Geongangbuja-Tang Decoction and Its Active Ingredient, Aconiti Lateralis Radix Preparata, Exerts Inhibitory Effects on Heat Stress-Induced Inflammation in Mice

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Abstract: Heat stress induces the hypothalamic-pituitary-adrenal (HPA) axis activation, influences biological responses, and reduces energy metabolism. Geongangbuja-tang (GBT) and its components, Zingiberis Rhizoma (ZOR) and Aconiti Lateralis Radix Preparata (ALRP) have been used to induce energy metabolism; however, the effects of GBT and its ingredients on heat-induced inflammatory responses have not yet been investigated. In this study, we performed an open-field test to evaluate locomotor activity in mice. To assess the effects of GBT and its ingredients on inflammation, the protein levels of c-fos, pro-inflammatory cytokines, and cortisol were measured in the mouse hypothalamus and serum. The results showed that GBT alleviated locomotive activity and reduced c-fos levels in a dose-dependent manner under the heat exposure. After investigating the active constituent of GBT, we found that compared to GBT and ZOR, ALRP significantly suppressed c-fos expression under heat stress. Subsequently, ALRP decreased the expression of pro-inflammatory cytokines, such as interleukin-9 and -13 and prostaglandin, under the heat stress in the mouse hypothalamus. Moreover, treatment with ALRP inhibited cortisol secretion in the mouse serum following heat exposure. These results indicate that GBT and its active component, ALRP, could be the thermoregulatory agents that regulate the HPA axis.

Keywords: heat stress; Geongangbuja-tang; Aconiti Lateralis Radix Preparata; hypothalamic-pituitary-adrenal axis; c-fos

1. Introduction

As global temperature has been gradually rising, the issue of pathophysiological responses in living organisms has been raised. Heat stress has been reported to be a critical factor causing hyperthermia, anorexia, weight loss, hypoglycemia, gastric hemorrhage, spermatogenesis dysfunction, heatstroke, and neurological disorders [1,2]. These symptoms result from systemic metabolic disorders induced by reduced food intake and hormone secretion levels. Physiological responses to stress are regulated by the hypothalamic-pituitary-adrenal (HPA) axis and the sympatho-adrenomedullary system axis [3]. The HPA axis, which includes the autonomic nervous system, plays a crucial role in regulating metabolic dysregulation by thermal stress in response to stressful stimuli, which can self-activate through neurotransmitters [4]. Hormones, such as glucocorticoids and corticosterone, are controlled by the HPA axis and secreted in response to stress, which results in suppression of the systemic immune response and stimulation of pro-inflammatory cytokines [5].

Geongangbuja-tang decoction (GBT), consisting of the rhizome of Zingiber officinale Roscoe (Zingiberis Rhizoma; ZOR) and daughter root of Aconitum carmichaeli Debeaux (Aconiti Lateralis Radix Preparata; ALRP), is a representative traditional decoction used...
to alleviate immune system dysregulation in East Asia. Several reports investigating its pharmacological effects related to stress responses have revealed that GBT activated Na\(^+\)-K\(^+\)-ATPase and Ca\(^2+\)-Mg\(^2+\)-ATPase transport at cellular levels, resulting in induction of energy metabolism [6]. In addition, activation of energy metabolism by GBT promoted cardioprotective effects by regulating the peroxisome proliferator-activated receptor \(\alpha\) (PPAR\(\alpha\))/peroxisome proliferator-activated receptor gamma coactivator-1\(\alpha\) (PGC-1\(\alpha\))/sirtuin3 pathway [7]. Interestingly, previous studies on ZOR and ALRP have also explored their effects on thermally stressed conditions. ZOR is effective at regulating chicken growth and antioxidant activities under heat stress conditions [8,9]. In addition, several reports have revealed that ZOR has suppressive effects against systemic inflammatory conditions [10–12]. For example, oral administration of ZOR promoted anti-inflammatory reactions, inhibited the proinflammatory cytokines including interferon-\(\gamma\) and interleukin (IL)-6 in the serum, and blocked the inducible nitric oxide synthase and cyclooxygenase-2 in the liver of mice with lipopolysaccharide-induced hepatic injury [10]. Conversely, ALRP has been shown to suppress decreased rectal temperature and increased serum cortisol levels, expression of PPAR-\(\gamma\), PGC-1\(\alpha\), and uncoupling protein1 under cold stress conditions [13]. Moreover, Makino et al. reported that oral treatment with a processed ALRP increased the activity of Na\(^+\)-K\(^+\)-ATPase and Ca\(^2+\)-Mg\(^2+\)-ATPase transport in natural killer cells isolated from cold stressed mice [14].

