Korean ginseng extract ameliorates abnormal immune response through the regulation of inflammatory constituents in Sprague Dawley rat subjected to environmental heat stress

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Abstract

Background: Increases in the average global temperature cause heat stress—induced disorders by disrupting homeostasis. Excessive heat stress triggers an imbalance in the immune system; thus protection against heat stress is important to maintain immune homeostasis. Korean ginseng (Panax ginseng Meyer) has been used as a herbal medicine and displays beneficial biological properties.

Methods: We investigated the protective effects of Korean ginseng extracts (KGEs) against heat stress in a rat model. Following acclimatization for 1 week, rats were housed at room temperature for 2 weeks and then exposed to heat stress (40°C/2 h/day) for 4 weeks. Rats were treated with three KGEs from the beginning of the second week to the end of the experiment.

Results: Heat stress dramatically increased secretion of inflammatory factors, and this was significantly reduced in the KGE-treated groups. Levels of inflammatory factors such as heat shock protein 70, interleukin 6, inducible nitric oxide synthase, and tumor necrosis factor-alpha were increased in the spleen and muscle upon heat stress. KGEs inhibited these increases by down-regulating heat shock protein 70 and the associated nuclear factor-κB and mitogen-activated protein kinase signaling pathways. Consequently, KGEs suppressed activation of T-cells and B-cells.

Conclusion: KGEs suppress the immune response upon heat stress and decrease the production of inflammatory cytokines in muscle and spleen. We suggest that KGEs protect against heat stress by inhibiting inflammation and maintaining immune homeostasis.

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1. Introduction

Excessive exposure to high temperatures increases body temperature and causes stress. Heat stress leads to various conditions such as heat stroke, cardiovascular diseases, nerve damage, and immune disorders [1–4]. The immune system protects hosts against external pathogens. Immune homeostasis must be maintained upon heat stress to avoid excessive inflammation, autoimmune diseases [5], and cancer [6].

Various plant products have been used to protect against heat stress [7,8]. In particular, Korean ginseng has been employed to treat a variety of conditions, including heat stress, obesity, diabetes, and oxidative stress, and improves immune function [9–11]. The saponin constituents of Korean ginseng, ginsenosides such as Rb1, Rg1, and Rf, elicit beneficial effects and differ according to the extraction method [12,13]. However, the mechanisms by which Korean ginseng influences the effects of heat stress on the immune system remain unclear.

Several studies report that various environmental stresses, including heat stress, induce expression of heat shock protein 70 (HSP70) and that this protein has both proinflammatory and anti-inflammatory functions [14–16]. To elicit proinflammatory effects,
HSP70 translocates into the nucleus and its expression increases [17]. HSP70 can interact with membrane receptors of antigen-presenting cells (APCs) such as macrophages and dendritic cells. This protein can also complex with peptides, leading to antigen cross-presentation in the extracellular environment [15,18,19]. Consequently, HSP70 stimulates pattern recognition receptors such as toll-like receptors (TLRs) on the surface of APCs. TLR signaling induces phosphorylation and nuclear translocation of nuclear factor (NF)-κB to increase expression of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6. TLR signaling also induces phosphorylation of Activator protein 1 (AP1) by increasing that of p38, extracellular signal-regulated kinase, and c-Jun N-terminal kinase via mitogen-activated protein kinase (MAPK) signaling [20,21]. APCs activated by the NF-κB and MAPK signaling pathways cause inflammation [22].

Inflammation is an innate immune response that is important to maintain the immune homeostasis and leads to adaptive immune responses. Inflammatory cytokines activate APCs, which lead to increased secretion of these cytokines [23]. T-cells and B-cells trigger adaptive immune responses in response to inflammatory cytokines [24]. Various cytokines such as IL-2 and IL-6 induce the differentiation of T-cells [25]. Extracellular HSP70 complexed with peptides induces the differentiation of CD8+ and CD4+ T-cells, which are termed cytotoxic and helper T-cells, respectively [26]. In addition, activated T-cells stimulate the secretion of immunoglobulins and cytokines by B-cells.

