen saturation (SpO_2) was significantly increased in 18 amyotrophic lateral sclerosis patients with respiratory dysfunction who were treated with lung tonification [19].

4.2. Saam Acupuncture Clinical Research. Clinical studies of Saam acupuncture were mainly performed in relation to musculoskeletal pain and autonomic nervous system regulation (Table 4). Meridian identification was predominantly used when Saam acupuncture was applied to musculoskeletal pain. For example, when leg pain and numbness were present due to a herniated disc, bladder tonification was selected if the symptom occurred towards the back of the leg, and gall bladder tonification was used if the symptom occurred towards the lateral side of the leg, according to the flowing area of the meridian [20–22]. This principle was applied equally to knee pain derived from osteoarthritis [23], posterior ear pain due to Bell's palsy [24], and chronic tension headache [25]. Musculoskeletal pain-related clinical researches of Saam acupuncture mentioned above showed good results.

In clinical trials related to autonomic nervous system dysfunction, visceral pattern identification was used as the diagnostic criteria. For example, in case of Hwa-byung (Korean somatization disorder) [26–28], the heart or pericardium meridian, which is associated with psychological states in TCM, was selected. A tonification of pericardium was effective on the treatment of Hwa-byung. When the balance of the autonomic nervous system was disrupted by night-shift working [29], the gallbladder meridian that fits the psychological proportion was selected. A tonification of gallbladder could attenuate the imbalance between sympathetic and parasympathetic activities. When the face temperature dropped because of smoking [30], the fire acupoints of a fire organ (heart) and water organ (kidney) (HT8 and KI2, resp.) were selected as the treatment points. 5 of the 7 subjects showed increased temperatures after fire tonification treatment. In order to decrease hypertension in stroke patients [31], the bladder meridian, which has water and cold attributes, was applied. After 30 minutes of treatment,

Table 4: Saam acupuncture clinical research.

Year	Disease	*N*	Period	Method
1999	Lumbar HNP	28	5 weeks	SA + GBA/GBA
2002	Sciatica and back pain	40	10 days	SA + GBA/GBA
2002	Lumbar HNP	29	1–10 weeks	SA + GBA/GBA
2003	Dysarthria	20	10 days	SA/GBA
2004	Hypertension	60	1 hour	SA/BR
2004	Posterior ear pain	30	10 days	SA/GBA
2006	OA of knee	78	4 weeks	SA/ShA (Park sham needle)
2007	Chronic tension headache	26	2 weeks	SA/ShA (nonacupoint)
2007	Dysmenorrhea	8	1 month	SA/ShA (acupoint not related to gynecological disorders)
2007	Obesity	60	4 weeks	SA/ShA (Kim sham needle)/NA
2007	Fatigue	56	4 weeks	SA/ShA (nonacupoint)
2007	Hwa-byung	23	2 weeks	SA/ShA (nonacupoint)
2008	Hwa-byung	52	2 weeks	SA/ShA (nonacupoint)
2011	Autonomic nerve disorder	6	6 days	SA/ShA (Park sham needle)
2011	Hwa-byung	50	2 weeks	SA/ShA (nonacupoint)
2012	Facial temperature	7	6 days	SA/ShA (Kim sham needle)
2013	Peripheral neuropathy	10	8 weeks	SA/NA

HNP: herniated nucleus pulposus; OA: osteoarthritis, SA: Saam acupuncture; GBA: general body acupuncture; ShA: sham acupuncture; BR: bed rest; NA: no acupuncture; *N*: number.

a tonification of the bladder meridian significantly depressed the systolic and diastolic blood pressure.

Although the method of setting the test and control groups differed slightly for each study, the use of a sham needle device for the control group appears to be the most desirable condition for scientific research. The Park sham needle was used to blind the participants [32], and the Kim sham needle was used to blind both the practitioner and participants [30].

Comparing the simultaneous application of Saam acupuncture and general body acupuncture and a single application of general body acupuncture is not suitable [20–22] because the amount of stimulation is different. Treating the control group by needling a nonacupoint is also problematic because the selection of nonacupoint near the five shu points could result in similar neurobiological activity. Notably, no significant difference was observed between the test and control groups in the Hwa-byung studies [26, 27]. In fatigue studies, the test group as well as the control group exhibited statistically significant effects in the multidimensional fatigue scale [33].

5. Neurobiological Mechanisms of Saam Acupuncture for Clinical Application

Saam acupuncture principally uses the acupoint below the elbow and knee joint. The five shu points occupy large areas in the cortical representation in the postcentral sensory gyrus in the brain. Cho et al. [34] stated that, based on their knowledge of Western medicine, it is difficult to believe that acupuncture treats organ-related disorders and diseases by direct control of organs. Acupuncture may first stimulate or activate the corresponding brain cortex via the central nervous system, thereby controlling chemical or hormone release to the diseased or disordered organs for treatment. From this point of view, inserting a needle in the distal limb may be more advantageous for inducing physiological activity caused by acupuncture than needling in the trunk.

Acupuncture likely has an effect on homeostasis via the somatic autonomic reflex [35]. This homeostasis between the sympathetic and parasympathetic nerve is frequently believed to be the scientific basis for the concept of the balance between yin and yang in TCM. The neural pathways involved in this acupuncture action have been well investigated in animal studies [36, 37].

Sato [37] showed that acupuncture stimulation in the extremity facilitates gastric motility through a somatic parasympathetic reflex associated with the vagal nerve in animal studies. In his studies, the response disappeared after either spinal transaction at the cervical level or bilateral severance of the vagal nerves. This suggests that a mechanism exists at the brain level related to a somatic parasympathetic reflex through the vagal nerve.

Nishijo et al. [38] revealed that acupuncture stimulation at PC4 decreased the heart rate as a result of the reciprocal coordination of an increase in cardiac vagal activity and a decrease in cardiac sympathetic activity in healthy humans. In this case, the reflex pathway might involve an afferent somatic nerve that goes to the brain and spreads to various structures in the hypothalamus, the midbrain, the medulla, and eventually to the autonomic efferent nerve.

Acupuncture stimulation on the abdomen impedes gastric motility [37]. Studies also showed that the response was maintained after spinal transection at the cervical level but was absent after bilateral severance of the gastric sympathetic nerve. This suggests that the reflex pathway is propagated within the spinal level, and acupuncture points within certain

spinal segments in the trunk tend to affect the functioning of the organs that receive autonomic innervation from the same spinal segments through the sympathetic nerve.

The effect of acupuncture stimulation varies depending on the depth of the insertion, the respiratory phase, stimulation dose, and observation period. Mori et al. [39] showed that needling at TE5 induced pupillary constriction by increasing parasympathetic nerve action. An increase in parasympathetic nerve function was observed after gentle, superficial acupuncture stimulation. Bäcker et al. [40] revealed that, when inserting the needle at LI4, high-dose stimulation resulted in a greater increase in sympathetic nerve activity than low-dose stimulation.

Haker et al. showed that stimulation of the thenar muscle (LI11) resulted in a significant increase in sympathetic and the parasympathetic activity during the stimulation period and during the poststimulation period. A significant decrease in the heart rate at the end of the poststimulation period was also demonstrated. Superficial needle insertion into the skin overlaying the right thenar muscle caused a pronounced increase in the balance of both the sympathetic and parasympathetic activities during the 60 min poststimulation period [41].

Tanaka et al. demonstrated that SES (superficial needling exhalation phase in a sitting position) stimulation significantly decreased headache intensity, with a strong trend towards decreasing static electromyography (EMG) activity compared to CONT (without considering the respiratory phase) stimulation [42]. This suggests that the effect of acupuncture is derived from point selection of matching symptoms as well as consideration and utilization of the patient's respiratory phase during stimulation. The above finding suggests that, when applying Saam acupuncture for parasympathetic nerve activation or autonomic nervous system homeostasis, inserting a needle into the skin layer while considering the respiratory phase and stimulating the needle gently and lightly is the most effective.

In the neurobiological model, point specificity does not appear to exist with regard to musculoskeletal action, but five shu points, including PC5, PC6, and ST36, have some point specificity with regard to systemic action at the brain level. This acupuncture-point specificity has been attributed to the presence of a deep nerve [43]. However, other acupuncture points overlying the same deep nerve are not effective.

The Saam acupuncture method includes a variety of combinations of five shu points, and each combination is believed to have a distinct effect. However, the specificity of each Saam acupuncture treatment has not been yet identified from a neurobiological perspective. Therefore, when applying Saam acupuncture in the clinic as medical acupuncture, an approach that focuses on the balance and homeostasis of the autonomic nervous system appears to be desirable. Further, revealing the difference between the Yin meridian and Yang meridian treatments, which are anatomically divided into the medial and lateral sides of the body, is necessary from the viewpoint of the neurobiological mechanism.

6. Conclusion

The original Korean acupuncture method, Saam acupuncture, was invented 400 years ago by a Korean Buddhist monk based on the foundation of Dongeuibogam and Chimgoogyeong-heombang. He embodied Nan-jing's theory as an acupuncture treatment. Because Saam acupuncture was created on the basis of the theory of oriental medicine, approaching Saam acupuncture in the clinic via the oriental medical diagnosis is easy. However, for Saam acupuncture to be recognized as a medical acupuncture and to be used as a universal treatment in many countries, the mechanism of Saam acupuncture should be explained through scientific verification. Saam acupuncture, which operates with five shu points as a main aspect of treatment, has the advantage of increasing parasympathetic nerve activity and adjusting the balance of the autonomic nervous system. To maximize this effect, inserting a needle into the skin layer and providing gentle and light stimulation while considering the respiratory phase may be desirable.

Conflict of Interests

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

Acknowledgments

This study was supported by Wonkwang University in 2015.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 203612, 9 pages
http://dx.doi.org/10.1155/2015/203612

Research Article

Anxiety and Anger Symptoms in Hwabyung Patients Improved More following 4 Weeks of the Emotional Freedom Technique Program Compared to the Progressive Muscle Relaxation Program: A Randomized Controlled Trial

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Received 17 February 2015; Accepted 9 June 2015

Academic Editor: David Mischoulon

Background. The Emotional Freedom Technique (EFT) is a meridian-based psychological therapy. The present clinical trial investigates the effectiveness of EFT as a new treatment option for Hwabyung (HB) patients experiencing anger and compares the efficacy to the Progressive Muscle Relaxation (PMR), the conventional meditation technique. *Methods*. The EFT and progressive muscle relaxation (PMR) methods were performed on 27 HB patients, and their capacities to alleviate anxiety, anger, and emotional status were compared. After a 4-week program, a survey was conducted; patients then completed a self-training program for 4 weeks, followed by a second survey. *Results*. During the initial 4 weeks, the EFT group experienced a significant decrease in the HB symptom scale, anger state, and paranoia ideation ($p < 0.05$). Over the entire 9-week interval, there were significant decreases in the HB symptom scale, anxiety state, anger state, anger trait, somatization, anxiety, hostility, and so on in EFT group ($p < 0.05$). *Conclusion*. The EFT group showed improved psychological symptoms and physical symptoms greater than those observed in the PMR group. EFT more effectively alleviated HB symptoms compared to PMR. EFT group showed better maintenance during self-training, suggesting good model of self-control treatment in HB patients.

1. Background

Hwabyung is a cultural syndrome in Korea and is well described in the Glossary of Culture-Bound Syndrome of Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV, Appendix 1) [1]. The term HB, which means "anger or fire" and "disease," is known as a chronic anger syndrome. HB patients experience chronic suppressed anger and express unique symptoms including feelings of unfairness, subjective anger, external anger, heat sensation, pushing-up in the chest, dry mouth, and sighing and psychological symptoms such as feelings of unfairness and resentment [2]. HB is reported in 4.1% of the Korean general population and is more frequent in low-income middle-aged or older housewives [3]. As studies investigating HB treatment increase, nonpharmacologic treatment is receiving attention as a research target.

Progressive muscle relaxation (PMR) is a widely known relaxation technique introduced by Jacobson that aims to reduce residual tension and ultimately achieve a zero firing threshold through a progressive process of muscle relaxation. Jacobson's progressive muscle relaxation model was originally designed to relax approximately 30 muscles over an extended period of time, but a more widely used muscle relaxation technique developed by Bernstein is comparatively simple, relaxing just 16 muscles [4]. PMR can decrease physiological strain and heart acceleration through parasympathetic activation. This mechanism is the basis of evidence for its utility in heart disease prevention, cancer prevention, and rehabilitation [5]. Conventional studies have shown that

PMR reduces various physical symptoms stemming from several psychological diseases. PMR has been shown to decrease anxiety and is especially effective at reducing insomnia, depression, and anxiety in elderly patients, as well as preventing both affective and behavioral disorders. PMR also reduces anxiety and psychological distress while improving subjective well-being in patients with schizophrenia [6]. It is also known to reduce heart rate and blood pressure [7]. PMR is currently utilized in HB patients, and studies have been conducted supporting its use [8].

The Emotional Freedom Technique (EFT) is a meridian-based psychological therapy that alleviates psychologic and psychosomatic conditions by applying tapping stimulations at certain meridian acupoints. This technique utilizes psychotherapy techniques such as assurance, while applying a tapping stimulation onto acupoints for meridian stimulation. Goodheart performed a meridian tapping therapy called emotional acupuncture; Thought Field Therapy (TFT) was later formalized by Callahan through numerous systematic studies investigating certain emotional problems such as fear and anxiety. In 1987, Carrigton created a technique of uniformly tapping 14 stimulation points called Acutap. Craig introduced a similar treatment in 1990, which became EFT in its present form. Those treatments were then generalized as Energy Psychotherapy and Meridian Tapping Therapy. In recent studies, EFT has been proven effective in alleviating symptoms such as headache [9], trauma [10], depression [11], phobia [12], insomnia [13], and anxiety [14]. Because these symptoms share certain similarities with those in HB, EFT is expected to be effective in HB patients. But because the time and frequency of the therapies can be changed variously, the standardization is needed to perform expected effect.

By accessing past memories and trauma, EFT lessens their impact on the patient in the present. Through this process, EFT is expected to reduce emotional trauma, a known HB trigger, in affected patients. PMR is thought to alleviate HB symptoms by fostering overall body relaxation. Therefore, PMR is expected to access trauma and lessen its influence in a manner different from EFT.

Accordingly, the present clinical trial investigates the effectiveness of EFT as a new treatment option for HB patients experiencing anger as a primary symptom and compares the efficacy to that of the conventional meditation technique of PMR.

2. Methods

2.1. Study Subjects and Trial Period. Between November 2013 and February 2014, a total of 26 people participated in this clinical trial. The participants were patients diagnosed with HB verified using the HB SCID during a screening period. In total, 27 participants were eligible based on the HB SCID and were classified as subjects for the study. Participants were assigned to the EFT treatment group ($n = 15$) or the PMR treatment group ($n = 12$) by random allocation; 26 participants completed the study, and one subject in the EFT group dropped out in the middle of the study (Figure 1). The number of participants in the two groups differed because a random allocation was performed on two separate occasions.

Table 1: The Emotional Freedom Technique (EFT) program performed on the EFT group (EFT Borrowing Benefit).

Weeks	Program contents
1st week	(i) Education on Hwabyung (ii) Introduction of basic EFT (iii) Goal setting and confirming the problem (iv) Set-up (v) The sequence (vi) The 9 Gamut sequence (vii) Treat present symptom (viii) Share experiences with Hwabyung (ix) Distribute worksheets, self-training CD, and homework assignment
2nd week	(i) Review week 1 program (ii) Share experiences from the previous week (iii) Treat past trauma (iv) Inquire about the person and the events that caused Hwabyung (v) Education on EFT reframing (vi) Distribute worksheets, self-training CD, and homework assignment
3rd week	(i) Review weeks 1 and 2 programs (ii) Share experiences from the previous week (iii) Treat distorted self-image (iv) Distribute worksheets, self-training CD, and homework assignment
4th week	(i) Review weeks 1–3 programs (ii) Share experiences from the previous week (iii) Treat the doubtful future (iv) Distribute work sheets, self-training CD, and homework assignment

This clinical trial was approved by the Institutional Review Board of Kyung Hee University Hospital at Gangdong, Korea (KHNMCOH 2013-01-012).

2.2. Program Protocol. Each subject group convened together for 1 hour each week to undergo EFT or PMR training. By providing education on HB, the basics concepts of meditation, and time for practice; the training program aimed to reduce the symptoms of HB.

The programs were performed over a 4-week period, and afterwards participants were instructed to complete homework using the distributed worksheets and self-training CD. These group programs are each summarized in Tables 1 and 2.

All groups were instructed to practice the program daily using the distributed text and mp3 files. All subjects recorded the number of daily practices and stressful events treated with the therapy. The EFT group ($n = 15$) and PMR group ($n = 12$) both performed the program.

Group Treatment. Both groups gathered weekly at the same time and location to receive group therapy and self-training goals.

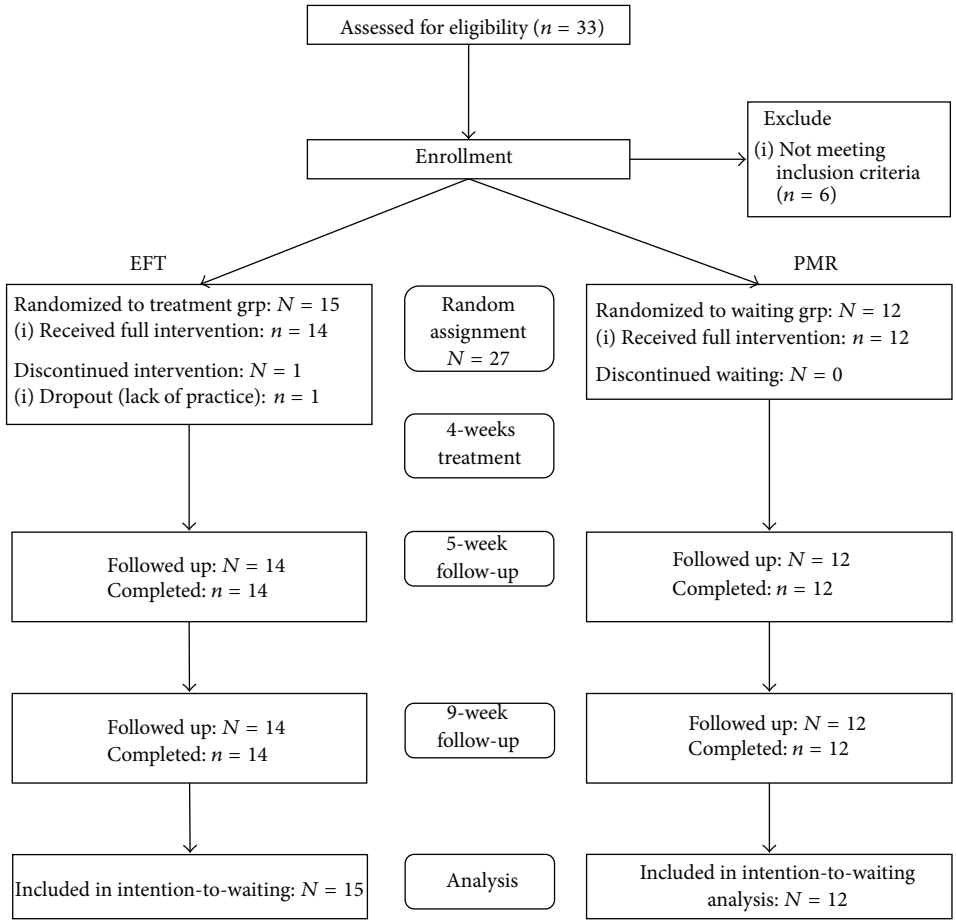

Figure 1: Schematic of the entire EFT and PMR program protocol.

The program in the EFT group followed the EFT Borrowing Benefit protocol [15] as follows:

Goal setting and confirming the problem.

Set-up.

Sequence.

The 9 Gamut sequence.

Sequence.

EFT reframing.

2.3. Symptom Assessment and Measurement Tools. To assess the condition of HB patients, the Hwabyung scale, STAI, STAXI, and SCL-90-R were utilized. Among these, the Hwabyung scale was used to assess HB-related symptoms and the severity of the psychological status. STAI and STAXI were employed to analyze the degree of anxiety and anger in the patients. Finally, the SCL-90-R was used to assess the overall psychological status of the participants.

2.3.1. Hwabyung Scale. The Hwabyung scale is a tool used to measure the severity of Hwabyung-related symptoms in Hwabyung patients. It is the first self-reported survey measuring Hwabyung and was constructed by Kwon et al. [16]. All items on the Hwabyung scale as well as subscales of Hwabyung characteristics and Hwabyung symptoms have a relatively high degree of internal consistency, and the symptoms of Hwabyung differ significantly between Hwabyung groups and depression groups. The scale assesses symptoms of Hwabyung during primary screening up to 30 points [16].

2.3.2. STAI. The STAI is used to measure the anxiety state of HB patients. Developed by Spielberger in 1964, STAI was designed as a subjective and easy-to-use self-reported scale that can measure both the anxiety state and trait simultaneously. Our study is based on the Korean version of the STAI [17].

2.3.3. STAXI. The STAXI is a tool for assessing the status of anger. STAXI was developed by Spielberger in 1987 as a tool to measure components of anger and can be utilized to analyze normal and abnormal personality characteristics. A state of anger is defined as a diverse range of subjective feelings ranging from feeling hotly indignant to displaying outrageous anger. The trait of anger is defined as a tendency to interpret a wide range of conditions as anger provoking or frustrating. Eight questions each were also included focusing on the expression of rage, known as anger-out, the suppression of

Table 2: Progressive muscle relaxation (PMR) performed on the PMR group [15].

Weeks	Program contents
1st week	(i) Education on Hwabyung (ii) Introduction of PMR and meridian muscle points (iii) Practice PMR on the upper body (iv) Share experiences with Hwabyung (v) Distribute worksheets, self-training CD, and homework assignment
2nd week	(i) Review week 1 program (ii) Share experiences from the previous week (iii) Practice PMR of face and chest (iv) Distribute worksheets, self-training CD, and homework assignment
3rd week	(i) Review weeks 1 and 2 programs (ii) Share experiences from the previous week (iii) Practice PMR of the lower body (iv) Application in stressful circumstances (v) Distribute worksheets, self-training CD, and homework assignment
4th week	(i) Review weeks 1–3 programs and all practices of the entire body (ii) Share experiences from the previous week (iii) Application in stressful circumstances (iv) Distribute worksheets, self-training CD, and homework assignment

rage, called anger-in, and the attempted regulation of rage, called anger-control [18].

2.3.4. The Symptom Checklist 90-R (SCL-90-R). First developed and improved by Derogetis as a self-reported multidimensional clinical checklist examination, this test comprises nine symptom scales measuring somatization (SOM), obsessive-compulsive (OC), interpersonal sensitivity (IS), depression (DEP), anxiety (ANX), hostility (HOS), phobic anxiety (PHOB), paranoid ideation, and psychoticism (PSY) symptoms and three overall scales measuring the global severity index (GSI), positive symptom distress index (PSDI), and the positive symptom total (PST). This type of self-report is effective in detailing the subjective experiences of patients that an observer cannot detect and is therefore used as a primary screening tool in patients requiring intervention. Because this test requires patients to self-assess their own conditions, it enables patients to organize their symptoms and clinicians to readily assess the patient condition relatively quickly [19].

2.4. Statistical Analysis. Survey data were analyzed using SPSS (version 18) and Microsoft Excel (2010). Pre- and posttreatment comparisons within each group were performed using average value correspondence analysis, and an independent analysis was used for the pre- and posttreatment comparisons between the groups. Data from each scale were evaluated to determine whether they were nonparametric using the Shapiro-Wilk test. When the data were nonparametric (STAI anxiety state scale, STAXI anger state scale, OC of SCL-90-R, ANX, PHOB, HOS, PST, and PSDI scales), the Mann-Whitney U nonparametric test was performed to determine significance compared to baseline in each group. When a periodic variation was nonparametric (all of STAI, all of STAXI, SOM in SCL-90-R, HOS, PHOB, IS, DEP, ANX, PSY, GSI, and PST), the Wilcoxon matched pairs signed ranks test was performed to assess the significance of changes. For the T-score of the SCL-90-R, a nonparametric test, the Wilcoxon matched pairs signed ranks test, was performed for the SOM, IS, DEP, ANX, HOS, PHOB, PSY, GSI, and PST scales.

In the pre- and posttreatment comparisons within groups, changes occurred between the weeks 1 and 5 (termination of clinical education), and weeks 1 and 9 (termination of follow-up) were analyzed using the standard of verification for effectiveness. Statistical significance in each scale was designated as $p < 0.05$. An intention-to-treat analysis and per-protocol analysis was performed in both groups. The statistical analysis was performed by a blinded controlled specialist.

3. Results and Discussion

3.1. Results

3.1.1. Differences within Each Group. There were no significant differences in the EFT and PMR groups compared to their respective baselines, except for the PHOB scale, which showed some difference. However, because the overall PHOB score was less than 70, which is the severe point of the T-score, it can be concluded that there was no significant clinical difference in this scale as well. There was no significant difference in the mean age between the EFT (53.53 ± 9.64 years) and PMR groups (59.00 ± 8.22 years, $p = 0.131$).

3.1.2. Significant Differences in the Hwabyung Scale. There was a significant decrease in the Hwabyung symptom scale ($p = 0.031$) between baseline and week 5 in the EFT group. After concluding the program, there was a highly significant decrease in the Hwabyung symptom scale between baseline and week 9 ($p = 0.001$).

In the PMR group, there was a significant decrease in the Hwabyung symptom scale between baseline and week 5 ($p = 0.049$). Between baseline and week 9, there was also a significant decrease in the Hwabyung symptom scale in the PMR group ($p = 0.041$, Figure 2).

There were no significant differences between the two groups in differences in values before and after.

3.1.3. Significant Differences in STAI. In the EFT group, there was no significant decrease in both the anxiety state and trait between baseline and week 5. After concluding the program, there was a significant decrease in the anxiety state between baseline and week 9 ($p = 0.046$, Figure 3).

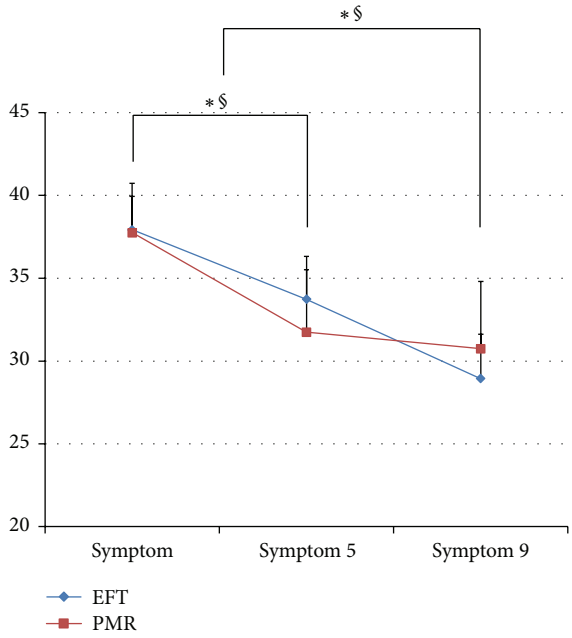

Figure 2: The Hwabyung symptom scale compared between the EFT and PMR groups over time. $* = p < 0.05$ (EFT group); $\S = p < 0.05$ (PMR group); symptom: baseline survey; symptom 5; 5th week survey; symptom 9: 9th week survey.

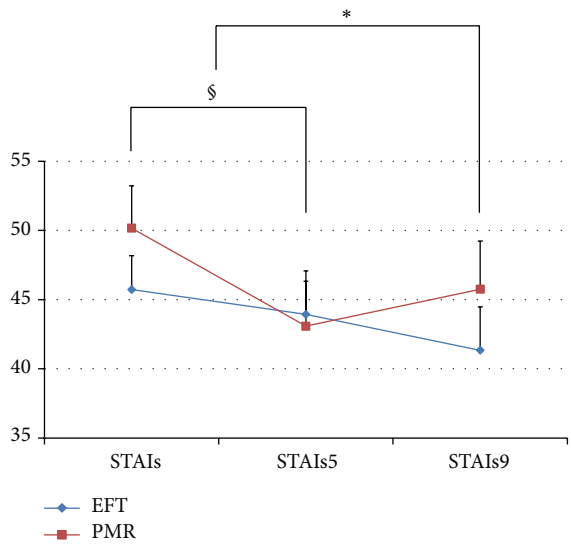

Figure 3: The STAI anxiety state scale compared between the EFT and PMR groups over time. $* = p < 0.05$ (EFT group); $\S = p < 0.05$ (PMR group); STAIs: baseline survey; STAIs5: 5th week survey; STAIs9: 9th week survey. The STAI anxiety state scale was analyzed using the Wilcoxon signed ranks test.

In the PMR group, there was a significant decrease in both the anxiety state ($p = 0.035$) and trait ($p = 0.032$) between baseline and week 5. After completing the program, there was no significant change between baseline and week 9.

No significant pre-post change differences in STAI were found between the two groups.

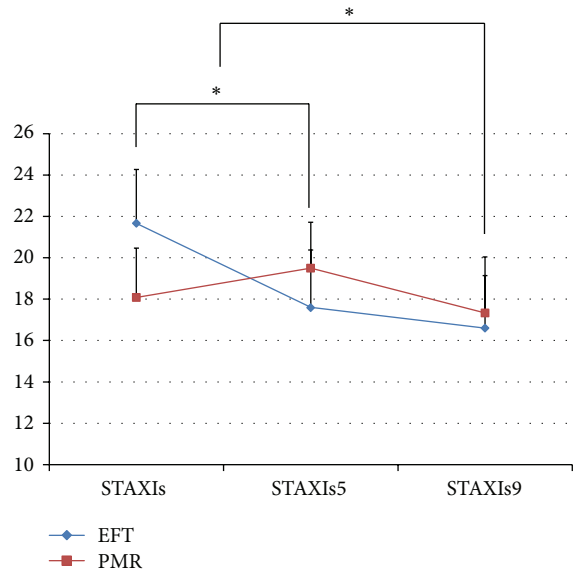

Figure 4: The STAXI anger state scale compared between the EFT and PMR groups over time. $* = p < 0.05$ (EFT group); STAXIs: baseline survey; STAXIs5: 5th week survey; STAXIs9: 9th week survey.

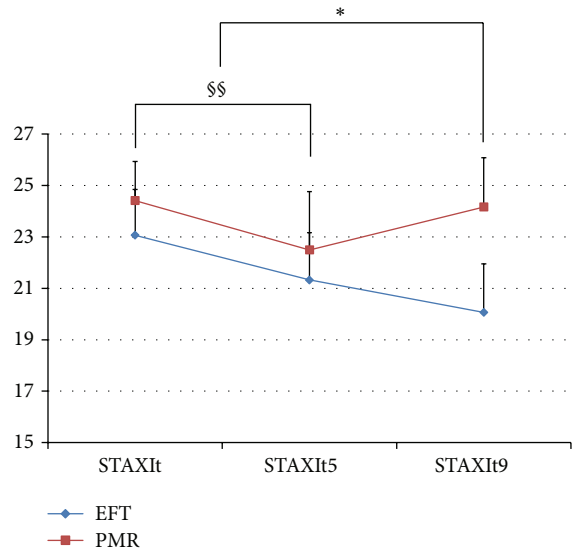

Figure 5: The STAXI anger trait scale compared between the EFT and PMR groups over time. $* = p < 0.05$ (EFT group); $\S = p < 0.05$ (PMR group); $\S\S = 0.05 < p < 0.1$ (PMR group); STAXIt: baseline survey; STAXIt5: 5th week survey; STAXIt9: 9th week survey.

3.1.4. Significant Differences in STAXI. In the EFT group, there was a significant decrease in the anger state between baseline and week 5 ($p = 0.021$). After completing the program, there was a significant decrease in both the anger state ($p = 0.006$) and trait ($p = 0.006$, Figures 4 and 5).

In the PMR group, there was a decrease in the anger trait between baseline and week 5, while no significant difference

was found between baseline and week 9 at the conclusion of the program.

No significant pre-post change differences in the anger trait in STAXI were found between the two groups. However, there was a significant difference in the anger state between the two groups between baseline and week 5 ($p = 0.026$) and baseline and week 9 ($p = 0.047$).

3.1.5. Significant Differences in SCL-90-R. In the EFT group, there was a significant change in the paranoid ideation between baseline and week 5 ($p = 0.012$). After conclusion of the program, the EFT group showed significant decreases in somatization ($p = 0.005$), interpersonal sensitivity ($p = 0.003$), anxiety ($p = 0.047$), hostility ($p = 0.003$), paranoid ideation ($p = 0.001$), depression ($p = 0.046$), psychoticism ($p = 0.047$), the global severity index ($p = 0.010$), and the positive symptom total ($p = 0.011$) of SCL-90-R between baseline and week 9.

In the PMR group, there was a decrease in the hostility scale ($p = 0.055$) between baseline and week 5. After the conclusion of the program, the PMR group showed only a decreasing tendency in the hostility scale between baseline and week 9.

3.1.6. T-Score Assessment of SCL-90-R. In the EFT group, the SCL-90-R T-score only showed a significant decrease in the paranoid scale between baseline and week 5 ($p = 0.016$). However, between baseline and week 9, there was a significant decrease in somatization ($p = 0.004$), interpersonal sensitivity ($p = -0.003$), depression ($p = 0.046$), anxiety ($p = 0.047$), hostility ($p = 0.003$), paranoia ($p = 0.001$), psychoticism ($p = 0.038$), the global severity index ($p = 0.014$), and the positive symptom total ($p = 0.010$, Figure 6).

In the PMR group, the SCL-90-R T-score only showed a decreasing tendency in the hostility scale between baseline and week 5 ($p = 0.068$) and between baseline and week 9 ($p = 0.074$). The other scales showed no significant difference.

No significant pre-post change differences were observed in the T-score between the two groups.

3.2. Discussion. Hwabyung, an abbreviated form of Ulhwabyung, is a psychological syndrome where emotion such as anger explodes after being accumulated without appropriate resolution. Physical symptoms are characterized by chest pressure, a hot rash, and a feeling of constriction in the neck and xiphoid process. Feelings of resentment, anger, haan, and hard feelings characterize the psychological symptoms. The term is medically interpreted as somatization symptoms caused by prolonged exposure to stress. Because HB is characterized by emotional anger, it is often referred to as an anger syndrome. In Korean culture, especially, the concept of "haan" is occasionally linked to anger as an explanation of this disease.

Studies of Hwabyung have been performed to evaluate their psychological symptoms such as anxiety, anger, and depression. According to the reports, HB patients have difficulty in controlling anger caused by extreme anger suppression, with severe anxiety or depressive mood. These HB patients' psychological problems cause multiple symptoms including insomnia and heart palpitations. Various meditation techniques have been reported as effective in controlling these symptoms in numerous cases [20].

Several recent clinical trials have investigated the effects of EFT on symptoms of anxiety disorder including phobia, tensional headache, depression, anxiety, and insomnia [8–13], and a review reported that the technique was effective against emotional trauma such as PTSD [21]. Considering these therapeutic successes, EFT is expected to be more effective at controlling psychological problems rather than the physical symptoms.

As one of the most well-known relaxation therapies, PMR is widely used to alleviate pain in cardiac and other diseases. This behavioral treatment aims to relax muscles by repeatedly applying tension and relaxation, which reduces the physical symptoms of participants with relative ease. Randomized controlled trials have been conducted assessing the effect of PMR on adenomyosis patients [22] and breast cancer patients [23]. The trials reported significant improvements in the quality of life and alleviation of physical and psychological symptoms.

In this study, the PMR group was expected to show a greater decrease in physical symptoms compared to the EFT group. However, the EFT group improved more in physical symptoms, as well as the overall psychological statuses of anxiety and anger compared to the PMR group. Particularly on the Hwabyung symptom scale, EFT was confirmed to be a highly effective Hwabyung treatment in clinical conditions; the EFT group showed a mean score of less than 30 after 9 weeks of follow-up, which is considered marginal Hwabyung [24].

Participants in the EFT group were more effective at controlling Hwabyung symptoms, especially anger and anxiety, compared to the PMR group. This is clearly explained by the STAI, STAXI, and somatization, anxiety scales of the SCL-90-R. Therefore, the EFT program could be suggested as a method of controlling anger and anxiety symptoms in patients suffering from HB or other diseases. The significant decrease in the anger state scale of STAXI before and after treatment in both groups indicates that EFT is clearly effective for controlling a current state of anger.

In this study, we anticipated that patients in the EFT group would experience a feeling of group homogeneity during treatment. To utilize this effect in the analysis, the study was designed to maximize this synergistic effect. In order to match this study design, the PMR group was also subjected to group therapy. During the first 5 weeks of the study, patients were required to participate in the group sessions, and they then continued self-therapy without the group sessions during weeks 5–9. When the participants were analyzed through week 5, the EFT group showed significant improvements in more criteria than the PMR group did. In addition, a comparison between the baseline and final assessments showed that the EFT group experienced greater alleviation of symptoms than the PMR group did. While patients in the PMR group demonstrated a low level of symptom control after the group sessions, patients in the EFT group showed a continuous decrease in symptoms even

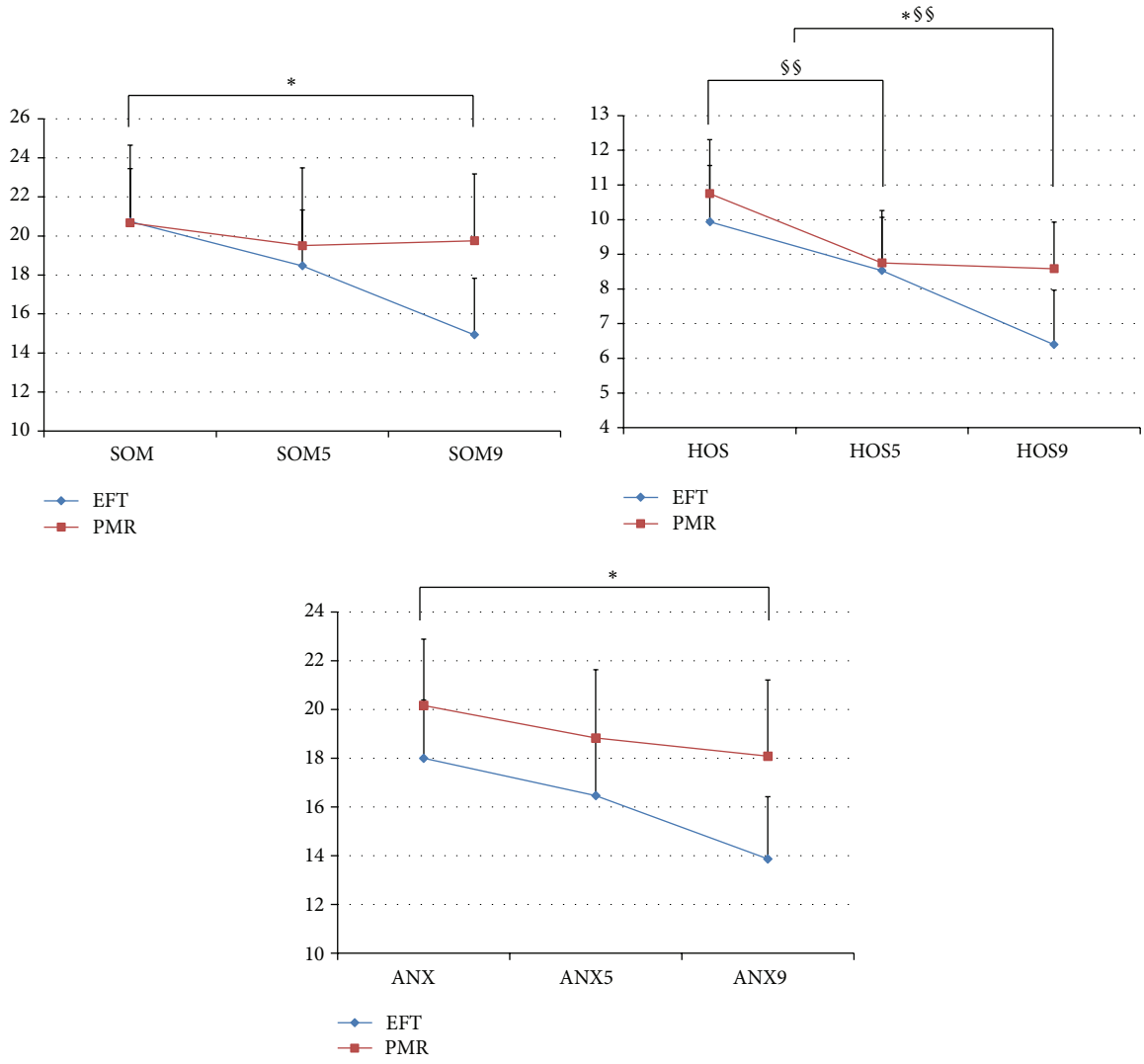

Figure 6: The SCL-90-R scales compared between the EFT and PMR groups over time. $* = p < 0.05$ (EFT group); § = $p < 0.05$ (PMR group); §§ = $0.05 < p < 0.1$ (PMR group); SOM: somatization baseline survey; SOM5: somatization 5th week survey; SOM9: somatization 9th week survey; HOS: hostility baseline scale; HOS5: hostility 5th week scale; HOS9: hostility 9th week scale; ANX: anxiety baseline scale; ANX5: anxiety 5th week scale; ANX9: anxiety 9th week scale.

during the self-training period without intervention. This clearly demonstrates that the EFT caused much improved self-training effect compared to PMR. The effect of group therapy was similar between both groups.

In addition, the effect of the group program in the EFT group continued to prevail during self-training after the fifth week. In other words, it means that EFT is more suitable at being performed on patients' own than PMR technique. It could be also inferred that EFT program itself was more effective considering the fact that homework distributed and CD shared an identical format. And because there were significant changes in several scales in week 9 compared to week 1, the program is expected to require a period longer than 8 weeks to show a full effect.

3.3. Limitations. The T-score was less than 70 in the SCL-90-R test, which means that the participants could actually be classified as normal emotional state. Further studies should be conducted in patients with severe psychological problems. In addition, the small number of participants limits the statistical significance of the findings; this limitation should be alleviated in further studies with big subjects. There is also an uncertainty and lack of control in programs conducted in the home after the group session is over.

4. Conclusions

(1) The 4-week EFT program shows more effect to improve scales of psychological symptoms including anxiety and anger in HB patients than PMR program. The significant improvements in physical symptoms are also found in EFT group.

(2) The EFT group showed greater decreases in HB symptoms compared to the PMR group. The EFT group

also showed improvement or sustained improvement during the self-training period of weeks 5–9. This demonstrates that EFT is an effective self-control treatment for HB.

