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Research Article

Korean Red Ginseng alleviates neuroinflammation and promotes cell survival in the intermittent heat stress-induced rat brain by suppressing oxidative stress via estrogen receptor beta and brain-derived neurotrophic factor upregulation

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ABSTRACT

Background: Heat stress orchestrates neurodegenerative disorders and results in the formation of reactive oxygen species that leads to cell death. Although the immunomodulatory effects of ginseng are well studied, the mechanism by which ginseng alleviates heat stress in the brain remains elusive.
Methods: Rats were exposed to intermittent heat stress for 6 months, and brain samples were examined to elucidate survival and antiinflammatory effect after Korean Red Ginseng (KRG) treatment.
Results: Intermittent long-term heat stress (ILTHS) upregulated the expression of cyclooxygenase 2 and inducible nitric oxide synthase, increasing infiltration of inflammatory cells (hematoxylin and eosin staining) and the level of proinflammatory cytokines [tumor necrosis factor α, interferon gamma (IFN-γ), interleukin (IL)-1β, IL-6], leading to cell death (terminal deoxynucleotidyl transferase−mediated dUTP nick-end labeling assay) and elevated markers of oxidative stress damage (myeloperoxidase and malondialdehyde), resulting in the downregulation of antiapoptotic markers (Bcl-2 and Bcl-x$_L$) and expression of estrogen receptor beta and brain-derived neurotrophic factor, key factors in regulating neuronal cell survival. In contrast, KRG mitigated ILTHS-induced release of proinflammatory mediators, upregulated the mRNA level of the antiinflammatory cytokine IL-10, and increased myeloperoxidase and malondialdehyde levels. In addition, KRG significantly decreased the expression of the proapoptotic marker (Bax), did not affect caspase-3 expression, but increased the expression of antiapoptotic markers (Bcl-2 and Bcl-x$_L$). Furthermore, KRG significantly activated the expression of both estrogen receptor beta and brain-derived neurotrophic factor.
Conclusion: ILTHS induced oxidative stress responses and inflammatory molecules, which can lead to impaired neurogenesis and ultimately neuronal death, whereas, KRG, being the antioxidant, inhibited neuronal damage and increased cell viability.

1. Introduction

Korean Red Ginseng (KRG) is a steamed and dried product of *Panax ginseng* (PG), which belongs to the *Araliaceae* family, and is used to treat a numerous symptoms, including heat stress [1]. Interestingly, in the USA, the effect of American ginseng, *Panax quinquefolius* (PQ), has been publicized as dissimilar from that of Korean ginseng (PG), suggesting that PG has the potential to raise

body temperature and is thus inappropriate for people living in hot climate or for those with hypertension, whereas PQ is devoid of such side effects and is therefore recommended to be used in hot climates. Such reports have been continuously spread to customers without any scientific evidence [2]. Indeed, clinical trials showed that KRG can treat cold hypersensitivity in the hands and feet without changing body temperature [3–5]. Moreover, animal studies also revealed that PG has no role in raising body

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temperature [2,3,6,7]. This was more evidently proved where PG maintained a constant state of temperature in both hypothermically and hyperthermically stimulated mice [8]. Furthermore, in a separate animal model, PG administration resulted in faster recovery from acute hypothermia [9], and even at a dose of 300 mg/kg, it also did not alter body temperature and thermogenesis-related factors in rats after short- and long-term treatments [2]. This notion was more supported by Park et al [10], who showed that a single dose of 1000 mg/kg PQ reduced body temperature and that there was no significant effect of ginseng treatment on core body temperature after chronic treatment (200 mg/kg/day) for 4 weeks.

Although the brain accounts for approximately 2% of body mass, it requires nearly 20% of blood flow from the heart to maintain continuous supply of fresh oxygen and energy [11]. Because the brain is characterized by a relatively larger amount of iron deposit and lipid content of myelin sheaths, a high rate of oxidative metabolism, and limited antioxidant capacity, it is highly vulnerable to oxidative damage compared with the other organs [12]. Thus, oxidative stress, including that caused by free radicals and reactive oxygen species (ROS), has been implicated as a major pathological mechanism of brain disorders such as Alzheimer disease, brain trauma, and Parkinson disease [13,14]. Subsequent generation of free radicals and formation of ROS impairs DNA, oxidizes protein and lipid content, and finally results in neural degeneration and cell death [15]. Therefore, protection from free radicals contributing to the pathogenesis of neurodegenerative disorders is required to cope with various stress factors.