This study investigated the effects of three Korean ginseng extracts (KGEs) on the immune response to heat stress in Sprague Dawley rats.

Fig. 1. Preparation of KGEs. (A) Procedure for the preparation of JP5. (B) Procedure for the preparation of BG1. KGEs, Korean ginseng extracts.
2. Materials and methods

2.1. Preparation of KGEs

Korean fresh ginseng samples (4-year-old ginseng) were purchased from Jeonbuk Ginseng Nonghyup in Jinan province, Korea. Korean fresh ginseng samples were steamed and dried for 2–4 h at 85°C twice to obtain Korean Red Ginseng. Korean Red Ginseng was also steamed and dried for 2–4 h at 50–80°C for 2–5 times to obtain Korean black ginseng. Korean Red ginseng and Korean black ginseng extracts were extracted with deionized water (4–8 h at 85–95°C, two times) and 30–70% ethanol (4–8 h at 85–95°C, three times). The resulting extracts were filtered, concentrated to 65 brix, and sterilized at 98–100°C. The Korean Red Ginseng and Korean black ginseng extracts were termed JP5 and BG1, respectively. Their preparation procedures are summarized in Fig. 1, and their compositions are shown in Table 1. JKJ, another KGE, was purchased from Korea Ginseng Corporation (Daejeon, Korea).

2.2. Reverse-phase HPLC ginsenoside analysis

Ginsenosides were analyzed with a Shimadzu 10 Avp HPLC system (Tokyo, Japan) equipped with a ultraviolet detector (SPD-10Avp, Shimadzu, Tokyo, Japan) and gradient pump. The detection wavelength was 203 nm. A Grasesmart column (250 mm × 4.6 mm, 5 μm; Grace, Columbia, MD, USA) was used at 40°C. A 10 μL aliquot of the sample was injected, and a mixed mobile phase of distilled water (solvent A) and acetonitrile (solvent B) was used under gradient conditions. The gradient elution was as follows: 0–10 min, 20% B; 10–42 min, 20–30% B; 42–67 min, 30–40% B; 67–70 min, 40–47% B; 70–80 min, 47–80% B; 80–93 min, 80% B; 93–95 min, 80–20% B; and 95–115 min, 20% B. The flow rates of solvents A and B were 1.2 mL/min⁻¹. Nineteen ginsenosides, Rg1, Re, Rf, 20(S)-ginsenoside Rg2, Rb1, F1, Rc, Rb2, Rb3, Rd, F2, 20(S)-ginsenoside Rg3, 20(R)-ginsenoside Rg3, Rk1, Rg5, 20(S)-ginsenoside Rh2, and 20(R)-ginsenoside Rh2, were used as standards. Ginsenoside standards were purchased from Chromadex (Irvine, CA, USA).

2.3. Chemicals and reagents

IL-2 and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were purchased from Koma Biotech (Seoul, Korea). IgM and IgA ELISA kits were purchased from Affymetrix/eBioscience (San Diego, CA, USA). A cortisol ELISA kit was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Antibodies against CD4, CD8, IL-6, and glyceraldehyde 3-phosphate dehydrogenase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p-NF-kB and anti-p-p38 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Alexa Fluor 488-conjugated antibodies and Hoechst 33342 were obtained from Thermo Fisher Scientific (San Jose, CA, USA). Unless noted otherwise, all chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