(3) PMR was proven effective on the Hwabyung scale symptoms. This is consistent with results from conventional studies showing that PMR is effective in alleviating physical symptoms.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

Jin Woo Suh and Jung Hwan Lee set up the EFT and PMR program protocol for this clinical trial. Jin Woo Suh, Sang Young Kim, and Jong Woo Kim wrote the first paper for this trial and Sun Yong Chung calculated the sample size. Jin Woo Suh and Jong Woo Kim performed the EFT and PMR methods on HB patients. After the program, Sang Young Kim conducted surveys. Jong Woo Kim initiated and directed this study. Sun Yong Chung edited the first paper. All authors read and approved the final paper.

Acknowledgment

This study was supported by the Ministry of Health & Welfare through the Korea Health Industry Development Institute (KHIDI: HI12C1933010013).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 737631, 6 pages
http://dx.doi.org/10.1155/2015/737631

Research Article

Serum Levels of Stress Hormones and Oxidative Stress Biomarkers Differ according to Sasang Constitutional Type

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Received 18 January 2015; Revised 24 March 2015; Accepted 26 March 2015

Academic Editor: Salih Mollahaliloglu

Objectives. This study investigated whether Sasang constitutional type is associated with differences in the serum levels of stress hormones and oxidative stress. *Methods.* A total of 236 participants (77 males and 159 females) were enrolled. The serum levels of cortisol, adrenaline, reactive oxygen species (ROS), and malondialdehyde (MDA) were analyzed. *Results.* The distribution of Sasang constitutional types was as follows: Taeumin, 35.6%; Soumin, 33.0%; and Soyangin, 31.4%. The serum cortisol levels of Taeumin were significantly lower than Soumin ($p < 0.1$ in both sexes) and Soyangin ($p < 0.05$ in males and $p < 0.1$ in females). The adrenaline levels were also significantly lower in Taeumin than in Soumin ($p < 0.05$ in males and $p < 0.1$ in females) and Soyangin ($p < 0.1$ in males). Serum ROS levels were significantly higher in Soyangin than in Taeumin and Soumin ($p < 0.05$ in males), whereas MDA levels were significantly lower in Taeumin compared with Soumin and Soyangin ($p < 0.05$ in males and $p < 0.1$ in females). *Conclusion.* Taeumin type may tolerate psychological or oxidative stress better than other types, which suggests a biological mechanism to explain the different pathophysiological features of Sasang constitutional types.

1. Introduction

Sasang constitutional medicine (SCM) is a major branch of traditional Korean medicine that emphasizes the role of inherited psychological and physical traits in the development and treatment of diseases [1]. SCM was established and popularized by Lee (1837–1900), a Korean doctor, through his book *Donguisusebowon* [2], and it has since been widely adopted as a diagnostic and therapeutic tool in traditional Korean medicine [3].

SCM classifies people into four types according to their constitution: Taeyangin, Soyangin, Taeumin, and Soumin. In this context, "constitution" includes the body's structural and functional features, including psychological characteristics [4]. Current genetic science has shown that the genome critically affects the complex processes involved in health and disease [5], and the SCM classification is presumably related to macrolevel genomic differences among individuals [6]. Moreover, recent studies have found that certain Sasang types are significantly more susceptible to certain diseases; specifically, those with the Taeumin type are especially susceptible to metabolic syndrome, and those with the Soumin type are especially susceptible to irritable bowel syndrome (IBS) [7, 8].

Psychological or emotional stress, which is inevitable in modern life, is associated with both mental and physical health [9]. Moreover, psychological characteristics have been associated with Sasang constitutional types [10]. In addition, evidence has shown a strong connection between psychological stress and oxidative stress, prominent features of pathophysiological processes in a wide range of disorders or aging [11, 12]. Therefore, we hypothesized that Sasang constitutional type would affect the serum levels of stress hormones, especially cortisol and adrenaline, and oxidative stress biomarkers including reactive oxygen species (ROS) and its lipid oxidation byproduct.

To contribute to elucidating the biological explanation for SCM, we initially investigated whether the serum level of stress hormones and the degree of oxidative stress differ according to Sasang constitution.

2. Methods

2.1. Subjects. Self-reported healthy adults were recruited in South Korea, and those who had an established illness or were taking medication were excluded from the study. This study also excluded night workers, alcoholics, and subjects with self-reported severe psychological stress. Blood samples were collected at least 4 hours after participants' last meal before noon. Informed consent was obtained from each subject and the Ethics Committee of Daejeon University Hospital approved the study protocol (authorization number: DJOMC-119).

2.2. Classification of Sasang Constitution. The grouping of participants according to type of Sasang constitution was based on consideration of an integrative combination of facial, body shape, vocal, and questionnaire response features using Sasang constitutional analytic tool (SCAT). This automated Sasang constitution classification system developed by the Korea Institute of Oriental Medicine [13]. Every participant was classified as Taeumin, Soumin, Taeyangin, or Soyangin.

2.3. Determination of Serum Cortisol and Adrenaline Levels. Serum cortisol and adrenaline levels were determined using a cortisol ELISA kit (LDN GmbH & Co., KG, Nordhorn, Germany) and an adrenalin ELISA kit (LDN GmbH & Co, KG, Nordhorn, Germany), respectively, according to the manufacturer's protocol. Absorbance was measured using a spectrophotometer (Molecular Devices, Sunnyvale, CA).

2.4. Determination of Serum ROS Levels. The total quantity of ROS in the serum was determined according to Hayashi' method [14]. Briefly, N,N-diethyl-para-phenylenediamine (DEPPD) and ferrous sulfate solutions were prepared in advance. Five μL of standard solution or serum was added to 140 μL of 0.1 M sodium acetate buffer (pH 4.8) in 96-well plates and incubated at 37°C for 5 min. A total of 100 μL of DEPPD and ferrous mixture solution was added to each well, and the amount of ROS was determined at the saturation point at 505 nm using a spectrophotometer. Hydrogen peroxide was used to generate the calibration curve, and the standard and the results were expressed as equivalent to levels of hydrogen peroxide (μmol H_2O_2 equiv/L).

2.5. Determination of Serum Malondialdehyde (MDA) Levels. Serum lipid peroxide levels were determined using thiobarbituric acid (TBA) reactive substances (TBARS) as previously described [15]. Briefly, 250 μL of serum or standard solution was added to 2.5 mL of 20% trichloroacetic acid (TCA). This was then mixed with 1 mL of 0.67% thiobarbituric acid (TBA) and heated at 10°C for 30 min, followed by cooling on ice. After centrifugation at 3000 ×g for 20 min, the absorbance of the upper organic layer was measured at 535 nm with a spectrophotometer and compared with a 1,1,3,3-tetraethoxypropane (TEP) standard curve.

2.6. Statistical Analysis. Statistical analysis was performed using SAS statistical software (SAS Rel. 8.02; SAS Institute, Inc., Cary, NC, USA). Comparisons among Sasang constitution groups were performed by one-way ANOVA followed by paired Student's t-tests. p values < 0.1 were considered statistically significant. All data are expressed as means ± standard deviations (SDs).

3. Results

3.1. Characteristics of Participants. A total of 236 adults, including 77 males (median age: 23 yr, range: 18–63) and 159 females (median age: 21 yr, range 18–77), participated in this study. The proportions of Taeumin, Soumin, and Soyangin among total participants were 35.6%, 33.0%, and 31.3% respectively. The sample included no participants with a Taeyangin constitution. Among males, 23.4%, 45.5%, and 31.2% were of Taeumin, Soumin, and Soyangin constitution, respectively; among females, 41.5%, 27.0%, and 31.5% were of Taeumin, Soumin, and Soyangin constitution, respectively (Table 1).

The average heights of the three Sasang types were similar; however, the body weights of differed notably among categories. The average body mass index (BMI) differed significantly according to Sasang constitution among male, female, and total participants ($p < 0.01$). The BMI of those with a Taeumin constitution was significantly higher than that of participants with a Soumin ($p < 0.01$ in male, female, and total subjects) or a Soyangin ($p < 0.01$ in male, female, and total subjects) constitution, and the BMI of those with a Soumin constitution was significantly lower than that of participants with a Soyangin constitution ($p < 0.05$ in male and total subjects, Table 1).

3.2. Serum Levels of Cortisol. The serum cortisol level of males was significantly higher than that of females (20.0 ± 8.9 versus 16.3 ± 7.5 ng/mL, $p = 0.002$). Males with a Taeumin constitution (16.8 ± 6.2 ng/mL) had a significantly lower serum cortisol level than those with a Soumin (21.0 ± 11.1 ng/mL, $p = 0.091$) or a Soyangin (20.9 ± 6.5 ng/mL, $p = 0.049$) constitution. This pattern was repeated in female: Taeumin (14.9 ± 5.5 ng/mL) comparing to Soumin (16.9 ± 5.9 ng/mL, $p = 0.081$) and Soyangin (17.6 ± 10.3 ng/mL, $p = 0.096$), respectively (Figure 1(a)). No significant difference in serum cortisol was observed between Soumin and Soyangin constitutional groups ($p = 0.685$).

3.3. Serum Adrenaline Levels. The serum adrenaline level of males was slightly higher than that of females (296.1 ± 126.4 versus 284.4 ± 85.4 pg/mL, $p = 0.604$). Males with a Taeumin constitution (250.9 ± 50.4 pg/mL) had a significantly lower level of serum adrenaline than males with a Soumin (321.3 ± 165.3 ng/mL, $p = 0.027$) or Soyangin (291.0 ± 230.9 pg/mL, $p = 0.069$) constitution. A statistically significant difference

Table 1: Characteristics of participants according to the Sasang classification.

Sex (number)	Characteristics	Taeumin	Soumin	Soyangin
Male (77)	Number (%)	18 (23.4%)	35 (45.4%)	24 (31.2%)
	Median age (yr, range)	23 (18–64)	21 (17–77)	21 (17–77)
	Average height (cm)	172.9 ± 5.2	172.6 ± 6.6	174.6 ± 5.9
	Average weight (kg)	81.7 ± 8.2	67.3 ± 7.7	73.3 ± 7.3
	Mean BMI***	27.3 ± 2.2	22.5 ± 1.7	24.3 ± 2.5
Female (159)	Number (%)	66 (41.5%)	43 (27.0%)	50 (31.5%)
	Median age (yr, range)	23 (18–64)	21 (17–77)	21 (17–77)
	Average height (cm)	162.6 ± 5.8	160.5 ± 4.8	159.8 ± 4.8
	Average weight (kg)	63.1 ± 12.1	51.1 ± 7.4	54.8 ± 5.4
	Average BMI***	24.0 ± 4.3	18.8 ± 2.6	21.4 ± 1.7
Total (236)	Number (%)	84 (35.6%)	78 (33.0%)	74 (31.4%)
	Median age (yr, range)	23 (18–64)	21 (17–77)	21 (17–77)
	Average height (cm)	164.4 ± 6.2	166.0 ± 8.3	164.6 ± 8.7
	Average weight (kg)	67.1 ± 13.7	58.4 ± 11.1	60.9 ± 10.8
	Mean BMI***	24.7 ± 4.2	21.0 ± 2.6	22.3 ± 2.4

***Comparisons of the BMIs of those with different Sasang constitutions ($p < 0.01$) were performed with ANOVAs. Those with a Taeumin constitution had significantly higher BMIs than those with a Soumin ($p < 0.01$ in male, female, and total subjects) or Soyangin ($p < 0.01$ in male, female, and total subjects) constitution, and those with a Soumin constitution had significantly lower BMIs than those with Soyangin constitution ($p < 0.05$ in male and total subjects). BMI: body mass index.

was observed between Taeumin (246.9 ± 55.4 pg/mL) and Soumin (333.2 ± 118.0 ng/mL, $p = 0.084$), but Soyangin (290.3 ± 54.4 ng/mL, $p = 0.198$), for serum adrenaline in females (Figure 1(b)).

3.4. Serum ROS Levels. Males and females had almost identical serum ROS levels overall (178.6 ± 81.4 versus 189.3 ± 89.8 μM, $p = 0.382$). Soyangin males (233.3 ± 93.5 μM) had a significantly higher level than Taeumin (171.5 ± 73.9 μM, $p < 0.05$) and Soumin (168.9 ± 86.5 μM, $p < 0.05$) males, but no significant differences were observed in females (Taeumin 181.0 ± 93.8 versus Soumin 177.2 ± 69.8 versus Soyangin 176.5 ± 75.0 μM, resp., Figure 1(c)).

3.5. Serum MDA Levels. The overall serum MDA levels of males and females were very similar (6.4 ± 5.6 versus 6.0 ± 6.8 μM, $p = 0.415$). Taeumin males (4.1 ± 4.8 μM) had a significantly lower value compared with Soumin (6.7 ± 6.6 μM, $p = 0.039$) and Soyangin (7.6 ± 6.2 μM, $p = 0.040$) males, and this pattern was repeated in females (Taeumin: 3.2 ± 4.0 μM; Soumin: 5.4 ± 5.5 μM, $p = 0.081$; Soyangin: 6.8 ± 3.8 μM, $p = 0.062$, Figure 1(d)). No significant difference was observed between Soumin and Soyangin constitutions in males ($p = 0.594$) or females ($p = 0.732$).

4. Discussion

Inherited genomic variance is a critical contributor to individual differences in the development of various diseases [16]. SCM stresses the importance of inborn physical and psychological characteristics, which might be linked to macrogenomic features [17, 18]. Several studies have found that the high incidence of certain disorders and different drug responses are associated with Sasang constitution-dependent genomic features, likely the significantly different distributions of Pro12Ala polymorphism or haplotypes of multidrug resistance 1 (MDR1) gene [19, 20].

We examined whether the serum levels of two representative stress hormones and oxidative stress markers differ according to Sasang constitution. Psychological stress and oxidative stress have been established as general contributors to various disorders, including cancer and immunological and age-related diseases [21, 22]. The total proportions of Taeumin, Soumin, and Soyangin constitutional types in this sample were 35.6%, 33.0%, and 31.3%, respectively (Table 1). No participant was classified as Taeyangin, probably because this type is extremely rare in the Korean population [23].

Taeumin males and females showed relatively lower concentrations of serum cortisol and adrenaline compared with Soumin and Soyangin males and females (Figures 1(a) and 1(b)). Cortisol and adrenaline are considered typical stress hormones that are released via activation of the hypothalamic–pituitary–adrenal (HPA) axis under stress [9]. These stress hormones have a harmful effect on multiple target organs and systems, with outcomes including chronic pain, immunosuppression, and psychological disorders [24, 25]. Our results suggest a partial explanation of clinical data showing that Soumins are vulnerable to stress and irritable bowel syndrome (IBS) [8]. Individuals with IBS were known to have the sustained HPA axis activity [26], and then Soumins showed the highest levels of both cortisol and adrenaline in our study. Additionally, activation of the HPA axis is associated with acceleration in oxidative stress via unbalanced redox, including excessive production of mitochondrial ROS [27, 28]. In our study, the serum levels of ROS

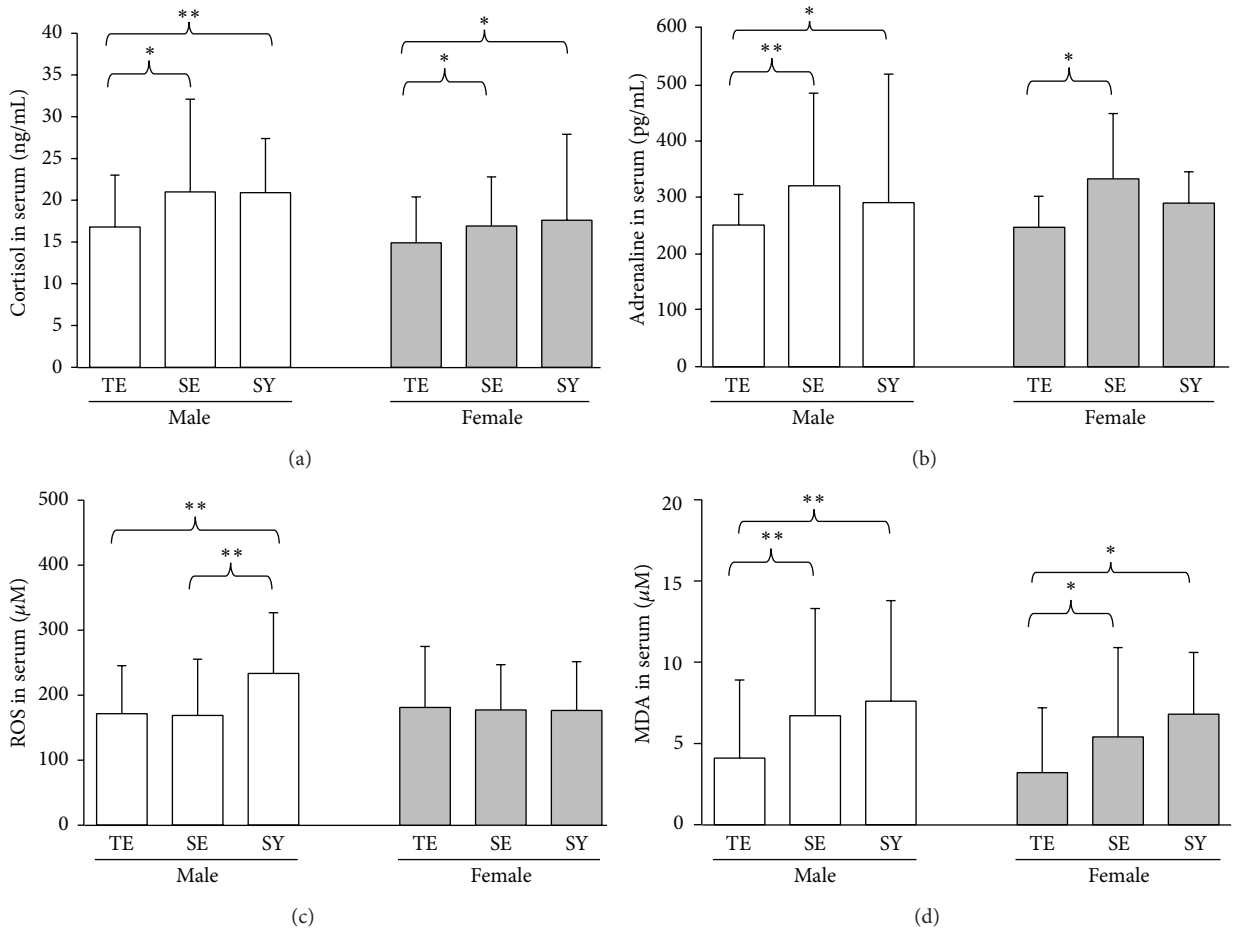

Figure 1: Serum levels of stress-related hormones and oxidative stress biomarkers. Blood was collected from healthy adults. The serum levels of cortisol (a) and adrenalin (b), reactive oxidative species (ROS (c)), and malondialdehyde (MDA (d)) were measured. Results are expressed as means ± SDs. Statistical significance was set at $^{*}p < 0.1$ and $^{**}p < 0.05$.

and its lipid peroxidation product MDA were low-high in Taeumins but high in Soumins and Soyangins (Figures 1(c) and 1(d)). Oxidative stress is implicated in diverse pathophysiological conditions, including inflammation, neurodegenerative diseases, and cancer [29]. Our previous study found that the incidence of cancer was significantly lower in Taeumins than in Soumins or Soyangins [30]. The serum cortisol levels however would be affected by time point of blood sample because circadian rhythm in the release of cortisol and adrenaline is well known [31, 32].

The analysis of physical features showed that BMIs differed significantly according to Sasang constitutional type; those with the Taeumin type had the highest BMIs, followed by those with the Soyangin type and those with the Soumin type (Table 1), which is consistent with results from other researches [33]. BMI is well known as a risk factor for type 2 diabetes; however, one study found that Taeumin constitution is an independent risk factor for this disease regardless of BMI score [34]. In our study, BMI values did not reflect a pathogenic level; the maximum value was 27.3 ± 2.2 (in Taeumins), and the minimal value was 22.5 ± 1.7 (in Soumins). Moreover, a huge cohort study with 120,700 East Asians found that, unlike Westerners, Koreans with a BMI between 22.6 and 27.5 showed the lowest risk of death [35]. Obesity is generally positively associated with oxidative stress [36]; however, our results did not show the correlation between BMI values and levels of ROS or MDA.

Our study has some limitations such as relatively small number and young age of participants. We also adopted $p = 0.1$ as the cutoff for statistical significance. Clinical studies may accept $p < 0.1$ as the threshold for statistical significance if doing so makes scientific sense but does not cause harm, as may be the case in drug tests [37]. The choice of $p = 0.1$, however, could increase the possibility of type 1 error; therefore we need to interpret our data with care.

In conclusion, our results carefully propose the differences in the serum levels of stress hormones and the oxidative stress markers across Sasang constitutional types, especially in Taeumins compared with Soumins and Soyangins. This finding would contribute to SCM-based practices by informing future research regarding the mechanisms underpinning such differences.

Conflict of Interests

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

Acknowledgment

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Ministry of Science, ICT & Future Planning (no. 2006-2005173).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 950829, 7 pages
http://dx.doi.org/10.1155/2015/950829

Research Article

Effect of a Traditional Herbal Prescription, Kyung-Ok-Ko, on Male Mouse Spermatogenic Ability after Heat-Induced Damage

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Received 26 June 2015; Revised 7 September 2015; Accepted 10 September 2015

Academic Editor: Angelo A. Izzo

Kyung-Ok-Ko (KOK), a well-known traditional Korean medicinal formula, has long been used to invigorate the essential *qi*. This use of KOK may be associated with reproductive ability as a more modern concept. The protective effect of KOK was evaluated against deterioration of testicular function induced by heat exposure in male mice. Male fertility was disrupted by scrotal heat stress at 43°C for 5 weeks. KOK (0.25, 0.50, and 2.00 g/kg/day) was administered orally at 3 h after the stress. To evaluate the protective effect of KOK, body weight, testicular weight, sperm count, sperm motility, and histopathological changes in the testes were evaluated. KOK-treated mice significantly recovered their general health, as evidenced by body weight. KOK-treated mice also showed significantly higher testes weights, sperm counts, and sperm motility than did the heat stress group. KOK-treated mice significantly recovered the morphological appearance of the seminiferous tubules and seminiferous epithelium. Furthermore, KOK-treated mice significantly increased antioxidant enzyme activities and reduced the protein expressions of apoptosis in the testes. KOK significantly protects against heat-induced damage to testicular function in male mice by inhibiting oxidative stress and apoptosis, indicating that KOK may be an effective agent for treatment of heat-induced male infertility.

1. Introduction

Infertility affects 15% of couples worldwide. The male contribution is 45–50%, and males are the sole cause in 20% of cases [1, 2]. The causes of male infertility are multifactorial which include anatomical and genetic defects, testicular injury and disease, sperm disorders, hormonal dysfunction, aging, and environmental- and lifestyle-related factors [3–5]. Among environmental and lifestyle factors, genital heat stress appears to be a major contributor to impairment of male reproductive health [6]. For example, sitting in a hot bath or car for a long time is associated with significantly higher scrotal temperatures, resulting in downregulation of spermatogenesis with declines in semen volume, sperm motility, and sperm morphology [3–5].

Kyung-Ok-Ko (KOK), also known as Qiong-yu-gao in China, is a traditional Korean medicinal formula composed of *Rehmannia glutinosa* var. *purpurea*, *Panax ginseng*, *Poria cocos*, *Lycium chinense*, *Aquilaria agallocha*, and honey [7, 8]. KOK has long been used to maintain health and increase longevity [8]. Based on traditional medicine physiology, male infertility is closely related to kidney storage of the essence *qi*, which corresponds roughly to the modern concept of the male gametes [9]. Therefore, herbal prescriptions that increase the essential *qi* have been used to treat impotence and sterility secondary to male infertility. KOK contains

phytochemicals, such as valine, aspartic acid, and arginine, which are antioxidants and exhibit oxidase inhibition, tyrosinase inhibition, nitric oxide inhibition, and superoxide dismutase-like activities [9]. In addition, some studies have examined the effects of KOK on age-related disorders as well as the biological properties of KOK, including its antioxidant, anti-inflammatory, antifatigue, and immunological activities [9, 10]. These biological properties are associated with medical therapy that aims to improve sperm parameters in male infertility [11–13].

Based on the above mentioned effects of KOK and its use in traditional medicine, and previous reports, it is hypothesized that KOK might be effective to treat male infertility. Hence, the aim of this study is to evaluate the effects of KOK on sperm quality parameters, such as sperm count, sperm motility, and testicular weight, as well as the histopathology, antioxidant, and apoptotic changes in male mice with infertility induced by heat exposure.

2. Materials and Methods

2.1. Materials. M199 medium was purchased from Gibco Industries, Inc. (Auckland, NZ). Phosphate-buffered saline (PBS), sodium chloride (NaCl), bovine serum albumin (BSA), hematoxylin, and eosin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tetramethylethylenediamine, protein assay kit, tween 20, ammonium persulfate, acrylamide, ECL reagent, and skim milk were purchased from Bio-Rad Lab. (Hercules, CA, USA). B-cell lymphoma-associated X protein (Bax), cytochrome c, and β-actin antibodies were obtained from Santa Cruz Biotechnology, Inc., (Delaware Avenue, CA, USA). Cleaved caspase-3 and HRP-conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The total glutathione (GSH) assay kit and the oxidized glutathione (GSSG)/GSH Quantification Kit were purchased from Dojindo Molecular Tech. (Tokyo, Japan). KOK was the same as that used in the previous study [14] in which chemical profiling and standardization of KOK had been performed and KOK (Lot No. SU12) was donated by Kwang Dong Pharmaceutical Co. (Pyongtaek, Korea).

2.2. Animals and Heat Exposure. Male ICR mice (7 weeks, 30–32 g) were purchased from Daehan Biolink (Eumseong, Korea). The mice were divided randomly into five groups of eight mice each: (1) normal group, (2) heat exposure (HE) group, (3) HE + KOK 0.25 g group, (4) HE + KOK 0.50 g group, and (5) HE + KOK 2.00 g group. The lower body, including the scrotum, in groups (2) to (5) was exposed to heat at 43°C for 10 min twice per day at 10 min intervals (6 days per week) for 5 weeks in a thermostatically controlled water bath. KOK was dissolved in distilled water and administered orally at 0.25, 0.50, or 2.00 g/kg/day, 3 h after the heat stress. The gavage doses of KOK were derived from the previous study [14, 15] and the normal group and HE group were treated with the same volume of distilled water. Four animals were housed in a single cage and had free access to water and food. The animals were kept at a constant temperature (23 ± 1°C) and humidity (60 ± 10%) and maintained under a 12 h light/dark cycle. Animal treatment and maintenance were carried out in accordance with the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University, Seoul, Korea. The animals were weighed twice per week to determine the gavage volume and monitor their general health.

2.3. Sperm Analysis and Testes Weight. The epididymal sperm motility and count were evaluated as described in previous research with some modifications [16]. The sperm analysis was performed using a hemocytometer (Superior, Marienfeld, Germany). The mice were anesthetized with Rumpun and Zoletil solution (3 : 1 ratio, 1 mL/kg) intramuscularly on the day after the last KOK treatment. The epididymis was rapidly washed in PBS, minced in M199 medium containing 0.5% BSA, and incubated for 5 min at 37°C. Sperm were scored as motile if any movement was detected, and the total number of sperm was counted. Additionally, the entire testis from each mouse was rapidly washed in PBS and weighed. The testes were then stored at −80°C until use.

2.4. Histology. Frozen tissues were cut along the coronal plane (5 μm) using a cryostat (Leica, Nussloch, Germany). The sections were mounted on gelatin-coated slides and stained with hematoxylin and eosin (H&E). The images were obtained using a research microscope (BX51T-32F01; Olympus Corporation, Tokyo, Japan). The effect of KOK on testicular tissue was quantified by measuring the optical density of ROIs in seminiferous tubule using the ImageJ software and the mean optical densities of each group are presented as percentages of the normal group values.

2.5. Total Glutathione Quantification and Oxidized Glutathione Quantification. Total GSH and GSSG levels were detected using the GSSG/GSH quantification kit with the reagent for GSH masking according to the instruction manuals. Briefly, frozen tissues were lysed in 10 mmol/L hydrochloric acid solution by freezing and thawing. To measure total GSH level, they were further treated with 5% 5-sulfosalicylic acid. 20 μL coenzyme working solution, 120 μL buffer solution, and 20 μL enzyme working solution were added to each well at 37°C for 5 min. Then, 20 μL GSH standard solution, 20 μL sample solution, and 20 μL substrate working solution were added for 10 min each. Absorbance was measured using a spectrophotometer at a wavelength of 405 nm, and concentrations of GSH were determined in the sample solution using a GSH standard calibration curve. To measure GSSG level, they were treated with 5% 5-sulfosalicylic acid. 40 μL GSSG standard solution and 40 μL sample solution with 2% masking solution each were incubated with 120 μL buffer solution at 37°C for 60 min. Then, 20 μL substrate working solution, 20 μL coenzyme working solution, and 20 μL enzyme working solution were added for 10 min each. Absorbance was measured using a spectrophotometer at a wavelength of 415 nm, and concentrations of GSSG were

Table 1: Body and testicular weights and sperm parameters in mice after heat stress and/or KOK treatment for 5 weeks.

Groups	Normal	HE	HE + KOK 0.25 g	HE + KOK 0.5 g	HE + KOK 2 g
Body weight (g)	35.22 ± 1.09	32.91 ± 0.49*	31.55 ± 0.39	34.03 ± 0.76	33.18 ± 0.34
Testes weight (mg)	117.75 ± 2.83	47.27 ± 1.51***	40.76 ± 1.04$^{\#}$	79.76 ± 2.74$^{\#\#\#}$	73.88 ± 4.23$^{\#\#\#}$
Relative testes weight (%)	0.34 ± 0.02	0.14 ± 0.01***	0.12 ± 0.01	0.24 ± 0.01$^{\#\#\#}$	0.22 ± 0.01$^{\#\#\#}$
Sperm count ($\times 10^6$)	61.22 ± 1.77	12.15 ± 0.49***	40.76 ± 1.40	20.98 ± 1.35$^{\#\#\#}$	24.83 ± 1.97$^{\#\#\#}$
Sperm motility (%)	49.11 ± 0.93	24.22 ± 1.01***	30.80 ± 0.46	36.16 ± 0.68$^{\#\#\#}$	39.22 ± 0.65$^{\#\#\#}$

The data represents the mean ± SEM (n = 6–8). $^{***}P < 0.001$, $^{*}P < 0.05$ compared with the normal group; $^{\#\#\#}P < 0.001$, $^{\#}P < 0.05$ compared with the heat stress group.

determined in the sample solution using a GSSG standard calibration curve.

2.6. Western Blotting. Frozen tissues were lysed using a protein assay kit according to the manufacturer's instructions. The lysates (protein 25 μg) were separated by 10% or 12% SDS-polyacrylamide gel electrophoresis, and then transferred to a membrane. The membranes were incubated with 5% skim milk in TBST for 1 h and then with primary antibody (1 : 500 dilution) overnight at 4°C, prior to incubation with HRP-conjugated secondary antibody for 1 h. Immunoreactive bands were detected using an ECL detection kit and an LAS-4000 mini system (Fujifilm, Tokyo, Japan) was used for visualization. The intensities of the bands were normalized to the β-actin intensity using Multi Gauge software (Fujifilm, Tokyo, Japan).

2.7. Statistical Analysis. All statistical parameters were calculated using the GraphPad Prism 5.0 software (San Diego, CA). Values are expressed as the means ± standard error of the mean (SEM). Results were analyzed by one-way analysis of variance followed by Tukey's *post hoc* test. Differences with a P value of <0.05 were considered as statistical significance.

3. Results

3.1. Effects of KOK on Heat Exposure-Induced Reduction of Body and Testicular Weights. Mice with heat stress showed significant reduced weight compared with the control. However, KOK-treated mice subjected to heat stress recovered this reduction more efficiently than mice in the HE group (Table 1). In addition, heat stress induced a greater loss of testicular weight (weight, 47.27 ± 1.51 mg) compared to the control group (weight, 117.75 ± 2.83 mg). However, mice treated with KOK at 0.50 and 2.00 g/kg for 5 weeks showed recovery of testicular weight (79.76±2.74 and 73.88±4.23 mg, resp.) (Table 1).

3.2. Effects of KOK on Sperm Parameters against Heat Stress. To investigate the effect of KOK on the epididymal sperm count and motility, sperm parameters were measured. The sperm count of mice exposed to heat treatment was decreased significantly to 14.41% ± 0.79% of that of the control mice. However, mice treated with KOK at 0.25 to 2.00 g/kg showed an increase in sperm count to 19.85% ± 2.295 to 40.56% ± 3.22% of that of the controls. In addition, KOK-treated mice significantly recovered their sperm motility after the heat exposure to 62.73% ± 0.94% to 79.87% ± 1.32% of that of the controls, whereas heat stress reduced sperm motility in mice to 49.33% ± 2.05% of that of the controls (Table 1).

3.3. Effects of KOK on Histopathological Change in Testes against Heat Stress. To determine the effect of KOK on seminiferous tubules in testes, we performed H&E staining. A normal morphological appearance of the seminiferous tubules and spermatocytes was evident in the testes of control mice, whereas the heat-exposed testes exhibited degenerated and disorganized features and reduced spermatocyte numbers. However, KOK-treated mice significantly recovered the morphological appearance of the seminiferous tubules and seminiferous epithelium (Figure 1).

3.4. Effects of KOK on GSH Depletion and Apoptotic Protein Expressions in Testes against Heat Stress. To examine the effect of KOK on heat stress-induced oxidative stress and apoptosis, the levels of total GSH and GSSH and apoptotic protein expressions were measured. In the GSH and GSSG quantification assays, treatment with heat stress reduced GSH level and increased GSSG level in the testes. However, KOK treatment recovered them (Figure 2). In addition, the testes of mice exposed to heat treatment showed increase of Bax and cytochrome c protein expressions to 265.78% ± 7.75% and 304.32% ± 9.76%, respectively, of that of the control mice. However, mice treated with KOK at 0.5 and 2.00 g/kg recovered these increases. In addition, KOK-treated mice significantly inhibited the heat treatment induced-increase in cleaved caspase-3 expression levels in the testes (Figure 3).

4. Discussion

It is widely accepted that heat stress adversely affects spermatogenesis, resulting in infertility. In humans, scrotal heat treatment by occupational exposure, lifestyle, or clothing is correlated with reduced sperm concentrations, sperm motility, and normal morphology [2]. In this study, the body weight, testicular weight, sperm number, and sperm motility were reduced in male mice after heat stress, 43°C water bath for 10 min twice per day (6 days per week for 5 weeks), which corresponds with previous studies on the effect of heat stress [17–19]. However, KOK-treated mice recovered the reduction of body weights induced by heat stress more efficiently than did mice in the HE group. And, mice treated with KOK at

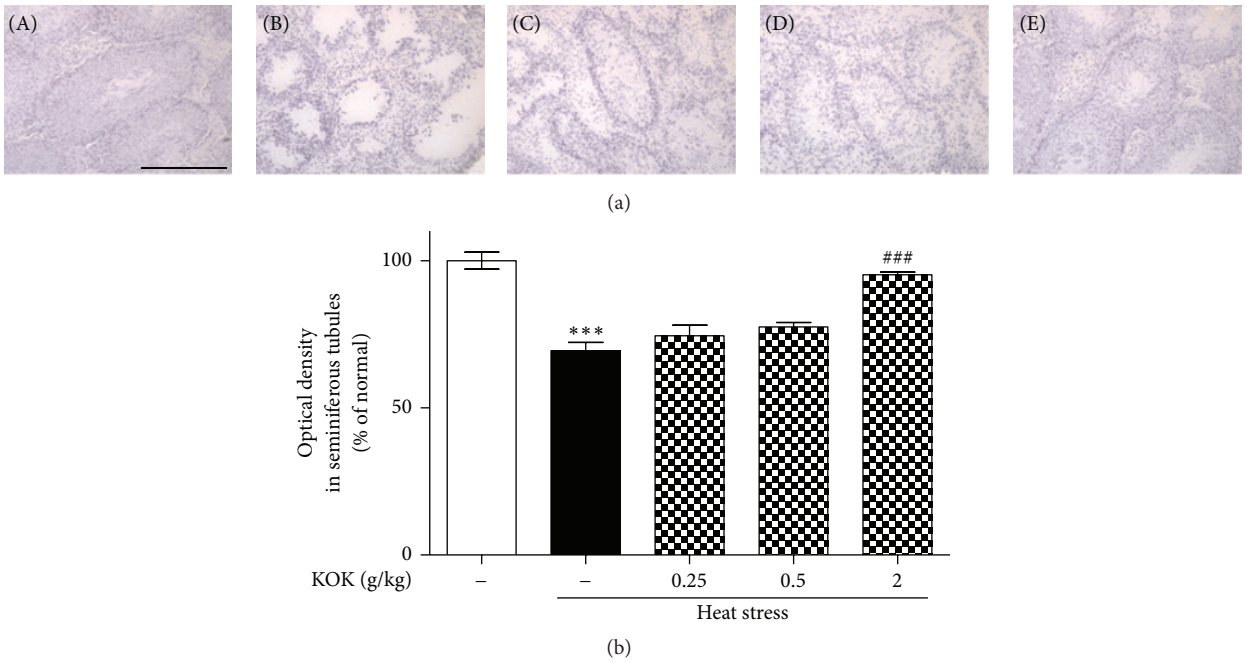

Figure 1: Effect of KOK on histological changes in testes of mice with heat stress-induced infertility. H&E staining was performed using testicular tissue after heat stress and/or KOK treatment for 5 weeks. Representative photomicrographs are shown in (a), and the mean optical density of seminiferous tubules was measured in (b). (A) Normal group; (B) heat stress group; (C–E) heat stress and KOK treatment at 0.25, 0.50, and 2.00 g/kg, respectively. Scale bar = 200 μm. Each column represents the mean ± SEM ($n = 6$). $^{***}P < 0.001$ compared with the normal group; $^{###}P < 0.001$ compared with the heat stress group.

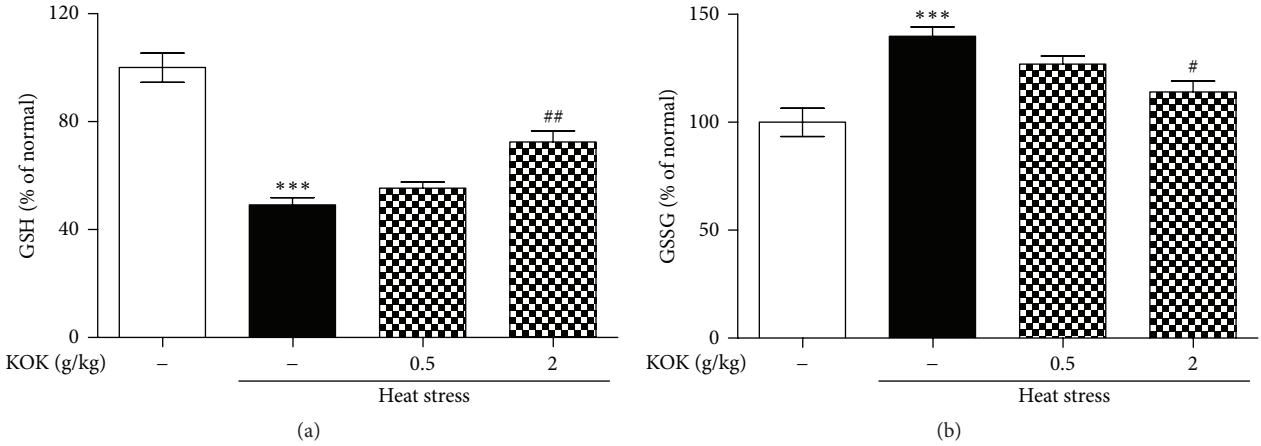

Figure 2: Effect of KOK on glutathione level variation in testes of mice with heat stress-induced infertility. Glutathione kit assays were performed using testicular tissue after heat stress and/or KOK treatment for 5 weeks. The total GSH level (a) and GSSG level (b) were determined. Each column represents the mean ± SEM ($n = 4$). $^{***}P < 0.001$ compared with the normal group; $^{##}P < 0.01$, $^{#}P < 0.05$ compared with the heat stress group.

0.50 and 2.00 g/kg showed recovery of testicular weight from the heat-induced damage. In addition, mice treated with KOK significantly showed an increase in sperm count and sperm motility after heat exposure.

The epididymal sperm and testicular germ cells are sensitive to damage by heat stress [20]. Thus, seminiferous tubules from testes after heat stress showed pathological morphologies including degenerating cells of primarily spermatocyte origin and condensed chromatin in germ cell nuclei, resulting in disruption of spermatogenesis [21]. The present study showed that KOK-treated mice significantly recovered the morphological appearance of the seminiferous tubules and epithelium.