Heat stress is an environmental factor known for causing oxidative stress and damage [12,16], which causes deleterious effects on the peripheral immune system and neuroinflammatory responses in the brain, leading to increased microglial activation and cytokine production [16].

Several studies were conducted in rodent models and *in vitro* cell lines to elucidate the protective effect of ginseng against heat stress. Rb1 and Rg3 were shown to reduce microglial cell death in a murine model of *Streptococcus pneumoniae* bacterial meningitis and promote survival through estrogen receptor beta (ER-β)/brain-derived neurotrophic factor (BDNF) signaling [17]. In oxidatively stressed human neuroblastoma SK-N-SH cells, pretreatment with KRG downregulates the expression of the proapoptotic proteins p-p53 and caspase-3 but upregulates the expression of the anti-apoptotic protein Bcl-2, thus inhibiting apoptosis via ER-β upregulation and the PI3K/Akt signaling pathway [18]. Environmental heat stress could be mitigated by KRG via downregulation of cellular damage–associated hepatic genes and lipid peroxidation [19]. KRG potently attenuates heat stress–induced testicular damage [20] and subsequently inhibits apoptosis in an immobilization stress–induced gene expression model *via* ER-β upregulation [21]. Furthermore, KRG represses peptidyl arginine deiminase type IV via tumor necrosis factor α (TNF-α) convertase and nuclear factor-κB (NF-κB) in brain cells, thus preventing ROS production and ultimately protecting microglial cell death [22].

Although the molecular mechanisms underlying neuroinflammation-related stress remain unclear, proinflammatory mediators have been suggested to be released from endothelial cells and immune cells through activation of ROS and reactive nitrogen species (RNS), during which astrocytes and microglia are activated [16], resulting in neuronal and glial cell death, axonal destruction, and functional loss [23]. Therefore, management of elevated stress response after thermal induction via inhibition of microglial activation and blockage of inflammatory reaction is important to prevent tissue damage and cell death. Previous *in vivo* stress-related studies have underlined the therapeutic potential of KRG for short-term exposure in the brain and other tissues, whereas we aimed our attention to elucidate the neuroprotective effect of KRG in the heat stress–induced model exposed for long-term effect.

To confirm this hypothesis, we developed a rat model of intermittent long-term heat stress (ILTHS) to address the pathophysiological effect of oxidative damage in the brain and confirm the antioxidative and antiinflammatory effects of KRG and its possible mechanism of action.

2. Materials and methods

2.1. Preparations of KRG water extract

Fresh PG roots were supplied by Korea Ginseng Corporation (Seoul, Korea) and extracted as described previously [24]. In brief, the 6-year-old root was washed with tap water, steamed at 98°C for 90 min, and dried at 70°C for 72 h. The dried material was subjected to hot water extraction by heating four times at 85°C for 8 h with 10 volumes of water, followed by filtration. The filtrate was pooled and concentrated under reduced pressure to obtain a dark brown syrup, referred to as KRG water extract (KRG-WE). The representative chromatogram of KRG-WE, as obtained by ultraperformance liquid chromatography, revealed 11 identifiable ginsenoside peaks (mg/g): Rg1 (0.35), Re (0.45), Rf (1.15), Rh1 (1.50), Rg2s (1.71), Rb1 (4.68), Rc (2.00), Rb2 (1.71), Rd (0.96), Rg3s (3.32), and Rg3r (1.57), as described previously [20].

2.2. Animals, heat exposure, and drug treatments

Male Sprague–Dawley rats (4 weeks old, 60–70 g) were purchased from Samtako Bio Korea, Inc. (Osan, Korea). Within 1 week of arrival, the rats were acclimated for 7 days in an animal facility to adapt to their surroundings. The animals were housed under a constant temperature of $23 \pm 1°C$ and relative humidity of $55 \pm 5\%$, with *ad libitum* access to food and water and a 12-h light/dark cycle. The rats were divided randomly into four treatment groups: normothermic control, heat-stressed control (HC), heat-stressed plus KRG 100 mg/kg, and heat-stressed plus KRG 200 mg/kg. Each dose of KRG (100 and 200 mg/kg) was mixed separately with a regular pellet diet and administered orally for 25 weeks starting at 1 week prior to heat exposure. Rats in the normothermic control group were maintained at 25°C, whereas those in the HC, HK100, and heat-stressed plus KRG 200 mg/kg groups, to induce heat stress, the rats were exposed to a high temperature ($32 \pm 1°C$, 2 h/day) for 6 months. Animal maintenance and treatments were performed in the Regional Innovation Centre Experimental Animal Facility, Konkuk University, South Korea, in accordance with the Animal Care and Use Committee Guidelines.