2.4. Animals and study design

Eighty-four 8-week-old male Sprague Dawley rats were purchased from Orient Bio (Gyeonggi-do, Korea) and housed in standard conditions (12 h light/dark cycle, 23–26°C, 50–55% humidity, light intensity of 100–200 Lux, and access to sterilized tap water and commercial rodent food ad libitum). All experimental procedures were performed in accordance with the guidelines for animal experimentation provided by the Faculty of Agriculture, CHA University (Seongnam, Gyeonggi-do, Korea) (IACUC 160072). After acclimatization for 1 week, rats were randomly assigned to one of six groups (n = 14): room temperature group (RT), heat stress group (HS), heat stress + JKJ group (JKJ, 300 mg/kg), heat stress + JP5 low-dose group (JP5-L, 100 mg/kg), heat stress + JP5 high-dose group (JP5-H, 300 mg/kg), and heat stress + BG1 group (BG1, 300 mg/kg). Rats in the RT group were housed at room temperature for 6 weeks, while those in the other groups were housed at room temperature for 2 weeks and then exposed to heat stress (40 ± 1°C/2 h/day) for 4 weeks. Equipment (LIH-1043G) for heat stress treatment was purchased from Lassele (Ansan, Korea). Animals were treated with KGEs from the beginning of the second week to the end of the experiment. The experimental design is summarized in Fig. 2. At the end of the experiment, blood was harvested and stored at −20°C until analysis. Subsequently, spleen and muscle tissues were excised from each rat and cut into small pieces for western blotting and reverse transcription polymerase chain reaction (RT-PCR) or sliced into sections. All tissues were stored at −80°C until analysis.

2.5. Biochemical analysis

Serum was separated by centrifugation at 950 g for 30 min at 4°C. Concentrations of adrenocorticotrophic hormone (ACTH), cortisol, TNF-α, IL-2, IgM, and IgA were measured using ELISA kits according to the manufacturers’ instructions. After adding stop
solution, absorbance at 450 nm was measured using a Powerwave HT ELISA reader (BioTek, Winooski, VT, USA). All assays were performed in duplicate to ensure accuracy.

2.6. Histological examination

The spleen was frozen in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, Netherlands) with optimal cutting temperature compound, immersed in liquid nitrogen until completely frozen, and sliced into 10 μm thick sections using a cryotome. The sections were placed onto glass slides and fixed in methanol cooled to −20 °C. The slides were rinsed with 1x phosphate buffered saline (PBS) and incubated in blocking buffer (Dulbecco’s Modified Eagle’s Medium containing 10% fetal bovine serum) for 1 h. Diluted anti-CD4 and anti-CD8 primary antibodies were applied in sufficient quantities to cover the tissue, and samples were incubated overnight at 4 °C. Thereafter, slides were washed three times with PBS. Diluted Alexa Fluor 488-conjugated secondary antibodies were applied in sufficient quantities to cover the tissue, and samples were incubated for 1 h. Finally, slides were counterstained with Hoechst 33342 for 1 h.

2.7. RNA isolation and RT-PCR

Muscle and spleen tissues were homogenized in 500 μL of TRIzol reagent, and RNA was extracted according to the TRIzol protocol. Thereafter, 1 μg of total RNA was reverse-transcribed into cDNA. The following primers were used for RT-PCR: 18S forward (5'-GGCCCGAAGCGTTTACTTTGAA-3') and reverse (5'-GATGGGAAGCAGGCTAC-3'), HSP90 forward (5'-CCGGTTGCGTTAGTACGT-3') and reverse (5'-TCCAGACGGTC- TGAAGATGGA-3'), Heat shock factor 1 (HSF1) forward (5'-CACCTTGAGATGAGTAA-3') and reverse (5'-GGCTTGAGATGGACTGATA-3'), c-Jun forward (5'-GTGGAACACCTCTAC-3') and reverse (5'-GTTGAACTTGCTGAGGTG-3'), inducible nitric oxide synthase (iNOS) forward (5'-CCAGAAGAGTTACACATCTG-3') and reverse (5'-CAAAGTTGCCAGTGGTGGTGTTC-3'), TNF-α forward (5'-AACAGCTGTCTCTC-3') and reverse (5'-GGAGGCTGACTTTCTCC-TGAATGAAA-3'), and CD11b forward (5'-CATCACCGTGAGTTCCACAC-3') and reverse (5'-GAGAACTGGTTCTGGCTTGC-3'). The PCR products were run on 1.5% agarose gels, stained with ethidium bromide, and photographed. Densitometric analysis was performed using ImageJ software (National Institute of Health, Bethesda, MD, USA).