Generally, germ cell death and decreased sperm motility secondary to heat stress appear to be caused by oxidative stress and apoptosis which involve reactive oxygen species (ROS), the tumor suppressor protein p53, nitric oxide synthase (NOS), translocation of the proapoptotic factor Bax,

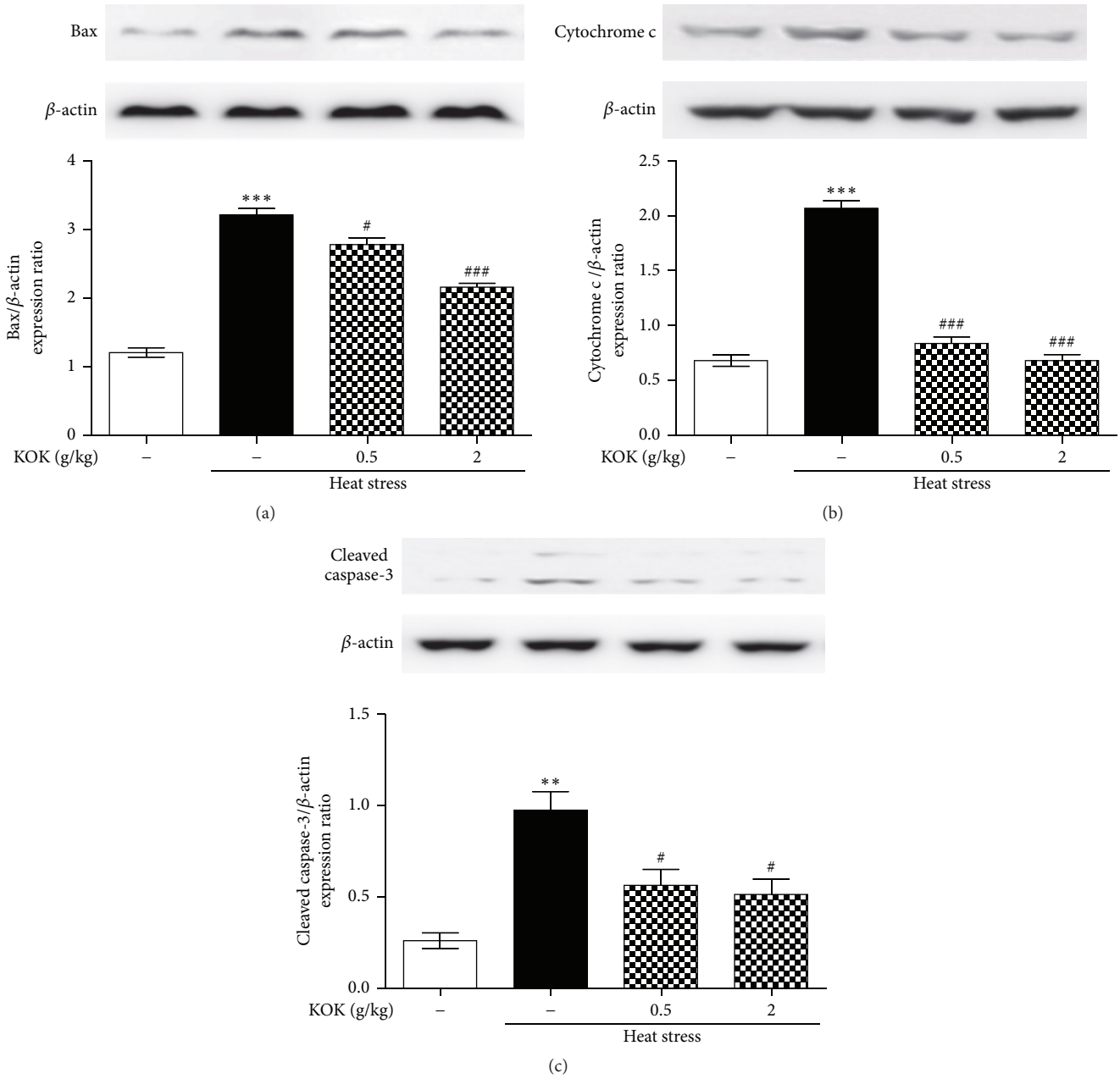

Figure 3: Effect of KOK on apoptotic protein expressions in testes of mice with heat stress-induced infertility. Western blotting was performed using testicular tissue after heat stress and/or KOK treatment for 5 weeks. Apoptosis factors such as Bax (a), cytochrome c (b), and cleaved caspase-3 (c) were presented. Each column represents the mean ± SEM ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the normal group; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared with the heat stress group.

release of cytochrome *c* from mitochondria, and several caspases [22, 23]. In addition, antioxidants have a significant effect on sperm oxidative stress and DNA damage in infertile patients and improve sperm motility [22, 24]. Thus, inhibition of oxidative stress and apoptosis could be protective in male infertility. In the present study, KOK treatment protected depletion of glutathione and increase of proapoptotic protein expressions in testes under heat stress condition. Taken together, these findings indicate that KOK-treated mice significantly improved male infertility induced by heat via antioxidant and antiapoptotic activities.

KOK contains ingredients that exert beneficial effects on male infertility. In a previous study, KOK treatment exerted its protective effect on polycystic ovarian syndrome induced by dehydroepiandrosterone via inhibition of inflammatory responses [15] which is also related to male fertility because infertile patients with infection have a heightened inflammatory response and parallel alterations in sperm parameters

[13]. KOK was also found to inhibit the expression of IL-1β, a proinflammatory cytokine, thus showing anti-inflammatory properties [14]. In addition, processed rhizome of *Rehmannia glutinosa* inhibits TNF-α secretion by inhibiting IL-1 secretion and has anti-inflammatory activity [25].

Moreover, Ginseng Radix, the root of *Panax ginseng,* improves the motility and total number of sperm by activating cAMP-responsive element modulator [26]. Treatment with Ginseng Radix also resulted in significantly enhanced sperm counts and glial cell-derived neurotrophic factor (GDNF) mRNA and protein levels, suggesting that it induces spermatogenesis and GDNF activation in rat testes [27]. In addition, the fruit of *Lycium chinense* has been used as a traditional remedy for male infertility [28]; it possesses antioxidant activity due to its inhibition of malondialdehyde formation and activation of superoxide anion scavenging and antisuperoxide formation [29]. Furthermore, KOK possesses potential bioactive components which might protect or treat spermatogenic ability, such as valine, aspartic acid, and arginine, which are antioxidants and exhibit oxidase inhibition, nitric oxide inhibition, and superoxide dismutase-like activities [9]. Therefore, the properties of KOK and its constituent compounds, including their antioxidant, anti-inflammatory, antiapoptotic, and spermatogenesis activities, likely contributed to the effects seen in this study. We believe that some medicinal herbs may improve male fertility with relatively few side effects.

5. Conclusions

In this study, KOK significantly protects against heat-induced damage in male mouse testes. These results suggested that KOK may be useful for the treatment of environmental and lifestyle-related male infertility.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

This research was supported by a grant from the Korea Institute of Oriental Medicine (KIOM) [K13301] and the Bio-Synergy Research Project (NRF-2012M3A9C4048795) of the Ministry of Science, ICT, and Future Planning through the National Research Foundation.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 483980, 6 pages
http://dx.doi.org/10.1155/2015/483980

Research Article

Protective Effect of *Artemisia asiatica* Extract and Its Active Compound Eupatilin against Cisplatin-Induced Renal Damage

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Received 13 April 2015; Accepted 14 June 2015

Academic Editor: Avni Sali

The present study investigated the renoprotective effect of an *Artemisia asiatica* extract and eupatilin in kidney epithelial (LLC-PK1) cells. Although cisplatin is effective against several cancers, its use is limited due to severe nephrotoxicity. Eupatilin is a flavonoid compound isolated from the *Artemisia* plant and possesses antioxidant as well as potent anticancer properties. In the LLC-PK1 cellular model, the decline in cell viability induced by oxidative stress, such as that induced by cisplatin, was significantly and dose-dependently inhibited by the *A. asiatica* extract and eupatilin. The increased protein expressions of phosphorylated JNK and p38 by cisplatin in cells were markedly reduced after *A. asiatica* extract or eupatilin cotreatment. The elevated expression of cleaved caspase-3 was significantly reduced by *A. asiatica* extract and eupatilin, and the elevated percentage of apoptotic cells after cisplatin treatment in LLC-PK1 cells was markedly decreased by cotreatment with *A. asiatica* extract or eupatilin. Taken together, these results suggest that *A. asiatica* extract and eupatilin could cure or prevent cisplatin-induced renal toxicity without any adverse effect; thus, it can be used in combination with cisplatin to prevent nephrotoxicity.

1. Introduction

Cisplatin is a potent chemotherapeutic agent for the treatment of multiple human malignancies [1, 2]. It accumulates in all segments of nephron but is predominantly taken up by the proximal tubule cells, which then provokes severe damage [3]. The efficacy of cisplatin is dose dependent, but the side effect in kidney limits the use of higher doses to improve its chemotherapeutic effects [4, 5]. The toxic effects of cisplatin mainly occur via oxidative stress and DNA damage [6, 7], ultimately leading to apoptotic pathways in tumour cells [8] and also in renal cells [4, 9, 10].

For centuries, many natural products have been identified for the prevention and/or treatment of kidney diseases because they are believed to have nephroprotective effects. They are widely used in clinical practice in many parts of the world. For example, *Silybum marianum* was found to attenuate nephrotoxicity induced by gentamicin in dogs [11]. A water extract of *Kalanchoe pinnata* leaves protected rat kidneys from gentamicin-induced nephrotoxicity [12]. Salviae Radix extract exerted a protective effect against cisplatin-induced renal cell injury, and its effect might be mediated by its antioxidant effect [13].

Artemisia asiatica Nakai is a traditional oriental medicine and it has been used for the treatment of several inflammatory disorders. Recent studies revealed that *A. asiatica* has antioxidative and anti-inflammatory effects contributing to its protective effects against various pathophysiological conditions including gastric damage [14], liver damage [15], experimental pancreatitis [16], and tumor promotion [17].

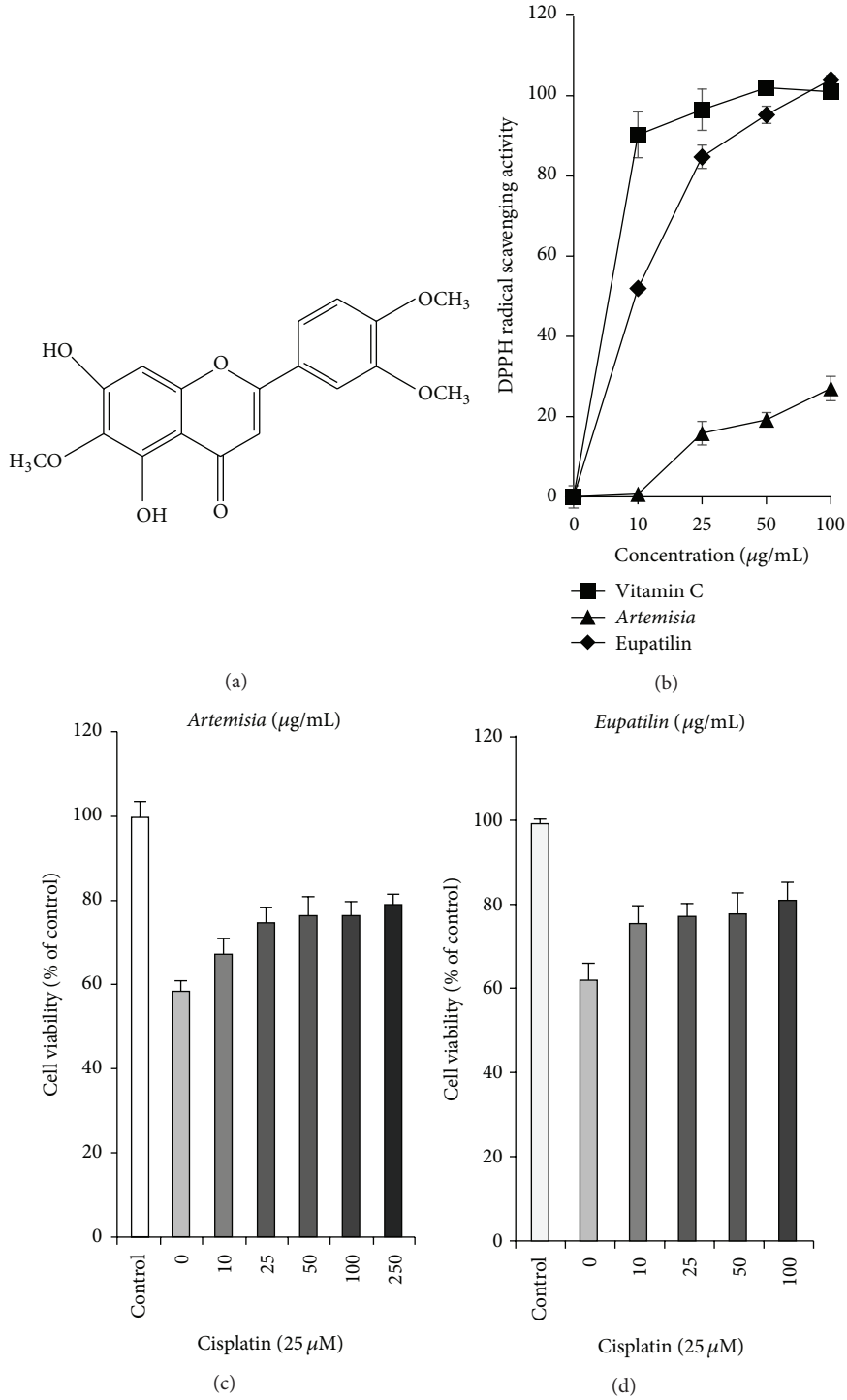

Figure 1: Effects of *A. asiatica* extract and eupatilin on cisplatin-induced nephrotoxicity in LLC-PK1 cells. (a) Structure of eupatilin. (b) Comparison of DPPH radical scavenging effects of *A. asiatica* extract, eupatilin, and vitamin C. (c) Dose-dependent protective effect of *A. asiatica* extract against cisplatin-induced nephrotoxicity in cells. (d) Dose-dependent protective effect of eupatilin against cisplatin-induced nephrotoxicity in cells.

Stillen is a commercially available extract from *A. asiatica*. Eupatilin (Figure 1(a)), an active compound isolated from *A. asiatica*, has been reported to treat peptic ulcers and gastritis. It has antioxidative and anti-inflammatory effects against gastric mucosal injury [18, 19]. Various inflammatory mediators such as cytokines and oxidative stress that can affect gastric mucosal injury are thought to be involved in its action mechanism [20, 21]. Eupatilin was also reported to

have therapeutic potential for the treatment of gastric cancer [22, 23].

Although cisplatin-induced nephrotoxicity has been well documented, the effects of *A. asiatica* and eupatilin on apoptosis in kidney cells after cisplatin exposure remain under active investigation.

2. Materials and Methods

2.1. Chemicals and Reagents. An ethanolic extract of *A. asiatica* and its active compound eupatilin were prepared as reported previously [17, 18]. Cisplatin and 1,1-diphenyl-2-picryl-hydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The stock solution of chemicals was prepared in 100% dimethylsulfoxide (DMSO) and stored at −20°C until use. Antibodies for p38, p-p38, JNK, p-JNK, ERK, p-ERK, cleaved caspase-3, and GAPDH were purchased from Cell Signaling (Boston, MA, USA).

2.2. Protective Effect against Cisplatin-Induced Nephrotoxicity in Cells. Possible renoprotective effects against cisplatin-induced damage were evaluated in LLC-PK1 cells as reported previously [24]. In brief, LLC-PK1 cells were seeded in 96-well culture plates at 1×10^4 cells per well and the test sample and/or radical donor, 25 μM cisplatin, were added to the culture medium. Twenty-four hours later, the cell viability was measured by using a microplate reader (PowerWave XS; Bio-Tek Instruments, Winooski, VT, USA).

2.3. DPPH Radical Scavenging Assay. The radical scavenging activity of *A. asiatica* and eupatilin against DPPH was determined spectrophotometrically. In microwells, 100 μL of an aqueous solution of the completely dissolved sample (control: 100 μL DW) was added to an ethanolic solution of DPPH (100 μL, 60 μM) according to the reported method [25]. The final concentrations of the tested samples in the assayed solution were 10, 25, 50, and 100 μg/mL. Vitamin C was used as the standard for comparison. The ability to scavenge DPPH radicals was calculated in terms of percentage of inhibition according to the following equation: % inhibition = $[(A_0 - A_1)/A_0 \times 100]$, where A_0 is the absorbance of the control (without extract) and A_1 is the absorbance in the presence of the extract.

2.4. Western Blot Analysis. Proteins (whole cell extracts, 30 μg/lane) were separated by electrophoresis in a precast 4–15% Mini-PROTEAN TGX gel (Bio-Rad, CA, USA) blotted onto PVDF transfer membranes as reported previously [26]. Bound antibodies were visualized using ECL Advance Western Blotting Detection Reagents (GE Healthcare, UK) and a LAS 4000 imaging system (Fujifilm, Japan).

2.5. Image-Based Cytometric Assay. To determine the portion of the population that had become apoptotic, cells were stained with annexin V-Alexa Fluor 488 conjugate using a Tali image-based cytometer (Invitrogen, CA, USA) [27]. Propidium iodide (PI) was used to differentiate dead cells (annexin V-positive/PI positive or annexin V-negative/PI positive) from those that were apoptotic (annexin V-positive/PI negative). The percentages of the population reported as viable, apoptotic, and dead by the Tali cytometer were comparable with data from the same samples independently run on a flow cytometer.

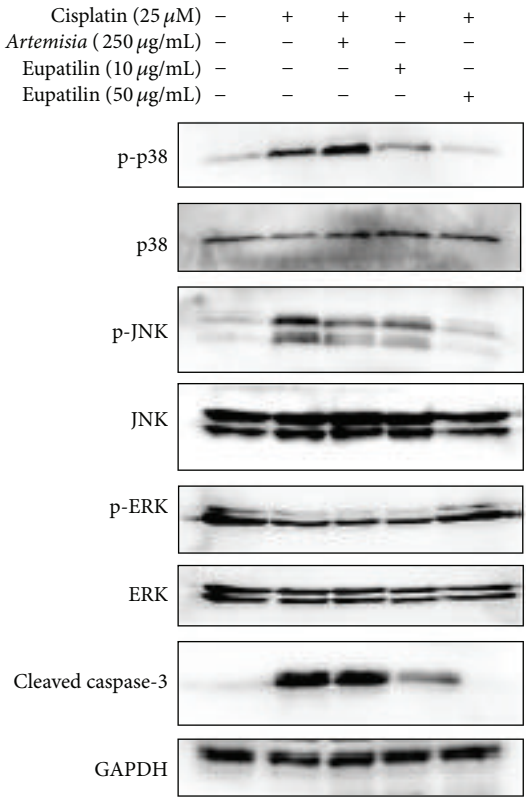

Figure 2: Involvement of the MAPKs-caspase-3 signaling pathway in the protective effect of *A. asiatica* extract and eupatilin against cytotoxicity in cultured LLC-PK1 cells. Results of the Western blot show the levels of p-p38, p38, p-JNK, JNK, p-ERK, ERK, and cleaved caspase-3 in LLC-PK1 cells treated with *A. asiatica* extract and eupatilin and/or cisplatin at different concentrations for 24 h. Whole cell lysates (20 μg) were separated by SDS-PAGE, transferred onto PVDF transfer membranes, and probed with the indicated antibodies. Proteins were visualized using an ECL detection system.

2.6. Statistical Analysis. Statistical significance was determined through analysis of variance (ANOVA). p values of less than 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Effects of A. asiatica Extract and Eupatilin on Cisplatin-Induced Nephrotoxicity in LLC-PK1 Cells. The antioxidant effects of *A. asiatica* and eupatilin were tested using DPPH, a stable free radical. DPPH decolorizes in the presence of antioxidants. The scavenging ability of *A. asiatica* and eupatilin was represented by a line diagram and compared with vitamin C (Figure 1(b)). This result suggests that eupatilin is the antioxidant and active component of *A. asiatica*. As shown in Figure 1, the cell viability was decreased

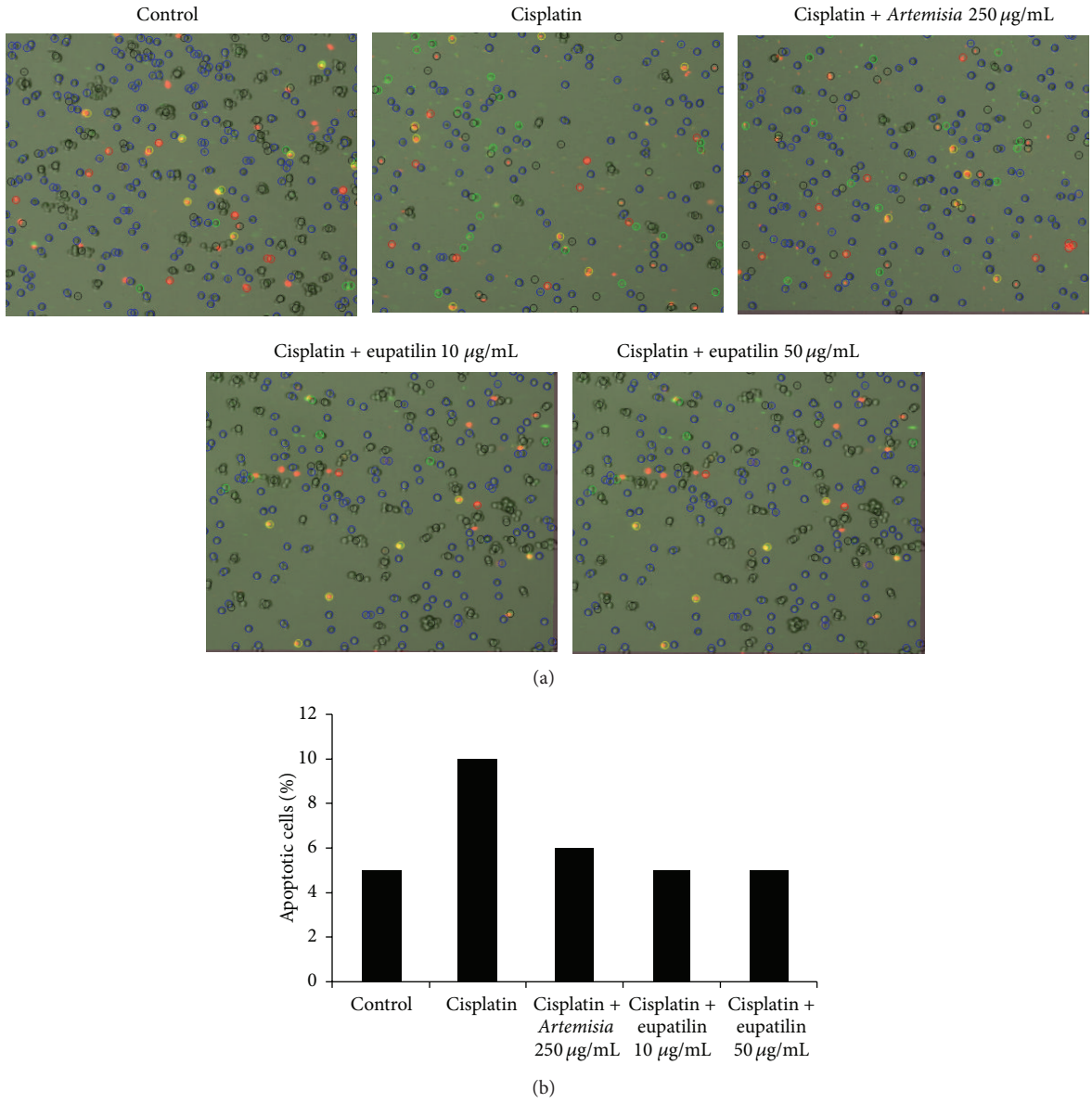

Figure 3: Effects of *A. asiatica* extract and eupatilin on apoptosis in LLC-PK1 cells. (a) Representative images of apoptosis detection. (b) Percentage of annexin V-positive-stained apoptotic cells. Dead and apoptotic cells were stained red and green, respectively. Apoptosis was determined by a Tali image-based cytometer.

significantly to about 60% of that of untreated control cells ($p = 0.0004$) after 25 μM cisplatin treatment. However, pretreatment with the *A. asiatica* extract and eupatilin markedly restored cell viability to 80 and 82%, respectively, in a dose-dependent manner (Figures 1(c) and 1(d)).

3.2. Involvement of MAPKs-Caspase-3 Signaling Pathway in the Protective Effect of A. asiatica Extract and Eupatilin against Cytotoxicity in Cultured LLC-PK1 Cells. Figure 2 shows the protein expressions of p38, p-p38, JNK, p-JNK, ERK, p-ERK, and cleaved caspase-3 after *A. asiatica* (250 μg/mL) and eupatilin (10 and 50 μg/mL) treatment. As shown in Figure 2, the phosphorylation of p38 and JNK was decreased in LLC-PK1 cells by *A. asiatica* and eupatilin treatments. In addition, the elevated protein expression of cleaved caspase-3 was also markedly reduced by *A. asiatica* and eupatilin treatments. ERK protein expression in the LLC-PK1 cells was slightly decreased by cisplatin treatment and increased by *A. asiatica* and eupatilin treatment; however, the differences were not of significant effect.

3.3. Effects of A. asiatica Extract and Eupatilin on Apoptosis in LLC-PK1 Cells. Figure 3 shows the effects of the *A. asiatica* extract and eupatilin on apoptosis in LLC-PK1 cells. As shown in Figure 3(a), the number of dead and apoptotic cells, which were stained with red or green colors, was increased by cisplatin treatment, whereas it was decreased after cotreatment with the *A. asiatica* extract ($p = 0.008$)

and more significantly eupatilin ($p = 0.003$). The elevated percentage of apoptotic cells after cisplatin treatment was markedly decreased after cotreatment with the *A. asiatica* extract and eupatilin (Figure 3(b)).

4. Discussion

The protective effect of the *A. asiatica* extract and eupatilin against cisplatin-induced nephrotoxicity was tested using LLC-PK1 cells, which are the most vulnerable renal tubular cells to oxidative stress [24, 28]. It is reported that reactive oxygen species (ROS) play vital biological roles in cellular homeostasis whereas the increased ROS levels are associated with apoptosis in cells [29–31]. Our result suggests that eupatilin is the antioxidant and active component of *A. asiatica*. In addition, pretreatment with the *A. asiatica* extract and eupatilin markedly ameliorated reduced LLC-PK1 cell viability by cisplatin in a dose-dependent manner.

It has been reported that ROS act as a second messenger initiating signal transduction cascades, including the MAPKs signaling pathway [32, 33]. The MAPKs are important mediators for apoptosis induction in response to anticancer drugs, in particular cisplatin [34, 35]. In the present study, the increased protein expressions of phosphorylated JNK and p38 by cisplatin in LLC-PK1 cells were markedly ameliorated after *A. asiatica* extract or eupatilin cotreatment. To further investigate the ability of *A. asiatica* and eupatilin to prevent apoptosis, we measured the expression levels of cleaved caspase-3, known as an index of apoptosis, in the kidney. As shown in the results, *A. asiatica* and eupatilin significantly reduced the expression of cleaved caspase-3. These results suggest that cisplatin-induced increases in the ROS level activate MAPKs in LLC-PK1 cells, whereas the *A. asiatica* extract and eupatilin inhibit the activation of p38 and JNK.

Cisplatin-induced renal cell damage is dependent on apoptosis induced by DNA damage [36, 37]. Apoptosis in renal tubular cells has been observed in several kinds of renal disorders [38]. The elevated protein level of cleaved caspase-3 decreased after treatment with *A. asiatica* extract and eupatilin.

In conclusion, our results suggest that the *A. asiatica* extract can ameliorate nephrotoxicity in LLC-PK1 cells. Eupatilin serves as one of the major components by blocking the MAPKs-caspase-3 signaling cascade.

Conflict of Interests

The authors declare no conflict of interests in this work.

Authors' Contribution

Jun Yeon Park and Dahae Lee contributed equally to the content of this paper.

Acknowledgments

This research was funded by the Gangneung Asan Hospital Biomedical Research Center Promotion Fund. This work was also supported by a grant from Korea Institute of Science and Technology, Republic of Korea (2Z04371).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 461295, 7 pages
http://dx.doi.org/10.1155/2015/461295

Research Article

Effects of Chung-Pae Inhalation Therapy on a Mouse Model of Chronic Obstructive Pulmonary Disease

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Received 25 June 2015; Accepted 13 August 2015

Academic Editor: Ken Yasukawa

Chung-pae (CP) inhalation therapy is a method frequently used in Korea to treat lung disease, especially chronic obstructive pulmonary disease (COPD). This study investigated the effects of CP inhalation on a COPD animal model. C57BL/6 mice received porcine pancreatic elastase (PPE) and lipopolysaccharide (LPS) alternately three times for 3 weeks to induce COPD. Then, CP (5 or 20 mg/kg) was administered every 2 h after the final LPS administration. The effect of CP was evaluated by bronchoalveolar lavage (BAL) fluid analysis, histological analysis of lung tissue, and reverse transcription polymerase chain reaction analysis of mRNA of interleukin- (IL-) 1β, tumor necrosis factor- (TNF-) α, IL-6, and tumor growth factor- (TGF-) β. Intratracheal CP administration reduced the number of leukocytes and neutrophils in BAL fluid, inhibited the histological appearance of lung damage, and decreased the mRNA levels of the proinflammatory cytokines IL-1β, TNF-α, IL-6, and TGF-β. Intratracheal CP administration effectively decreased the chronic inflammation and pathological changes in a PPE- and LPS-induced COPD mouse model. Therefore, we suggest that CP is a promising strategy for COPD.

1. Introduction

Inhalation therapy is a treatment technique for administering a variety of inhalable drugs to target lung tissue, airway secretion components, and microorganisms in the upper, central, and/or peripheral airways [1]. Such therapy is used widely to treat chronic obstructive pulmonary disease (COPD) in the respiratory tract [2]. Inhalation administration has an advantage over oral administration for treating respiratory disease in that it allows rapid and substantial drug absorption and has fewer side effects [3]. Typically, herbal medicines are administered orally in the form of decoction or granular extract; however, several studies have reported the direct delivery of herbal medicine to the airway via inhalation [4–7]. The current study employed the MicroSprayer, which generates a plume of liquid aerosol (mass median diameter (MMD) of 16–22 μm), enabling the administration of drugs directly to the lung via the trachea [8].

Chung-pae (CP), composed of *Ephedrae Herba*, *Caryophylli Flos*, *Pogostemonis (Agastachis) Herba*, and *Zingiberis Rhizoma Crudus*, is a representative aerosol agent used in the respiratory clinic at Kyung Hee Oriental Medicine Hospital, Seoul, Korea, for relieving the symptoms of patients with dyspnea and cough. Previously, we investigated the effect of intratracheal (i.t.) CP administration on lipopolysaccharide- (LPS-) induced acute lung injury (ALI) in a mouse model. We found that CP suppressed neutrophil infiltration to the lung and reduced the production of proinflammatory cytokines via decreased expression of the proinflammatory transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and activation of the anti-inflammatory factor, nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2) [7].

The current study investigated the activities of CP on chronic lung injuries including COPD. COPD was selected for the study because it is a prevalent chronic respiratory

disease that has become a major public health problem [9]. LPS and porcine pancreatic elastase (PPE) were used to induce COPD in a mouse model. Long-term administration of LPS by inhalation induces emphysema [10–12], and PPE augments the emphysematous changes that are critical characteristics of COPD [13, 14].

In the present study, the effect of CP on chronic lung injury was evaluated in a mouse model of COPD generated using LPS and PPE. The effect of CP was assessed by analyzing bronchoalveolar lavage (BAL) fluid, lung histology, and mRNA levels of proinflammatory cytokines.

2. Materials and Methods

2.1. Preparation of Chung-Pae Water Extract (CP). CP was prepared as described previously [7]. Briefly, 20.0 g Ephedrae, 20.0 g Ephedrae Herba, (Agastachis) Herba, 10.0 g Caryophylli Flos, and 10.0 g Zingiberis Rhizoma Crudus were boiled in 1 L distilled water for 2 h. The mixture was concentrated to 50 mL with a low-pressure evaporator and then freeze-dried to yield 6.0 g of powder.

2.2. Animals. Male C57BL/6 mice were supplied by Orient Bio Inc. (Seongnam, Korea) and were bred in a pathogen-free facility at Pusan National University, Yangsan, Korea. Animals were housed in certified standard laboratory cages and fed with food and water *ad libitum* prior to the experiments. All experimental procedures were approved by the Guidelines of the Institutional Animal Care and Use Committee of Pusan National University, Busan, Republic of Korea (protocol number: PNU-2010-00028).

2.3. COPD Mouse Model and Treatment. COPD was induced in mice using the method reported previously with some modifications [15]. A MicroSprayer (syringe assembly, MSA-250-m, the Penn Century Inc., PA, USA) was used to deliver all materials to the lungs via i.t. Mice (20–30 g) were exposed to 0.25 U of PPE (on days 1, 7, and 14) and 7.0 μg of LPS (on days 4, 11, and 18) for three consecutive weeks. In this manner, the treated mice received PPE and LPS alternately. Two doses of CP (low dose of 5 mg/kg and high dose of 20 mg/kg) in 25 μL of PBS were administered 2 h after every LPS administration. The vehicle-treated group was treated with 25 μL of PBS using the same method and treatment schedule as the CP-treated group. Normal, untreated non-COPD mice were included as a control in the analyses.

2.4. BAL Fluid Analysis. BAL fluid analysis was conducted on day 21. BAL was obtained using two consecutive instillations of PBS (1.0 mL) using a 24-gauge intravascular catheter. The total cell number was determined using a hemocytometer. Macrophages, lymphocytes, and neutrophils were counted by Hemacolor (Merck, Darmstadt, Germany) after centrifugation and staining; 100 cells were counted for each microscopic field, and the mean number of cells per field was reported.

2.5. Lung Histological Analysis. Mice were perfused with saline and the whole lung was inflated with fixatives. After paraffin embedding, lung tissue were cut in 5-μm thick slices and stained with hematoxylin and eosin (H&E). Three separate H&E-stained sections were evaluated in each mouse under a microscope using 100x magnification.

2.6. Isolation of Total RNA from Tissue and Reverse-Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was isolated with the QIAGEN RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Two micrograms of total RNA were reverse-transcribed by M-MLV reverse transcriptase (Promega, Madison, WI, USA), and single-stranded cDNA was amplified by PCR using specific primers. The forward and the reverse primers for interleukin- (IL-) 1β were 5′-TCATGGGATGATGATGATAACCTGCT-3′ and 5′-CCCATACTTTAGGAAGACACGGATT-3′, respectively; the primers for tumor necrosis factor- (TNF-) α were 5′-GGCAGGTCTACTTTGGAGTCATTGC-3′ and 5′-ACATTCGAGGCTCCAGTGAATTCGG-3′, respectively; the primers for IL-6 were 5′-CTGGTGACAACCACGGCCTTCCCTA-3′ and 5′-ATGCTTAGGCATAACGCACTAGGTT-3′, respectively; the primers for tumor growth factor- (TGF-) β were 5′-GCGGCAGCTGTACATTGACT-3′ and 5′-ACTGTGTGTCCAGGCTCCAA-3′, respectively; and the primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-GGAGCCAAAAGGGTCATCAT-3′ and 5′-GTGATGGCATGGACTGTGGT-3′, respectively. For PCR amplification, *Taq*PCRx DNA polymerase recombinant (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's protocol. The reaction conditions were as follows: initial denaturation at 95°C for 5 min followed by 22–30 cycles of denaturation for 40 sec at 95°C, annealing for 40 sec at 57°C, and extension for 50 sec at 72°C with a final extension for 7 min at 72°C. Amplicons were separated in 1.2% agarose gels in boric acid buffer at 100 V for 30 min, stained with ethidium bromide, and visualized under UV light. GAPDH was used as an internal control to evaluate the relative expressions of IL-1β, TNF-α, IL-6, and TGF-β.

2.7. Statistical Analysis. Group comparisons were performed using one-way analysis of variance (ANOVA) with Duncan's post hoc test. The analysis was conducted using SPSS 18.0 for Windows (SPSS, Chicago, IL, USA). p values < 0.05 were considered to indicate significant differences. All experiments were performed independently at least three times.

3. Results

3.1. Effect of CP on the Total Cell Count and Inflammatory Cell Numbers in the BAL Fluid of PPE- and LPS-Induced COPD Mice. The total cell and neutrophil counts in the BAL fluid of PPE- and LPS-induced COPD mice increased significantly compared to those in the normal group ($p < 0.01$, Figures 1(a) and 1(b)). CP treatment significantly decreased the total cell and neutrophil counts in the BAL fluid compared to the vehicle-treated group ($p < 0.05$, Figures 1(a) and 1(b)). However, no difference was detected between groups treated with 5 or 20 mg/kg CP.

The macrophage population in the vehicle-treated group increased significantly compared to that in the normal group

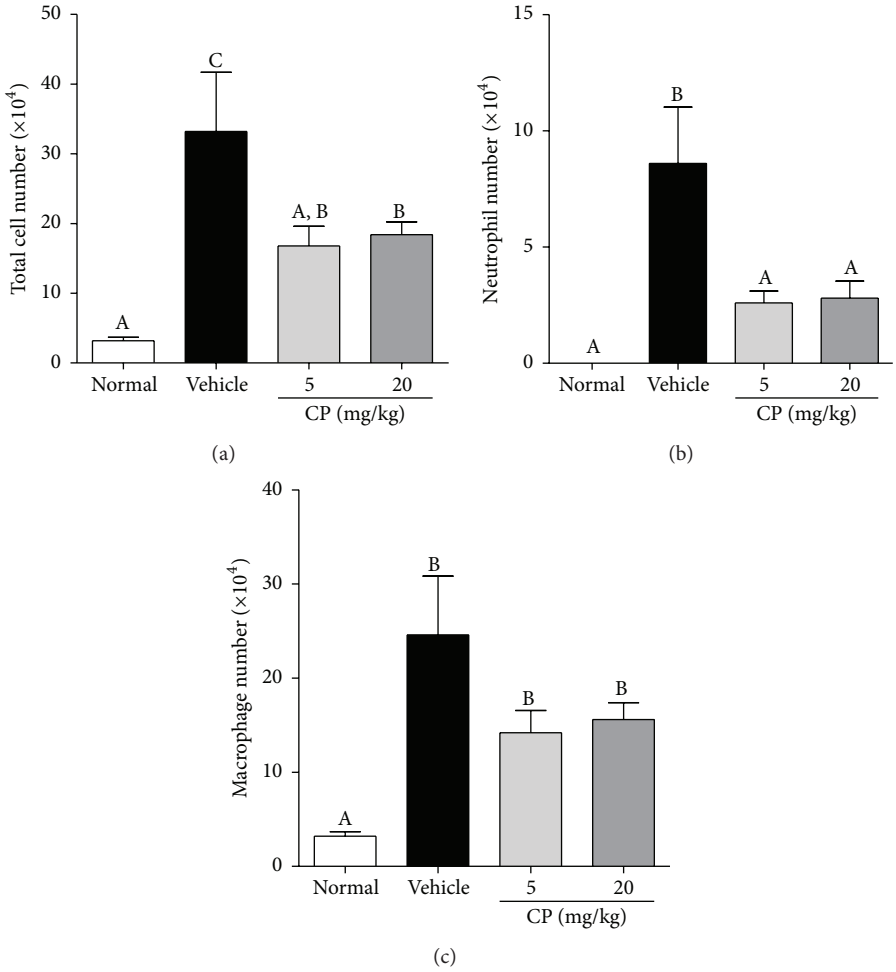

Figure 1: Effect of CP on the total cell number (a), number of neutrophils (b), and number of macrophages (c) in the BAL fluid of PPE- and LPS-induced COPD mice. Data are presented as means ± SEM ($n = 5$). Letters (A–C) indicate different levels of significance (95% level, Duncan's test).

($p < 0.01$, Figure 1(c)). However, CP did not decrease the macrophage population in the BAL fluid compared to the vehicle-treated group.

3.2. Effect of CP on the Histological Evidence of Lung Damage in PPE- and LPS-Induced COPD Mice. Larger vacuoles were present in the lung sections of vehicle-treated mice (Figure 2(b)) compared with the normal group (Figure 2(a)). Such enlarged air spaces suggested alveolar destruction due to emphysematous change. However, CP-treated COPD mice showed smaller vacuoles (Figures 2(c) and 2(d)) compared to the vehicle-treated group, suggesting that CP (5 or 20 mg/kg) ameliorated inflammation in the lung.

3.3. Effect of CP on the mRNA Expression Levels of Cytokines in the PPE- and LPS-Induced COPD Mice. CP (5 or 20 mg/kg) decreased the mRNA levels of IL-1β, TNF-α, and IL-6 in the lung (Figure 3), which was in agreement with the decreased numbers of inflammatory cells in the BAL fluid. However, a significant decrease in TGF-β expression was observed only in mice treated with 20 mg/kg CP.

4. Discussion

In the current study, i.t. administration of CP to PPE- and LPS-induced COPD mice reduced the number of leukocytes and neutrophils in the BAL fluid, inhibited lung injury, and decreased the mRNA levels of the proinflammatory cytokines IL-1β, TNF-α, IL-6, and TGF-β.

In our clinic, patients usually received CP at a daily dose of 5 mg/kg; however, long-term administration of 20 mg/kg of CP for 3 weeks showed no adverse effect on vital organs, including the liver and kidney (data not shown). Therefore, in this study, we administered CP at doses of 5 or 20 mg/kg in the PPE- and LPS-induced COPD mice. Infiltration of inflammatory cells in the BAL fluid was observed in the PPE- and LPS-induced COPD mice. Subsequent i.t. administration of CP reduced the total number of infiltrating cells, especially neutrophils, suggesting that CP could inhibit neutrophils, the most deleterious inflammatory mediator in COPD. However, CP did not significantly decrease the macrophage number compared to the vehicle-treated group (42.3% versus 37.6%); this result was consistent with a previous study [7].

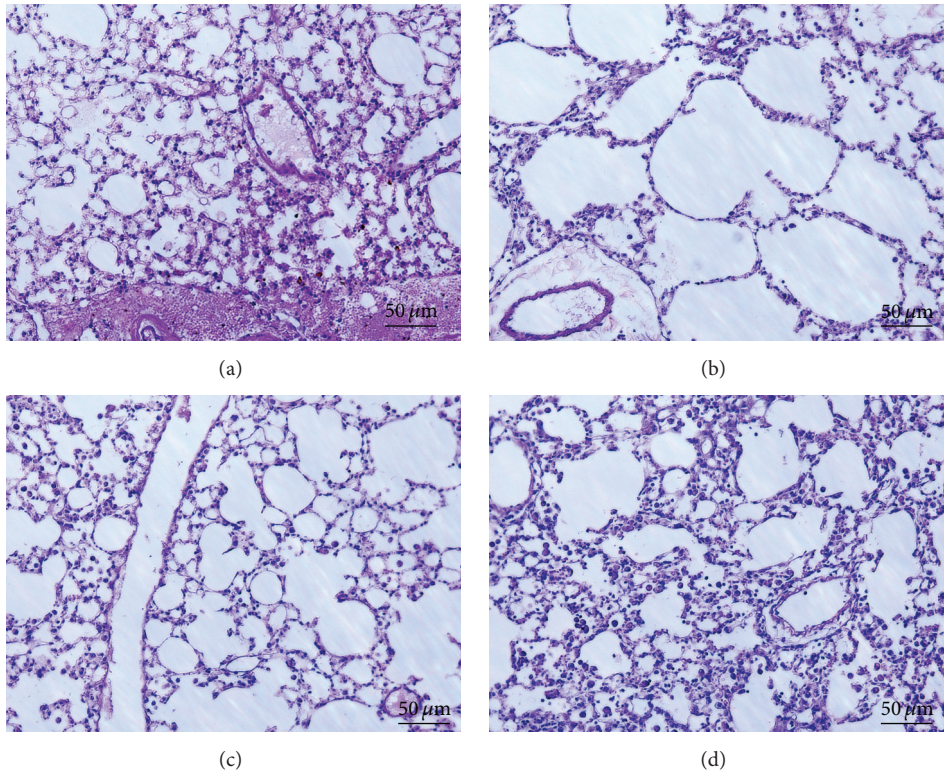

Figure 2: Effect of CP on the histological evidence of lung damage in the PPE- and LPS-induced COPD mice: (a) normal group; (b) vehicle-treated group; (c) CP-treated group (5 mg/kg); and (d) CP-treated group (20 mg/kg).

COPD is characterized mainly by increased levels of activated neutrophils, macrophages, and T-lymphocytes [16]. Macrophages mediate inflammation in COPD through the release of chemokines that attract neutrophils, monocytes, and T-cells [17]. Neutrophils are key mediators of COPD, as they migrate to the airway under the control of chemotactic factors and become activated [18, 19]. Activated neutrophils secrete proteolytic enzymes that can induce emphysema as well as numerous lung-damaging, proinflammatory cytokines and chemokines (e.g., matrix metalloproteinase-(MMP-) 8, 9, and 12) [20–22]. Moreover, increased numbers of neutrophils in the airway lumen and BAL fluid in individuals with COPD are correlated with disease severity [23, 24].