Based on a previous study, GBT and its constituents are regarded as agents for boosting biological metabolic pathways and suppressing inflammation. However, whether GBT regulates heat stress-induced systemic inflammation mediated by the HPA axis has not been demonstrated. Moreover, its biologically active ingredients against the HPA axis-mediated stress responses remain unclear. In this study, we aimed to explore the thermal-regulatory effects of GBT and to demonstrate its active components. First, we investigated the effects of GBT on the thermal stress-induced behavioral impairment and inflammation in mice. To address its active component, a comparative investigation of ALRP and ZOR on c-fos levels in the heat-stressed mouse hypothalamus was conducted. In addition, we measured the levels of IL-9, IL-13, and prostaglandin E 1 (PGE1), and PGE2 in the serum to evaluate its effects on systemic inflammation induced by heat exposure.

### 2. Materials and Methods

#### 2.1. Sample Preparation

Herbs were purchased from Omni Herb Inc. (Andong-si, Gyeongbuk, Korea). For the extraction of GBT, the fibrous powder of the ALRP and ZOR (1:1) with mixed condition was extracted in boiling distilled water for 2 h. GBT was successively extracted at a yield of 13.8%. We also extracted ALRP and ZOR alone under the same conditions, with yields of 10.45% and 21.1%, respectively. ALRP used in this study was the same extract which was qualified by the fingerprint using the reference compound aconitine utilized in the previous study and its chromatogram was published in the reference [13]. The samples were deposited at the College of Pharmacy, Kyung Hee University.

#### 2.2. Animals

All animal studies were performed in accordance with the “Guide for the Care and Use of Laboratory Animals, 8th edition” (National Institutes of Health, 2011) and approved by the “Animal Care and Use Guidelines” of Kyung Hee University, Seoul, Republic of Korea (approval number: KHP-2014-05-2). Seven-week-old male ICR mice (30–32 g) were purchased from the Orient Co., Ltd. (Seoul, Korea). The animals were housed in a cage (size 40 cm × 25 cm × 18 cm), with six individuals per cage. They had free access to water and food, and were maintained under constant temperature (23 ± 1 °C) and humidity (60 ± 10%), with a 12 h light/dark cycle. One week after arrival, the mice were acclimated to their surroundings for seven days and kept under the same conditions before the start of the study.
The mice were assigned to several groups as follows: control group (non-heat stress with vehicle treatment), heat stress group (heat stress with vehicle treatment), GBT 100 group (heat stress with GBT 100 mg/kg/day), GBT 300 group (heat stress with GBT 300 mg/kg/day), GBT 1000 group (heat stress with GBT 1000 mg/kg/day), ZOR 500 group (heat stress with ZOR 500 mg/kg/day), ALRP 30 group (heat stress with ALRP 30 mg/kg/day), ALRP 100 group (heat stress with ALRP 100 mg/kg/day), ALRP 300 group (heat stress with ALRP 300 mg/kg/day), ALRP 500 group (heat stress with ALRP 500 mg/kg/day), and ALRP 1000 group (heat stress with ALRP 1000 mg/kg/day).

Mice were subjected to acute heat exposure for 3 days as previously described [15]. Briefly, heat exposure was achieved by maintaining mice in a chamber maintained at 43 °C and 60 ± 10% humidity for 15 min. To avoid the influence of diurnal cycling, heat exposure began at approximately the same time each day. The vehicle or extracts of each sample dissolved in saline was administered by oral gavage for three days with 1 h before heat stress treatment (Figure 1).

Figure 1. Experimental schedule.

2.3. Open-Field Test

The open-field test is a classic method to measure the ambulation of mice. The mice were placed in a testing chamber (40 cm × 25 cm × 18 cm) with a black floor for 5 min adaptation, followed by a 120 min test period using a computerized automatic analysis system (Viewer; Biobserve, Bonn, Germany). The data collected by computer included the total distance traveled by tracking the center of the animal.

2.4. Measurement of Cortisol Level in Serum

A cortisol enzyme-linked immunosorbent assay (ELISA) assay (Enzo, New York, NY, USA) was performed according to the manufacturer’s protocol. Blood was collected from the mice on the day of decapitation, and centrifuged at 3000 rpm for 10 min to obtain serum samples, which were stored at −70 °C until use. Briefly, serum was mixed with diethyl ether. Then, ether mixture was evaporated using nitrogen, after which protease activity was detected using a microplate reader (VERS Amax, Sunnyvale, CA, USA), with filters set at 570 nm excitation and 590 nm emission.