2.8. Western blot analysis

Muscle and spleen tissues were washed with PBS, homogenized in 300 μL of radio immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/mL aprotinin, and 5 μg/mL leupeptin) and phosphatase inhibitors (1 mM Na3VO4 and 1 mM NaF), and centrifuged at 15,500 g for 10 min at 4 °C. The protein concentration was determined by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) using bovine serum albumin as the standard. Proteins (20 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue. The protein bands were visualized by exposure to UV light and were quantified using ImageJ software (National Institute of Health, Bethesda, MD, USA).

Fig. 3. Effects of heat stress and KGEs. (A) On water consumption of rats. (B) On body weight of rats. (C) On food intake of rats. (D) On survival rate of rats. Rats were housed at room temperature for 2 weeks and then exposed to heat stress for 4 weeks. Water consumption, body weight, food intake, and survival rate were monitored weekly. Results are expressed as means ± standard deviation (n = 7, repeated). Values with different letters are significantly different (p < 0.05).

HS, heat stress; JP5-L, JP5 low-dose; JP5-H, JP5 high-dose; KGEs, Korean ginseng extracts; RT, room temperature.
transferred to nitrocellulose membranes (Osmonics, Minnetonka, MN, USA). Membranes were incubated with a specific primary antiserum in tris-buffered saline containing 0.05% Tween-20 and 5% nonfat dry milk overnight at 4°C. After three washes with tris-buffered saline containing 0.05% Tween-20, membranes were incubated with peroxidase-conjugated IgG for 1 h at room temperature, visualized using enhanced chemiluminescence (Amer- sham Biosciences, Piscataway, NJ, USA), and photographed using the ChemiDoc system and Image Lab Software (Bio-Rad Laboratories, Hercules, CA, USA).

Fig. 4. Effects of heat stress and KGEs on CD4 and CD8 expression in the spleen. (A) Western blot analysis of CD4 and CD8 expression in spleen tissue of each group of rats. GAPDH was used as a loading control. The bar graphs show the band densities of CD4 and CD8 relative to that of GAPDH. (B) Histological examination of CD4 and CD8 expression in spleen tissue of each group of rats. 4',6-diamidino-2-phenylindole (DAPI) was used as a counterstain. The green-fluorescence shows the expression of CD4 and CD8. Scale bars, 50 μm. Original magnification 630×. Values with different letters are significantly different (p < 0.05).

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HS, heat stress; JP5-H, JP5 high-dose; JP5-L, JP5 low-dose; KGEs, Korean ginseng extracts; RT, room temperature.

Fig. 5. Effects of heat stress and KGEs on expression of HSP70 and inflammatory mediators in the spleen. (A) Western blot analysis of p-NF-κB and IL-6 expression in spleen tissue of each group of rats. GAPDH was used as a loading control. The bar graphs show the band densities of p-NF-κB and IL-6 relative to that of GAPDH. (B) RT-PCR analysis of HSP70, iNOS, and TNF-α expression in spleen tissue of each group of rats. 18S was used as a loading control. The bar graphs show the band densities of HSP70, iNOS and TNF-α relative to that of 18S. Values with different letters are significantly different (p < 0.05).

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HS, heat stress; HSP70, heat shock protein 70; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; JP5-H, JP5 high-dose; JP5-L, JP5 low-dose; KGEs, Korean ginseng extracts; NF-κB, nuclear factor-κB; RT, room temperature; TNF-α, tumor necrosis factor-alpha.
2.9. Statistical analysis

Differences among groups were determined by a one-way analysis of variance followed by Duncan’s multiple range test using SPSS software (SPSS for Windows, version 20; SPSS, Inc., Chicago, IL, USA). Values with different letters are significantly different ($p < 0.05$).

3. Results

3.1. Effects of heat stress and KGEs on body weight, food intake, water consumption, and survival rate of rats

Body weight, food intake, water consumption, and survival rate did not significantly differ among the groups over the first 2 weeks, during which all rats were housed at room temperature (Fig. 3). After exposure to heat stress, water consumption was much higher in the HS group than in the RT group, whereas food intake, body weight, and survival rate were significantly lower. However, these changes in water consumption, food intake, body weight, and survival rate upon heat stress were reduced in the KGE-treated groups.