The COPD model used in this study involved the inhalation of LPS and elastase to induce emphysematous change [14, 25]. Generally, emphysema is induced by a proteolytic-antiproteolytic imbalance. Proteolytic enzymes may augment the inflammatory cell influx into airspaces, which causes destruction of alveolar septa and increased airspaces [10, 26]. Thus, air space enlargement is a criterion used for measuring the severity of emphysematous change [10, 27, 28]. In the current study, CP reduced the vacuole size compared to that in the vehicle group, suggesting that it prevented alveolar destruction. Histological analysis of lung tissue showed increased cell and neutrophil numbers in the BAL fluid.

Numerous cytokines play important roles in the pathological processes of COPD through the recruitment, activation, and survival of inflammatory cells. TNF-α and IL-1β have long been known to be classical proinflammatory cytokines that contribute to the development of COPD [29–31]. IL-6 is stimulated by TNF-α and IL-1β and also plays a critical role in the pathogenesis of emphysematous change [32]. These proinflammatory cytokines influence one another and amplify the inflammatory response in COPD [16, 33]. TGF-β, a profibrotic cytokine, is one of the main mediators involved in tissue remodeling in the lungs and contributes to architectural changes in the lungs in COPD [34–37]. The blocking of TGF-β improves emphysematous changes [38, 39], although a low concentration of activated TGF-β is required to maintain alveolar homeostasis and prevent the development of emphysema [40, 41]. Therefore, inhibition of proinflammatory cytokines is one of the most promising treatments for COPD [42]. In this study, CP reduced the mRNA levels of these cytokines in the lung, suggesting the suppression of chronic inflammation and pathological changes as well as the associated neutrophil infiltration in the lung.

5. Conclusion

Previously, we have demonstrated the therapeutic effect of i.t. CP administration on ALI [7]. The current study provides experimental evidence that long-term administration of CP has a therapeutic effect on chronic lung injury in a COPD mouse model induced by PPE and LPS. The anti-inflammatory effect exerted by i.t. CP administration suggests that it could be a new therapeutic formula and that inhalation

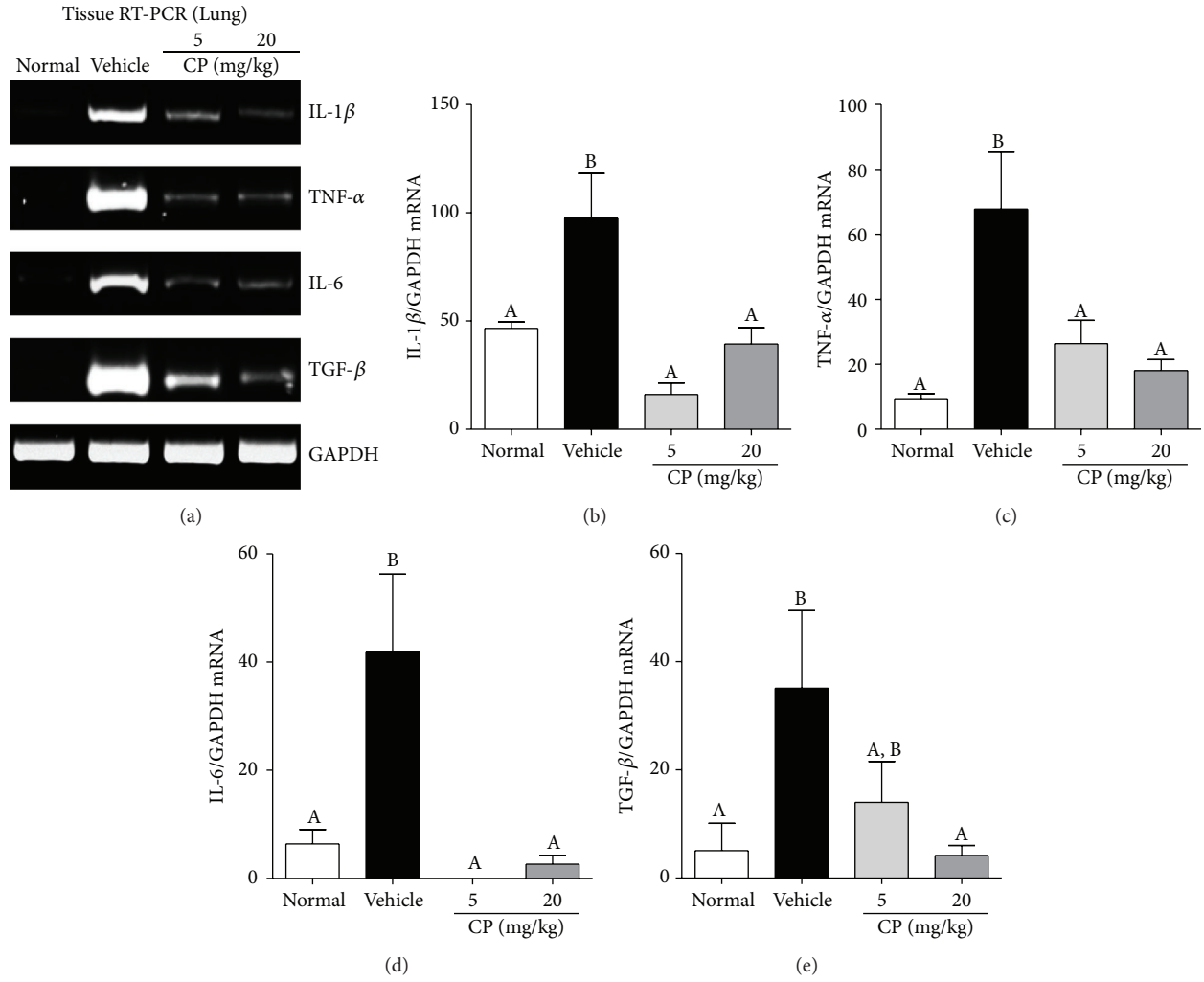

Figure 3: Effect of CP on the mRNA levels of cytokines in the lung of PPE- and LPS-induced COPD mice. Mice were exposed to PPE (on days 1, 7, and 14) and LPS (on days 4, 11, and 18) and administered 5 mg/kg or 20 mg/kg of CP 2 h after every LPS administration. The lungs of variously treated mice were harvested on day 21 for RT-PCR analysis. The intensity of each PCR band was measured by densitometric analysis (a), and relative expression of each gene was calculated over GAPDH. CP reduced the mRNA level of these cytokines (b–e). Data are presented as means ± SEM ($n = 5$). Letters (A–C) indicate different levels of significance (95% level; Duncan's test).

of herbal medicine can be a promising strategy for treatment of COPD.

Conflict of Interests

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

Authors' Contribution

Myungsoo Joo and Sung-Ki Jung designed research and contributed to the editing of the paper. Joon-Ho Hwang and Beom-Joon Lee contributed equally as first authors. All authors read and approved the final paper.

Acknowledgment

This work was supported by a Grant to Korean Medical Science Research Center for Healthy Aging from the National Research Foundation of Korean Government (2014R1A5A2009936).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 457071, 7 pages
http://dx.doi.org/10.1155/2015/457071

Research Article

Spatial Patterns of the Indications of Acupoints Using Data Mining in Classic Medical Text: A Possible Visualization of the Meridian System

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Received 28 April 2015; Revised 1 September 2015; Accepted 2 September 2015

Academic Editor: Ciara Hughes

The indications of acupoints are thought to be highly associated with the lines of the meridian systems. The present study used data mining methods to analyze the characteristics of the indications of each acupoint and to visualize the relationships between the acupoints and disease sites in the classic Korean medical text *Chimgoogyeongheombang*. Using a term frequency-inverse document frequency (tf-idf) scheme, the present study extracted valuable data regarding the indications of each acupoint according to the frequency of the cooccurrences of eight Source points and eighteen disease sites. Furthermore, the spatial patterns of the indications of each acupoint on a body map were visualized according to the tf-idf values. Each acupoint along the different meridians exhibited different constellation patterns at various disease sites. Additionally, the spatial patterns of the indications of each acupoint were highly associated with the route of the corresponding meridian. The present findings demonstrate that the indications of each acupoint were primarily associated with the corresponding meridian system. Furthermore, these findings suggest that the routes of the meridians may have clinical implications in terms of identifying the constellations of the indications of acupoints.

1. Introduction

In many instances, one diagram is better than a thousand words. For example, a famous physician from the early stages of the Tang Dynasty, Sun Si-miao, declared that an acupoint could not be well located without graphic guidance. The ancient Chinese invented several methods for displaying information about acupoints and meridians on the human body surface by relying on ancient infographics of the meridian system rather than a detailed knowledge of human anatomy. Additionally, they described empirical clinical information in terms of the selection of appropriate acupoints for the treatment of specific diseases. However, when Western people were exposed to the Chinese acupuncture map in the late 17th century, it was necessary for them to understand the acupuncture point system using anatomical knowledge. As a result, to comply with the contemporary conventions of European anatomical atlases, the first versions of the Chinese acupuncture map for the Western world were embellished with dissected flaps of skin at the head [1]. From a historical point of view, the Eastern texts *Mingtang Diagram*, *Diagram of Meridian and Collaterals*, and *Bronze Statue* were also gradually influenced by Western-style anatomy. However, despite decades of research and a merging of Western and Eastern ideals, an anatomical map of the meridians on the human body has yet to be fully realized [2]. It has been suggested that high electrical conductance, acupuncture sensation patterns, and possible relationships with connective tissue planes could represent meridians or act as identifiers for meridians [3–6]. However, none of these studies can fully explain the relationships that exist between treatment at each acupoint and the subsequent clinical improvements. Thus, one must understand the origin and clinical significance

of the meridian system in order to fully understand the acupuncture process that is based on this system.

Traditional East Asian medical techniques can be used to diagnose diseases of visceral organs, such as the stomach or kidneys, by the simple application of the appropriate palpations on the arteries around particular body sites. There are intriguing similarities between Western medicine and traditional bloodletting sites and acupoints, but Hippocratic treatments using venesection have largely lost their topographical importance and disappeared from the Western medicine [7]. In traditional East Asian medicine, on the other hand, the relationships among acupoints and disease sites are generally understood based on an empirical knowledge of the meridian system which functions as the underlying template for acupuncture treatment [8, 9]. In and of itself, the meridian system is also considered to be a diagnostic tool useful for the identification of diseases or symptoms along the meridian lines and for the association of the pathology of diseases or symptoms with the relevant organ [10, 11]. The route of a meridian system exemplifies the constellations of the indications of acupoints. In other words, the indications of acupoints have a high association with the lines of the meridian systems and, in this manner, the acupoints may be connected with a suspected disease or symptom to form a network of knowledge that is the meridian system [12, 13]. As a theoretical model of the indications of acupoints, the meridian system can further the current understanding of the interconnections that underlie the pathologies of particular diseases or symptoms [14]. Moreover, based on an ever-increasing amount of empirical clinical data regarding the indications of acupoints, the meridian system has been continually amended and developed in association with advances in medicine.

Given that the meridian system proposes that there are a series of connections among different areas, organs, and functions, this system may be visualized using the indications of acupoints. Whenever practitioners treat patients using acupuncture and moxibustion, the appropriate selection of acupoints is made based on three fundamental principles: (1) local acupoints near the area where the symptoms are occurring, (2) distant acupoints along the meridian, and (3) distant acupoints based on symptom differentiation [14, 15]. For example, when patients are suffering from a toothache and facial edema, the practitioner can consider diseases associated with the Large Intestine meridian and choose local acupoints such as LI 19 and LI 20 and distant acupoints such as LI 4 and LI 5. To clarify the unique characteristics of traditional East Asian medicinal techniques, several studies have used data mining and network science to characterize the relationships that exist among the symptoms of patients and East Asian treatment methods [14, 16–19]. In recent years, it has become the norm for acupuncture practitioners to develop a practical clinical decision prior to treatment using the modern methodologies of evidence-based medicine and the abundant information readily available in current scientific databases [20]. Additionally, the field of acupuncture has used data mining algorithms to uncover useful patterns of symptoms and treatment methods within various fields of research, including those from the classic texts of traditional East Asian medicine [21, 22].

Every disease or symptom can be treated by a wide assortment of acupoints and, accordingly, there are large variations in the choice of acupoints among different acupuncturists [23, 24]. The application of data mining technology to the clinical research literature of ancient acupuncture and moxibustion has revealed that many studies have analyzed the selection of meridians and acupoints used by different practitioners. These studies provide evidence supporting the traditional theories of acupuncture treatment for a variety of diseases including knee osteoarthritis, diarrhea, migraine, vertigo, and lumbar disc herniation [25–29]. Based on a comprehensive set of quantitative clinical data, a recent article from our laboratory proposed that acupoints should be depicted according to the frequency of their use for the treatment of low back pain [8]. However, to date, there has been a lack of systematic research aimed at furthering the current understanding of the characteristics of the indications of each acupoint or attempting to visualize the meridian systems in terms of disease location. Thus, using data mining methods, the present study analyzed the characteristics of the indications of Source points and attempted to characterize the relationships among these acupoints and the different disease sites described in the classic Korean medical text *Chimgoogyeongheombang*.

2. Methods

2.1. Source of the Data. Chimgoogyeongheombang (Experiential Prescriptions of Acupuncture and Moxibustion) is a representative Korean medical text written by the royal physician Heo Im in 1644 and was the first book to specialize in acupuncture and moxibustion treatment during the Joseon dynasty [30, 31]. As we can see according to its own title, this text contains prescriptions for acupuncture and moxibustion that were obtained from transmitted medical texts and Heo Im's own clinical experiences. In his practice, Heo Im initially referred to authoritative medical texts such as *Huangdineijing (Huangdi's Internal Classic)*, *Qianjinfang (Prescriptions Worth a Thousand Gold)*, *Tongrenjing (Classic of the Bronze Figure)*, *Zhenjiuzishengjing (Classic of Nourishing Life with Acupuncture and Moxibustion)*, *ShenyingJing (Classic of Wondrous Response)*, and *Qixiaoliangfang (Formulae of Miraculous Effect)* from China and *Dongeuibogam (Treasured Mirror of Eastern Medicine)* from Korea. After accumulating a wide range of knowledge concerning acupuncture and moxibustion, however, he compiled his own medical theories and prescriptions. Heo Im's understanding of the causes of disease was usually based on meridian theory and internal organ theory [30] and he provided optimized descriptions of his own clinical experiences using his own language. In addition to providing his theories of the causes of disease, Heo Im also proposed corresponding prescriptions for each disease using acupuncture and moxibustion based on his clinical experience.

2.2. Database Construction. Chimgoogyeongheombang can be divided into two parts: general theory and clinical

details. The clinical details section consists of 43 chapters that describe information regarding the acupoints that are associated with particular diseases or symptoms; 13 of these chapters are named according to disease sites (chapter of the head and face, chapter of the ears, chapter of the neck, etc.). All relevant information concerning the acupoints and disease sites used in the present study were extracted from the 13 chapters and categorized into the following 20 individual disease sites: head, face, ears, eyes, mouth, tongue, teeth, nose, throat, neck, chest, heart, hands, upper limbs, abdomen, flank, back, lower limbs, knees, and feet. A total of 471 acupoints were used to treat these 20 different disease sites and 110 acupoints remained after the removal of duplicates. As the Source point is the representative acupoint of the relationship between the spatial specificity of the meridian and the disease location, we analyzed the frequency of the cooccurrences of the Source points and disease sites among *Chimgoogyeongheombang*. Out of 12 Source points, 8 Source points were used to treat different disease sites. Of these 8 Source acupoints, none were related to the neck and the knee, and 18 disease sites were included in the final analyses.

2.3. Data Mining and Visualization of the Indications of Acupoints. Following construction of the database, data regarding the frequency of the co-occurrences of the 8 Source points and 18 disease sites were extracted to better understand the relationships among these factors. More specifically, the present study assessed which disease sites were meaningfully associated with a specific acupoint. To accomplish this, a term frequency-inverse document frequency (tf-idf) weighting scheme was applied to the cooccurrence table.

The tf-idf method is one of the most widely used weighting schemes in the data mining research field, especially for information retrieval systems [31], because it quantifies the significance of particular terms in a document. Additionally, the present study quantified the significance of the associations between the disease sites and acupoints. In the tf-idf scheme, term frequency ($\mathrm{tf}_{[t\cdot d]}$) refers to the number of times that term "t" occurs in document "d" and, therefore, $\mathrm{tf}_{(t,d)}$ represents how relevant term "t" is to document "d". Document frequency (df_t) is the number of documents that contain term "t" and, therefore, df_t represents the rarity of a term within the system of documents. Across the document system, rare terms are more informative than frequent terms and, thus, the inverse document frequency of "t" (idf_t) is positively related to the informativeness of "t". Arithmetically, idf is defined as $\log(N/\mathrm{df}_t)$ instead of N/df_t to where N is the number of whole documents in order to diminish the effect of idf. Tf-$\mathrm{idf}_{(d,a)}$ is defined by assigning "disease site" to "term" and "acupoint" to "document" so that it quantifies the significance of the relationship of a specific disease site with a specific acupoint. Based on the tf-$\mathrm{idf}_{(d,a)}$ values, each acupoint is represented by a vector of tf-idf weights in an 18-dimension vector space (18 disease sites). Finally, the calculated tf-idf weights of each acupoint were normalized by the following cosine normalization: $(1/\sqrt{w_1^2 + w_2^2 + w_3^2 \cdots + w_M^2})$. The relationships among the acupoints and disease sites were only described in the present study if they exhibited a tf-idf value greater than 0.4 (Figure 1).

The algorithm was intended to identify the disease sites that were related to the acupoints described in *Chimgoogyeongheombang*. All the tf-idf values were calculated using scikit-learn, which is a full-featured machine-learning package for the python programming language (http://scikit-learn.org/). The tf-$\mathrm{idf}_{(d,a)}$ values were overlaid on a human figure template using matplotlib, which is a plotting library for python (http://matplotlib.org/ [32]). A variety of relationships between a specific disease site and a specific acupoint were labeled according to the tf-$\mathrm{idf}_{(d,a)}$ values.

3. Results

3.1. Characteristics of the Indications of Acupoints. The 8 Source points are presented on the y-axis of the array in Figure 2. The 18 selected disease sites are presented on the x-axis of the array in Figure 2; the 8 Source points were highly associated with the 18 disease sites. Based on the Yin/Yang and Hand/Foot groupings, meridians can be categorized into the following three groups: Foot-Yin, Hand-Yin, and Hand-Yang.

In the Foot-Yin meridian, acupoint SP 3 showed superior tf-idf values with the abdomen (tf-idf: 0.85) and the chest (tf-idf: 0.53), acupoint KI 3 showed superior tf-idf values with the abdomen (tf-idf: 0.60), the lower limb (tf-idf: 0.40), and the throat (tf-idf: 0.40), and acupoint LR 3 showed superior tf-idf values with the abdomen (tf-idf: 0.52), the lower limb (tf-idf: 0.52), and the back (tf-idf: 0.40).

In the Hand-Yin meridian, acupoint LU 9 showed superior tf-idf values with the heart (tf-idf: 0.64), the upper limb (tf-idf: 0.57), and the chest (tf-idf: 0.51), acupoint HT 7 showed superior tf-idf values with the heart (tf-idf: 0.41) and the chest (tf-idf: 0.40), and acupoint PC 7 showed superior tf-idf values with the heart (tf-idf: 0.63), the chest (tf-idf: 0.50), and the face (tf-idf: 0.41).

In Hand-Yang meridian, acupoint LI 4 showed superior tf-idf values with the teeth (tf-idf: 0.60) and nose (tf-idf: 0.50) and acupoint SI 4 showed superior tf-idf values with the head (tf-idf: 0.64), the ears (tf-idf: 0.53), and the eyes (tf-idf: 0.46).

3.2. Visualization of the Indications of Acupoints on the Body Map. Eight Source points that represented each meridian system, as assessed using the tf-idf value, were visualized on a human body template. Thus, it was possible to visually display the spatial patterns of the various disease sites as they related to each acupoint (Figure 3).

The Source points on the left part (SP 3, KI 3, and LR 3), which are representative acupoints of the Foot-Yin meridian system, were highly associated with the truncus (including the abdomen and the chest) and the lower limbs. The Source points in the middle part (LU 9, HT 7, and PC 7), which are representative acupoints of the Hand-Yin meridian system, were highly associated with the upper truncus (including the heart and the chest) and upper limbs. The Source points in the right part (LI 4 and SI 4), which are representative acupoints

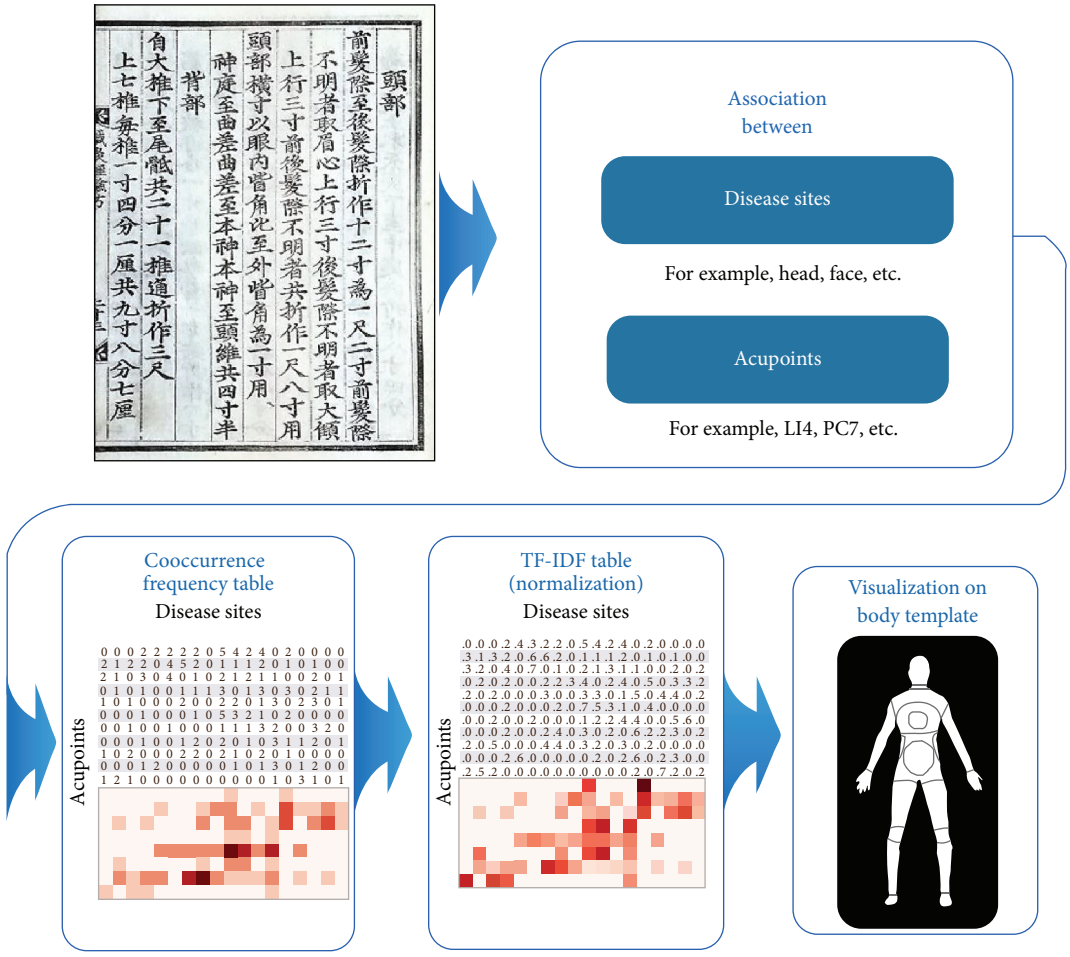

Figure 1: Procedures for data mining and the visualization of the indications of acupoints on the body map. The frequency of the cooccurrences of the 18 disease sites and the 8 Source points and the relationships among these factors provided valuable information regarding their clinical implications. To normalize the data, a term frequency-inverse document frequency (tf-idf) weighting scheme was applied to the cooccurrence table. The indications of each acupoint (tf-idf value) were visualized on a human body template.

of the Hand-Yang meridian system, were highly associated with the head regions (including the head, the face, the ears, the eyes, the mouth, the teeth, and the nose) and the upper limbs.

Additionally, there were similarities between the spatial patterns of the constellations of the indications of each acupoint and the routes of the corresponding meridians according to texts such as *Illustrations of Meridians and Collaterals in Ancient Times* and *Diagram of the Circulatory Course of Meridians*.

4. Discussion

The present study used data mining methods to analyze the characteristics of the indications of acupoints based on a classic text of Korean medicine, *Chimgoogyeongheombang*. Using the frequency of cooccurrences among 18 disease sites from *Chimgoogyeongheombang* and 8 Source points and the normalized relationships (tf-idf values) among these factors, the present study identified valuable data regarding the indications of each acupoint (Figure 2). For instance, the Source points of the Foot-Yin meridian, that is, SP 3, KI 3, and LR 3, were highly associated with the abdomen (tf-idf values: 0.85, 0.60, and 0.52, resp.). In contrast, the Source points of the Hand-Yin meridian, that is, LU 9, HT 7, and PC 7, were highly associated with diseases of the chest (tf-idf values: 0.51, 0.40, and 0.50, resp.) and heart (tf-idf values: 0.64, 0.41, and 0.63, resp.). Each of these relationships can be explained by the routes of the meridian systems. These findings suggest that the characteristics of the indications of each acupoint are primarily associated with their corresponding meridian system.

The present study also used tf-idf values to visualize the spatial patterns of the disease sites on the body as they related to each acupoint (Figure 3). For example, LI 4 in the Large Intestine meridian was highly associated with the constellations of the teeth and nose (tf-idf values: 0.60 and 0.50, resp.). The relationships between each of the acupoints and the constellations of the indications of the acupoints can be explained by the routes of the meridian systems. According to the classic text *Diagram of Meridians and Collaterals*, all

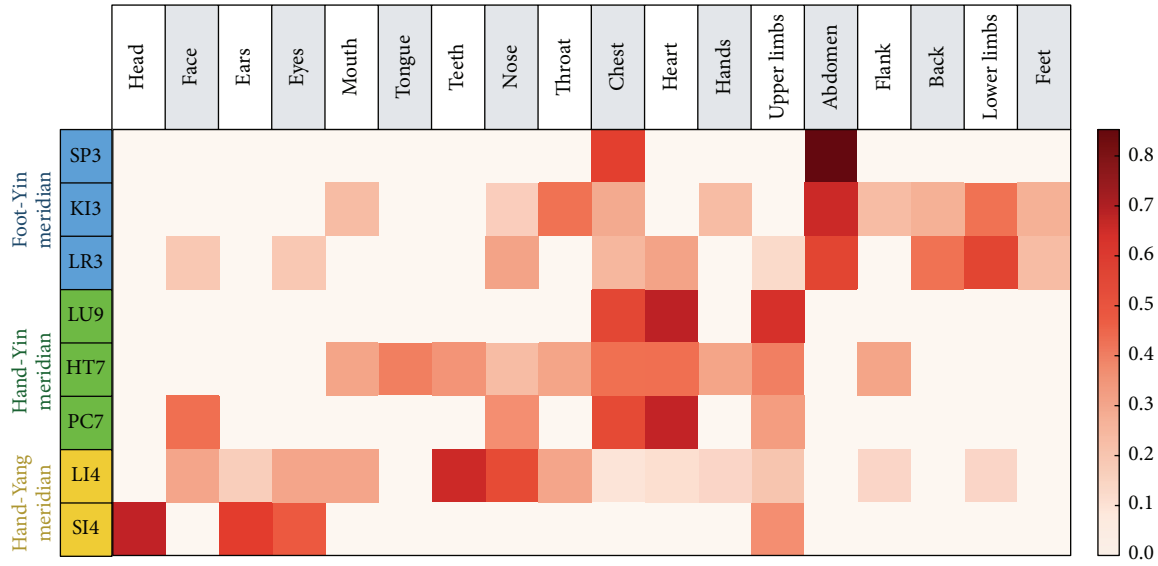

FIGURE 2: Characteristics of the indications of acupoints. The 8 Source points are presented on the y-axis of the array and the 18 selected disease sites are presented on the x-axis of the array. The 8 Source points were allocated with each meridian group (Foot-Yin, Hand-Yin, and Hand-Yang).

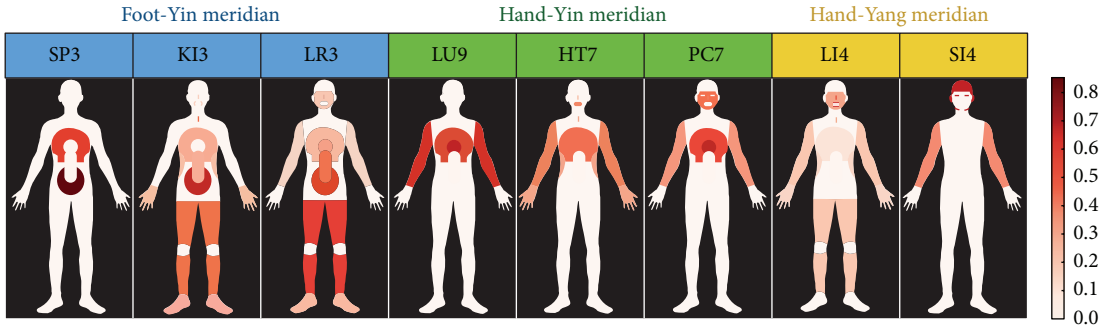

FIGURE 3: The visualization of the indications of the acupoints on a body map. Eight Source points representing the three meridian systems (Foot-Yin, Hand-Yin, and Hand-Yang meridian) and the indications of each acupoint (tf-idf value) were visualized on a human body template.

three Hand-Yin meridians extend from the upper truncus, including the heart and chest, to the medial part of hands; all three Hand-Yang meridians extend from the lateral part of the hands to the head; all three Foot-Yang meridians extend from the head to the anterior and lateral parts of the foot; and all three Foot-Yin meridians extend from the medial part of the foot to the lower truncus. A comparison of the spatial patterns of the constellations of the indications of each acupoint with the routes of their corresponding meridians according to *Diagram of Meridians and Collaterals* reveals that the routes of the meridian systems likely demonstrate the constellations of the indications of the acupoints.

The present study has several limitations. First, the characteristics and spatial patterns of the indications of the acupoints were extracted from a single classic Korean medical text with limited bibliographical data. There are large variations from text to text when describing the relationships between disease sites and acupoints. Thus, it is necessary to further investigate the similarities and/or differences of the indications of acupoints using a variety of classic medical books from several cultures. Second, the present study assumed that the spatial patterns of the indications of each acupoint in terms of the 18 selected disease sites were highly associated with the routes of corresponding meridian systems but the available spatial data for the body were limited and did not include detailed spatial location information, such as the medial or lateral parts of the hand. However, based on the global distribution of the spatial patterns of the indications of each acupoint, it is possible to utilize the characteristics of the meridian systems as medical infographics. Third, the present findings were obtained based on the description of one individual's clinical observations from a classic medical textbook and not using outcomes extracted from well-structured clinical data. In order to fully characterize the characteristics of the indications of each acupoint to properly inform clinical assessments, it is necessary to investigate the relationships among the disease sites and acupoints using detailed spatial information from large-scale clinical investigations. Last but

not least, when the Source points, that is, SP3 or LU 9 ($n < 4$), were not used frequently in the text, we cannot fully rule out the possibility of tf-idf values from the acupoints being exaggerated or distorted.

In conclusion, the present findings demonstrate that the indications of each acupoint are primarily associated with their corresponding meridian system. These findings also suggest that the routes of the meridian systems have clinical implications regarding the constellations of the indications of acupoints. The present authors strongly believe that the characteristics of the meridian system can act as an ancient infographic that will aid in the development and characterization of the clinical implications of acupoint selection for the treatment of various diseases.

Disclosure

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

Conflict of Interests

The authors declare that no competing financial interests or conflict of interests exists.

Acknowledgment

This research was supported by a Grant-in-Aid from the Korea Institute of Oriental Medicine (no. K15511).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 270380, 8 pages
http://dx.doi.org/10.1155/2015/270380

Research Article

Comparison of the Spasmolytic Effects of Jakyak-Gamcho Decoctions Derived via Different Extractants

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Received 26 June 2015; Revised 22 August 2015; Accepted 2 September 2015

Academic Editor: Hajime Nakae

Aim. To investigate whether differences in the amounts of effective index components in Jakyak-Gamcho decoctions derived via extraction with either water or ethanol were associated with differential spasmolytic effectiveness. *Methods*. The amounts of effective index components (paeoniflorin, benzoic acid, glycyrrhizin, and isoliquiritin) contained in water-extracted Jakyak-Gamcho decoction and 70% ethanol-extracted Jakyak-Gamcho decoction were compared by high-performance liquid chromatography. Muscle cramp reduction rates were compared between the two decoctions by comparing the degrees of muscle contraction, measured as the tension developed during electrical stimulation, before and 1 and 2 h after injection in rats. *Results*. The relative amounts of effective index components were, on average, about 43% higher in the 70% ethanol-extracted decoction than in the water-extracted decoction. Two hours after injection, 0.25 g/kg of 70% ethanol-extracted decoction produced a significantly greater spasmolytic effect than 0.25 g/kg of water-extracted Jakyak-Gamcho decoction or distilled water (both $p < 0.05$). *Conclusion*. Differences in the amounts of effective index components resulting from the use of different extractants were associated with differences in spasmolytic effectiveness. Hence, it may be worthwhile to investigate alternative extraction methods in terms of extraction efficiency and *in vivo* effectiveness for various herbal medicines in the future.

1. Introduction

Oriental medicine treatment modalities including acupuncture, moxibustion, and herbal medicines are effective for the treatment of musculoskeletal conditions [1–5]. Muscle cramping is a common musculoskeletal condition, and it is accompanied by instant intense pain [6]. Muscle cramps can be classified as true cramps, tetany, contractions, and dystonic cramps [7]. Tetany refers to a large constant contraction resulting from continuous single contractions induced by a repetitive stimulus applied to muscles. Low frequency stimulations result in incomplete tetanus that exhibits a serrated contraction curve, whereas high frequency stimulations result in longer contractions and are associated with complete tetanus exhibiting a curve with a gradual plateau [8]. The factors that cause muscle cramps are known to include intense exercise, metabolic disorders, electrolyte abnormalities, and pregnancy, but additional causes remain to be clarified [9]. Muscle relaxants and spasmolytic drugs are used to treat muscle cramps, but most are associated with side effects such as hepatotoxicity and central nervous system (CNS) depression [10].

Jakyak-Gamcho decoction is a commonly prescribed spasmolytic. It comprises Radix Paeoniae and Radix Glycyrrhizae in a 1 : 1 ratio and contains the index substances paeoniflorin, benzoylpaeoniflorin, albiflorin, glycyrrhizin, isoliquiritin, and liquiritigenin, and so forth. It is reportedly effective for the alleviation of skeletal muscle cramps caused by nervous stimulation and hemodialysis [11, 12]. In addition, it has been reported that Jakyak-Gamcho decoction can relieve intestinal cramps via smooth muscle relaxation and that it is effective for the alleviation of myodynia caused

by peripheral nerve damage or chemotherapy [13–15]. Particularly, among the constituents of Jakyak-Gamcho decoction, Radix Glycyrrhizae, which contains glycyrrhizin and isoliquiritin, has a significant spasmolytic effect [9]. Although the mechanism of the muscle-relaxing effect of Jakyak-Gamcho decoction remains unclear, its complex constituents interact with each other, thereby resulting in fewer CNS side effects compared to muscle relaxants that directly act on the CNS [9]. Recently, it was also reported to be effective against extrapyramidal symptoms, a side effect of antipsychotic agents [16]. Traditionally, Jakyak-Gamcho decoction is prepared with water as the extractant. However, in terms of extraction efficiency, water may not be an optimal extractant. In fact, 70% ethanol exhibits higher extraction efficiency than water [17].

Most previous studies investigating extraction efficiencies have been limited to the assessment of changes in the levels of effective index components according to different extraction methods [17]. Studies investigating whether the different levels of effective index components resulting from different methods of extraction actually lead to differential effectiveness in practice are lacking. *In vivo* studies that can elucidate optimal methods with regard to extraction efficiency in the preparation of herbal medicines are urgently needed.

This study aimed at investigating whether differences in the levels of effective index components in Jakyak-Gamcho decoctions resulting from the use of different extractants are associated with differences in its effectiveness as a spasmolytic agent in an experimental animal model. In other words, we tested the hypothesis that the higher amounts of effective index components in 70% ethanol-extracted Jakyak-Gamcho decoction than in water-extracted decoction resulted in the greater spasmolytic effects of the former *in vivo*. We also investigated whether Jakyak-Gamcho decoction is effective under physiological conditions. To this effect, we estimated the tension of complete tetanus (pathological muscle cramp) and that of incomplete tetanus (physiological contraction) and compared them. Jakyak-Gamcho decoctions were prepared via an ultrasound-reflux extraction method using either water or 70% ethanol as the extractant and injected into the duodenum of rats to compare gastrocnemius muscle tetanus reduction rates after the induction of muscle cramps.

2. Materials and Methods

2.1. Experimental Medicines and Anesthetics. Sixty grams of Radix Paeoniae (Hwalim Natural Drug Co., Ltd., Korea) and sixty grams of Radix Glycyrrhizae (Hwalim Natural Drug Co., Ltd., Korea) were used to prepare decoctions with 1.2 L of either water or 70% ethanol as the extractant, via reflux extraction for 3 hours using an ultrasonic extractor (POWER SONIC410, Hwashin, Korea, oscillation frequency: 40 KHz, temperature: 50°C). After reflux extraction, the extracts were subjected to pressure evaporation (RV10, IKA, Germany) and lyophilization via a freeze dryer (ILShin Lab Co., Ltd., Korea) and then stored in a freezer at −80°C in powder form. The powdered extracts were dissolved in distilled water (Young Lin Instrument Co., Ltd., Korea) prior to use. Isoflurane (Hana Pharm Co., Ltd., Korea) and urethane (Sigma-Aldrich, Co., St. Louis, USA) were used as anesthetics. The experimental medicines were injected into the duodenum 1 hour before the experiment. The doses of the medicines administered were as follows: water-extracted Jakyak-Gamcho decoction, 0.25 g/kg and 0.5 g/kg i.d.; 70% ethanol-extracted Jakyak-Gamcho decoction, 0.25 g/kg and 0.5 g/kg i.d.; isoflurane (99% oxygen, 5% isoflurane), inhalation anesthesia; urethane (25%), 1.5 mL i.p. The doses of water- and 70% ethanol-extracted decoctions were chosen based on the results of a preliminary study that showed that 70% ethanol-extracted Jakyak-Gamcho decoction had a spasmolytic effect at dose of 0.25 g/kg or higher, while for the water-extracted decoction, the dose had to be 0.5 g/kg or higher. In addition, a pharmacological study of Jakyak-Gamcho decoction showed that doses of 0.5 g/kg, 1 g/kg, and 2 g/kg have no effect on general behavior or CNS function [18].

2.2. Experimental Animals. Four male Wistar rats aged 7-8 weeks and weighing 172.5–183.1 g were used in a preliminary experiment to determine the initiating frequency. In the actual experiment, 5 male Wistar rats aged 7-8 weeks and weighing 178.6–195.8 g were used in each group. A distilled water injection (control) group, a water-extracted Jakyak-Gamcho decoction (0.25 g/kg, 0.5 g/kg) injection group, and a 70% ethanol-extracted Jakyak-Gamcho decoction (0.25 g/kg, 0.5 g/kg) injection group were included in the study. All experimental animals were adapted in a controlled environment (24°C ± 1°C, 60% ± 5% humidity, and 12-hour light/dark cycle), and water and food (Samtako Inc., Korea) were available *ad libitum*. All experiments were approved by the Institutional Review Board for Animal Studies of Dongshin University. After the completion of experiments, the animals were killed via a cardiac injection of a lethal dose of urethane.

2.3. High-Performance Liquid Chromatography Analysis. The Jakyak-Gamcho decoctions derived via different solvents were subjected to high-performance liquid chromatography (HPLC) analysis to investigate differences in the levels of effective index components. HPLC machine (Hewlett Packard 1100 Series, Agilent) at the Korea Basic Science Institute (Seoul) was utilized in the analysis (Column-G1316A, DAD-G1315A, QuatPump-G1311A, ALS-G1329A, and Degasser-G1322A). Paeoniflorin, benzoic acid, glycyrrhizin, and isoliquiritin were included as effective index components. Analysis conditions were as follows: column, Kinetex C18 (4.6 × 250 mm, 5 μ, Phenomenex); column temperature, 25°C; eluents, (A) 0.01% phosphoric acid and (B) acetonitrile; flow rate, 1.0 mL/min; run time, 60 min; wavelengths, paeoniflorin, benzoic acid, and glycyrrhizin 230 nm and isoliquiritin 360 nm; injection volume, 10 μL. Gradient details are shown in Table 1.

2.4. Electrical Stimulation Experiment

2.4.1. Surgery. After inducing inhalation anesthesia with isoflurane, 1.5 mL of 25% urethane was injected into the right abdominal cavity of rats, to maintain anesthesia. During

Table 1: Gradient details.

Time (min)	A (%)	B (%)
0	90	10
5	90	10
25	70	30
35	40	60
45	5	95
48	5	95
49	90	10
60	90	10

anesthesia and surgery, vital signs were monitored via the front legs (MouseOx Plus, Starr Life Science Corp., USA). Physiological saline solution was frequently applied to avoid drying of the surgical areas.

Surgery was performed in the left posterior lower limbs and abdomen. After removing hair in the surgical areas, skin from the calcaneus to the knee and from the knee to the hip joint was incised and separated from the subcutaneous muscles. The tensor fasciae latae and the gluteus maximus were then incised and removed, to expose the gastrocnemius and tibial nerves. In order to restrict electrical stimulation to the tibial nerves, potentially affected peroneal and sural nerves were incised and removed. In addition, a thin plastic film was placed underneath the exposed tibial nerve to prevent electrical stimulation from being transferred to peripheral tissues. The gastrocnemius muscle, a target for tension measurements, was located and separated and the soleus muscles attached underneath it were removed. The legs undergoing surgery were fixed to the operating table with pins, to induce isometric contraction. Pins were fixed to the left femur, knee, tibia, calcaneus, and soles (see Figure 1).

Surgery was also performed on the abdomen, to facilitate the administration of medicine. A 1.5 cm horizontal incision was made in the abdomen, and the duodenum was located for the injection of medicine. After the injection of medicine, the incision was closed using a stapler for animal surgery (Leica Biosystems Richmond Inc., USA).