2.5. Western Blot Analysis

Western blotting was performed according to the method described previously. Mouse hypothalamus was lysed using a protein assay kit according to the manufacturer’s instructions (Bio-Rad Laboratories, Hercules, CA, USA). The lysates were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride membrane (Millipore Bioscience Research). The membranes were incubated with 5% skim milk in Tris-buffered saline with Tween 20 (TBS-T) for 1 h and then with a c-fos antibody (1:1000; Santa Cruz, Pas Robles, CA, USA) overnight at 4 °C; this was followed by incubation with a horseradish peroxidase conjugated secondary antibody for 1 h. Immunoreactive-bands were detected using an enhanced chemiluminescence (ECL) detection kit (Bionote, Hwaseong, Korea), and a LAS-4000 Mini system (Fujifilm Corp., Tokyo, Japan) was used for visualization. Band intensities
were normalized to the β-actin band intensity using MultiGauge software (Fujifilm Corp., Tokyo, Japan).

2.6. Measurement of IL-9, IL-13, PGE1 and PGE2 Levels

The mouse IL-9, IL-13, PGE1 and PGE2 ELISA kits (Enzo, New York, NY, USA) were performed according to the manufacturer’s protocol. Briefly, the hypothalamic lysates were incubated with reaction buffer. The mixture was incubated for 2.5 h at room temperature before protease activity was detected using a microplate reader, with filters set at 360 nm excitation and 450 nm emission. The mixture was incubated for 45 min at room temperature before protease activity was detected using a microplate reader, with filters set at 570 nm.

2.7. Statistical Analysis

All statistical parameters were calculated using GraphPad Prism 8.0 software (Graphpad Software, San Diego, CA, USA). All quantifications for assays were repeated three times and values were expressed as the mean ± standard error of the mean (S.E.M.). All of data were analyzed by one-way analysis of variance followed by Dunnett’s multiple comparison test. Differences with a p value less than 0.05 were considered statistically significant.

3. Results

3.1. Effects of GBT on Heat-Induced Sickness Behaviors and C-Fos Expression in Mice

To evaluate the effects of GBT on heat stress-induced behavioral dysfunction, we examined locomotor activity in mice for 2 h (Figure 2A). The track length of the heat stress group (101 ± 20 m) was significantly lower than that of the control group (270 ± 31 m). However, mice treated with GBT showed a dose-dependent enhancement of locomotive behaviors. In the GBT1000 group, locomotor activity significantly increased compared to that of the heat stress group (183 ± 14 m).

Figure 2. Protective effect of GBT on heat stress-induced behavior impairment and hypothalamic c-fos overexpression. Open-field test (n = 6 per group) was performed 3 h after heat exposure (A). Quantification of densitometric analyses was performed by measuring the expression ratios of c-fos/β-actin (n = 6 per group) in the lysates of hypothalamus for investigating the effects of GBT (B). Values are expressed as means ± SEM. *** p < 0.001 as compared with the control group. # p < 0.05 as compared with the heat-exposed vehicle-treated group. GBT; Geongangbuja-tang decoction.

To evaluate the effects of GBT on heat stress-induced c-fos levels, we examined c-fos expression in the mouse hypothalamus (Figure 2B). The protein levels of c-fos in the mouse hypothalamus significantly increased in the heat-stressed vehicle-treated group (221 ± 14% that of control) compared to that in the control group. However, c-fos levels decreased in the GBT treatment groups than in the heat stress group. In the GBT 1000 group, the protein levels of c-fos significantly decreased compared to those in the heat stress group (171 ± 17%).
3.2. Effects of GBT and Its Components, ALRP and ZOR, on C-Fos Expression in the Mouse Hypothalamus

To determine the active component responsible for the thermoregulatory effects of GBT, we investigated the expression of c-fos in the mouse hypothalamus using additional comparative experimental set (Figure 3A). C-fos expression elevated under the heat stress treatment (198 ± 15% of control) were significantly decreased under GBT, ZOR, and ALRP treatments (120 ± 23%, 84.1 ± 9.2%, and 75.9 ± 4.6%, respectively). Among them, ALRP treatment showed the most suppressive effects against heat stress-induced c-fos levels in the mouse hypothalamus. Based on these results, we identified ALRP as the active ingredient of GBT.