3.2. Effects of heat stress and KGEs on T-cell activation in the spleen

To investigate the effects of heat stress on the immune system, we monitored expression of CD4 and CD8, which are surface markers of helper and cytotoxic T-cells, respectively, in the spleen. Expression of CD4 and CD8 was significantly higher in the HS group than in the RT group, but these increases were inhibited in the KGE-treated groups (Fig. 4A).

Immunohistochemistry of frozen spleen sections was performed to confirm that heat stress increased expression of CD4 and CD8 and that this was inhibited by KGEs. Fluorescence was stronger in the HS group than in the RT group, but weaker in the KGE-treated groups than in the HS group (Fig. 4B). Moreover, JP5 inhibited heat stress-induced expression of CD4 and CD8. These
results suggest that heat stress stimulates T-cell activation and that this is inhibited by KGEs.

3.3. Effects of heat stress and KGEs on inflammation in the spleen

In general, inflammation accompanies the activation of T-cells, and regulation of this inflammatory response helps to maintain immune homeostasis. To determine whether inflammation underlies the activation of T-cells upon heat stress, we examined gene and protein expression of inflammatory factors in the spleen. NF-κB signaling induces secretion of proinflammatory cytokines such as IL-6, TNF-α, and iNOS. Levels of p-NF-κB and IL-6 were increased in the HS group, and these increases were suppressed in the KGE-treated groups (Fig. 5A). In addition, gene expression of iNOS and

Fig. 7. Effects of heat stress and KGEs on gene and protein expression in muscle tissue. Western blot analysis in muscle tissue of each group of rats. (A) CD4 and CD8 expression. (B) p-p38, p-NF-κB, and IL-6 expression muscle tissue of each group of rats. GAPDH was used as a loading control. The bar graphs show the band densities of CD4, CD8, p-p38, p-NF-κB and IL-6 relative to that of GAPDH. RT-PCR analysis in muscle tissue of each group of rats. (C) HSP70, HSP90, and HSF1 expression. (D) c-Jun, iNOS, and TNF-α expression. (E) CD11b expression. 18S was used as a loading control. The bar graphs show the band densities of HSP70, HSP90, HSF1, c-Jun, iNOS, TNF-α and CD11b relative to that of 18S. Values with different letters are significantly different (p < 0.05).

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HS, heat stress; HSP, heat shock protein 70; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; JP5-H, JP5 high-dose; JP5-L, JP5 low-dose; KGEs, Korean ginseng extracts; NF-κB, nuclear factor-κB; RT, room temperature; TNF-α, tumor necrosis factor-alpha.
TNF-α was significantly increased in the HS group, and these increases were dramatically inhibited in the KGE-treated groups (Fig. 5B). Moreover, JP5 suppressed heat stress–induced expression of iNOS and TNF-α.

HSP70 stimulates secretion of TNF-α and IL-6 via the NF-κB signaling pathway. Furthermore, secretion of HSP70 induces expression of CD4 and CD8 in activated T-cells. Heat stress increased gene expression of HSP70, and this was inhibited by KGEs. Our data clearly demonstrate that KGEs can suppress inflammation induced by heat stress via HSP70 and its signaling pathway in the spleen (Fig. 5). In summary, KGEs prevent activation of T-cells and maintain immune homeostasis upon heat stress.

3.4. Effects of heat stress and KGEs on serum biomarkers

The effects of heat stress and KGEs on serum levels of hormones, inflammatory cytokines, and immunoglobulins were investigated. Serum levels of ACTH and cortisol, which are secreted under stress conditions, TNF-α and IL-2, which are inflammatory cytokines, and IgM and IgA were increased in the HS group (Fig. 6). However, these increases were inhibited in the KGE-treated groups.