2.4.2. Electrical Stimulation and Muscle Cramp Induction. Only complete tetanus was deemed to constitute muscle cramping, and incomplete tetanus was deemed to constitute physiological muscle contraction. Then, the initiating frequency where complete tetanus is induced varies for each individual rat, so a preliminary study was performed to ascertain some foundation parameters. In the preliminary study, electrical stimulation of 20, 40, 60, or 70 Hz was applied for 5 seconds at 5-minute intervals, and the degree of induced muscle contraction was measured in each case. Using a Dual Impedance Research Stimulator (Harvard Apparatus, USA) and bipolar electrode, stimulations of 20 Hz and 70 Hz (3 V, 0.3 ms) were applied to the tibial nerves for 5 seconds in order to induce gastrocnemius muscle contraction under physiological (20 Hz) and pathological (70 Hz) conditions. Incomplete tetanus was induced by stimulation of 20 Hz, and complete tetanus was induced by 70 Hz. A 20 Hz stimulation was applied first, and 70 Hz stimulation was then applied after a 5-minute rest, to prevent muscle fatigue [9]. Electrical

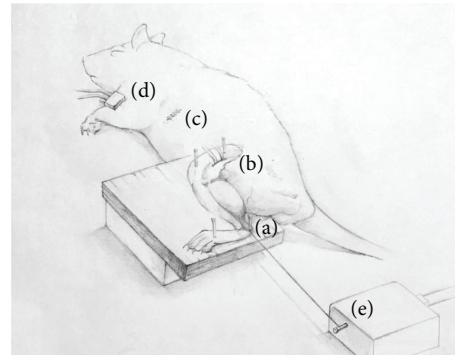

Figure 1: Schematic view of the experimental setup. A force transducer is attached to the gastrocnemius muscle by a silk thread on the distal Achilles tendon (a). Bipolar electrodes are attached to the tibial branch of the sciatic nerve (b). Medicine is directly injected into the duodenum (c). Vital signs are measured via the left front leg (d). Force transducer (e).

stimulations were applied before medicine injection, 1 hour after the injection, and 2 hours after the injection.

Silk threads were attached to the Achilles tendon and connected to a force transducer (Harvard Apparatus, USA), and the degree of muscle contraction induced in the gastrocnemius muscle by 20 Hz or 70 Hz electrical stimulation of the tibial nerve was measured. Then, based on the degree of muscle contraction before and at 1 and 2 h after the injection of water-extracted Jakyak-Gamcho decoction or 70% ethanol-extracted Jakyak-Gamcho decoction, muscle cramp reduction rates were compared (1). The thread was connected to the gastrocnemius muscle uniformly in each rat, with respect to its anatomical position. The force transducer was connected to MP100A-CE system (BIOPAC System Inc., USA), and the degree of muscle contraction was visualized and analyzed via Acqknowledge 3.9.1 software. Consider the following:

$$\text{Reduction rate} = \left(1 - \frac{\text{tension 1 h or 2 h after stimulation}}{\text{tension before stimulation}}\right) \times 100\,(\%)\,. \quad (1)$$

2.5. Statistical Analysis. Based on tension measurements before and after medicine injection, muscle cramp reduction rates were calculated and used in analyses. IBM SPSS Statistics 21 (IBM, USA) was used for statistical analyses. A normality test and equal variation assumption were satisfied, and reduction rates among groups were compared via ANOVA. Duncan's test was used for multiple comparisons. The significance level was set at $p < 0.05$.

3. Results

3.1. HPLC. HPLC results are shown in Figures 2 and 3 and Table 2. Larger amounts of all of the effective index components including paeoniflorin, benzoic acid, glycyrrhizin, and

Table 2: HPLC quantification results.

	Water-extracted decoction	70% ethanol-extracted decoction
Acquired amount (μg/mL)		
Paeoniflorin	383.626	451.842
Benzoic acid	17.664	17.755
Isoliquiritin	33.729	90.434
Glycyrrhizin	343.904	555.444

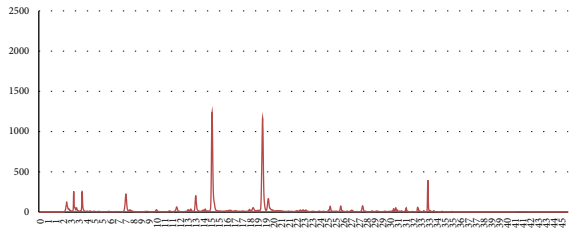

Figure 2: Chromatogram derived from a water-extracted Radix Glycyrrhizae/Radix Paeoniae Jakyak-Gamcho decoction.

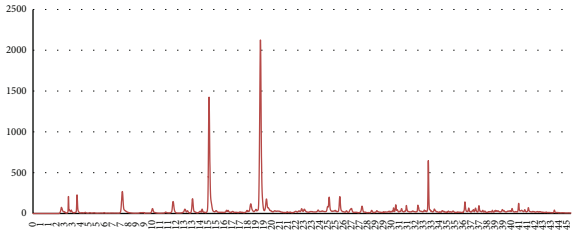

Figure 3: Chromatogram derived from a 70% ethanol-extracted Radix Glycyrrhizae/Radix Paeoniae Jakyak-Gamcho decoction.

isoliquiritin were extracted using 70% ethanol as an extractant, as compared to water. Compared to the amounts derived via water, 70% ethanol yielded 68.216 μg/mL more paeoniflorin, 0.091 μg/mL more benzoic acid, 208.540 μg/mL more glycyrrhizin, and 56.705 μg/mL more isoliquiritin. Notably, the extraction yields of glycyrrhizin and isoliquiritin, effective index components of Radix Glycyrrhizae, were 1.6 and 2.6 times greater, respectively, when using 70% ethanol as the extractant instead of water.

3.2. Initiating Frequency. Figure 4 shows the results of the preliminary study. Electrical stimulation of 20 Hz yielded a serrated contraction curve, and the contraction curves gradually became flatter as the intensity of stimulation was increased. In addition, the degree of muscle contraction increased with stronger electrical stimulation, and complete tetanus was induced at 70 Hz.

3.3. Muscle Cramp Reduction Rate. Compared to the time before medicine injection, muscle cramp reduction rates 1 hour after injection did not differ significantly at electrical stimulations of 20 Hz or 70 Hz in any group (data not shown). The degrees of muscle contraction at 20 Hz before medicine injection and 2 hours after medicine injection were compared, and the reduction rates are shown in Figure 5. The solvent used did not have a significant effect on muscle cramp reduction rate (Figures 5(a) and 5(b)) and neither did the medicine dose (Figures 5(c) and 5(d)).

The degrees of muscle cramping at 70 Hz before medicine injection and 2 hours after medicine injection were compared and the reduction rates are shown in Figure 6. With regard to water-extracted Jakyak-Gamcho decoction, 0.25 g/kg did not yield a significant spasmolytic effect compared to the control group (distilled water) ($p > 0.05$). Conversely, 0.25 g/kg of 70% ethanol-extracted Jakyak-Gamcho decoction yielded a significant spasmolytic effect compared to both the control group and 0.25 g/kg of water-extracted Jakyak-Gamcho decoction ($p < 0.05$ for both comparisons) (a). At the higher dose of 0.5 g/kg, both water-extracted and 70% ethanol-extracted Jakyak-Gamcho decoctions yielded significant reductions in muscle cramping, as compared to the control group ($p < 0.05$ for both comparisons) (b).

With regard to medicine dose, 0.25 g/kg of water-extracted Jakyak-Gamcho decoction did not yield a significant reduction in the rate of muscle cramping compared to the control group (distilled water) ($p > 0.05$). On the other hand, 0.5 g/kg exhibited a significant spasmolytic effect compared to both the 0.25 g/kg group and the control group ($p < 0.05$ for both comparisons) (c). Both 0.25 g/kg and 0.5 g/kg of 70% ethanol-extracted Jakyak-Gamcho decoctions yielded significant spasmolytic effects compared to the control group ($p < 0.05$ for both comparisons) (d).

4. Discussion

Muscle cramping is a condition that occurs suddenly, and the duration of it is short [6]. In this regard, muscle cramps are hard to predict and hard to manage. Therefore, preventive approaches that can reduce the degree and frequency of unpredicted muscle cramps are required rather than administration of therapeutic agents every time they occur. Thus, in order to investigate muscle cramp prevention, Jakyak-Gamcho decoctions were injected 1 hour prior to the experimental induction of cramping in this study. We designed an intuitive muscle cramp induction model to use in this study. As each individual rat possesses different muscle force, it is not appropriate to simply compare the muscle cramp-induced gastrocnemius muscle tension between different groups of rats. Therefore, in this study gastrocnemius muscle tension was measured before and after injection; then these measurements were used to calculate reduction rates. By controlling for the individual differences in muscle capacity in each rat, muscle cramp reduction rates were able to be compared via groups, and significant outcomes were obtained.

While the spasmolytic mechanisms of Jakyak-Gamcho decoctions are not clearly understood, it has been suggested that paeoniflorin desensitizes nicotine acetylcholine receptors, and by acting together with glycyrrhizin, contractile Ca^{2+} mobilization is inhibited and neuromuscular transmission is blocked [11]. In this current study, the amounts of all

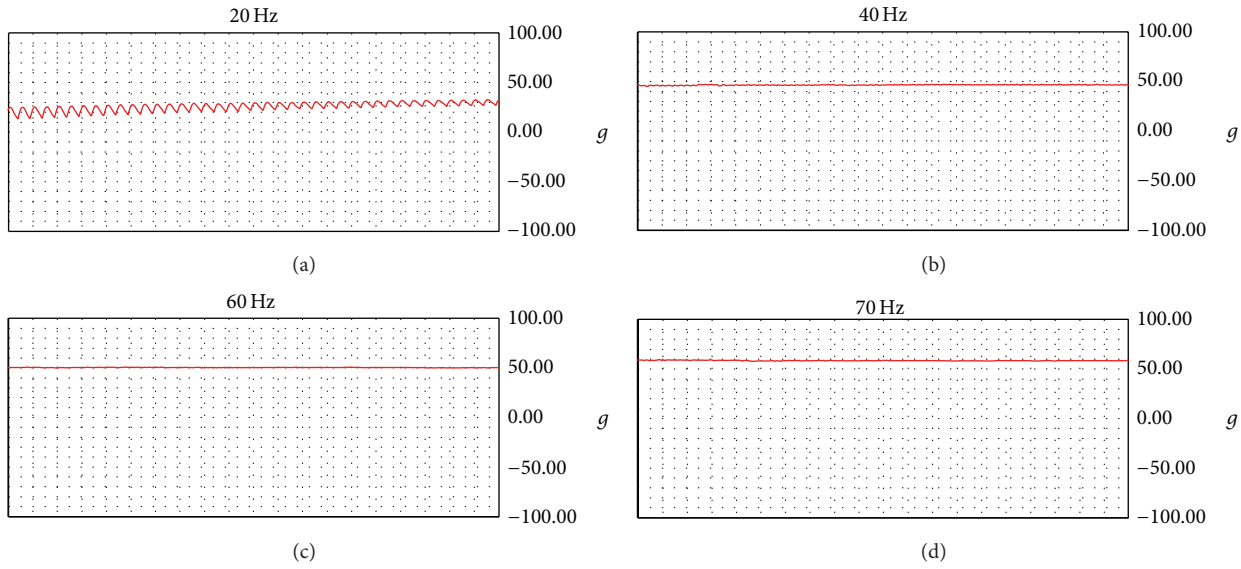

Figure 4: Initiating frequency.

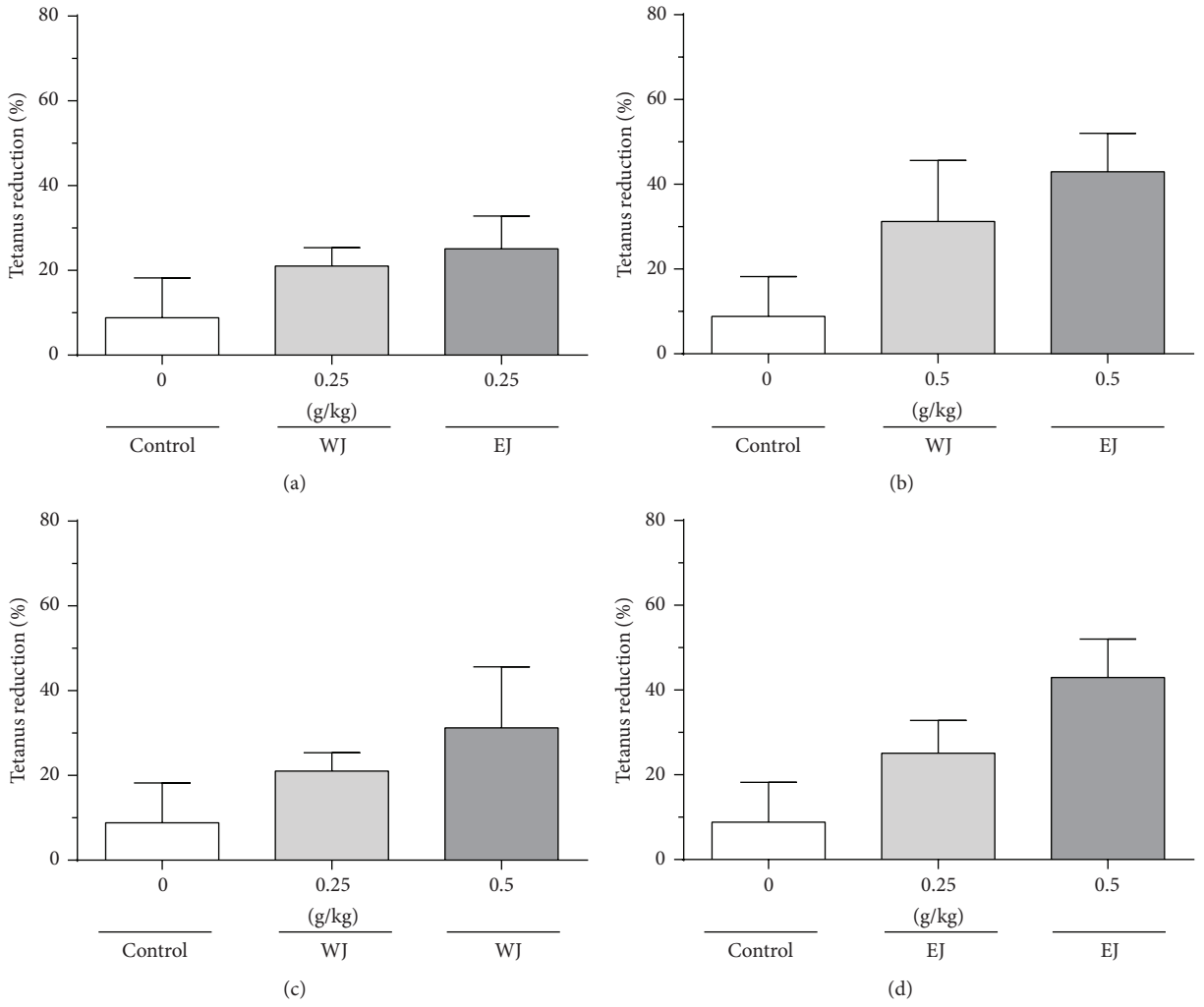

Figure 5: Muscle cramp reduction rates with electrical stimulation of 20 Hz 2 hours after medicine injection, by solvent (a, b) and medicine dose (c, d). Each bar represents mean + SEM of 5 animals.

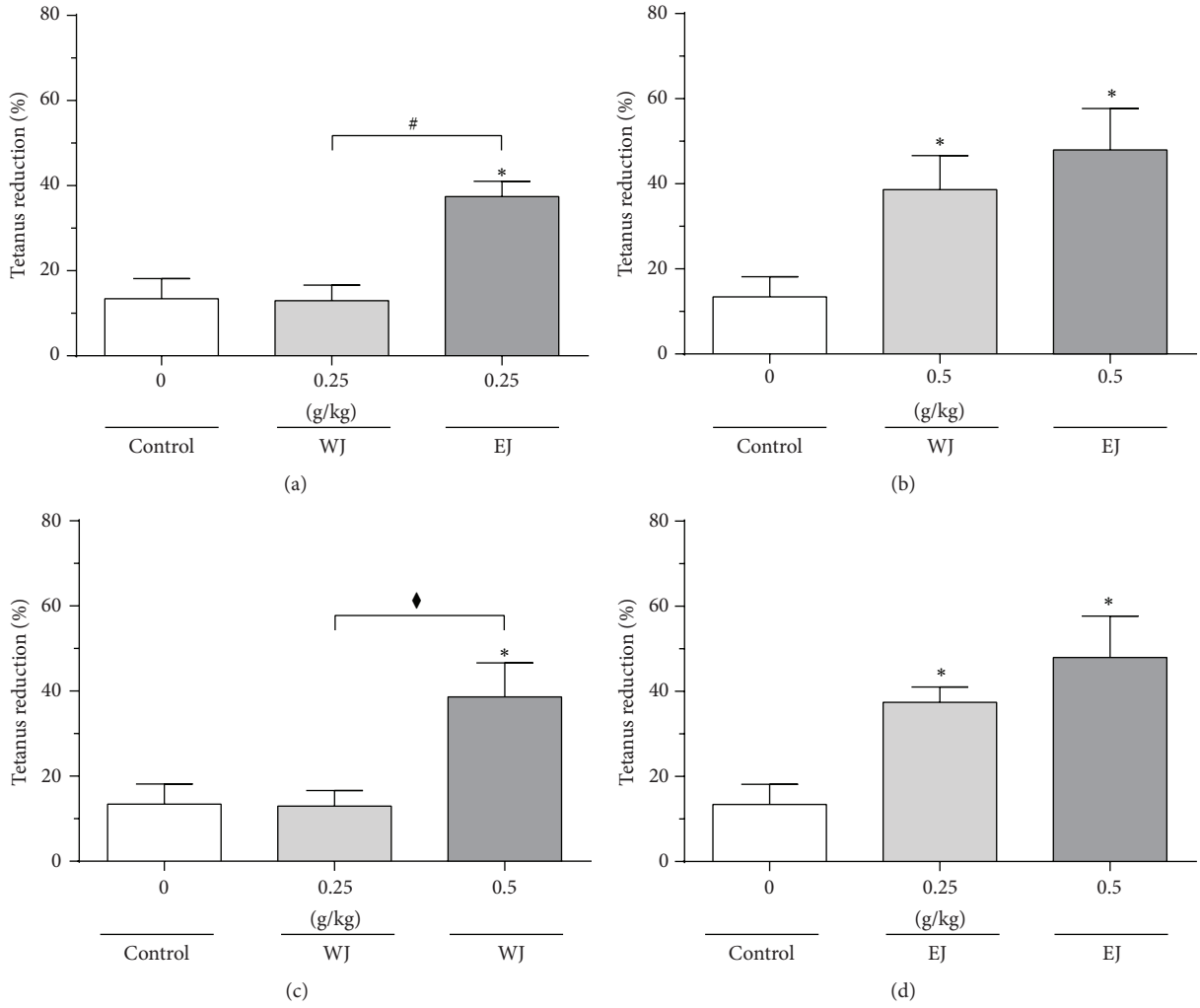

Figure 6: Muscle cramp reduction rates with electrical stimulation of 70 Hz 2 hours after medicine injection, by solvent (a, b) and medicine dose (c, d). Each bar represents mean + SEM of 5 animals. $^{*}p < 0.05$ compared to the control group by ANOVA and Duncan's test. $^{\#}p < 0.05$; comparison between the WJ and EJ groups by Duncan's test. $^{\blacklozenge}p < 0.05$; comparison between the 0.25 g/kg and 0.5 g/kg WJ groups by Duncan's test.

four effective index components were higher in 70% ethanol-extracted Jakyak-Gamcho decoction than water-extracted Jakyak-Gamcho decoction, as determined by HPLC analysis. This is consistent with the results of a previous study investigating the extraction efficiency of Jakyak-Gamcho decoctions [17]. Particularly, among the constituents of Jakyak-Gamcho decoction, Radix Glycyrrhizae has significant spasmolytic activity [9]. In this current study, the yields of the Radix Glycyrrhizae constituents glycyrrhizin and isoliquiritin were 1.6 and 2.6 times higher, respectively, when extracted with 70% ethanol than when extracted with water. This confirmed that different extractants can yield different amounts of active components related to the spasmolytic effects of Jakyak-Gamcho decoctions.

In herbal medicines, intracellular contents from which active components are derived are extracted through cell walls or by breaking down cell walls [19, 20]. In the case of ethanol extraction in herbal medicine, ethanol solvents break down the cell walls of medicinal ingredients, facilitating the extraction of active components from within the cells [21, 22]. In the present study, higher amounts of the active components of Jakyak-Gamcho decoction were extracted by 70% ethanol than by water. We suggest that this is because ethanol breaks down the cell walls of the ingredients of oriental medicines, ultimately enhancing extraction efficiency.

Since ethanol can explode when heated, a traditional boiling method was not used in the extraction of Jakyak-Gamcho decoction with ethanol in this study. Instead, ultrasound-reflux extraction, which is known to be effective for herbal medicines [19, 20], was used. To ensure an accurate comparison between the two solvents investigated, the same ultrasound-reflux extraction method was used for the water-extracted Jakyak-Gamcho decoction.

Several studies have investigated the threshold frequency at which complete tetanus starts to develop and continue [23–25]. However, the threshold frequency at which complete

tetanus reportedly started in these previous studies differed from the initiating frequency observed in this current study [9]. This is likely due to differences in experimental conditions and inconsistent operational definitions of complete tetanus. As muscle cramping is an instant musculoskeletal condition and is accompanied by instant intense pain, we designed an electrical stimulation experiment that can induce maximum muscle contraction instantly in spite of its short duration. Accordingly, an experiment investigating initiating frequency was performed, and complete tetanus was confirmed to develop at 70 Hz.

Neither the muscle contraction rates induced by electrical stimulation with 20 Hz nor those induced by 70 Hz were reduced significantly by any of the decoctions in any group, 1 hour after the injection of medicine. This might have been because glycyrrhetinic acid, which is glycyrrhizin's hydrolysis product, had not yet been absorbed sufficiently to yield an effect. Glycyrrhetinic acid is a major component for spasmolytic effect [9]. It has been reported that the highest plasma concentration of glycyrrhetinic acid is observed 8–10 hours after oral administration [26]. Although intraduodenal administration can increase the rate of absorption, 1 hour might not be enough to yield a spasmolytic effect. Two hours after injection, muscle cramps induced by electrical stimulation of 20 Hz were not significantly reduced, indicating that Jakyak-Gamcho decoctions were not effective in the muscle contraction of incomplete tetanus, which occurs before complete tetanus. Conversely, a significant difference was observed when an electrical stimulation of 70 Hz was applied, suggesting that Jakyak-Gamcho decoctions can be effective for muscle cramp alleviation associated with complete tetanus. However, further studies are needed to elucidate the pharmacological mechanism of Jakyak-Gamcho decoction, which makes it effective as a spasmolytic agent in case of pathological muscle cramps but not for physiological muscle contraction.

With regard to electrical stimulation of 70 Hz that was applied 2 hours after medicine injection, 0.25 g/kg of water-extracted Jakyak-Gamcho decoction did not show a significant spasmolytic effect compared to distilled water, but 0.25 g/kg of 70% ethanol-extracted Jakyak-Gamcho decoction did. Even at an equivalent dose (0.25 g/kg), the effects of 70% ethanol-extracted Jakyak-Gamcho decoction were more significant than those of water-extracted Jakyak-Gamcho decoction ($p < 0.05$) and therefore the difference in the amounts of effective index components evidently results in different effects in practice. Only when 0.5 g/kg of water-extracted Jakyak-Gamcho decoction was injected did it exhibit a spasmolytic effect similar to that of 0.25 g/kg of 70% ethanol-extracted Jakyak-Gamcho decoction. With regard to 70% ethanol-extracted Jakyak-Gamcho decoctions, differences between the effects of 0.25 g/kg and 0.5 g/kg were not significant ($p > 0.05$), which may be because 0.25 g/kg achieves the maximum possible level of effectiveness of Jakyak-Gamcho decoction [27, 28].

Limitations of this study include that an electrical stimulation differs from general muscle cramp causes. Electrical stimulation-induced muscle cramps may possess different physiological characteristics to general muscle cramps. In addition, the surgery was invasive, which may have led to additional differences between the physiological conditions in the study and those of naturally occurring muscle cramps. However, direct muscle tension measurement is required in order to measure muscle cramps, so invasive surgery procedures were unavoidable.

In the present study, we increased the efficiency of Jakyak-Gamcho extraction by using an alternative extractant to water and confirmed that this corresponded with an increase in the spasmolytic effectiveness of Jakyak-Gamcho decoction in an animal model. To date, Jakyak-Gamcho decoctions have been shown to be effective for muscle relaxation and muscle pain alleviation [9, 12–14, 29]. In this current *in vivo* study, ethanol-extracted Jakyak-Gamcho decoction was superior to water-extracted Jakyak-Gamcho decoction with regard to both extraction efficiency and spasmolytic effects. The elucidation of changes in the dosages of herbal medicines in the form of powders is required, for the quantitative standardization of their active components. The results of this current study suggest changes in extraction methods when Jakyak-Gamcho decoctions are prepared and converted into powder form. Moreover, the results suggest that it may be worthwhile to investigate alternative extraction methods in terms of extraction efficiency and *in vivo* effectiveness for other herbal medicines besides Jakyak-Gamcho decoction.

5. Conclusion

This study confirmed that differences in the amounts of effective index components in Jakyak-Gamcho decoctions resulted in differences in their effects *in vivo*. Jakyak-Gamcho decoction extracted with 70% ethanol exhibited higher amounts of effective index components than that extracted with water. In addition, 2 hours after medicine injection, the injection of 70% ethanol-extracted Jakyak-Gamcho decoction, which contained relatively higher amounts of effective index components, was associated with greater reductions in muscle cramping. The results of this study suggest that it may be worthwhile to investigate alternative extraction methods in terms of extraction efficiency and *in vivo* effectiveness for other herbal medicines in the future.

Conflict of Interests

The authors declare no conflict of interests, and all authors have approved the final paper.

Authors' Contribution

Dongwook Kwak, Changwoo Lee, and Inseong Kong contributed equally to this work.

Acknowledgment

This study was supported by a grant from the 2014 KIOM Undergraduate Research Program of the Korea Institute of Oriental Medicine.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 682735, 14 pages
http://dx.doi.org/10.1155/2015/682735

Review Article

Essential Experimental Methods for Identifying Bonghan Systems as a Basis for Korean Medicine: Focusing on Visual Materials from Original Papers and Modern Outcomes

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Received 24 June 2015; Accepted 16 August 2015

Academic Editor: Morry Silberstein

In the 1960s, through studies on Korean Medicine, Bonghan Kim proposed the Bonghan systems (BS) as the anatomical reality of the acupuncture meridians based on various experimental data. Since 2002, several groups, mainly led by a team at Seoul National University, who renamed the BS as the primo vascular system (PVS), have published around 70 papers showing biological structures corresponding to the BS. However, it is still difficult for other researchers to find them, especially under the skin, which Bonghan Kim first reported as acupuncture points, due to similar-looking biological tissues, for example, the lymphatic vessels, and such artifacts as blood clots or fascia debris. To solve these drawbacks, we examined the main methods for identifying the BS by comparing the original papers with the modern outcomes in terms of the common physical/chemical characteristics of the BS. In addition, effective methods of staining and microscopic observations discovered by modern teams are synthetically explained using visual materials such as diagrams and photos. Through the essentially organized methods in this review paper, we suggest that one can find the BS under the skin as putative acupuncture points by tracing the intraexternal BS, from which a new Korean Medicine will be born.

1. Introduction

Korean Medicine [1, 2] has developed in parallel with Chinese medicine. These two types of medicine have been established in the same root in which the meridian system has been considered as a vital channel holistically orchestrating the human body with the yin and yang. However, beyond the philosophical approach to the meridian system in China, in the 1960s a Korean named Bonghan Kim proposed the Bonghan system as an anatomical reality of the acupuncture meridian (Kyungrak in Korean) based on the various kinds of experimental data. A team led by Bonghan Kim at the Academy of Kyungrak, which was formerly called the Kyungrak Research Institute, published five research papers showing detailed anatomical/histological structures and biochemical components inside them for elucidating the new systems they found [3–7]. They called the structures Bonghan corpuscles (BCs) and Bonghan ducts (BDs) corresponding to the acupuncture points and meridian pathways, respectively. Although the team abruptly disappeared with their papers during the late 1960s in North Korea, and its claim was neglected in the academic field for about 40 years, Bonghan Kim remains the only scientist who has claimed to find the physical substances of the meridian systems thoroughly throughout the body using western scientific methodology.

Since 2002, however, a research group, called the Laboratory of Biomedical Physics for Korean Medicine in the Department of Physics, Seoul National University in South

Korea, has taken the lead in representing the systems, publishing about 70 research papers in domestic and international journals [8]. In 2010, the team renamed the Bonghan system, the primo vascular system (PVS), and until recently, they reported many scientific evidences corresponding to the structures and contents of the BCs and BDs, as well as new findings regarding their distributions and functions that Bonghan Kim's research team (BRT) had not mentioned [9]. In addition, in the 2010s, other domestic and international teams, influenced by the PVS team, started to report the potential functions of the Bonghan system, which was related to tumor development [10], cancer metastasis [11, 12], and the origin of adult stem cells [13–15]. In 2013, the BRT's claim was academically reevaluated by international researchers, through the perspective of oriental medicine as well as western science, as a special issue titled "Primo Vascular System: Past, Present, and Future" with 18 articles in Evidence-Based Complementary and Alternative Medicine (http://www.hindawi.com/journals/ecam/si/954751/).

It is natural that recent researchers are mainly interested in the biological functions of the Bonghan systems. Particularly, the researchers of oriental medicine get to pay attention to the BRT's claim because it would imply the physical evidence of Qi circulation, which has been the basic premise of medical cures such as acupuncture and medicinal herbs for a long time.

However, for subsequent researchers, it is first necessary to determine the existence of the Bonghan systems themselves. Despite this necessity, there are some critical problems disturbing the formation of positive attitudes regarding the presence of the Bonghan systems. First, the BRT did not describe the methods used for identifying them, only revealing their locations within the body and their characteristics using experimental instruments such as several types of microscopes and dyes. For example, the BRT insisted that they first found the new anatomical tissues under some acupuncture points in the skin in the first paper without explaining experiment methods, and thereafter they showed them connected to the most organs in the second and third papers. Therefore, one can know whether the new structures are the real Bonghan systems after comparing the physical/chemical characteristics to those of the BRT papers, through numerous trial-and-error methods, such as in the works of the modern research teams (MRTs), particularly the PVS team. Second, even if one studies the methods identified from the papers of MRTs, there may still be difficulty in exactly identifying the Bonghan systems due to not only the existing biological tissues similar to them, but also the several kinds of artifacts brought about during the experiments. To find the Bonghan systems efficiently over such obstacles, it is necessary to have knowledge regarding the essential characteristics of the Bonghan systems through the papers by the BRT as well as the effective methods distinguishing them from artifacts suggested by the MRTs.

The purpose of this paper is to review the papers not only of the BRT but also of the MRTs to solve these problems. Comparing two kinds of papers, particularly using visual materials, the authors proposed the common essential characteristics about the Bonghan systems. In addition, the authors presented useful methods, through a summarizing of the MRTs' papers mainly with PVS team's ones, to avoid misjudgments from similar tissues and artifacts. This review paper may help subsequent researchers deeply study the Bonghan systems in the future.

2. Change from "Kyungrak" to "Bonghan Systems"

According to the second and third papers of the BRT, there are four kinds of Bonghan systems, that is, internal ones inside the blood and lymphatic vessels, intraexternal ones mainly on the surfaces of the internal organs, external ones in the skin (also independently named superficial systems) and through the outside vessels, and neural ones on the surfaces of the brain and inside the ventricles as well as in the central canal of the spinal cord, connected to the peripheral nervous systems. The BRT used the term "Kyungrak" for its newly discovered systems in the titles and texts throughout four of the papers, with the exception of the last one, which suggested the fundamental formation process for blood and white cells. Interestingly, however, the terms regarding the acupuncture points and meridian pathways in the text appeared almost only in the first paper, including sangyang (商陽) and igan (二間) on sooyangmyung daijanggyung (手陽明大腸經), joksamri (足三理) on jokyangmyung wikyung (足陽明胃經), rokoong (勞宮), haryum (下廉), soosamri (手三里), and hapkok (合谷) referring to *Dongeuibogam* (東醫寶鑑), which was written by a famous Korean medical doctor in 1610. Instead, the terms "Kyungrak" and "Bonghan systems" were used simultaneously in the second, third, and fourth papers without specific terms of the meridian systems, with the exception of joksamri, which was mentioned once in the second paper.

We guess that the change of naming was caused by the change in perception of the BRT through the new findings. In the first paper, the BRT reported that it tried to "clarify the material foundation of the basic theory" of oriental medicine and finally found the physical substance under the acupuncture points in the skin which were known to functionally be connected with internal organs such as the stomach and intestine. In the second paper, however, the BRT claimed that it observed a complex network throughout the whole body with a simple anatomical unit of BC and BD and the existence of the structure inside the blood and lymphatic vessels that "no one has ever conceived of." To the BRT, these characteristics would clearly seem to be different from those of the meridian systems mentioned in traditional meridian literature.

Ironically, however, the MRTs were unable to find the external (and superficial) ones until recently. Therefore, in this paper, we investigated the three Bonghan systems and summarized the common characteristics reported by the BRT and MRTs. Also, we selected the first three papers [3–5] of the BRT for a comparison because the anatomical/histological structures as well as the biochemical components are mainly introduced in them. English versions, which were disclosed to the public online (http://www.ispvs.org/), were used because

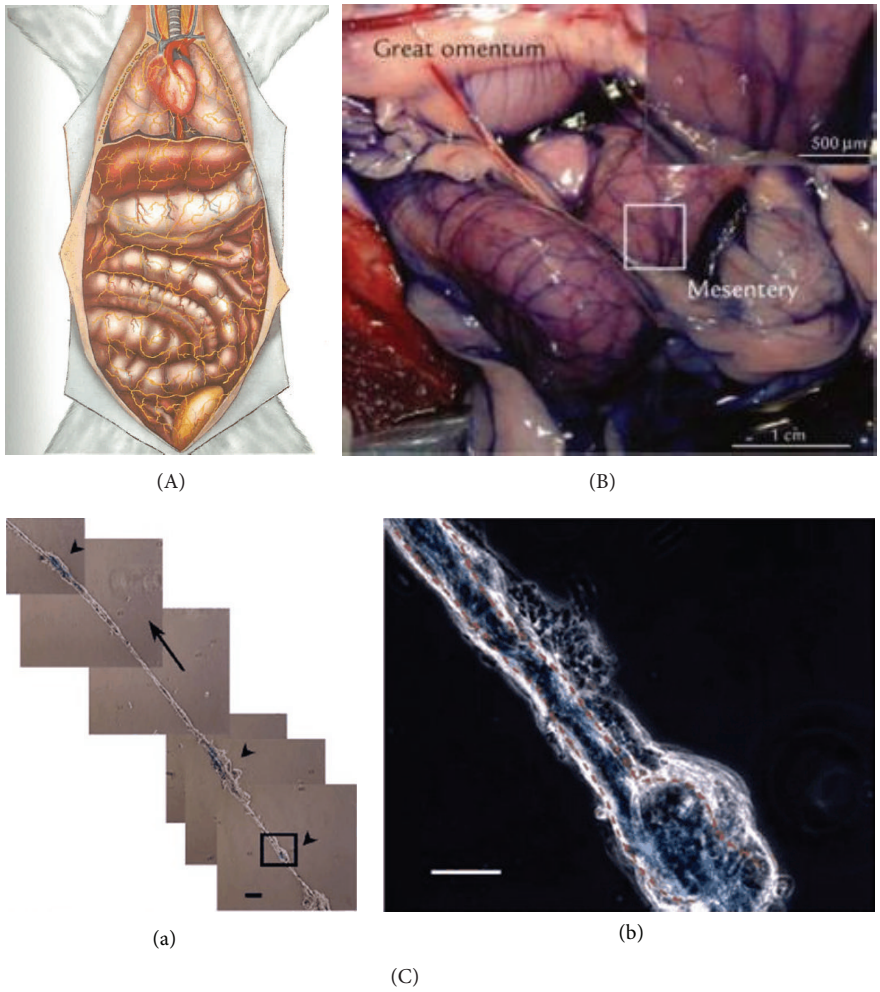


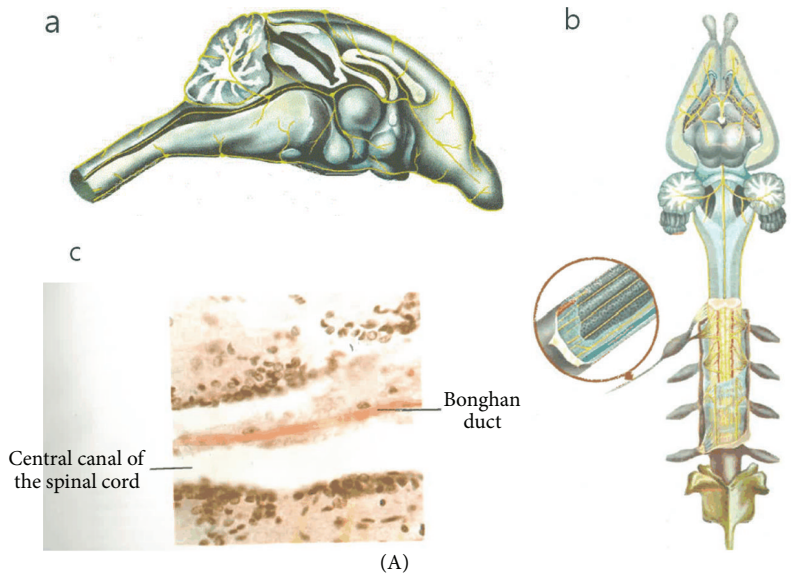
Figure 1: The intraexternal Bonghan systems. (A) The intraexternal Bonghan systems by the Bonghan research team. Diagram of the intraexternal Bonghan systems. The Bonghan ducts and corpuscles are distributed, independent of the paths of vessels and nerves, all throughout the body like a network. The Bonghan ducts exist in a free state, but they adhere to the surfaces of the organs and the walls of the blood vessels only in the region where they branch off [5]. (B) The intraexternal Bonghan systems stained by Trypan blue. Trypan blue-stained primo vascular system network on the mesentery of dog. The inset shows a high-magnified primo node (arrows) located at the joints of the primo vessels [16]. (C) The flow of liquor in the intraexternal Bonghan systems. The flow of Alcian blue (thick arrow, a) through a Bonghan duct connected to the corpuscles (arrowheads) on the surface of a rabbit's abdomen organ and a magnified view (b) of the boxed segment showing blue fluid inside the Bonghan duct and corpuscle (dotted red line) under a phase-contrast microscope. All scale bars are 50 μm [17].

they contained colorful photos and diagrams unlike the Korean versions. For the papers of the MRT, the authors selected some critical photos clearly showing the characteristics of the Bonghan systems.

3. The Structures and Contents of the Bonghan Systems

3.1. The Basic Structures of the BD and BC Regarding Three Bonghan Systems. According to the BRT's first three papers, the basic unit of a Bonghan System consists of the BC and threadlike BDs. One BC connects with two or more BCs through the BDs, and all of them are semitransparent and look somewhat yellowish.

There are three major anatomical/histological characteristics of the BD. First, the number of BDs connected to one BC and their existing states differ depending on their locations in the body. Only two BDs are connected to one BC freely floating in the liquor, in case of those inside the blood and lymphatic vessels. However, in case of those on the surfaces of the abdominal organs, two or more BDs are reticulately connected to one BC spreading some branches into the organs (Figure 1(A)). For the neural Bonghan system, the BRT described the number of BDs as being only two to four. According to the visual materials, the spreading pattern inside the brain would be similar to the latter (Figure 2(A)(a-b)), and the pattern inside the central canal of the spinal cord would be analogous to the former (Figure 2(A)(b-c)). In



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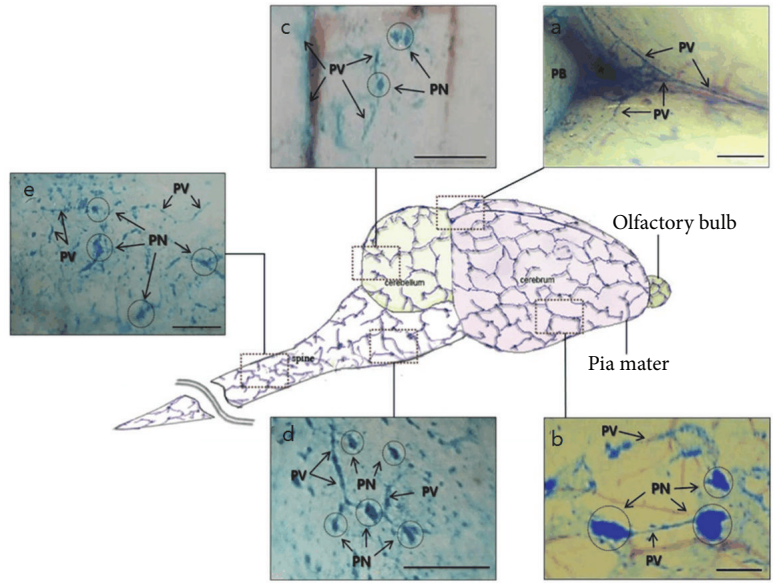

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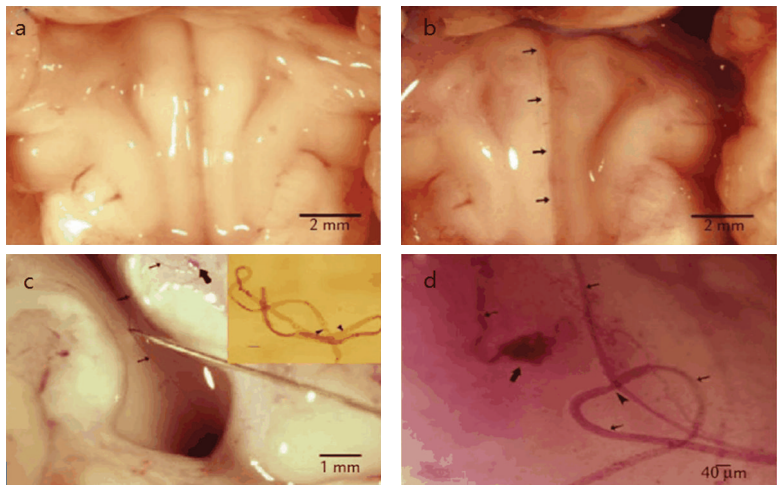

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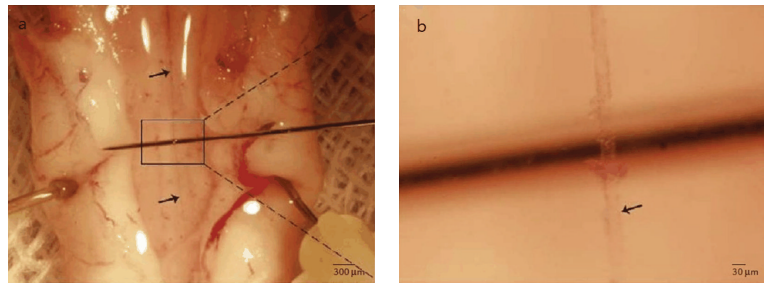

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Figure 2: The neural Bonghan systems. (A) The neural Bonghan systems determined by the Bonghan research team. Diagrams and a photo of the neural Bonghan systems. In the central nervous systems, the Bonghan ducts (a, b) are distributed in the brain and spinal cord in a free state through the cerebral ventricles, the central canal, and the subarachnoid space along the circulating route of the cerebrospinal liquor. In addition, they enter under the perineurium and between the nerve fibers in the peripheral nervous systems. The Bonghan duct in the central canal of the spinal cord (c) is surrounded by unknown tissues [5]. (B) The Bonghan ducts and corpuscles on the surfaces of the brain stained by Alcian blue. Illustration of a network of the Bonghan ducts (PV) and corpuscles (PN) above the pia mater of the brain and the spine of rats stained by Alcian blue under a stereomicroscope. Stereoscopic images are visualized by spraying Alcian blue into the pia mater of the brain (a, b) and by injecting it into the lateral ventricles (c, d, e). The red-colored blood vessels are not stained (a, b, c). All scale bars are 500 μm [18]. (C) The Bonghan duct in the brain ventricles stained by hematoxylin. The Bonghan duct in the brain ventricles of rabbits under a stereomicroscope. Image (a) at the bottom of the fourth ventricle beneath the cerebellum of a rabbit did not show any threadlike structures. However, after applying hematoxylin in the same region (b), the Bonghan duct (arrows) emerged near the sulcus. In addition, the Bonghan duct is stained by hematoxylin in the aqueduct and the third ventricle (c) and lifted using a needle to show that it was floating in the cerebrospinal fluid. The inset shows a wound state of the Bonghan duct specimen demonstrating its elastic nature and two Bonghan corpuscles (arrowheads). The scale bar of the inset is 60 μm. Image (d) shows a Bonghan duct (arrows) with a corpuscle (thick arrow) and a node (arrowhead). One end of the structure was cut at the front part of the third ventricle [19]. (D) The Bonghan duct in the central canal of the spinal cord. The Bonghan duct (arrows, a) and its magnified view (b) inside an opened central canal of the spinal cord of a rabbit without dye treatment under a stereomicroscope [19].

addition, the pattern inside the heart, expressed only through a diagram, would be like the latter (Figure 3(A)(a)).