Figure 3. Suppressive effect of GBT and its constituents on heat stress-induced c-fos expression. Quantification of densitometric analyses was performed by measuring the expression ratios of c-fos/β-actin (n = 6 per group) in the lysates of hypothalamus for investigating the effects of GBT and its ingredients, ZOR and ALRP (A) and ALRP in a dose-dependent manner (B). Values are expressed as means ± SEM. * p < 0.05 and *** p < 0.001 as compared with the control group. # p < 0.05, ## p < 0.01 and ### p < 0.001 as compared with the heat-stressed vehicle-treated group. GBT; Geongangbuja-tang decoction. ZOR; Zingiber officinale Radix. ALRP; Aconiti Lateralis Radix Preparata.

To evaluate the effects of ALRP on heat stress-induced c-fos levels, we examined c-fos expression in the mouse hypothalamus (Figure 3B). The protein levels of c-fos in the mouse hypothalamus significantly increased in the heat-stressed vehicle-treated group (155 ± 16% of control) than in the control group. However, ALRP treatment decreased c-fos levels, starting at 30 mg/kg/day or more. As below, the protein levels of c-fos in ALRP treated group (30, 100, 300 and 1000) were significantly decreased, compared to the corresponding protein level of the heat stress group (91.8 ± 8.3%, 78.5 ± 3.2%, 88.4 ± 12.3% and 76.4 ± 6.9%, respectively).

3.3. Effects of ALRP on Heat Stress-Induced Production of Inflammatory Mediators in the Mouse Hypothalamus

To determine the effects of ALRP on heat stress-induced inflammatory cytokines in the hypothalamus, we examined the expression levels of IL-9, IL-13, PGE1, and PEG2. Levels of IL-9 significantly increased in the hypothalamic homogenates of mice subjected to heat stress (168 ± 3% of control). However, IL-9 expression significantly decreased in the ALRP-treated groups at 300 and 1000 mg/kg/day than in the heat stress group (147 ± 3% and 142 ± 3%, respectively; Figure 4A). Moreover, IL-13 expression significantly increased in the heat stress group than in the control group (164 ± 1% of control). The IL-13 levels significantly decreased in all of the ALRP-treated groups than in the heat stress group, showing a dose-dependent tendency (155 ± 1%, 156 ± 1%, 139 ± 1%, and
134 ± 1%, respectively; Figure 4B). PGE2 is abundantly produced in the brain in response to inflammation and mimics stress responses, whereas PGE1 plays an antagonistic role as an anti-inflammatory agent. To evaluate the effects of ALRP on PGE levels in the hypothalamus, we examined the ratio of PGE2 to PGE1 using ELISA, and found it to be significantly upregulated after heat exposure (113 ± 3% of control); however, this ratio was significantly restored by ALRP at 300 mg/kg/day (100 ± 5%, Figure 4C).

Figure 4. Inhibitory effect of ALRP on increases of IL-9, IL-13 and ratio of PGE2/PGE1. In hypothalamus of mice after heat exposure, the presences of IL-9 (A), IL-13 (B) and ratio of PGE2/PGE1 (C) were determined using sandwich ELISA kit (n = 6 per group). Values are expressed as means ± SEM. * p < 0.05 and *** p < 0.001 as compared with the control group. # p < 0.05, ## p < 0.01 and ### p < 0.001 as compared with the heat-stressed vehicle-treated group. ALRP; Aconiti Lateralis Radix Preparata. IL-9; interleukin-9. IL-13; interleukin-13. PGE1; prostaglandin 1. PGE2; prostaglandin 2.

3.4. Effects of ALRP on Heat Stress-Induced Secretion of Cortisol

To evaluate the effects of ALRP on hyperthermia-induced release of cortisol, we examined cortisol levels in mouse serum (Figure 5). The cortisol levels in mouse serum were significantly increased in the heat stress group (204 ± 2% of control) than in the control group. However, ALRP treatment decreased cortisol levels in a dose-dependent manner. Compared to the ALRP 1000 group, the level of cortisol was significantly decreased in the heat stress group (146 ± 4%).

Figure 5. Suppressive effect of ALRP on heat exposure-induced increase of cortisol secretion (n = 6 per group). Values are expressed as means ± SEM. *** p < 0.001 as compared with the control group. ### p < 0.001 as compared with the heat-stressed vehicle-treated group.