3.5. Effects of heat stress and KGEs in muscle tissue

Muscles comprise a large proportion of the body and are greatly affected by heat stress. We performed Western blotting and RT-PCR to investigate the effects of heat stress and KGEs on inflammatory and immunological markers in muscle tissue. Protein expression of CD4 and CD8 in muscle tissue was increased by heat stress, but these increases were inhibited in the JKJ, JP5-H, and BG1 groups (Fig. 7A). Similar effects were observed on protein expression of p-NF-κB, p-p38, and IL-6, which are major inflammatory factors (Fig. 7B). To confirm that KGEs also protect against heat stress in muscle, we investigated gene expression of HSPs, inflammatory factors, and APC markers via RT-PCR. Levels of these genes were significantly increased in the HS group, but these increases were inhibited in the KGE-treated groups (Figs. 7C–7E). These results suggest that heat stress induces an inflammatory response and that this is inhibited by KGEs.

4. Discussion

Heat stress alters the expression of various genes, leading to many physiological changes in the body. These changes in gene expression affect immune regulation [27,28]. In particular, heat stress increases expression of HSP70. This protein is an intracellular chaperone that plays a role in protein folding and inhibits apoptosis and inflammation [29]. However, when released from the cytoplasm into the extracellular milieu, HSP70 modulates proinflammatory cytokines. Specifically, extracellular HSP70 stimulates inflammatory signaling in APCs by binding to various receptors [15]. We demonstrated that gene expression of HSP70 was increased in muscle tissue of rats exposed to heat stress, and that of HSP90 and HSF1, which trimerize with HSP70, was also increased. However, these increases were inhibited by treatment with KGEs prior to heat stress. Similar effects were observed on the secretion of inflammatory cytokines, such as IL-6 and TNF-α, as well as on phosphorylation of NF-κB and p38. In summary, KGEs inhibited expression of HSP70, which induces an inflammatory response and disrupts immune homeostasis upon heat stress.

Heat stress and KGEs elicited similar effects on expression of CD11b, a surface marker of APCs, suggesting that APCs infiltrate muscle tissue in response to inflammatory cytokines upon heat stress. In addition, heat stress and KGEs had the same effects on protein expression of CD4 and CD8, which are markers of activated T-cells. This suggests that T-cells that are already activated or activated by APCs infiltrate muscle tissue in response to cytokines upon heat stress.

Serum levels of the stress hormones ACTH and cortisol were lower in rats administered with KGEs prior to heat stress than in those exposed to heat stress alone. Thus, KGEs inhibit both inflammation and stress caused by high temperatures. The increased serum levels of inflammatory cytokines upon heat stress suggest that muscle tissue is damaged and the inflammatory response is triggered. In particular, IL-2 markedly affects the differentiation and proliferation of T-cells [25,30]. Secretion of IL-2 was significantly higher in the HS group than in the RT group, but this increase was dramatically suppressed in the KGE-treated groups. This suggests that IL-2 secreted into blood upon heat stress affects T-cells. IgM is the first antibody to be produced upon antigen exposure and is generated in large amounts by the spleen.

**Fig. 8. Proposed mechanism whereby KGEs ameliorate abnormal immune response through the regulation of inflammatory constituents.** KGEs ameliorate abnormal immune response through inhibition of the HSP70, NF-κB, and MAPK signaling pathways. → indicates stimulation; ←, inhibition. HSF, heat shock protein; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; KGEs, Korean ginseng extracts; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; TLR, toll-like receptor; TNF-α, tumor necrosis factor-alpha; ERK, extracellular signal–regulated kinase; JNK, c-Jun N-terminal kinase.
IgA binds to and thereby activates APCs, which induces an inflammatory response [31,32]. Levels of IgM and IgA were increased in the HS group, presumably due to the inflammatory response, but these increases were inhibited in the KGE-treated groups.

In conclusion, KGEs ameliorate environmental heat stress–mediated abnormal immune response in Sprague Dawley rats through inhibition of the HSP70, NF-κB, and MAPK signaling pathways (Fig. 8). Therefore, we anticipate that KGEs might be useful functional food ingredients to maintain a balance of immune homeostasis under environmental heat stress condition.

**Conflicts of interest**

The authors declare no conflict of interest.

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