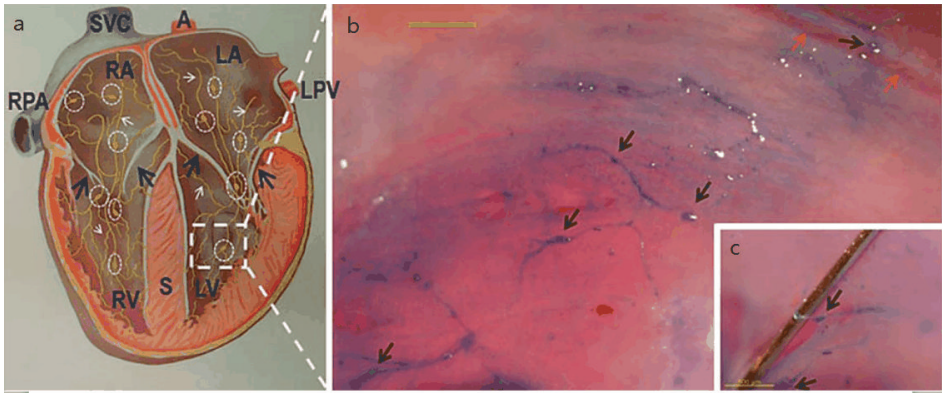
Second, a BD consists of a bundle of ductules (Figure 4(A)(a–d)). Each ductule has two layers, an endothelial layer and an outer membrane. These are totally surrounded by the other layer, the periductium. Generally, the diameter of a ductule reaches 5–15 μm, but it sometimes varies by 1–50 μm (Table 1). The BRT also showed a cross section of the internal BD under an electron microscope (Figure 4(A)(c)). The authors believe that the bundle structure is important to distinguish from other biological tissues and systems because the blood and lymphatic vessels consist of only one duct, and the nervous system and connective tissues are not a duct system.

Third, the three layers mainly consist of some kinds of cells, which have peculiar types of nuclei, respectively. Among them, the nuclei of the endothelial cells, 15–20 μm in length, take a rod shape with pointed ends and are more intensively stained than the others (Figure 4(A)(a-b, d)). These nuclei align longitudinally along the ductule. The authors guess that the shape of the nuclei and the type of arrangement within the endothelial layer is another distinct point between the BD and existing circulatory vessels. The endothelial cells of the blood and lymphatic vessels have somewhat rod-shaped nuclei but with blunt ends, and the nuclei align vertically as well as longitudinally through the vessels.

Meanwhile, the BC's form is oval or spindle-like (Figure 4(A)(e)) and is surrounded by one layer. Generally, the layer of biological tissues can consist of cells, fibers made of proteins, or a mixed form of them. The BRT, however, did not indicate the exact ingredients about the BC layer, just mentioning that there is a thin membrane made of connective tissues containing oval or spindle-like nuclei of cells for the neural BC in the third paper.

The Bonghan ductules inside the BC become enlarged and form sinuses, which become ramified and tangled. Around the sinuses, there are undifferentiated reticular tissues such as those of hemopoietic organs. The size of the BCs varies depending on their locations in the body (Table 1).

3.2. The Contents of the BD and BC in Three Bonghan Systems. According to BRT's first three papers, one of the most abundant elements inside the ductules is nucleic acid, that is, DNA and RNA. They are categorized by three types by the size: basophile granules, basophile structures of various shapes, and nucleus-like structures. Among them, in the fourth paper, spherical basophile granules are called "Sanals," meaning "live eggs" in Korean, whose center consists of abundant DNA surrounded by RNA, ranging from 1.2 to 1.5 μm in diameter. The BRT confirmed the presence of nucleic acids in the granules and structures inside not only the ductules but also the BC, by a Feulgen reaction and Brachet reaction (or Unna-Pappenheim reaction), which has been available for identifying DNA and RNA in a nucleus, respectively. In addition, the BRT used acridine orange for identifying the DNA elements inside the superficial Bonghan systems in the second paper (in the fourth paper, the BRT produced the content and base composition of DNA and RNA in the Sanals through a quantitative analysis). Another interesting discovery within the ductules is the presence



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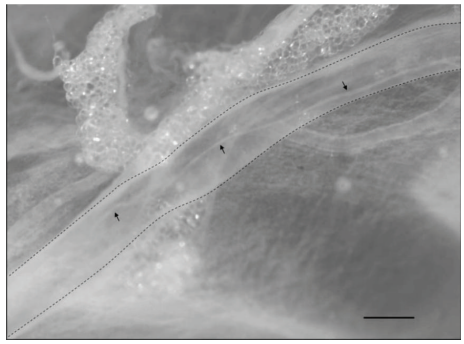

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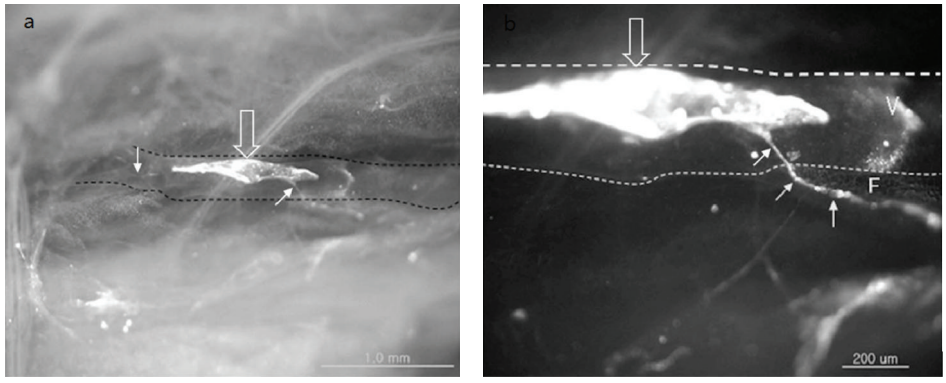
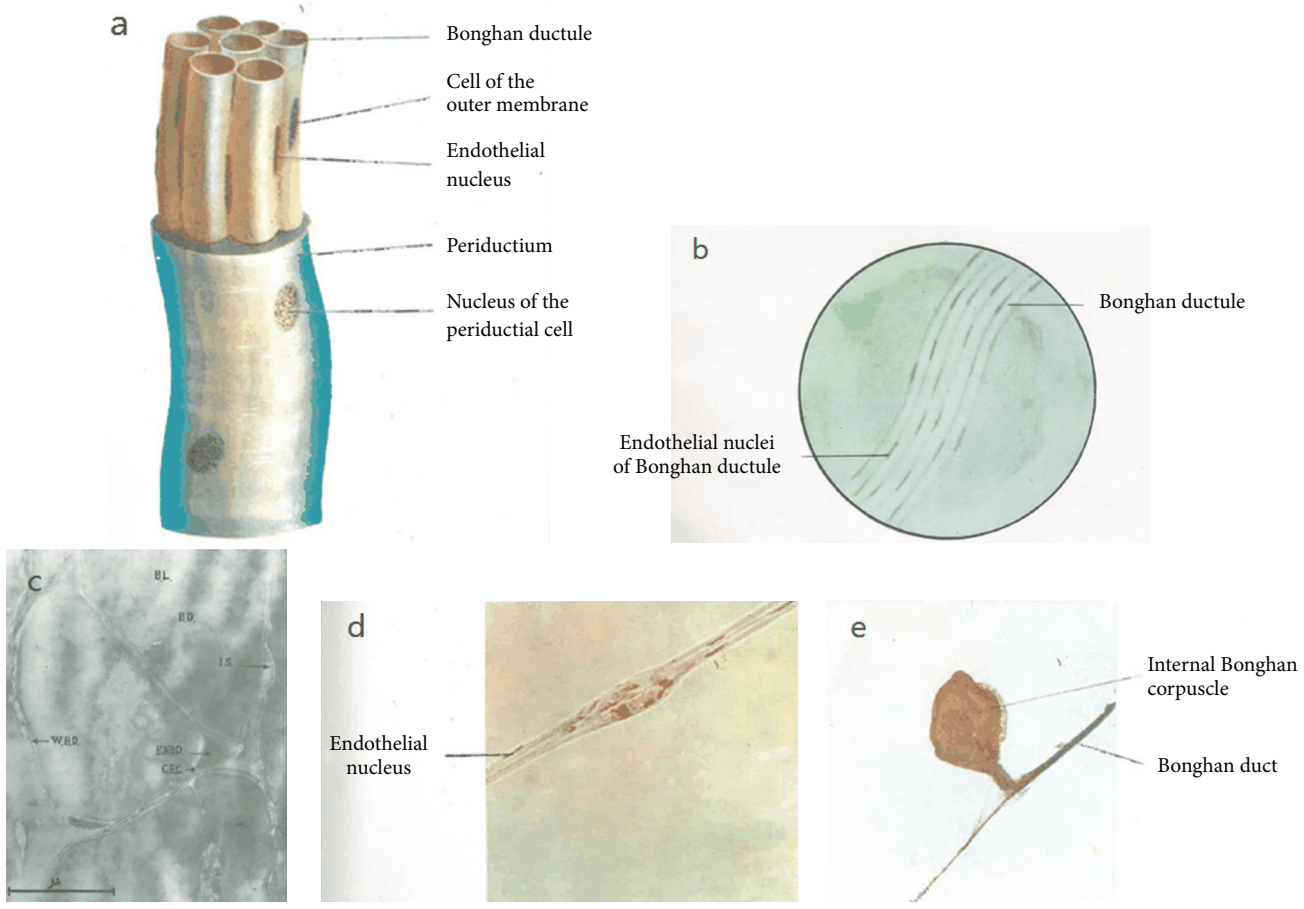


(C)

Figure 3: The internal Bonghan systems. (A) The Bonghan systems inside the heart stained by Trypan blue. Diagram from the third paper by the Bonghan research team (a), and photo images inside the bovine heart treating Trypan blue under a stereomicroscope by a modern research team (b and c). The Bonghan ducts (white arrows, a) and corpuscles (dotted circles) are indicated by the modern research team. SVC = superior vena cava; A = aorta; LA = left atrium; RA = right atrium; LPV = left pulmonary vein; RPA = right pulmonary artery; S = septum; LV = left ventricle; RV = right ventricle. The Bonghan corpuscles (black arrows, b) connected to the Bonghan ducts are stained blue, whereas the blood capillaries (red arrows, b) are not stained. The scale bar is 1 mm. The inset (c) demonstrates the lifting of a Bonghan duct using a needle showing the floating state of the Bonghan duct on the endocardium. The scale bar is 500 μm [20]. (B) The Bonghan duct in the lymphatic vessel without staining. The Bonghan duct (arrows) in the lymphatic vessel (dotted line) on the caudal vena cava of a rabbit under a stereomicroscope using halogen red light. The scale bar is 500 μm [21]. (C) The Bonghan duct in the lymphatic vessel stained by DiI. The Bonghan corpuscle (open arrow, a) and duct (arrows) in the lymphatic vessel (dotted line) on the caudal vena cava of a rabbit stained by DiI under a stereomicroscope. These are merges of bright-field and fluorescent images. A magnified view (b) shows that a Bonghan duct came out through the lymphatic vessel wall and entered the surrounding fat tissue (F). V is a valve weakly stained by DiI [21].



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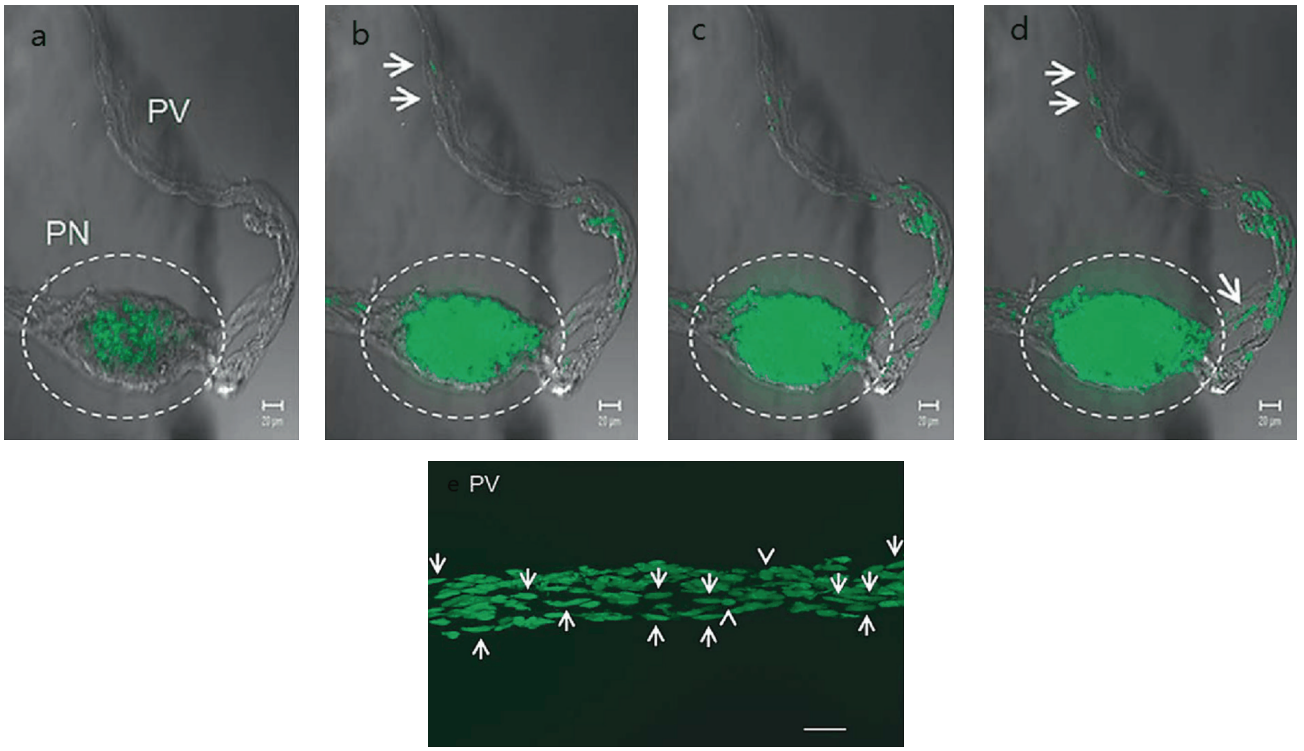

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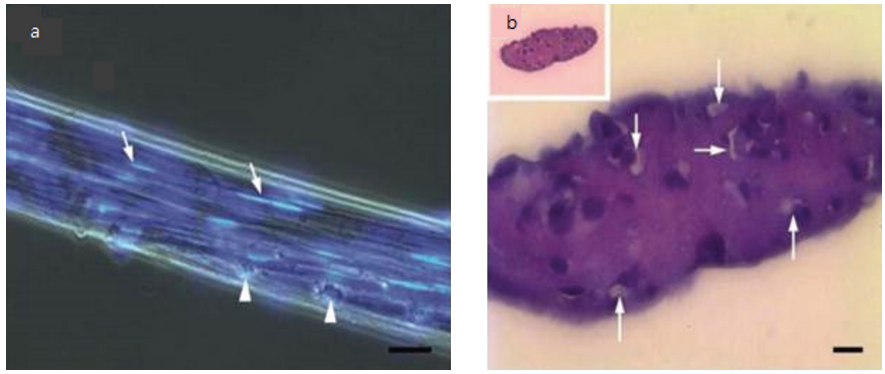


(C)

Figure 4: The structure of the Bonghan duct and corpuscle. (A) The structure of a Bonghan duct determined by the Bonghan research team. Diagram and photos showing the structure of the Bonghan duct and corpuscle. The Bonghan duct (a) consists of several subducts (Bonghan ductules) and three layers, and each layer has peculiar nuclei. Under a phase-contrast microscope, subducts and rod-shaped nuclei of endothelial cells (b) are observed. An electron micrograph of the internal BD (c) through a cross section (×42,000). BL = Bonghan liquor; BD = Bonghan ductule; IS = interstitial substance; WBD = wall of Bonghan ductule; ENBD = endothelial nucleus of Bonghan ductule; CEC = cytoplasm of endothelial cell. Photo image showing the rod-shaped nuclei of endothelial cells (d) from the intraexternal Bonghan duct stained using Feulgen reaction under a stereomicroscope (×400). Photo image (e) of the internal Bonghan duct and corpuscle (×63) [5]. (B) The Bonghan duct and corpuscle stained using acridine orange. The Bonghan duct (PV) and corpuscle (PV, dotted circle) inside the bovine heart stained by acridine orange under a confocal laser scanning microscope. The rod-shaped nuclei (arrows) are aligned longitudinally to the bundle of the Bonghan duct. Round nuclei (arrowheads, e) also exist. All scale bars are 20 μm [20]. (C) The structure of the Bonghan duct. Histological observation of the Bonghan duct, whole specimen and cross-sectioned. Photomicrograph (a) of merged phase-contrast and fluorescent image of a Bonghan duct, which shows the bundle of several ductules (arrows) with characteristic rod-shaped nuclei stained with DAPI (bright blue) and immune cells (arrowheads) on duct surface. The scale bar is 50 μm. Cross-sectioned Bonghan duct (b) also presents several ductules (arrows). The scale bar is 10 μm [22].

Table 1: The size of a Bonghan corpuscle and ductule determined by the Bonghan research team.

	Bonghan corpuscle			Bonghan ductule
	Internal	Intraexternal	Neural	
Size	0.1–0.2 mm	Connecting to two Bonghan ducts 0.3–1.0 × 0.1–0.5 mm	0.5–1.0 × 0.2–0.5 mm	Generally 5–15 μm
		Connecting to three or over Bonghan ducts 0.6–2.5 × 0.3–1.5 mm		Totally 1–50 μm

of chromaffin granules, which appeared to be a positive response of adrenalin reaction by Hillarp's method.

In the BCs, there are granules and structures consisting of nucleic acids and chromaffin granules. However, certain kinds of cells are observed in the BCs unlike in the ductules. One of them is the chromaffin cell, which has plenty of chromaffin granules inside. The existence of chromaffin granules and cells suggests that the Bonghan systems contain adrenomedullary hormones such as adrenalin and noradrenalin. In the third paper, the BRT reported that there are more adrenalin and noradrenalin than any other biological tissues and organs as a result of analyzing the liquor components of the Bonghan systems. Moreover, an adrenal cortical hormone like corticosteroid and a sex hormone like estrogen are found there. Another kind of cell, just observed in the internal BCs, is the family of hematopoietic cells such as myelopoietic and lymphogenetic cells in different stages of differentiation, that is, granulopoietic, monopoietic, erythrogenic, and lymphopoietic elements and megakaryocytes. Moreover, only in the internal BCs, there is a group of cells similar to the peculiar parenchymal cells of the organs. The types of the cells depend on the positions of the internal BCs. For example, in the internal BCs of the liver, there are cells similar to the liver parenchymal cells.

According to the third paper, hyaluronic acid, a kind of mucopolysaccharides, is another rich component inside the Bonghan systems. In general, it has been known that hyaluronic acid significantly contributes to cell proliferation and migration and distributes throughout connective, epithelial, and neural tissues. The BRT mentioned that there are more hyaluronic acids in the Bonghan systems compared to other biological tissues and organs but did not explain how they had come to notice this. The other materials contained in the Bonghan systems are biogenic elements for

Table 2: The experimental procedure for identifying and confirming the Bonghan systems.

Stage	Target	Checkpoint	Experimental methods	
			Stain	Microscopy
Identifying the BS				
From the other tissues	Inside the lymphatic vessel	—	—	Stereomicroscope with a red light
	On the surfaces of the organs	Preferentially stained	Trypan blue	Stereomicroscope, light microscope
	In the brain	Preferentially stained	Hematoxylin	Stereomicroscope
	Inside the lymphatic vessel and in the brain	Preferentially stained (especially from Reissner's fibers)	DiI	Stereomicroscope
	Inside the lymphatic vessel and on the brain	Inner fluid stained	Alcian blue	Stereomicroscope
From the artifacts	In the blood clots and the fascia	Preferentially stained	Chromium-hematoxylin	Stereomicroscope, light microscope
Confirming the BS	In the Bonghan duct	Ductules in a duct containing the rod-shaped nuclei of endothelial cells	Feulgen, acridine orange, and Janus Green B	Stereomicroscope, CLSM, SEM, and TEM
	In the Bonghan ductule	DNA stained	Feulgen, DAPI, and yoyo-1	Stereomicroscope, fluorescent microscope
	In the BS	Chromaffin substance stained	Chromium-hematoxylin	Light microscope

cell metabolism such as proteins, lipids, carbohydrates, free amino acids, and mononucleotides.

4. How to Distinguish the Bonghan Systems from the Surrounding Tissues

In general, Bonghan systems could be obtained from live animals. The BRT anaesthetized animals such as rabbits, rats, and mice and found these systems under living conditions. The authors conjecture that it may be very difficult to posthumously identify the Bonghan systems because they rapidly deteriorate through the postmortal changes. For example, the BDs inside the blood and lymphatic vessels may aggregate among themselves or stick on the vessels while the liquor flow in the vessels gradually stops. However, the sampling inside the heart inevitably needs to proceed after slaughter. In this case, the state of the heart should be prepared as freshly as possible [20]. In the case of the sampling for the brain Bonghan systems, it is generally possible to obtain them after decapitation, but the sample could be obtained after piercing holes and injecting some dyes into the head of an anaesthetized animal for minimizing the biological change brought by decapitation [23].

Although the BRT mentioned the sizes and shapes of the BDs and BCs, it would be hard to discriminate them from the surrounding tissues such as lymphatic capillaries and lymphoid nodules with similar sizes and semitransparency like threads and corpuscles. In fact, it has been pointed out for a long time in the academic fields that the MRTs as well as the BRT had misjudged the lymphatic systems as new structures. In addition, the fascia, thin membrane surrounding all organs in the body, is another biological tissue that makes it difficult to find the Bonghan systems, because the BDs and BCs are inserted into the semitransparent fascia exposing only some parts of them outside. For instance, the peritoneum, which is one of the serous membranes, consists of three layers: superficial fascia, deep fascia, and subserous fascia. In the case of the central nervous systems, the dura mater, arachnoid mater, and pia mater protect the brain and the spinal cord. The Bonghan systems are distributed through the interior and the surface of these kinds of fascia, as suggested in a MRT's model [24, 25]. Sometimes, the mesentery, a fold of the anterior and posterior walls of the peritoneum, looks like a threadlike structure under the naked eye or a stereomicroscopic view.

The BRT indicated that the anatomical/histological structures of the Bonghan systems are different from those of the lymphatic systems and the fascia but did not inform how to exactly recognize them. Therefore, the MRTs had to find the Bonghan systems developing its own specialized microscopic instruments and dyes.

One of the methods to distinguish Bonghan systems from the lymphatic systems would be to observe the former inside the latter simultaneously *in situ* through a microscope without any dyes. One MRT found a way to do so in the lymphatic vessel of rabbits (Figure 3(B)), using a stereomicroscope with a red light from a halogen light source [21]. Without that light, the lymphatic vessel under a stereomicroscope was observed to be almost transparent, containing nothing inside. This method cannot be applied for a blood vessel because of its red constituents (Table 2).

In addition, the MRTs developed some dyes selectively staining the Bonghan systems not only in the lymphatic

vessels but also in the fascia of the organs. A prime example is Trypan blue, which has been useful to identify the Bonghan systems on the surfaces of the organs such as the heart (Figure 3(A)), the intestine (Figure 1(B)), and the brain [16, 20, 23] under a stereomicroscope or a light microscope. Through the experimental processes, the MRTs concluded that Trypan blue could preferentially stain the Bonghan systems rather than the lymphatic vessels and the fascia as well as the blood vessels, nerves, and adipose tissues. However, the MRTs did not explain what the mechanism of staining was in the reports. Nevertheless, the fact that Trypan blue has been available for preferentially staining the Bonghan systems is interesting because it has been used to separate live cells from dead cells by staining the proteins of the latter in the biological fields. In general, Trypan blue has been known to not pass through the membranes of viable cells.

In addition, one MRT found that a dye, hematoxylin (Figure 2(C)), which forms salts with basophilic compounds such as DNA and RNA, could preferentially stain the BD in the brains of rabbits [19]. The BRT had used hematoxylin for staining the nuclei of endothelial layer's cells of the ductules in the third paper. The idea of the MRT came from the concept that the nucleic acids inside the Bonghan systems can also be discriminatively stained by hematoxylin. In the same experiment, the MRT was sometimes able to find the BD in the central canal of the spinal cord without hematoxylin, which was connected along the BD from the ventricles of the brain (Figure 2(D)).

The MRT developed another dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), to selectively reveal the membranes of the Bonghan systems. DiI is a fluorescent lipophilic dye and has often been used for tracing the nervous systems as they have lots of lipophilic components. The MRT conceived that DiI (Figure 3(C)) could stain the phospholipids of the membranes and successfully presented the Bonghan systems in the lymphatic vessels of rabbit [21] as well as rat [26] and brain [19] under a stereomicroscope. Some images stained by DiI were particularly notable because they showed that the BDs enter into the nearby tissues or organs by passing through the lymphatic wall.

Moreover, the MRTs paid attention to the mentions of the BRT in which there is more hyaluronic acid than any other biological tissues in the fluid inside the BDs. The MRTs discovered that Alcian blue, a dye used for staining some types of acidic mucopolysaccharides, could make the fluid of BD blue and clearly showed the stained BDs (Figure 2(B)) on the surface of the brain [18] and in the lymphatic vessels [21, 27] under a stereomicroscope by injection or spraying.

5. How to Distinguish the Bonghan Systems from the Potential Artifacts

5.1. Identifying Process in the Sampling Stage. The most common artifact in the sampling stage is made from blood clots. When the blood vessels are damaged during surgical processes, the biological self-defense mechanism forming thrombus immediately is activated in the body. Fibrins, threadlike proteins polymerized from fibrinogens, form a network trapping the blood cells. Therefore, it is difficult to distinguish the Bonghan systems from blood clots. This situation frequently comes along in finding Bonghan systems in the blood vessel systems including the heart. Thus, it would be practically reliable to get them in the relatively thick blood vessels such as the caudal vena cava, iliac vein, and thoracic aorta. The thrombus, however, forms right after incising the blood vessels during a surgery to find the Bonghan systems. Moreover, blood clots can be generated in the lymphatic vessels. For example, the threadlike structures removed from the lymphatic vessels could be a mixture of fibrins in the lymph with lymphocytes. However, it should be noted that the Bonghan systems would exist while mixed with the thrombus.

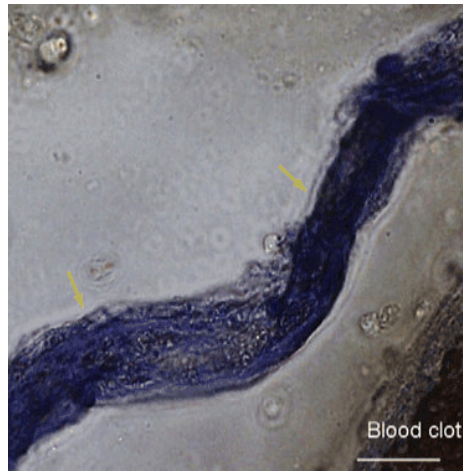


Figure 5: The Bonghan duct and blood clot stained using Cr-Hx. The Bonghan duct (arrows) in the venous sinuses of a rat brain protruded from a blood clot is stained blue by Cr-Hx under a light microscope. Cr-Hx nearly does not stain the blood clot. The scale bar is 12 μm [28].

Another typical artifact, particularly on the surface area of the visceral organs, is the threadlike structures generated from the fascia during dissection. Generally, the fascia contains not only cells such as fibroblasts and fibrocytes, but also fibers made of proteins such as collagen fibers, reticular fibers, and elastic fibers. If one incises the fascia to find the Bonghan systems on the surfaces of the organs, they can usually observe a threadlike mass around the incision sites due to the aggregation of cells and fibers.

Beyond those artifacts, are there any reliable methods to identify the Bonghan systems? One MRT thought that the Bonghan systems can be preferentially stained by some dyes rather than the artifacts, which have no nuclei. One of the dyes discovered by the MRT was chromium-hematoxylin (Cr-Hx), which strongly stains chromaffin granules or cells as well as acid materials such as nucleic acids. The MRT found that Cr-Hx could preferentially stain the Bonghan systems compared to blood clots (Figure 5) under a light microscope [28]. The authors think that treating Cr-Hx is available in

the experiment sites not only for identifying the Bonghan systems in blood clots, but also for ignoring something with no staining such as blood clots without Bonghan systems and the fascia fibers made of proteins. In addition, as mentioned above, Trypan blue and DiI are useful for distinguishing Bonghan systems from the fascia.

5.2. Confirming the Presence of the Ductules and Rod-Shaped Nuclei. Although one can observe the Bonghan systems using some specific microscopes and staining suggested by the MRTs, the possibility of causing artifacts still remains in the process of obtaining "pure" systems. One of the reasons for this is that it is difficult to adjust the proper incubation time of the dyes. When the incubation time for staining is too long, nearby biological tissues can be contaminated in addition to the Bonghan systems. In this case, one could get the wrong tissues, for example, the threadlike and corpuscle structures made by fibers and fibroblasts of the fascia. In addition, it is possible that the chemical reactions between some dyes and existing vessels could bring about unsuspected byproducts. For example, uncertain threadlike structures can be formed owing to the chemical reactions between the dyes and inner walls of the blood or lymphatic vessels.

To isolate the pure Bonghan systems over such artifacts, it is necessary to first confirm whether the threadlike structures of the sample have ductules containing rod-shaped nuclei. The BRT showed several visual images of the ductules containing the rod-shaped nuclei with pointed ends of endothelial cells through the diagrams, the photos under a phase-contrast microscope, and the photos stained by some dyes under a stereomicroscope (Figure 4(A)(a–d)). The BRT mainly used a Feulgen reaction and hematoxylin to stain the nuclei of endothelial cells.

The MRTs also showed rod-shaped nuclei with pointed ends within the bundle by an advanced instrument, a confocal laser scanning microscope (CLSM), presenting high-resolution images from selected depths. The MRTs observed the Bonghan systems using staining methods such as a Feulgen reaction [29], acridine orange [20, 21, 30], and Janus Green B [31] treatment. The photo images (Figures 4(B) and 4(C)) show that the BD is different from the blood or lymphatic vessels because there are ductules forming a bundle and the rod-shaped nuclei inside aligned longitudinally through the ductules. The existence of rod-shaped nuclei also implies that this structure is not a thrombus formed from the aggregation of fibrins or fascia fibers with blood cells, not only because there is no nucleus in the red blood cells and platelets, but also because the shapes of the white blood cells are round or irregular. On the other hand, the MRTs presented the lumens or sinuses inside the BD [17, 18, 20, 22, 31] under several kinds of electron microscopes such as a scanning electron microscope (SEM), cryo-SEM, focused-ion-beam-SEM, and transmission electron microscope (TEM). In addition to the above criteria for confirming real primo vessels, it would be much more reliable to consider that primo vessels should be distinguished from inflammatory substance as Wang et al. suggested [32].

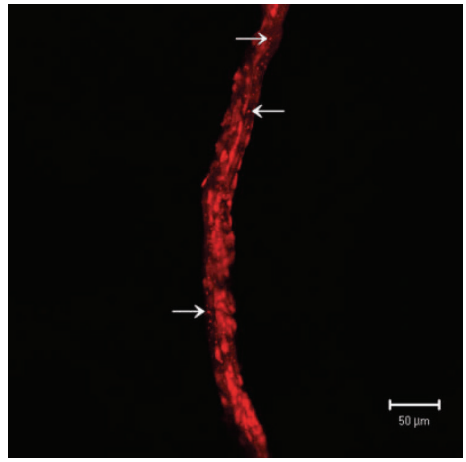
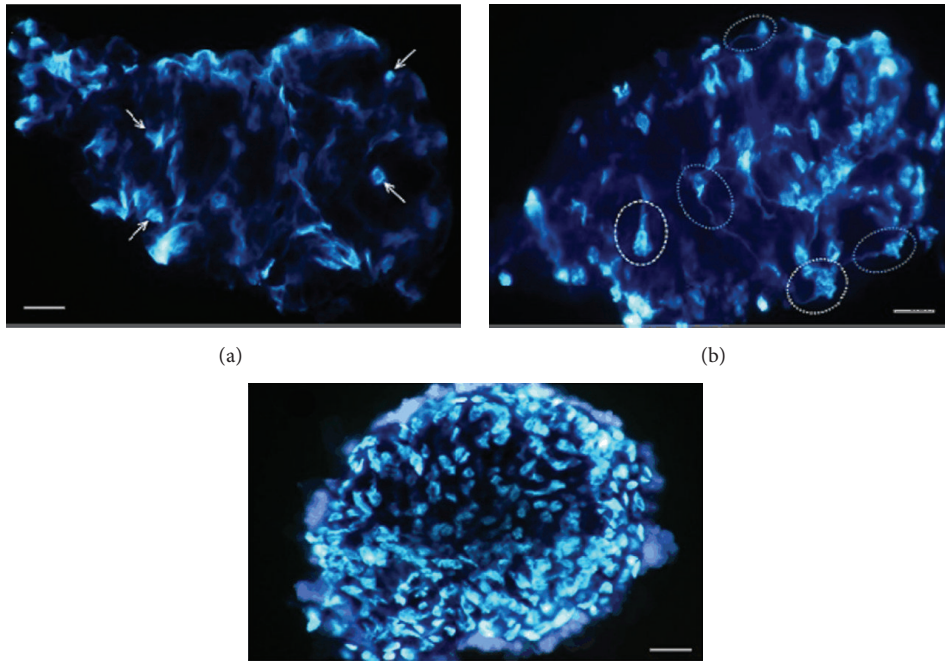


Figure 6: The DNA substances inside the Bonghan duct stained through a Feulgen reaction. The 1-2 μm granules (arrows) revealing the DNA content inside the subducts of a Bonghan duct at a rabbit's abdomen surface, stained using a Feulgen reaction under a stereomicroscope [29].

5.3. Confirming the Presence of the DNA and Chromaffin Substances inside the Bonghan Systems. The MRTs have found some types of DNA components in the Bonghan systems using a qualitative analysis. For example, one MRT noticed granules of 1-2 μm in size in the subducts in the BD of the abdomen surfaces of rabbits (Figure 6) using a Feulgen reaction [29]. In addition, inside the BC of the rats' brain and spine, another MRT [18] observed three types of extracellular DNA (eDNA) stained by 4′,6-diamidino-2-phenylindole (DAPI), a fluorescent dye binding DNA, under a fluorescence microscope (Figure 7). This observation implies that the DNA substance inside the BC can transform into nucleus-like structures.

The existence of DNA components, inside the Bonghan systems as well as in the endothelial cells of the ductules, means that the staining methods for nuclei can distinguish Reissner's fibers, the glycoprotein fibrous structures from the subcommissural organ through inside the ventricles and central canal of the spinal cord, from the Bonghan systems. Because they are found in the central nervous systems of most vertebrates, their main function not being well known, they could be confused with the Bonghan systems during the experiments. They mainly consist of carbohydrates and proteins without cells. The MRT showed that the structures they had found were well stained with dyes for nuclei, such as DAPI and yoyo-1, and confirmed that they were not Reissner's fibers. In addition, another MRT observed that the structures were well stained by DiI. Because Reissner's fibers have no cell membranes, they are not stained by DiI [19].

The presence of chromaffin substance would be proved because one MRT clearly stained the BD and BC by Cr-Hx [28], which stains the chromaffin granules or cells, as mentioned above. In addition, the MRTs reported the presence of cells of a mixed type with adrenalin and noradrenalin [33] or chromaffin cells [34] on the surface of the mammals' abdomen organs in the BCs.



(a) (b)

(c)

Figure 7: The dynamics of DNA substances inside a Bonghan corpuscle stained using DAPI. Three kinds of extracellular DNA (eDNA) in the BC above the pia mater of a rat brain and spine, stained using DAPI under a stereomicroscope. The BC has eDNA with rare nucleus-like forms (arrows, a). Most eDNA images appear amorphous. The BC has many nucleus-like forms (dotted circles, b) connected to threadlike structures. The eDNA images show normal nuclei (c). The scale bars are 25 μm, 28 μm, and 29 μm, respectively [18].

6. Conclusions

Although the BRT claimed that it found the anatomical/histological structures corresponding to acupuncture points and meridian pathways referring to the knowledge of Korean Medicine [1, 2], it is still unclear whether BCs and BDs are really the parts of meridian systems or not [35]. In fact, the BRT showed only a few examples for meridian systems associated with the structures in the skin it found in the first paper. Besides, the BRT reported that the distribution of the structures coincides with acupuncture points indicated in *Dongeuibogam* "as a whole," not concretely. The BRT even said that there are some more acupuncture points not reported before.

If the MRTs including the researchers on Korean Medicine succeeded in finding the BCs which the BRT reported in the skin, the relationship between the Bonghan systems and the meridian systems would be clearly determined. However, until recently, the Bonghan systems in the skin have not reported in the academic fields.

We guess that one of the ways to find the superficial BCs is the backtracking method. For example, one could trace the structures connected from the intraexternal Bonghan system to near the skin using some dyes or radioisotopes. To do this, the basic step is to identify Bonghan systems correctly without any artifacts in the experimental process. We also think that the relationship itself between the traditional acupuncture points and the BCs is not the critical problem for Korean Medicine. Instead, the idea of the close relationship among four Bonghan systems in the body is more meaningful to the fields of diagnosis and cure for Korean Medicine [1, 36], which has almost the same concept with the BRT's.

Although the BRT described in detail the characteristics of the Bonghan systems through its first three papers, it has not been repeatedly possible to find them in animals. The main reason seems that the Bonghan systems usually exist within the existing biological tissues or organs, that is, inside the blood vessels, the lymphatic vessels, the heart, the ventricles, and the central canal of the spinal cord. Some parts of them appear on the surface of the organs such as the intestines and brains, but many of them exist in the fascia. No one has been able to easily find these new structures, which have not been reported in the academic fields. Another reason is the possibility of artifacts during the surgery process. One can be confronted with several kinds of artifacts caused by physical damage or chemical reaction with the dyes, from the artifacts themselves such as blood clots to the mixed forms of the Bonghan systems, with artifacts such as the BDs surrounded by erythrocytes or lymphocytes in the vascular and nervous systems. Even the BDs connected to the corpuscle-like blood clots can be found. To escape from such difficulties, the authors tried to present the main methods for identifying the Bonghan systems more easily in this document, comparing the works of the MRTs to those of the BRT with the visual materials.

As for the three Bonghan systems, the authors have confirmed that the MRTs can find most of the basic anatomical/histological structures and inner biochemical components corresponding to the research results of the BRT. However, until recently, many things reported by the BRT can be found only in part or not at all. For instance, the Bonghan systems in the peripheral nervous systems and the RNA substance inside the BD or BC have not been reported. In addition, the MRTs found some kinds of cells, which partly resembled the cells from the BRT paper inside the internal Bonghan systems, such as immune cells and hematopoietic cells through a TEM observation or immunohistochemical analysis [13, 14].

In addition, the point most stressed by the BRT is the function of the DNA granules, Sanals, which grow into new cells and are generated from the existing cells flowing through the entire body. The BRT insisted that the circulation of the liquor containing Sanals be confirmed through the dosimetry of radioactivity and microradioautography [4–7]. Although one MRT [17] partly showed that the liquor in the BD flows in one direction (Figure 1(C)), a presentation regarding the circulation of Sanals and liquor still remains an important problem to be proved. To examine the structures and functions of the Bonghan systems more deeply, we suggest that the first step for exactly identifying the Bonghan systems free from artifacts at the experimental sites is critical. In addition, we recommend that, for data consistency, one should use and show the same samples taken from the Bonghan systems through all of the processes.