4. Discussion

In the present study, we investigated whether GBT exerts effects on behavioral impairment induced by heat stress by inducing antifebrile effects in mice. This study also showed that ALRP has potent antipyretic properties, suppressing systemic inflammation after heat exposure. To reveal the anti-inflammatory effects of ALPR against heat stress, we demonstrated the downregulation of levels of IL-9, IL-13, PGE1, and PGE2 levels in the mouse hypothalamus, which resulted in the secretion of cortisol in mice. Thus, after treated with GBT, the amelioration of the HPA axis-mediated systemic inflammation appears to be mediated by the effects of ALRP at least.
The immune inflammatory response is closely associated with stress response. Activation of the HPA axis produces marked inhibitory effects on the immune inflammatory response, as virtually all the components of the immune response are inhibited by cortisol, an important factor in glucocorticoid hormone synthesis in the hypothalamus. Numerous studies have identified the central pathways mediating the stress response by mapping neuronal activation using c-fos as a dependable indicator [16,17]. In addition, intense expression of c-fos in the hypothalamus is closely related to corticotropin-releasing factor (CRF), which activates the release of corticosterone [17].

Corticosterone secretion is related to an increase in the synthesis of the proinflammatory prostaglandin PGE2 in the brain. In the central nervous system, PGE2 has been related to the control of neuroinflammatory processes, synaptic transmission, brain plasticity, regulation of fever, and the HPA axis activation [18]. In turn, cytokines, such as IL-9 and IL-13, also stimulate the HPA axis, which can influence virtually every pathophysologic domain relevant to depression, including neuroendocrine function, neurotransmitter metabolism, regional brain activity, and ultimately, behavior [19]. In this study, we evaluated locomotive activities reduced by heat stress, which could be the phenotype underlying depressive behavior, and studied the associated effects of GBT.

The effects of ALRP on thermal stress-induced inflammation have been previously reported. Studies have investigated the effects of ALRP on cold stress-induced homeostasis dysregulation according to its traditional functions in alleviating energy metabolism [20]. In addition, reports have identified regulatory mechanisms, such as enhancement of natural killer cell activity and normalization of the HPA axis [13,14]. According to Kim et al., ALRP is regulated in the mouse hypothalamus, which resulting in the inhibition of systemic inflammation, including neuroinflammation [13]. Interestingly, these anti-inflammatory effects were only observed in thermal stress-induced thermo-dysregulation. This indicates that ALRP inhibits the production of cytokines and pro-inflammatory factors by regulating the HPA axis. Thus, further studies using other inflammatory models are needed to demonstrate the effects of ALRP on inflammation and elucidate its precise mechanisms. Moreover, alterations in locomotive activity according to heat exposure has been happened to a decrease in neurotransmitter activity in the brain, which is regulated by c-fos mRNA levels [21]. The inhibitory effects of ALRP on molecular changes including c-fos expression could induce an increase in the locomotive activity and related studies should be needed further.

One of the major components of ALRP, aconitine, is a toxic component that induces paralysis and hypotension [22]. Therefore, when applying ALRP to a living thing, side effects caused by aconitine should be considered. ALRP used in this study contained 0.12 mg of aconitine per 1 g [13], which could be interpreted as taking 0.12 mg/kg of aconitine/kg of aconitine based on 1000 mg/kg, the maximum dose used. As considering the LD50 dosage of aconitine in the mouse as 1.8 mg/kg [23], toxicity-related side effects for treating ALRP might be rare. Moreover, an appropriate amount of aconitine shows effective pharmacological effects, such as anti-inflammatory and immune-modulating actions, rather than toxicity [24,25]. Thus, ALRP with a safe dose could increase pharmacological activity as well as reduce the risk of side effects.

ALRP was presented as an active ingredient of GBT; however, as in Figure 3, ZOR also played a role in significantly suppressing c-fos expression. Based on the previous reports that ZOR acted on the various responses caused by heat exposure [8,9], active ingredients of ZOR also affected the action of GBT. Both 6-gingerol and 6-shogaol, representative active ingredients of ZOR, have shown that they could improve energy metabolism to increase the level of AMPK-dependent pathway in 3T3-L1 adipocytes by heat exposure [9,12]. Furthermore, 6-shogaol and zingerone have been known to show excellent anti-neuroinflammatory effects in various LPS-treated mouse and cell models [10,11]. Thus, further studies on the effect of ZOR on the efficacy of GBT are needed.
5. Conclusions

GBT exhibited effects on heat stress-induced behavioral impairments and hypothalamic c-fos expression in mice. In addition, ALRP suppressed cortisol levels by reducing pro-inflammatory factors. Though the precise mechanisms of GBT and ALRP on thermal stress-induced inflammation should be studied further, we found that these anti-inflammatory effects were regulated by HPA axis. These results suggest that GBT and ALRP could be potent agents for heat stress-induced disorders such as depressive behaviors via inhibition of hypothalamic stress responses.

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