Conflict of Interests

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

Acknowledgment

This study was supported by Korea Atomic Energy Research Institute.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 379268, 11 pages
http://dx.doi.org/10.1155/2015/379268

Review Article

Intercultural Usage of Mori Folium: Comparison Review from a Korean Medical Perspective

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Received 26 June 2015; Revised 8 August 2015; Accepted 9 August 2015

Academic Editor: Rainer W. Bussmann

Objectives. A review on studies related to the use of Mori folium, the leaves of *Morus alba*, was conducted with the goal of identifying new clinical applications in Korean medicine. *Methods*. Global literature search was conducted using three electronic databases up to January 2015 with the term *Morus alba* and its Korean terms. KM literatures including textbooks and standard pharmacopoeia were separately hand-searched and reviewed to provide comparison. Data were extracted according to predetermined criteria, and clinical uses were standardized with ICD-10 categories. *Results*. 159 potentially relevant studies were identified, and 18 articles including 12 ethnopharmacologic and 6 clinical studies were finally included in this analysis. Ethnopharmacologic studies from 8 countries provided 17 clinical uses. We found that five out of six clinical trials were related to diabetes and suggested a moderate short-term to mild long-term effect. And 43 Korean texts also provided 156 clinical uses in 35 categories including ocular and respiratory disorders. *Discussion and Conclusions*. Though majority of the clinical uses were also found in Korean medicine literature, treatment of infertility, jaundice, cognitive disorder, and hyperpigmentation was found to be effective and diabetes with *Morus alba* was recognized to have clinical importance.

1. Introduction

Morus alba Linne is a deciduous tree that belongs to the Moraceae family. Mulberry trees are usually grown throughout the world including Korea and have been used in many ways in traditional medicine for a long time [1].

Mori folium is also used agriculturally for feeding the silkworms, and in many countries like Turkey and Greece, Mori fructus has a role in food supply [2]. In Asia, Africa, Europe, and South and North America, *Morus alba* is widely used to treat diseases for its antimicrobial and antioxidant properties [3, 4], however, mainly for antidiabetic, immunomodulatory, antimicrobial, antioxidant, and anticancer purposes [5]. The major phytochemical component of Mori folium is rutin, and Mulberry leaves are known to have antipyretic, antitussive, anti-inflammatory, and hepatoprotective effects [6]. Mori folium is also frequently used in Korea for treating common cold, cough, headaches, and red swollen eyes [6]. Korea has a dual medical system of conventional Western medicine and indigenously developed traditional Korean medicine (KM) and tries to combine these for more integrative medical services [7]. Traditional Korean medicine has a clinical experience of more than five thousand years and has influence of traditional Chinese medicine with more emphasis on Person-Centered Medicine [8]. A 2014 nationwide survey by the Korean Ministry of Health and Welfare found that 27.1% of the general respondents received KM treatment within the past 3 years, and 68.8% were found to have intention to use KM for treatment [9]. Considering that Mori folium is an abundant, economical, and versatile herbal resource, expanding its clinical application range in the KM system would therefore be beneficial both economically and

medically. There is a difference in applications of Mori folium in KM and other medical systems, but studies addressing this gap are not available yet.

The current study thus aims to identify potential clinical areas of additional use of Mori folium in KM practice by reviewing ethnopharmacologic and clinical literature from other medical systems and comparing the results with a narrative review of Korean medical literatures. The specific procedures and methods are described in the following section.

2. Materials and Methods

2.1. Search Strategy and Data Sources. Three electronic databases including PubMed (http://www.ncbi.nlm.nih.gov/pubmed/), ScienceDirect (http://www.sciencedirect.com/), and Korean studies Information Service System (KISS, http://kiss.kstudy.com/) were searched for materials made available up to January 2015. The keywords entered were "*Morus alba*" in all databases and the Korean terms of Mori folium and *Morus alba* in KISS.

Herbs are usually administered as a multiherbal prescription and seldom used as single herb in KM. Assessment of full texts provided only one Korean ethnopharmacologic study of Mori folium in single herb. Since this was deemed insufficient to serve as a basis for comparison, a narrative review on effect of Mori folium in KM was separately performed.

2.2. Article Selection and Data Extraction

2.2.1. Inclusion and Exclusion Criteria. Ethnopharmacologic studies providing uses and effects attributed specifically to Mori folium alone were included, but multiherbal prescriptions were excluded. Studies providing secondhand information only were excluded to avoid duplication.

Clinical studies on isolated substances from Mori folium were excluded for this review since they cannot represent the whole range of substances present in the material as used in KM practice. Research on Mori folium products with certain fortified substances was included only when new substances were not included. Case reports were excluded since they were deemed not to represent usage. Other disputes were settled through consensus among authors.

Out of 3,421 articles identified in the search process, one KM ethnopharmacologic field study was analyzed separately in the KM narrative review, and 18 articles were included in this review.

2.2.2. Data Extraction. All articles were independently reviewed by three reviewers (Jeon, Lim, and Joh) and data was extracted by predefined criteria (see Table 1). These criteria were modified from previous reviews on ethnopharmacology and/or clinical trials of similar nature [10, 11]. Due to disparities in description of quality and cultural context, uses were standardized and coded as the closest matching category listed on the 10th International Classification of Diseases (ICD-10) [12]. Only firsthand data was used from each study.

2.2.3. Methodological Quality Assessment. There is currently no validated tool for assessing ethnopharmacologic research quality and methodological quality of clinical studies was assessed with JADAD scale [13]. The JADAD scale has been used for assessing the methodological quality of randomized controlled clinical trials depending on its description of randomization, blinding, and others with score range of 0 to 5 [13]. Studies with 3–5 points were regarded as good methodological quality and 0–2 points were poor methodological quality.

2.2.4. Data Analysis. The data analysis was conducted using the following process. Clinical uses and nationality or cultural origin of research subjects in ethnopharmacologic studies were analyzed and summarized. Clinical trials were first grouped according to ICD-10 subchapters of usage. Subsequently, exploration of the significant factors for the clinical characteristics of each group was conducted.

2.3. KM Literature Review. A literature review was separately performed as previously mentioned in Section 2.1. KM pharmacology textbooks currently used in one or more colleges and 10 classics recognized by Korean Food and Drug Administration as standard pharmacopoeias were searched for uses of Mori folium. Literature from the previous database search containing first- or secondhand information on KM use of Mori folium, including one ethnopharmacologic field study, was also retrieved for review in this part. Furthermore, the references in all the located articles were manually searched for additional relevant article search. Data extraction was performed according to the predefined criteria in Table 1, with the exception of originating culture since all pertained to KM.

Clinical usage data attributed to Mori folium were extracted, but effects of multiherbal prescriptions were excluded. To preserve the cultural intonations of the data, uses were first standardized into 2nd Korean Standard Classification of Diseases-Oriental Medicine (KCD-OM2) codes which employ KM diagnostic terminology and then translated into pertaining ICD-10 category codes based on the diagnosis matching chart of KCD-OM2. Closest matches were used in both coding steps. The same ICD-10 categories appearing multiple times in a single text were counted as one use.

3. Results

3421 potentially relevant articles for the global literature review were found on initial search of databases. Figure 1 shows the data extraction process, and the results are summarized in Tables 2 and 3. The detailed analyses were presented in the following sections.

3.1. Ethnopharmacologic Research. 17 records of clinical uses in 13 categories were found from 12 non-Korean ethnopharmacologic studies with 8 cultural origins. Table 2 provides a summary for these.

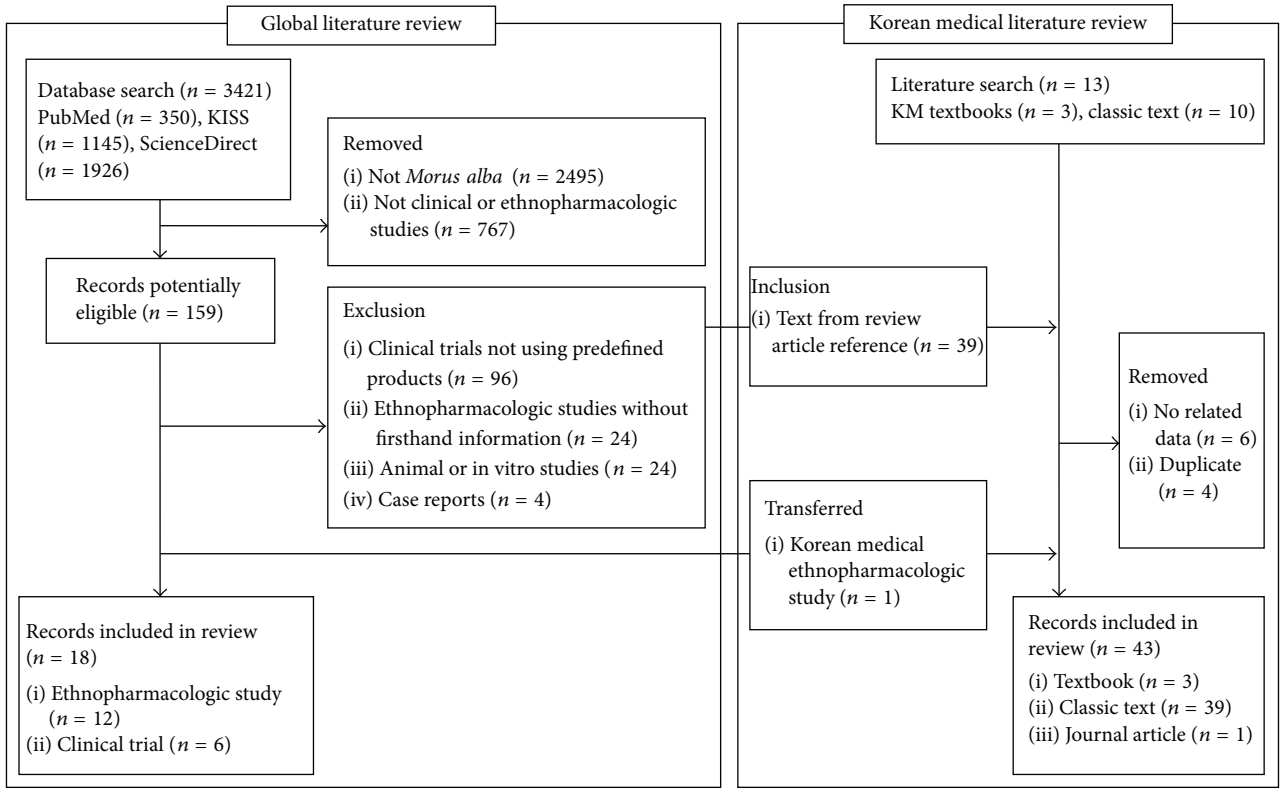

Figure 1: Data extraction process of the current review.

Table 1: Predefined criteria for data extraction.

Ethnopharmacologic studies				
Study & text details	Uses[†]	Culture or nationality		
ID (e.g., first author) Year of publication	Disorder treatment Symptom treatment Preventive purposes Tonic purposes	Nationality of subjects		
Clinical studies				
Study details	Uses	Population	Material	Outcome measures & results
ID (e.g., first author) Year of publication Study design Research quality	Disorder treatment Symptom treatment Preventive purposes Tonic purposes	Diagnosis Clinical setting Sample sizes (cases and controls or cohort size, including recruitment success rates) Other important participant characteristics	Material used (intervention & control) Dosage Administration schedule	Primary outcome measures Laboratory results Questionnaire scores Physical symptoms Incidence rates of given event within population

[†]Only single use of Mori folium herb was retrieved.

Mori folium was used in multiple studies for respiratory tract disorders at Pakistan. It was used in 3 instances such as an expectorant and sore throat treatment [14] and to relieve cough due to throat pain [15]. Two studies described the clinical use for ocular disorders including relieving effect on sore and inflamed eyes [14] and blindness treatment [16]. Substances in Mori folium such as rutin, choline, and folic acid have anti-inflammatory effects which might be a reason for these clinical effects [6].

Other uses include dizziness and vertigo treatment, antipyretic, analgesic [14], antivenom [17], antihypertensive [18], anti-infertility [19], antidiabetic [20], anticancer [21], reducer [22], neuritis treatment [23], jaundice treatment [24], and hyperpigmentation treatment [25]. Chlorogenic acid has neuroprotective functions which might be in play [26]. Eugenol is a local antiseptic and anaesthetic [27] and has been demonstrated to have anticancer activities against certain human cancer cell lines in vitro and in vivo [28]. Chlorogenic

Table 2: Ethnopharmacologic studies on Mori folium.

Author (year)	Relevant ICD-10 category	Description of use in original article	Culture/nationality
Menale and Muoio (2014) [18]	I10 essential (primary) hypertension	High blood pressure	Italy
Bibi et al. (2014) [15]	R05 cough	The leaves were boiled in water (Joshanda) and given for cough due to throat pain	Pakistan
Ullah et al. (2013) [14]	J40 bronchitis, not specified as acute or chronic R50 fever H57 other disorders of eye and adnexa J03 acute tonsillitis R51 headache R42 dizziness and giddiness	White mulberry leaves are expectorant, encouraging the loosing and coughing up of catarrh, and are prescribed in China as a treatment for coughs. The leaves are also taken to treat fever, sore and inflamed eyes, sore throats, headache, dizziness, and vertigo	Pakistan
Tetik et al. (2013) [22]	n/a	Reducing[†]	Turkey
Gakuya et al. (2013) [16]	H54 visual impairment including blindness	Blindness	Kenya
Oliveira et al. (2012) [21]	C80 malignant neoplasm, without specification of site	Cancer	Brazil
Sathiyaraj et al. (2012) [19]	N46 male infertility[††]	Anti-infertility	India
Sharma et al. (2012) [24]	R17 unspecified jaundice	Leaf juice mixed with one cup of curd is given once a day till the patient is cured	India
Adhikari et al. (2008) [25]	L81 other disorders of pigmentation	Hyperpigmentation	Nepal
Au et al. (2008) [23]	M79 other soft tissue disorders	Neuritis	China
Arýkan et al. (2009) [20]	E10 type 1 diabetes mellitus	Type 1 diabetes in children	Turkey
Samy et al. (2008) [17]	T63 toxic effect of contact with venomous animals	Snakebite treatment	India

[†]It might refer to weight reduction, fetal reduction, and use in conjunction with orthopedic reduction, fever reduction, or cicatrizer.
[††]Gender is not explicitly mentioned, but study describes male infertility.

acid plays a role in cancer prevention and protection in animal models [29], possibly through increasing DNA repair rates. Folic acid is an important substance in the reproductive process of both men and women [30]. Folic acid also plays a controversial role in cancer patients; diets high in folate are associated with decreased risk of colorectal cancer [31], but the quickly multiplying cancer cells require folate for growth and reducing its availability to cancer cells is a major pathway in mechanisms of commonly used chemotherapy agents such as methotrexate. The actions of these substances might provide at least some parts of medicinal effects of Mori folium in humans.

3.2. Clinical Trials. Six clinical trials within predetermined criteria were found and had small or moderate sample size. Five studies were on diabetes or postprandial glucose suppression, and one study was on cognitive function. Table 3 provides a summary of these clinical trials. Various Mori folium-derived products were used around the globe in herbal supplements for blood glucose management, and the present results reflected this fact. The key active substance for this application is the glucose analogue 1-deoxynojirimycin (DNJ). It is the most abundant iminosugar in Mori folium and is an α-glycosidase inhibitor [32]. HPLC fluorescent detection of DNJ in Mori folium from *Morus alba* L. showed the content to be at 0.12% [33]. Most of the studies attempted to use materials with higher concentrations of DNJ, ranging from 0.36% to 1.5% of dry weight. Single dose studies for postprandial glucose suppression had more robust results, while long-term studies on plasma glucose provided weaker results.

The single study on cognitive function was a randomized clinical trial with poor methodological quality. It suggested a possible improvement of cognition in mild cognitive impairment patients [34], but the small number of subjects and poor methodological quality made the evidence weak.

3.3. Korean Medical Literature Review. One ethnopharmacologic study provided a record of treatment of sore throat [35]. 40 classics and contemporary texts retrieved from the references of a literature review on Mori folium provided the majority of clinical uses [36]. 156 mentions of clinical uses in 35 ICD-10 categories were found from 43 texts. The most prevalent uses were for ocular disorders ($n = 33$), respiratory disorders ($n = 24$), analgesic purposes ($n = 16$), treating excessive sweating ($n = 15$), joint disorders ($n = 11$), and gastrointestinal tract disorders ($n = 11$). Table 4 provides a summary of the literature review.

4. Discussion

The current study examined global and Korean literature on medical use of Mori folium to discover additional clinical applications in traditional Korean medicine. In traditional

Table 3: Clinical trials using Mori folium or derived products.

Author & year	Study type	Condition & ICD-10	Participants & sample size	Material used	Dosage & duration	JADAD score	Primary outcome measures	Main results
Banu et al. (2015) [41]	CC	Type 2 diabetes (E11)	48 type 2 diabetics on oral medication (28 intervention + 20 black tea placebo)	Tea (*Mulbericha green* variety)	1 cup (70 mL), single dose	—	Plasma glucose change before meal with tea and 90 minutes afterwards	Plasma glucose levels in control and intervention groups before meal and 90 minutes after being changed from 178.55 ± 35.61 to 287.20 ± 56.37 (control) and 153.50 ± 48.10 to 210.21 ± 58.73 (intervention) ($p < 0.001$, effect size 1.31)
Chung et al. (2013) [42]	RCT	Glucose suppression (n/a)	50 healthy people between ages 20 and 50 (10 + 10 + 10 + 10 + 10)†	Aqueous extract (0.36% DNJ)	0 g, 1.25 g, 2.5 g, and 5 g extract, single dose	3 (1 + 0 + 1 + 0 + 1)	Plasma glucose level at 0, 15, 30, 60, 90, and 120 minutes after maltose intake	Intake of 2.5 g or 5 g with maltose suppressed glucose elevation significantly compared to control ($p < 0.05$). 5 g before and after treatment showed no significant difference.
Asai et al. (2011) [32]	RCT + CR††	Diabetes NOS (E14)	76 adults with 110–140 mg/dL FPG (38 intervention + 38 placebo)	Enriched extract (1.5% DNJ)	6 mg DNJ equivalent extract, t.i.d., 12 weeks	4 (1 + 0 + 1 + 1 + 1)	Fasting plasma glucose, insulin, HbA1c, glycated albumin, 1,5-anhydroglucitol at weeks 0, 4, 8, 12, and 16	No significant difference was found between groups except for 1, 5 AG at weeks 8 & 12 ($p < 0.05$), but difference was not maintained after treatment at week 16.
Srichaikul (2012) [34]	RCT	Other mental disorders (F06)	20 women with mild cognitive impairment (5 + 5 + 5 + 5)†††	Extract (*Burirum-60* variety)	200 mg, q.d., 3 months	2 (1 + 0 + 1 + 0 + 0)	SAGE, MMSE scale score changes at start and end of treatment period	Mean SAGE score rank changed from 14.1, 12.7, 7.3, and 7.9 to 17.70, 8.7, 11.0, and 4.6. Mean MMSE score rank changed from 17.4, 10.5, 6.7, and 7.4 to 17.6, 10.1, 8.5, and 5.8.
Nakamura et al. (2009) [43]	CR	Glucose suppression (n/a)	10 healthy volunteers	Ethanol extract (0.77% DNJ)	1.2 or 3 g, single dose	—	Plasma glucose (Glu.) & insulin (Ins.) (every 30 minutes for 3 hours)	Glucose and insulin elevation was suppressed in 1.2 and 3 g group compared to control at different time points (1.2 g Glu.: 30, 120 min/Ins.: 30 min/3 g Glu.: 30.90, 120 min/Ins.: 30 min/all $p < 0.05$)
Yang and Han (2006) [44]	RCT	Type 2 diabetes (E11)	25 type 2 diabetics on oral medication (14 intervention + 9 control)	Aqueous extract (0.5% DNJ)	500 mg b.i.d., 12 weeks	4 (1 + 0 + 1 + 1 + 1)	Fasting plasma glucose, HbA1c, serum lipids at start and end of treatment period	HbA1c, LDL-C, and TG decreased in intervention compared to control ($p < 0.05$, 0.05, 0.01). FBS >140 mg/dL or HbA1c >8% subjects showed FBS or HbA1c decrease (<0.05, <0.05) but <140 mg/dL or <8% subjects did not.

RCT, randomized controlled trial; CR, crossover; CC, case-controlled. All medications were taken orally.

†Group 1: 0 g extract with 75 g maltose. Group 2: 1.25 g extract with 75 g maltose. Group 3: 2.5 g extract with 75 g maltose. Group 4: 5 g extract with 75 g maltose. Group 5: 5 g extract 30 minutes before 75 g maltose. Group 5 cannot be considered as part of double-blind design.

††Dual phase design; only the RCT part is analyzed in this table.

†††Group 1: silkworm weavers given Mori folium extracts (SWE). Group 2: silkworm weavers given placebo (SWP). Group 3: nonsilkworm weavers given Mori folium extracts (NSWE). Group 4: nonsilkworm weavers given placebo (NSWP).

Table 4: Korean medical literature on Mori folium.

Author	Published	Name of the book	Relevant ICD-10 category
Unknown	0th–2nd	*Divine Agrarian's Canon of Materia Medica*	R50 fever of other and unknown origin
Ge Hong	3rd	*Eating Your Way to Immortality*	A09 other gastroenteritis and colitis of infectious and unspecified origin T14 injury of unspecified body region
Tao Hongjing	2nd-3rd	*Supplementary Records of Famous Physicians*	T63 toxic effect of contact with venomous animals
Su Jing	7th	*Newly Revised Materia Medica*	R52 pain, not elsewhere classified K59 other functional intestinal disorders
Meng Shen	7-8th	*Materia Medica for Successful Dietary Therapy*	R63 symptoms and signs concerning food and fluid intake
Chen Zangqi	8th	*Chen Zangqi's Materia Medica*	K52 other noninfective gastroenteritis and colitis T14 injury of unspecified body region
Cao Beng	8th	*Four Tones Materia Medica*	K52 other noninfective gastroenteritis and colitis
Rihuazi	10th	*Materia Medica by Rihuazi*	M25 other joint disorders, not elsewhere classified M06 other rheumatoid arthritis R52 pain, not elsewhere classified
Unknown	10–13th	*Illustrated Canon of Materia Medica*	M06 other rheumatoid arthritis
Unknown	14th	*Dan Xi's Words*	R61 hyperhidrosis
Yu Hyo-Tong	15th	*Compilation of Native Korean Prescriptions*	N61 inflammatory disorders of breast G24 dystonia K29 gastritis and duodenitis T30 burn and corrosion, body region unspecified
Liu Wentai	16th	*Collection of the Essential Medical Herbs of Materia Medica*	A09 other gastroenteritis and colitis of infectious and unspecified origin T14 injury of unspecified body region T63 toxic effect of contact with venomous animals
Chén Jiä-Mó	16th	*Coverage of the Materia Medica*	H04 disorders of lacrimal system T63 toxic effect of contact with venomous animals R52 pain, not elsewhere classified M25 other joint disorders, not elsewhere classified K52 other noninfective gastroenteritis and colitis M06 other rheumatoid arthritis T14 injury of unspecified body region
Li Chan	16th	*Introduction to Medicine*	H04 disorders of lacrimal system T63 toxic effect of contact with venomous animals R52 pain, not elsewhere classified M25 other joint disorders, not elsewhere classified K52 other noninfective gastroenteritis and colitis M06 other rheumatoid arthritis T14 injury of unspecified body region
Li Shizhen	16th	*Materia Medica Outline*	R05 cough H54 visual impairment including blindness (binocular or monocular) L67 hair colour and hair shaft abnormalities E14 unspecified diabetes mellitus
Miu Xi-Yong	17th	*Annotations to the Divine Husbandman's Classic of Materia Medica*	R61 hyperhidrosis R52 pain, not elsewhere classified H54 visual impairment including blindness (binocular or monocular) R63 symptoms and signs concerning food and fluid intake L67 hair colour and hair shaft abnormalities K92 other diseases of digestive system

Table 4: Continued.

Author	Published	Name of the book	Relevant ICD-10 category
Miu Xi-Yong	17th	*Wide-Rangings Medical Notes from the First-Awakened Studio*	H11 other disorders of conjunctiva
Wu, Youxing	17th	*Ben Cao Sheng Ya Ban Jie*	R50 fever of other and unknown origin
Heo Jun	17th	*Treasured Mirror of Eastern Medicines*	K52 other noninfective gastroenteritis and colitis R52 pain, not elsewhere classified M25 other joint disorders, not elsewhere classified N61 inflammatory disorders of breast L02 cutaneous abscess, furuncle, and carbuncle R61 hyperhidrosis
Lun Zhu	18th	*Poem Collection of Materia Medica*	H04 disorders of lacrimal system L02 cutaneous abscess, furuncle, and carbuncle
Wú Yíluò	18th	*Renewed Materia Medica*	T14 injury of unspecified body region H54 visual impairment including blindness (binocular or monocular) L67 hair color and hair shaft abnormalities H04 disorders of lacrimal system M06 other rheumatoid arthritis E14 unspecified diabetes mellitus R61 hyperhidrosis
Huang Gongxiu	18th	*Truth-Seeking Herbal Foundation*	H54 visual impairment including blindness (binocular or monocular)
Guö Rû-Cóng	19th	*Combined Annotations of Three Experts on the Classic of Materia Medica*	R61 hyperhidrosis
Unknown	19th	*Ben Cao Shu Zheng*	R50 fever of other and unknown origin
Yang Shitai	19th	*An Expoundation on the Origin of the Herbal*	H11 other disorders of conjunctiva
Chen Qirui	19th	*Ben Cao Cuo Yao*	H54 visual impairment including blindness (binocular or monocular) R61 hyperhidrosis H04 disorders of lacrimal system L02 cutaneous abscess, furuncle, and carbuncle
Beijing Institute of Chinese Medicine	20th	*Yao Xing Ge Kuo Si Bai Wei Bao Hua He*	H54 visual impairment including blindness (binocular or monocular) J00 acute nasopharyngitis [common cold] R42 dizziness and giddiness R04 haemorrhage from respiratory passages J02 acute pharyngitis
Szechuan Chinese Materia Medica Editing Committee	20th	*Szechuan Chinese Materia Medica*	H54 visual impairment including blindness (binocular or monocular) J00 acute nasopharyngitis [common cold] H10 conjunctivitis
Shin Gil-Gu	20th	*Shin's Materia Medica*	R50 fever of other and unknown origin R61 hyperhidrosis R52 pain, not elsewhere classified R60 edema, not elsewhere classified T63 toxic effect of contact with venomous animals R63 symptoms and signs concerning food and fluid intake T14 injury of unspecified body region K52 other noninfectious gastroenteritis and colitis R05 cough H54 visual impairment including blindness (binocular or monocular) R61 hyperhidrosis E14 unspecified diabetes mellitus H10 conjunctivitis

Table 4: Continued.

Author	Published	Name of the book	Relevant ICD-10 category
Lee Sang-In	20th	*Herbology*	H54 visual impairment including blindness (binocular or monocular) R61 hyperhidrosis E14 unspecified diabetes mellitus R05 cough H10 conjunctivitis T14 injury of unspecified body region R52 pain, not elsewhere classified T63 toxic effect of contact with venomous animals
Nan Jing Traditional Chinese Medical School	20th	*Chinese Medical Great Dictionary*	J00 acute nasopharyngitis [common cold] R51 headache H54 visual impairment including blindness (binocular or monocular) R63 symptoms and signs concerning food and fluid intake R05 cough M06 other rheumatoid arthritis D04 carcinoma in situ of skin L23 allergic contact dermatitis
Lee Sang-In	20th	*Clinical Application of Korean Medical Herbs*	J00 acute nasopharyngitis [common cold] H54 visual impairment including blindness (binocular or monocular)
Zhen Xunying	20th	*Illustrated Chinese Medical Great Dictionary*	J00 acute nasopharyngitis [common cold] R51 headache H10 conjunctivitis H54 visual impairment including blindness (binocular or monocular) R63 symptoms and signs concerning food and fluid intake I10 essential (primary) hypertension R60 edema, not elsewhere classified M06 other rheumatoid arthritis D04 carcinoma in situ of skin L23 allergic contact dermatitis
Nationwide Chinese Medical Herb Compilation Committee	20th	*Nationwide Compilation of Chinese Medical Herbs*	J00 acute nasopharyngitis [common cold] R51 headache H10 conjunctivitis J02 acute pharyngitis
Jiangxi Health and Welfare Ministry	20th	*Jiangxi Herbal Preparation Guideline*	J00 acute nasopharyngitis [common cold] R51 headache R42 dizziness and giddiness H10 conjunctivitis J02 acute pharyngitis
Zhou jinzhong	20th	*Essential in Collection and Use of Chinese Herbs*	J00 acute nasopharyngitis [common cold] R51 headache R42 dizziness and giddiness H10 conjunctivitis J02 acute pharyngitis H04 disorders of lacrimal system
Lin Tongguo	20th	*Practical Chinese Medicine Guidelines for Clinical Syndromes*	J00 acute nasopharyngitis [common cold] R63 symptoms and signs concerning food and fluid intake H54 visual impairment including blindness (binocular or monocular)
Color Illustrated Chinese Medicine Pharmacopoeia Editing Committee	20th	*Color Illustrated Chinese Medicine Pharmacopoeia*	J00 acute nasopharyngitis [common cold] R51 headache R42 dizziness and giddiness H10 conjunctivitis
Editing Committee	20th	*Chinese Herbology*	J00 acute nasopharyngitis [common cold] R42 dizziness and giddiness H10 conjunctivitis

Table 4: Continued.

Author	Published	Name of the book	Relevant ICD-10 category
Great Collection of Chinese Medicine Editing Committee	20th	*Great Collection of Chinese Medicine*	J00 acute nasopharyngitis [common cold] R51 headache R42 dizziness and giddiness H10 conjunctivitis
National Korean Medical College Textbook Editing Committee	20th	*Herbology*	J00 acute nasopharyngitis [common cold] R51 headache H10 conjunctivitis
Kim Ho-Chul	21th	*Korean Medicine Pharmacology*	R50 fever of other and unknown origin J00 acute nasopharyngitis [common cold] H10 conjunctivitis H43 disorders of vitreous body
Song and Kim	21th	*Ethnomedicinal Application of Plants in the Western Plain region of North Jeolla Province in Korea*	J02 acute pharyngitis

English translations of author and text names were used whenever available.

Korean medicine (Table 4), Mori folium clears heat of the lungs which can be understood as the inflammation or congestion in the upper body, respiratory system, and skin. The anti-inflammatory effects might explain the clinical reports in the world, which showed antipyretic, analgesic, antivenom, antihypertensive, and anti-infertility properties that can be used in respiratory and ocular disorders, neuritis, jaundice, and hyperpigmentation (Tables 2 and 3). These clinical uses can be easily understood when we compare the reports using the standardized ICD-10 codes.

However there are some other clinical uses uniquely for traditional Korean medicine including treatment of hyperhidrosis, gastrointestinal tract, and joint disorders and use as hair tonic that can be adopted in other medical systems. Along with these, treatments of infertility [19], jaundice [24], cognitive disorder [34], and hyperpigmentation [25] were not or seldom described in Korean medical classics and texts (Tables 3 and 4).

There have been many clinical reports of Mori folium on blood glucose and diabetes (Tables 3 and 4). Though different research settings and low research quality of these reports lower the reliability of these, they showed strong possibility of dose-dependent suppression of postprandial glucose elevation that can have long-term effect on patients with severe glucose metabolism impairment. There still lies a need for well-designed clinical study, which may provide pivotal methods for diabetes treatment.

As for the summary and analysis in this study, the authors coded reported clinical uses into ICD-10 categories to compare their clinical effects. Though ethnopharmacologic studies sometimes have used ICD-10 chapters to categorize medicinal effects [37–39], the present study used categories since chapters and subchapters were too broad to incorporate all the clinical uses of Mori folium in the world. Also, the classification into chapters would cause overrepresentation of the system chapter since many ethnopharmacologic records were just simple mention of symptom names.

Although the ICD-10 coding system has universal use and usefulness for comparing studies from the world in scientific ways, there might be inevitable losses during the translation due to the nature of Western medicine underneath the coding system. For example, ICD-10 does not differentiate fever caused by liver heat and kidney deficiency, which has different accompanying signs and symptoms, and warrants completely different ways of treatment. Therefore, it should be noted that the findings in this study should be thoroughly explored with respect to their originating culture or nationality before the clinical application. And, the indicators of clinical importance and reliability were also not provided for the majority of studies. Therefore, all the potential clinical uses found in this review should be reexamined with further studies regardless of citation frequencies in here.

In this study, we excluded the clinical reports with multiherbal decoction including Mori folium as one of the ingredients and rather included description from medical textbooks, classics, and pharmacopoeia, since clinical action and effectiveness of a given herb may vary according to its role in the prescription and so it is hard to analyze its clinical usefulness. As the medical herbs are usually used as a multiherbal decoction in traditional Korean and Chinese medicine, there would be a possibility that Korean ethnopharmacologic usage and Chinese clinical studies might be unsatisfactory in this review [40]. Also, limitation with accessible clinical research databases might also have happened as for the local literatures with other languages not included here.

In conclusion, we performed a review on clinical use of Mori folium with three database and traditional Korean medical textbooks and pharmacopoeia and analyzed its clinical use with ICD-10 code. From 159 relevant studies and 17 clinical usages, infertility, jaundice, cognitive disorder, and hyperpigmentation were identified as potential clinical uses, and diabetes was the one deserving more emphasis. This study would contribute to the thorough understanding on

the clinical usefulness of *Morus alba* and Mori folium with carefully designed researches on its clinical applications.

Conflict of Interests

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

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 817324, 8 pages
http://dx.doi.org/10.1155/2015/817324

Research Article

Trigonellae Semen Enhances Sperm Motility and the Expression of the Cation Sperm Channel Proteins in Mouse Testes

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Received 25 June 2015; Revised 25 August 2015; Accepted 26 August 2015

Academic Editor: Raffaele Capasso

Genetic defects during spermatogenesis can lead to a reduction in sperm motility and cause male infertility. The cation channels of sperm (CatSper) play a role in the regulation of hyperactivated sperm motility in mouse testes. The effect of *Trigonellae* Semen (TS) on the male reproductive system and CatSper protein in mouse testes during spermatogenesis was examined. C57BL/c mice were divided into the following five groups: normal, cyclophosphamide- (CP-) only treated (control group), and three groups treated with varying concentrations of TS with CP (100, 500, and 1000 mg/kg TS and 100 mg/kg CP). Real-time PCR, western blot analysis, and a testosterone immunoassay were performed to assess CatSper protein levels in the five groups. Additionally, sperm cell counts and motility were examined. Results indicate that sperm motility and sperm counts increased in the TS treated groups in a dose-dependent manner ($p < 0.01$). CatSper levels were also significantly higher in the TS treated groups compared to that of the control group ($p < 0.001$). Therefore, TS treatment could enhance sperm function by promoting spermatogenesis and the expression of CatSper proteins in mouse testes.

1. Introduction

Spermatogenesis is a complex process of male germ cell proliferation, differentiation, and maturation from diploid spermatogonia to haploid spermatozoa in the seminiferous tubules of the testes [1]. The biological process of sperm production is regulated hormonally through feedback mechanisms using Leydig cells to promote testosterone production and cell signaling such as a Sertoli and Leydig cell [2]. This paracrine and endocrine regulation of germ cell development requires spermatogenic stage- and cell-specific gene expression [3]. It has been estimated that over 2000 proteins are involved in the specialized regulation of spermatogenesis [4, 5]. Genetic disorders such as chromosomal abnormalities or single gene mutations can impair spermatogenesis or reduce sperm cell function and can result in male infertility [5–8]. The development of assisted reproductive technologies can overcome male infertility; however, the genetic defects may still be passed to the male's offspring. Therefore, it is necessary to investigate the effects of controlled drugs for testis-specific gene expression or identify novel genetic biomarkers of normal spermatogenesis.

Cation channels of sperm (CatSper) are composed of four separate pore-forming α subunits (CatSper 1–4) and auxiliary subunits (β, γ, and δ) [9]. CatSper 1–4 transcripts are differentially expressed at the time of spermatogenesis in the testes and are localized to the principal piece of the sperm tail. The expression of CatSper 1, 3, and 4 is restricted to spermatids, whereas CatSper 2 is transcribed in the early stages of spermatogenesis (pachytene spermatocytes) [10, 11]. CatSper channels are also named as Ca^{2+} ion channels that mediate sperm hyperactivation [12]. CatSper gene expression levels were significantly lower in males with low sperm motility than males with normal fertility [13]. A recent study reported that male mice with CatSper 3 and 4 genes knocked out displayed infertility due to a lack of hyperactivated sperm motility, despite the initial presence of normal sperm counts and motility [14].

Trigonellae Semen (TS) is derived from the dry and ripe seeds of *Trigonella foenum-graecum* L., which belongs to

Leguminosae family. It has commonly been used in medicine to tonify the kidneys and provide pain relief. Additionally, it has also been reported to have antidiabetic activity [15, 16], anticholesterolemic effects [17, 18], a curative gastric antiulcer action [19], and antibacterial [20], anthelmintic [21], and antinociceptive effects [22]. The broad biological and pharmacological actions of TS are attributed to the variety of its constituents, namely, steroids, *n*-compounds, polyphenolic substances, volatile constituents, and amino acids [23–25]. TS is a medicinal herb used for the treatment of infertility and impotence in Korean medicine. However, the effect of TS on spermatogenesis-related gene expression and levels of encoded protein in mouse testes have yet to be determined.

This study investigated the effects of the TS extract on spermatogenesis and CatSper gene expression in mice using cyclophosphamide (CP) to induce testicular toxicity. Sperm count and motility, serum testosterone levels, and CatSper protein levels were assessed to evaluate the effects of TS on spermatogenesis.

2. Materials and Methods

2.1. Preparation of Trigonellae Semen Extract. TS, the seed of *T. foenum-graecum*, was purchased from Wonkwang Herbal Drug Co. Ltd. (Korea). Three hundred grams of dried TS was boiled in 6 L of water for 2 h at 100°C. The suspension was filtered and concentrated under reduced pressure. The filtrate was then lyophilized and yielded 60.27 g (20.09%) of powder, which was stored at 4°C.

2.2. Animals and Experimental Protocol. Five-week-old male C57BL/c mice were purchased from SLC Inc. (Japan). The animals were housed in a specific pathogen-free environment with a 12 h light : dark cycle at the Center for Laboratory Animal Care and Use at Kyung Hee University. Animal care and experimental procedures conformed to the "Guide for the Care and Use of Laboratory Animals" (Department of Health, Education, and Welfare, NIH publication # 78-23, 1996). Animals had free access to standard rodent pellets (Purina, Korea) and water.

After 7 days of adaptation to the environment, the mice were divided into the following five groups: normal group (N: vehicle-treated, $n = 8$), control group (C: cyclophosphamide (CP) 100 mg/kg, i.p. only treated, $n = 10$), and groups treated with three concentrations of TS and CP (CP + TS: 100, 500, and 1000 mg/kg TS and CP 100 mg/kg, $n = 10$/group). The animals were weighed weekly to adjust the gavage volume and to monitor their general health. At the end of the treatment period, the mice were anesthetized with urethane (100 mg/kg, i.p.). Serum was separated from the whole blood collected by cardiac puncture and stored in the deep-freezer until required for quantitative serum testosterone analysis. The testes were removed and cleared of the adhering tissues and weighed. The epididymis was removed for use for sperm analysis. The testes samples were frozen for use in real-time PCR and western blotting assays.

2.3. Sperm Cell Count and Motility. To obtain the sperm cell count, the entire epididymis from the mouse was minced in a sperm washing medium and incubated for 30 min at 37°C. Total epididymal sperm cell counts and motility were evaluated using the Computer Assisted Semen Analysis (CASA) system (Hamilton Thorne, USA). Sperm cells were scored as motile if any movement was detected.

2.4. Testosterone Immunoassay. Testosterone levels were determined using serum samples and a testosterone immunoassay kit following the manufacturer's protocols (R&D systems, USA). The samples were tested in triplicate and compared to two testosterone control standards.

2.5. Real-Time PCR Analysis. Total RNA was extracted from each mouse. cDNA synthesis was performed using 5 μg of total RNA with MMLV reverse transcriptase and oligo-dT primers for 1 h at 42°C. Real-time PCR was performed using a total reaction volume of 20 μL containing the following: 2 μL (200 ng) of cDNA, 10 μL of PCR master mix, 1 μL of each TaqMan probe, and 7 μL of diethyl pyrocarbonate-treated water. The samples were tested using the Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems, USA). The program parameters used were a 50°C hold for 2 min and a 95°C hold for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 60 s. The primers and probes for the CREM gene and the housekeeping gene GAPDH were predesigned by Applied Biosystems. As a control for the input amount, each cDNA sample was also amplified using the predesigned primers and probes (assay ID: Mm00460530_m1 (CatSper 1), Mm00467632_m1 (CatSper 2), Mm00712792_m1 (CatSper 3), Mm01190761_m1 (CatSper 4), and Mm99999915_g1 (GAPDH), Applied Biosystems, USA). Samples were amplified with GAPDH primers for determination of the initial relative quantity of cDNA in each sample, and then all PCR products were normalized to that amount. Nontemplate controls were used for each run. Samples were amplified in triplicate, the averages were calculated, and the differences in the relative quantity were evaluated using the StepOne Software v. 2.1 (Applied Biosystems, USA).

2.6. Western Blot Analysis. Proteins from homogenized testes were separated using a nuclear extraction kit following a modification of the manufacturer's protocol (Active & Motif, USA). SDS-PAGE and western blotting were performed as described previously [26]. Samples for protein extraction were half of the same testes used for RNA extractions. Equivalent amount (50 μg) of protein extracts was separated in 10% Tris-glycine gels by SDS-PAGE and transferred to nitrocellulose membranes using 25 mM Tris and 250 mM glycine buffer containing 20% methanol, pH 8.3. Transfer was performed at a constant voltage of 120 mA for 1 h. After transfer, the membranes were blocked in phosphate-buffered saline (PBS) containing 0.05% Tween (PBS-T) with 5% skim milk for 2 h at room temperature and incubated with the primary antibodies (1 : 1000) for CatSepr 1 (sc-21180), CatSper 2 (sc-98539), CatSper 3 (sc-98818), and CatSper 4 (sc-83126) in PBS-T overnight at 4°C. Following overnight incubation,

Table 1: Body and testicular weights following TS treatment.

Group[(1)]	Body weight (g)	Absolute testes weight (g)	Relative testes weight (%)
Normal	25.58 ± 2.81[(2)]	0.094 ± 0.004	0.37 ± 0.039
Control	20.93 ± 0.95	$0.031 \pm 0.002^{***}$	$0.15 \pm 0.004^{**}$
CP + TS 100	22.03 ± 1.35	$0.039 \pm 0.003^{\#}$	$0.17 \pm 0.008^{\#}$
CP + TS 500	23.20 ± 3.23	$0.045 \pm 0.006^{\#}$	$0.19 \pm 0.002^{\#\#\#}$
CP + TS 1000	22.90 ± 1.44	$0.046 \pm 0.005^{\#\#}$	$0.20 \pm 0.012^{\#\#}$

[(1)]Normal: vehicle-treated group.
Control: cyclophosphamide-only treated group (100 mg/kg, i.p., 5 weeks).
CP + TS: CP (100 mg/kg, i.p., 5 weeks) and TS (100, 500, and 1000 mg/kg/day, p.o., 5 weeks) treated group.
[(2)]Values are the means ± SD ($n = 8$).
$*$ indicates that the mean is significantly different from the normal value ($^{**}p < 0.01$, $^{***}p < 0.001$).
# indicates that the mean is significantly different from the control value ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$).

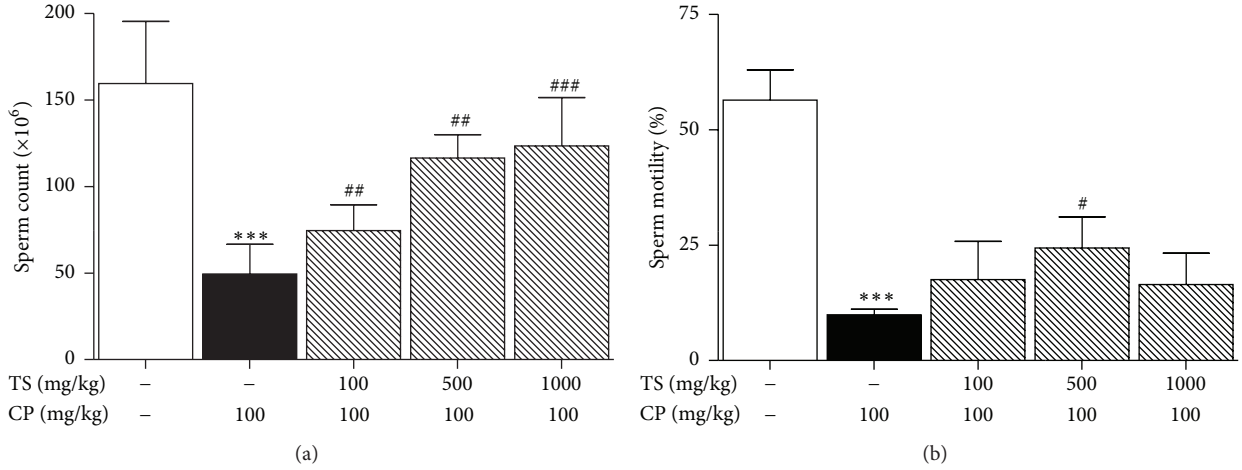

Figure 1: Effect of TS and CP on sperm count and motility. Normal, vehicle-treated group. Control, CP (100 mg/kg/week, i.p. 5 weeks) treated group. The CP and TS groups received CP (100 mg/kg) and TS (100, 500, and 1000 mg/kg, p.o., 5 weeks). (a) Sperm cell count and (b) sperm motility. Each column represents the mean ± SD ($n = 5$). $*$ means significantly different from the normal value ($^{***}p < 0.001$). # indicates that the mean is significantly different from the control value ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$).

the membranes were rinsed with 1x PBS three times and incubated with conjugated goat anti-rabbit IgG for 1 h at room temperature, followed by three additional washes with 1x PBS.

2.7. Statistical Analysis. The results were expressed as the means ± standard deviation (SD). Differences between the groups were assessed by one-way ANOVA using the SPSS software package for Windows. p values of <0.001, <0.01, and <0.05 were considered to indicate statistical significance.

3. Results

3.1. Body and Testes Weights after TS Treatment. The body and testes weights were measured on the day following full treatments (Table 1). The body weights and absolute and relative weights of testes in the CP and TS treated groups (100, 500, and 1000 mg/kg) were significantly increased compared to the control group (treated with CP only).

3.2. Sperm Counts and Motilities. The epididymal sperm counts of the CP and TS treated groups (100, 500, and 1000 mg/kg) were significantly higher than that of the control group (74.56 ± 14.91, 116.58 ± 13.47, and 123.44 ± 28.02 × 10^6, resp.; $p < 0.01$, Figure 1(a)). Additionally, sperm cell motility in the CP and TS groups (100, 500, and 1000 mg/kg) was greater than that in the control group (17.56 ± 8.24, 24.41 ± 6.69, and 16.49 ± 6.75%, resp.; $p < 0.05$, Figure 1(b)). Sperm velocity parameters such as average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), and amplitude of lateral head displacement (ALH) of control group have significantly decreased compared to normal group. CP and TS groups were increased compared to control group (Table 2).

3.3. Effect of TS on Serum Testosterone Levels. The serum testosterone levels of mice treated with CP significantly decreased by 65% when compared to the normal group (0.74 ± 0.04 versus 0.26 ± 0.09 nmol/L, $p < 0.01$). Furthermore, it was observed that samples treated with CP and TS also increased as the concentration of TS increased (0.26 ± 0.09 versus 0.58 ± 0.13, 2.25 ± 0.88, and 3.03 ± 0.92 nmol/L, respectively (Figure 2)).

Table 2: Sperm parameter with TS.

Groups[1]	VAP (μm/s)[2]			VSL (μm/s)[2]			VCL (μm/s)[2]			ALH (μm/s)[2]		
	Mean	±SD	p	Mean	±SD	t-test	Mean	±SD	p	Mean	±SD	p
Normal	63.23	4.22	—	45.88	3.18	—	110.10	7.68	—	7.45	0.35	—
Control	44.06	5.09	0.01	30.04	2.98	0.003	77.52	11.19	0.011	4.76	0.73	0.002
CP/TS 100 mg/kg	54.99	3.24	0.014	39.89	3.00	0.01	91.82	5.05	0.1	5.92	0.83	0.1
CP/TS 500 mg/kg	51.61	2.98	0.03	35.73	1.68	0.02	90.09	5.50	0.03	6.81	0.50	0.0011
CP/TS 1000 mg/kg	54.93	4.01	0.001	38.97	3.42	0.0003	93.27	6.64	0.01	6.52	0.24	0.01

[1]Normal: vehicle-treated group. Control: cyclophosphamide (CP) (100 mg/kg, i.p., 5 weeks) treated group. CP/TS: cyclophosphamide (100 mg/kg, i.p., 5 weeks), and *Trigonellae* Semen (100, 500, and 1000 mg/kg/day, p.o., 5 weeks) treated group.
[2]VAP, average path velocity (μm/s); VSL, straight line velocity (μm/s); VCL, curvilinear velocity (μm/s); ALH, amplitude of lateral head displacement (μm/s).

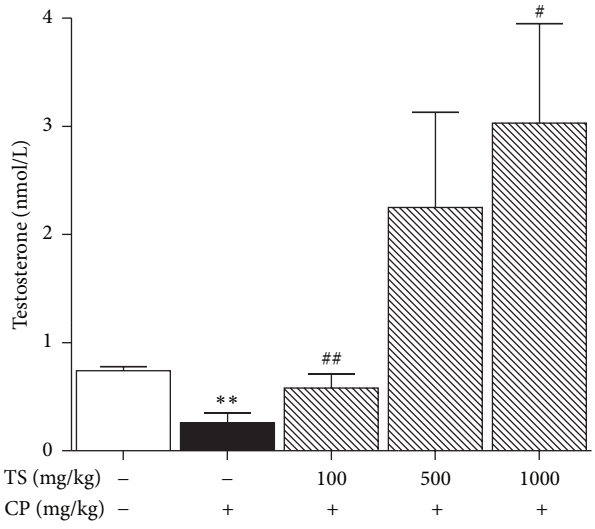

Figure 2: Effect of TS on serum testosterone levels in CP treated mice. Normal, vehicle-treated group. Control, CP (100 mg/kg/week, i.p. 5 weeks) treated group. The CP and TS groups received CP (100 mg/kg) and TS (100, 500, and 1000 mg/kg, p.o., 5 weeks). Each column represents the mean ± SD ($n = 5$). $*$ indicates that the mean is significantly different from the normal value ($^{*}p < 0.05$, $^{**}p < 0.01$). # indicates that the mean is significantly different from the control value ($^{\#\#}p < 0.01$).

3.4. Effect of TS on CatSper mRNA Levels in Mouse Testes. To determine the effect of TS on CatSper 1–4 mRNA levels in mouse testes, CatSper 1–4 mRNA levels were analyzed using real-time PCR. The normal group is regarded as the standard value (relative quantity; RQ = 1). The relative quantity for the control groups (CatSper 1–4) decreased significantly (RQ = 0.24, 0.13, 0.28, and 0.27, $p < 0.001$, resp.). CatSper 1 mRNA levels in mouse testes treated with CP and 500 mg/kg TS increased significantly, as did CatSper 2, 3, and 4 mRNA levels in mouse testes treated with CP and 1000 mg/kg TS, compared to the levels in the control group (Figure 3).

3.5. Effect of TS on CatSper Protein Levels in Mouse Testes. Samples treated with CP showed a decrease in CatSper protein levels ($p < 0.05$). However, the levels were recovered in samples treated with both CP and TS (Figure 4).

4. Discussion and Conclusions

The aim of this study was to investigate the effect of *Trigonellae* Semen on the male reproductive system and CatSper expressions in mouse testes during spermatogenesis. As an anticancer chemotherapeutic drug typically used as an immunosuppressive agent for organ transplantation, systemic lupus erythematosus, multiple sclerosis, and other benign diseases [27], cyclophosphamide (CP) was used to induce reproductive toxicity in the experimental rodents. The bioactivated metabolites of CP cause cross-linking of the DNA strands, preventing cell division and causing damaging to the testes [28].

The testes weights were measured the day following administration of the prescribed treatment. The absolute and relative testes weights in the CP treated group significantly decreased compared to the normal group. In contrast, the absolute and relative weights of testes in the CP and TS treated groups increased.

Sperm cell analysis and histopathological examination of the testes are the most effective methods for the detection of male reproductive disorders [29]. Sperm cell count and motility were estimated after isolation of sperm from mouse epididymis. The epididymal sperm count and motility of the mice treated with CP were significantly decreased compared to the control group. In contrast, the CP and TS treated groups showed an increased sperm count compared to the CP-only treated group. Notably, the number of sperm cells was significantly higher than that in the CP-only treated group. In results about sperm motion parameters, VAP, VSL, VCL, and ALH of control group have significantly decreased compared to normal group, while CP and TS treated groups were increased compared to control group. Similar to the testicular weights, this increase occurred in a dose-dependent manner.

The production of a normal number of spermatozoa is highly dependent on the regulation of gene expression in the germ cells, paracrine signaling and hormonal control of germ cell proliferation, and differentiation. The germ cells are supported structurally, nutritionally, and functionally by the Sertoli cells. The Leydig cells are adjacent to the Sertoli cells on the nonluminal side of the seminiferous tubules and produce testosterone [30]. The pituitary gonadotropin-luteinizing hormone (LH) stimulates testosterone synthesis

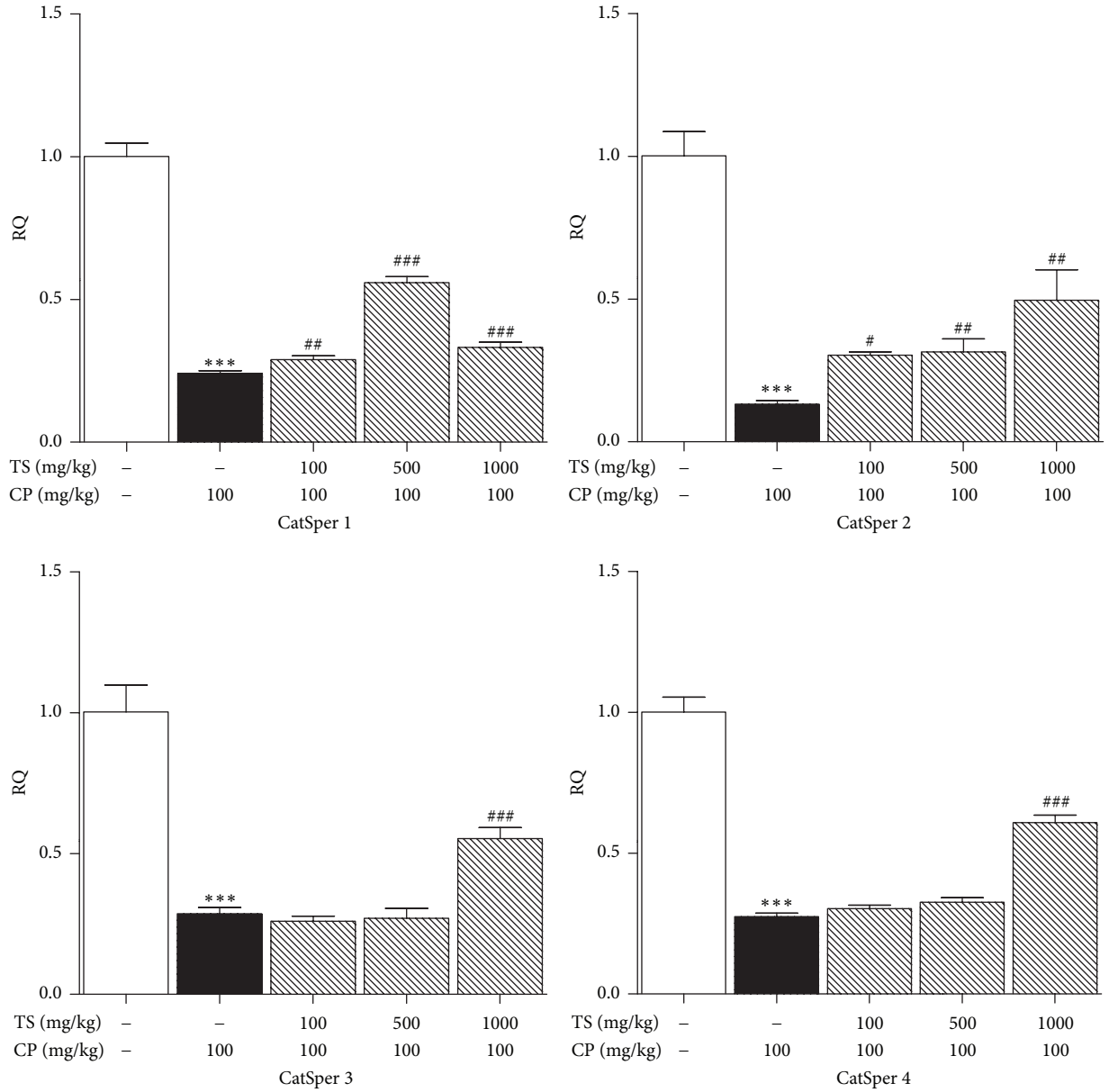

Figure 3: Real-time PCR analysis of CatSper 1–4 gene expression in TS and CP treated mice testes. Normal, vehicle-treated group. Control, CP (100 mg/kg/week, i.p. 5 weeks) treated group. The CP and TS groups received CP (100 mg/kg) and TS (100, 500, and 1000 mg/kg, p.o., 5 weeks). The level of CatSper mRNA was normalized to the GAPDH reference signal. RQ refers to the relative quantity of gene expression. Each column represents the mean ± SD ($n = 3$). ∗ indicates that the mean is significantly different from the normal value ($^{***}p < 0.001$). # indicates that the mean is significantly different from the control value ($^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$).

in the Leydig cells. As a primary regulator of spermatogenesis, testosterone, together with follicle-stimulating hormone (FSH), causes Sertoli cells to secrete the growth factors and peptides required for germ cell differentiation [31, 32]. It is, therefore, imperative to maintain the level of testosterone secretion by Leydig cells to ensure proper spermatogenesis [33]. In this study, the serum testosterone levels in the CP and TS treated groups increased in a dose-dependent manner.

Sperm motility is a significant indicator of fertilization capability. Spermatozoa differentiate to mature spermatozoon by testis-specific gene regulation during spermatogenesis [34]. For successful fertilization, hyperactivation, a type of sperm motility, is required. The hyperactivated sperm swim vigorously and generate enough force to penetrate the cumulus cells and zona pellucida of the egg cell during fertilization [35]. Hyperactivated sperm motility is regulated by the intracellular Ca^{2+} concentration. Ca^{2+} influx through CatSper channels induces hyperactivated sperm motility [36].

To investigate the effects of *Trigonellae* Semen on CatSper expression, real-time PCR and western blotting assays were performed. CatSper 1, 2, 3, and 4 protein levels decreased due to reproductive toxicity caused by CP. However, CatSper mRNA levels of mouse testes treated with CP and TS were

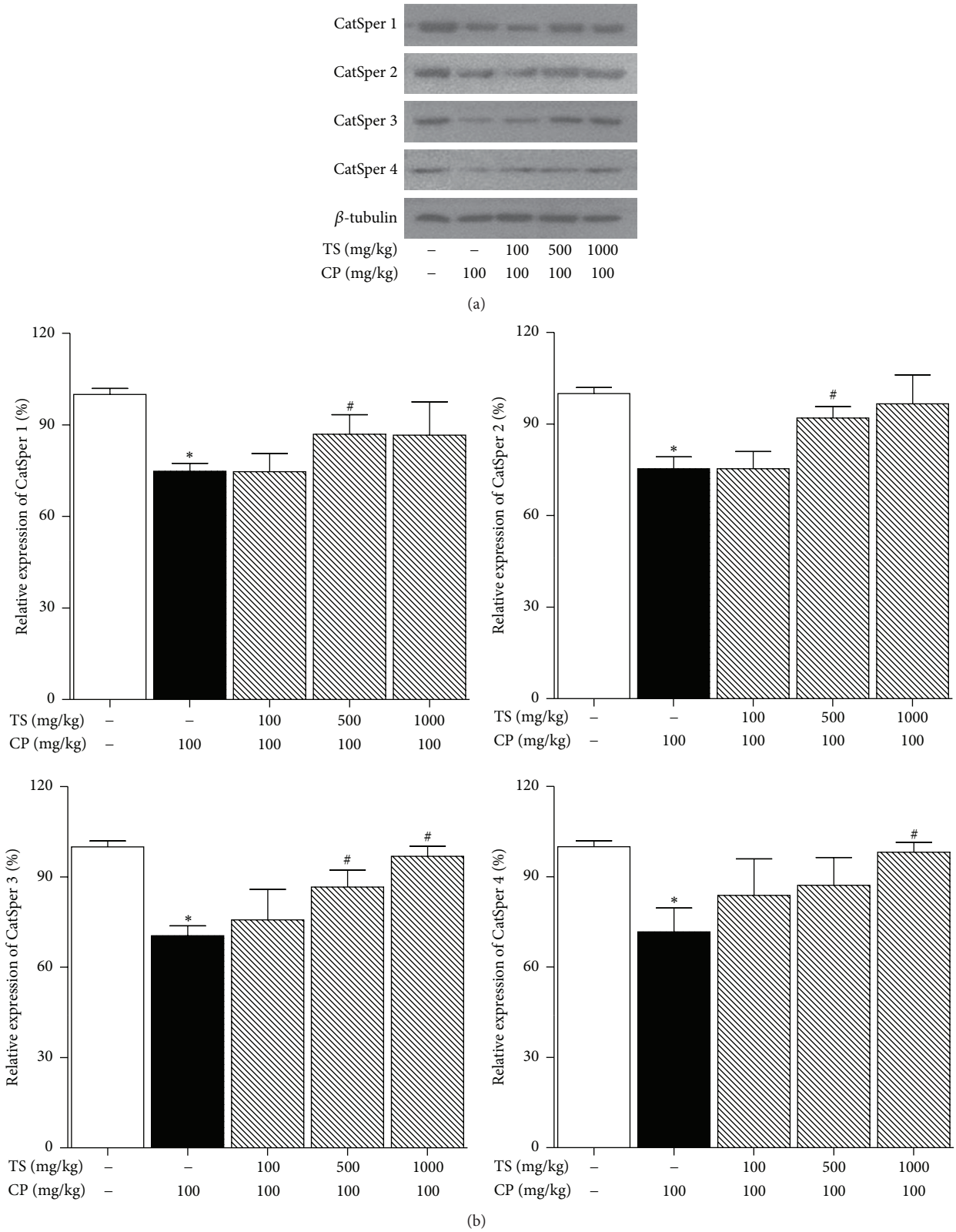

Figure 4: Effect of TS on CatSper 1–4 protein levels in TS and CP treated mice testes. Normal, vehicle-treated group. Control, CP (100 mg/kg/week, i.p. 5 weeks) treated group. The CP and TS groups received CP (100 mg/kg) and TS (100, 500, and 1000 mg/kg, p.o., 5 weeks). β-tubulin was used as an internal control. Each column represents the mean ± SD ($n = 3$). $*$ indicates that the mean is significantly different from the normal value ($^{*}p < 0.05$).

increased compared to that in the CP-only treated group (the control group). The western blot assay showed that the protein levels in the CP and TS treated groups were higher than those in the control group. These results indicate that TS stimulates the hyperactivity of sperm motility through activation of CatSper channels.

In conclusion, *Trigonellae* Semen has a protective effect on CP-induced infertile male mice by enhancing testosterone secretion and increasing sperm count and sperm motility. In addition, *Trigonellae* Semen upregulated CatSper mRNA and protein levels, thus protecting sperm motility against the damage caused by CP. Our results suggest that TS could be of help in subjects treated with CP in other diseases.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and future Planning (NRF-2014R1A2A1A11050419).

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2015, Article ID 827380, 7 pages
http://dx.doi.org/10.1155/2015/827380

Review Article

Discrimination and Proper Use of Polygoni Multiflori Radix, Cynanchi Wilfordii Radix, and Cynanchi Auriculati Radix in Korea: A Descriptive Review

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Received 26 June 2015; Revised 31 August 2015; Accepted 31 August 2015

Academic Editor: Salih Mollahaliloglu

Polygoni Multiflori Radix (PMR), Cynanchi Wilfordii Radix (CWR), and Cynanchi Auriculati Radix (CAR) are very popular herbal medicines in Traditional Korean Medicine, Traditional Chinese Medicine, and Kampo Medicine. However, the plant origins, efficacies, and traditional uses of these herbal medicines differ. In Korea, PMR is called *Ha Su O* (*He Shou Wu* in China), and CWR is called *Baek Ha Su O* or *Baek Su O* (*Bai Shou Wu* in China). *Baek Su O* refers to CWR in Korea and CAR in China. CAR has not been used as a traditional herbal medicine, and it cannot be legally used as a food or food ingredient in Korea. However, CAR is cultivated in Korea and imported from China. Because the morphology of CWR and CAR is very similar, they are often confused and misused in Korea. This review discusses the reasons for the confusion and misuse of these substances in Korea and provides the exact plant origins, efficacies, uses, components, and toxicities of PMR, CWR, and CAR so that they can be correctly understood and used.

1. Introduction

In Korea, the Ministry of Food and Drug Safety (MFDS) permits the use of 189 medicinal herbs, including Ginseng Radix, Angelicae Gigantis Radix, Polygoni Multiflori Radix (PMR), and Cynanchi Wilfordii Radix (CWR), for use in foods and as traditional herbal medicines [1]. However, various minor and major problems have arisen relating to their use owing to a lack of correct understanding of these medicinal herbs.

Traditional medicines have long been used in Korea, China, and Japan. Traditional Korean Medicine, Traditional Chinese Medicine (TCM), and Kampo Medicine have a number of similarities and differences due to their geographical, cultural, political, and climatic connections. In particular, there are many differences in their use of herbal medicines. PMR and CWR are two classic examples.

In 2015 in Korea, information regarding foods and functional health foods that contain CWR caused great social confusion and resulted in an economic loss. In recent years, functional health foods containing CWR have been popularly used for preventing or treating climacteric syndrome in women, as the MFDS permits complex extracts containing CWR as a functional ingredient for this purpose. These functional health foods created a revenue of more than $100 million in 2014. However, 301 cases of side effects from functional health foods containing CWR were reported by MDFS, which accounted for 17% of total reported side effects cases (1,733 cases) induced by functional health foods [2]. Furthermore, the MFDS reported that most of these functional foods contained Cynanchi Auriculati Radix (CAR) with CWR, and in some instances only CAR was present in place of CWR [2].

By law, CAR cannot be used as food or as a food ingredient in Korea because there is no evidence to support its use, and, in China, it has been reported to have toxicities. Therefore, people who consumed foods or functional foods containing CWR were subject to great confusion and this resulted in the mass return of such products and subsequent refunds.

The MFDS collected samples of all CWR-related products, which are produced in Korea, and discarded all

the products containing CAR after DNA analysis. In addition, in order to relieve the anxiety of the people, the MFDS publicly announced the names of companies and products that did not contain CAR, as well as the products for which presence of CWR could not be determined.

The primary cause of these events was the misunderstanding of PMR, CWR, and CAR and these three medicinal herbs often have been sold as *Ha Su O* or *Baek Su O* without distinction in the market.

PMR, which has long been used as a traditional herbal medicine, is called *Ha Su O* in Korea (*He Shou Wu* in China). However, there is some confusion of the use of this herbal medicine because of misunderstandings of the PMR name. In Korea, PMR is often separated into red PMR (*Jeok Ha Su O* in Korea) and white PMR (*Baek Ha Su O* or *Baek Su O* in Korea). Red PMR is a root tuber of *Polygonum multiflorum* (PM) Thunberg, while white PMR is the root tuber of *Cynanchum wilfordii* (CW) Hemsley [3–5]. Similarly, the plant origins of red PMR and white PMR are completely different, even though the Korean names are similar. Therefore, CWR is commonly misused as PMR in Korea. In addition, CAR is commonly misused as CWR or PMR because of its very similar external morphology to CWR. Furthermore, the plant origin of *Baek Su O* (*Bai Shou Wu* in China) differs between Korea and China. CWR is *Baek Su O* in Korea [4], while CAR is *Baek Su O* in China [6].

The plant origins, efficacies, and traditional uses of these herbal medicines differ from each other. Therefore, people should use these medicinal herbs appropriately after correctly understanding the differences between them. In the current review, we provide a comprehensive overview of PMR, CWR, and CAR, including their exact plant origins, efficacies, uses, components, and toxicities so that they can be correctly understood and used.

2. Summary of Terms

Chinese characters have long been used in the Korean language. Their meaning is the same, but their pronunciation is different. In the current review, the Korean names of the literature, herbal medicines, and prescriptions are written in English according to their Korean pronunciation, and the Chinese names of the literature and herbal medicines are written in English according to the Chinese pronunciation. *Ha Su O* is *He Shou Wu* in Chinese, and *Baek Su O* is *Bai Shou Wu* in Chinese. Other terms are written in English according to the international standard of the World Health Organization of traditional medicine terms in the western pacific region [7].

3. Origins

The root tuber of PM has been used for centuries in Korea, China, and Japan as the traditional medicine of PMR [5, 8, 9]. PMR was first mentioned as *He Shou Wu* in *Ri Hua Zi Ben Cao*, which is an ancient Chinese text that was written during the Wu Ddai Shi Guo [3, 6]. In Korea, PMR was first recorded as *Ha Su O* in *Dong Ui Bo Gam*, which is an ancient Korean text that was written during the Cho Sun dynasty. In addition, PMR was called *On Jo Rong* in the Gangwon province and *Sae Park Bul Hui* in the Hwanghae province [10]. Today, PM is registered as the plant origin of PMR in the Korean, Chinese, and Japanese pharmacopoeia [5, 8, 9].

The root tuber of CW has been used as CWR, which is a traditional medicine, in Korea [4]. CWR was first recorded as *Baek Ha Su O* in the traditional Korean texts *Bang Yak Hap Pyeon* (1884) and *Dong Ui Su Se Bo Won* (1894) [3]. CWR is recorded as *Baek Su O* in the Korean Herbal Pharmacopoeia [4]. Therefore, CWR is called *Baek Ha Su O* or *Baek Su O* in Korea. However, no traditional medical literature refers to CWR as *Baek Su O* in Korea. Confusingly, a herbal medicine that is called *Baek Su O* was first mentioned in *Shan Dong Zhong Yao* (1959) in China [3, 6]. The herbal plants that are currently called *Baek Su O* in Korea and China are different. The origin of the Korean *Baek Su O* is a root tuber of CW [4], while the origin of the Chinese *Baek Su O* is a root tuber of *Cynanchum auriculatum* (CA) Royle ex Wight and *Cynanchum bungei* Decne [6]. CAR, which is the Chinese *Baek Su O*, is called *Yi Yeop Wu Pi So*, and it is not registered in the Korean Pharmacopoeia and Korean Herbal Pharmacopoeia. CAR has not been used as a traditional medicine and food in Korea. Therefore, the MFDS of Korea prohibits the use of CAR as a food or food ingredient. In contrast, CAR is permitted for use as a drug or food in China. CWR, which is the Korean *Baek Su O*, is called *Ge Shan Xiao* in China, and it is not used nearly as much as a drug or food in China.

4. Morphological Botany

PM, CW, and CA are all climbing plants. PM is an herbaceous plant that belongs to the Polygonaceae family. Its stem twines to the right, and its leaves alternate. It does not contain a white milky liquid, and its roots are fusiform and reddish-brown like a sweet potato [11] (Figure 1(a)). CW and CA are herbaceous plants that belong to the Asclepiadaceae family. Their stems twine to the left, their leaves are opposite each other and contain a white milky liquid, and their roots are thickened and yellowish brown [11]. Therefore, CW and CA are easily distinguished from PM by these morphological characteristics.

The morphological characteristics of CW and CA are mostly similar (Figure 1). However, they can be distinguished by their stipule and flower color. A stipule is present in CW and absent in CA. The flower color of CW is yellow-green, while it is yellowish white in CA [11]. However, the external and internal morphology of CWR and CAR are very similar, except that CAR is generally longer and thicker than CWR is (Figure 1). Therefore, it is not easy to distinguish the roots of CW and CA, and it is almost impossible to distinguish CWR and CAR in the mixture.

5. Pharmacology

PMR has been used to treat sores and abscesses, scrofula, rubella, deficiencies, constipation due to intestinal dryness from the effects of detoxification, sallow complexions due to blood deficiency, dizziness, tinnitus, premature graying

Figure 1: The roots (one year old) of (a) *Polygonum multiflorum* (PM) Thunberg, (b) *Cynanchum wilfordii* (CW) Hemsley, and (c) *Cynanchum auriculatum* (CA) Royle ex Wight. Decocting pieces of (d) PM, (e) CW, and (f) CA.

of the hair and beard, weakness and pain in the waist and knees, paralysis, flooding and spotting, vaginal discharge, and hypercholesterolemia with tonifying of the liver and kidney; disperse abscesses; interrupt malaria; moisten the intestine; relax the bowels; nourish the essence and blood; blacken the beard and hair; and strengthen the sinew and bone after preparation with black bean juice in Korea and China [8, 10]. PMR has been reported to have various pharmacological activities, including acetylcholinesterase inhibition, neuroprotection, antioxidation, immunomodulation, antihyperlipidemia, hepatoprotection, anticancer effects, and anti-inflammation [12].

CAR has been used to treat weakness and pain in the waist and knee, impotence, spermatorrhea, dizziness, tinnitus, palpitations, insomnia, loss of appetite, infantile malnutrition with accumulation, postpartum milk decreases,

sores and abscesses, and snake bites with tonifying of the liver and kidney; strengthen the sinew and bone; nourish the essence and blood; fortify the spleen; and promote digestion, and it has been used for detoxification in TCM [6]. It has been reported to have a number of effects, including antioxidant, immunomodulating, antitumor, antihyperlipidemia, hair growth promoting [6], and antidepressant effects [13]. However, to the best of our knowledge, there are no descriptions of the prescribing of CAR as a medicine or food in Korea in the ancient literature or other references.

References to some prescriptions that contain CWR without descriptions of the characteristics of CWR, including its flavor, medicinal nature, or efficacy, appear in the ancient writing of *Bang Yak Hap Pyeon* (1884) in Traditional Korean Medicine. The list of prescriptions and their indications was as follows: *Oryeong-tang* for wind cold dampness impediment and tetanus; *Boanmanryeong-dan* for hemiparalysis; *Seungyangikgi-tang* for qi deficiency in Greater Yang syndrome in a Lesser Yin person; *Ogan-tang* for prolapse of the uterus; *Muki-hwan* for deficiency syndrome; *Gabil-hwan* for food accumulation, aggregation accumulation, vomiting, diarrhea, and cholera; *Paeo-tang* for static blood; *Homa-san* for wind-heat urticaria; and *Kyeoleum-dan* for refractory bloody stool. Another list of prescriptions that contain CWR was in the *Dong Ui Su Se Bo Won* (1894), and it included *Hyangsayukgunja-tang*, *Seungyangikgi-tang*, *Hwanggigyeji-tang*, *Palmulgunja-tang*, *Hyangbujapalmul-tang*, *Jeokbaekhaogwanjung-tang*, *Sanmil-tang*, and *Baekhaobujalijung-tang* for treating the syndromes of a Lesser Yin person. However, CWR is an herbal medicine that is known for treating dizziness, vertigo, insomnia, forgetfulness, early white beard and hair, impotence, spermatorrhea, weakness of the waist and knee, an absence of transport of spleen deficiency, abdominal distension, loss of appetite, diarrhea, postpartum milk lessening, and mouth sores with the effects of tonifying the liver and kidney; strengthening the sinew and bone; fortifying the spleen; and causing detoxification in TCM [6]. Recently, anti-inflammatory effects [14], antihyperlipidemia effects [15–17], and antihypertensive effects [18] of CWR have been elucidated.

6. Phytochemistry

The components of PMR are the following: resveratrol; polydatin; rhaponticoside; polygonumosides A, B, C, and D; emodin; chrysophanol; physcion; rhein; emodin-1,6-dimethyl ether; citreorosein; fallacinol; 2-acetylemodin; tricin; rutin; luteolin; quercetin; kaempferol; isoorientin; apigenin; hyperoside; vitexin; quercetin-3-O-arabinoside; polygonflavanol A; phosphatidylethanolamine; copaene; eicosane; hexanoic acid; hexadecanoic acid ethyl ester; squalene; catechin; epicatechin; 3-O-galloyl-procyanidin B2; gallic acid; methyl gallate; daucosterol; β-sitosterol; and schizandrin [12].

The components of CWR are as follows: cynandione A [19], cynanoneside B, p-hydroxyacetophenone, 2,5-dihydroxyacetophenone, 2,4-dihydroxyacetophenone, wilfoside K1N, wilfoside C1N [20], β-sitosterol, wilfoside C3N, methyleugenol, wilfoside C1G, cynauriculoside A, daucosterol, acetovanillone, sucrose, geniposide, succinic acid, bungeiside A [21], and cynanchone A [22].

The components of CAR are the following: cynandione A [19]; cynanoneside B; p-hydroxyacetophenone; 2,5-dihydroxyacetophenone; 2,4-dihydroxyacetophenone; wilfoside K1N; wilfoside C1N [20]; C21-steroidal glycoside [23]; cyanoauriculosides F, G, and H [24]; wilfoside C3N [25]; taraxasterol acetate; cynanchone A; succinic acid; betulinic acid; kidjoranin [25]; auriculoside A [26]; caudatin; metaplexigenin; azelaic acid; wilforibiose; sucrose; 1-O-hexadecanolenin; beta-amyrin acetate; acetylquinol; beta-sitosterol; and daucosterol [27].

7. Toxicology

PMR or prescriptions that include PMR have been reported as the cause of 450 cases of liver toxicity, including jaundice, fatigue, anorexia, or yellow or tawny urine, in 76 articles [28]. In addition, a 50% alcohol extract of PMR induced liver injury in a lipopolysaccharide-based idiosyncratic hepatotoxicity rat model [29]. The ethanol extract and water extract of PMR both show hepatotoxicity, while the hepatotoxicity from the ethanol extract is much stronger [30]. The oral administration of the water extract of raw PMR is much more toxic than the acetone extract in Kunming mice. In addition, the oral administration of the acetone extract of raw PMR is much more toxic than that of the acetone extract of processed PMR [31]. In addition to these reports, many studies have described the hepatotoxicity of PMR in China [32–34] and Korea [35–38]. Most of these reports were related to individuals who were taking PMR without a doctor's prescription or for a long time or those who overdosed. PMR is known in traditional medicine to have slight poisonous effects. Therefore, it is usually used after it is prepared with black bean juice that reduces its toxicity and increases its efficacy [8, 39]. Recently, Wu et al. reported that the toxicity of PMR is decreased after this preparation [31]. Therefore, the intake of PMR as an herbal medicine or food without preparation or a doctor's prescription has great potential for causing liver toxicity.

CWR has been reported to be toxic in rats and mice. The intraperitoneal administration of the 70% ethanol extract at doses of 50, 100, 200, and 300 mg/kg did not show toxicity, but doses of 500 (10%) and 1,000 mg/kg (20%) caused death [40]. In a subchronic toxicity study, the 4-week oral administration of the 70% ethanol extract of PMR in rats resulted in death in groups treated with 300, 500, and 1,000 mg/kg during the experimental period, and the toxicities were highly dose dependent [40].

CAR has also been reported to be toxic and cause increased saliva, vomiting, spasm, difficulty in breathing, and slowing of the heart beat in the China Plant Collection Database [41]. In addition, most of the *Cynanchum* family (Asclepiadaceae) is toxic, and the toxicities of the root and white milky liquid are greater than those of the other parts [41]. Lu et al. reported that CAR cannot be used as a food because the intake limit for humans (bodyweight, 60 kg) was inferred to be 2.4 g/day from the intake of rats of a rodent

diet that contained raw CAR and that caused severe weight loss and death [42]. Although this experiment had a number of problems and the quantity of CAR was higher in the diet, the rats did not eat much, and it still caused death. These results are important because the rats died, and a higher dose of CAR would have caused much more death. In addition, CAR is listed as a toxic plant in the Food and Drug Administration's Poisonous Plant Database in the US with reference to abortion activity in sows [43]. Therefore, the intake of large amounts of CAR should be avoided in China [6].

8. Future Perspectives and Conclusions

In Korea, PMR, CWR, and CAR are used as traditional herbal medicines, foods, or food ingredients. However, these substances can be confused and misused due to misunderstandings of these medicines. The plant origins, components, efficacies, potential applications, and toxicities of these medicines differ from each other. In addition, CWR is sometimes misused as PMR because of the similar Korean names of *Baek Ha Su O* (*Baek Su O*) and *Ha Su O*, respectively. CAR is also misused as CWR because of their similar morphology and its cheap price. CAR is even misused as PMR. All cases of imported *Ha Su O* from China are all CAR because *Baek Su O* is CAR in China. However, CWR is only referred to as *Baek Su O* in Korea. Therefore, the MFDS of Korea prohibits the use of CWR or CAR as PMR [5] and the use of CAR as CWR. In addition, no CA parts, including the root, can legally be used as food or food ingredients because its safety as a food has not been proved in Korea [44]. However, it can be used as an herbal medicine by Korean Medicine doctors.

PM and CW are widely distributed and have long been cultivated in Korea, but CA was distributed and cultivated only in China. CA seeds were first imported from China and cultivated since the 1990s in Youngju city in Kyungsangbuk-do province [45]. The external morphology of CAR is very similar to that of CWR. The growth rate of CAR is faster than that of CWR, and CA is much more resistant to blight and harmful insects. Therefore, farmers prefer to cultivate CAR over CWR. However, CAR cannot be used as a food or food ingredient, and it is not used as an herbal medicine in Korea. Thus, CAR is disguised as CWR or PMR or mixed with CWR. In addition, according to a presentation by the Ministry of Agriculture, Food and Rural Affairs, 187 tons of *Baek Su O* (CWR) was produced in Korea and 79 tons of *Baek Su O* was imported from China in 2014. However, Chinese *Baek Su O* is not CWR but CAR, and Chinese *Baek Su O* (CAR) is being sold as CWR or even as PMR in local markets.

Therefore, an exact understanding of these herbal medicines and their methods of distinction is needed for the correct use of these substances as a food or drug. However, it is difficult to distinguish between CWR and CAR because their external morphology and components are very similar. Li et al. suggested that conduritol F might be a unique marker compound that can be used for discriminating between CWR and CAR because it exists only in CWR [20]. CWR can therefore be distinguished from CAR by a high-performance liquid chromatography analysis of conduritol F, but it is impossible to identify CAR in the mixtures or products that contain both CWR and CAR. A DNA analysis can then be used to identify CAR in mixtures or products in Korea. However, this method is greatly limited because most mixtures or products are extracts after boiling. Thus, there is no way to identify CAR in the products that contain CWR and CAR after heating. For this reason, although the MFDS collected a total of 207 CWR-related products and conducted DNA analyses, they could not confirm whether CAR was present in 157 products. They further found that 40 products contained CAR and only 10 products did not contain CAR. As a result, the MFDS discarded all 40 products that contained CAR and allowed manufacturers to sell the other 157 products after proving that they did not contain CAR [2]. Therefore, phytochemical studies need to be conducted in order to find an index component that exists only in CAR.

PMR, CWR, and CAR all have some toxicity, which has been described in several case reports. PMR is used as an herbal medicine by doctors of traditional medicine after it is prepared with black bean juice, which reduces the toxicity and increases its efficacy in traditional medicine. CAR and CWR are also carefully used in clinics. Most of their toxicities were induced by their long-term use, overdose, or individuals taking it without a doctor's prescription. Case reports on their toxicities and side effects are useful for determining the safe use of herbal medicines. In Korea, many cases have been reported on the hepatotoxicity of PMR. Most of these toxic cases were also caused by individuals taking the drug without a doctor's prescription. However, most people consider CWR to be PMR. In addition, most people cannot distinguish between CWR and CAR and do not know the exact amount they had taken. Thus, these reports cannot be used as scientific proof of the safe use of herbal medicine. It is important that people understand the characteristics of PMR, CWR, and CAR and abstain from using these medicines on their own without a doctor's guidance. Careful toxicological studies of these medicines are needed in order to determine safety guidelines.

In conclusion, PMR and CWR are very popular traditional herbal medicines and food ingredients in Korea, and CAR is a popular traditional herbal medicine in China. Their plant origins, efficacies, uses, components, and toxicities differ from each other. However, they are often confused and misused because of the confusion in their drug names and their similar morphologies. Therefore, people should try to understand their characteristics and be able to distinguish between them. In addition, it is necessary to rename the drug names of CWR and CAR in Korea because of the confusion and misuse that are caused by the same appellative name being given to CAR and CWR. The name *Baek Su O* does not appear in traditional Korean literature but does appear in traditional Chinese literature. CWR was called *Baek Ha Su O* in traditional Korean literature. Therefore, the drug name of CWR should be changed to *Baek Ha Su O*, and the drug name of CAR should be changed to *Baek Su O* in Korea in order to prevent these misunderstandings and misuses. Further, more comparative studies of their efficacy, phytochemistry, and toxicity are needed. In particular, more phytochemical studies

are needed to find CAR in boiled herbal mixtures, along with further scientific and clinical research of the toxicology.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

This study was supported by a grant from the High Value-Added Food Technology Development Program, Ministry of Agriculture, Food and Rural Affairs (314071-03-1-HD020).

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