2.3. Sample collection

On Week 25, all animals were fasted for 24 h with *ad libitum* access to water, and they were immediately sacrificed in a CO_2 chamber. For experimental analysis, the brain samples were isolated and immediately frozen in liquid nitrogen and stored at −80°C until further use.

2.4. Histopathological analysis

The whole brain was harvested and immersed in 20% neutral buffered formalin solution for 24 h, dehydrated, and embedded in paraffin. Tissue sections (5–10-μm thickness) were then stained using hematoxylin and eosin (H&E) according to the manufacturer's instruction (GS Tech Korea Co., Ltd, 804-1, Top-dong, Gwonseon-gu, Suwon-si, Gyeonggi-do, South Korea). Morphological examination was performed according to the standard protocol

using a previously established histopathological scoring system [25] by light microscopy (Olympus BX51, Olympus U-TV0.63XC, 6E19749; Olympus, Tokyo, Japan).

2.5. Terminal deoxynucleotidyl transferase−mediated dUTP nick-end labeling assay

Apoptosis in the hippocampus was assessed using terminal deoxynucleotidyl transferase−mediated dUTP nick-end labeling (TUNEL) assay following the manufacturer's protocol (TUNEL test kit; Promega). Nuclei were counterstained with 4,6-diamidino-2-phenylindole. TUNEL-positive, apoptotic cells were detected by localized fluorescein isothiocyanate (FITC) (green).

2.6. Assessment of malondialdehyde activity

Malondialdehyde (MDA) was evaluated as described previously [26]. The rat brain sample was homogenized with ice-cold 1.15% KCl solution. After centrifugation, 100 μL of the tissue homogenate supernatant was combined with 1 mL of tricarboxylic acid and thiobarbituric acid−HCl reagent and mixed thoroughly. Absorbance of the supernatant was measured at 532 nm in 96-well plates using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.7. Assessment of myeloperoxidase activity

Myeloperoxidase (MPO) content in the brain was measured as described previously [27]. In brief, the brain sample was homogenized on ice in potassium phosphate buffer pH 6.5 containing 0.5% (w/v) hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO, USA). Following homogenization, the supernatant was added to a mixture of o-dianisidine hydrochloride and hydrogen peroxide, and change in absorbance per gram of tissue was measured at 460 nm using a microplate reader (Molecular Devices).

2.8. cDNA synthesis and real-time quantitative polymerase chain reaction

Total RNA (1 μg) was extracted from brain specimens using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and cDNA was purified using a minikit RNA and converted to cDNA using random hexamers (EcoDry™ Premix; Takara, Clontech, USA). Real-time polymerase chain reaction (PCR; Applied Biosystems, Foster City, CA, USA) was performed using the iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA, USA). The oligonucleotide primers (Cosmo Genetech, Seoul, Korea) designed to analyze gene expression levels in mouse are shown in Table 1. GAPDH was used as a housekeeping gene. Relative quantification of target genes from triplicate reactions was analyzed using GraphPad prism 5.0 software (GraphPad Software, San Diego, CA, USA).

2.9. Western blotting analysis

Brain tissue was lysed using a protein assay kit according to the manufacturer's instructions (Bio-Rad). The lysates were separated by 12.5%, 10%, and 7.5% sodium dodecyl sulfate−polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Each membrane was incubated with 5% skim milk in tris-buffered saline containing Tween® 20 for 1 h, further incubated with specific primary antibodies overnight at 4°C, washed with tris-buffered saline containing Tween® 20, and then incubated with the appropriate secondary antibodies (antimouse or antirabbit) for 1 h at room temperature. Immunoreactive bands were detected using an

Table 1
Primer sequences used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis in this study.

Name		Sequence (5′ to 3′)
COX-2	F	CACTACATCCTGACCCACTT
	R	ATGCTCCTGCTTGAGTATGT
iNOS	F	GGAGCCTTTAGACCTCAACAGA
	R	TGAACGAGGAGGGTGGTG
TNF-α	F	GCC TCTTCTCATTCCTGCTT
	R	TGGGAACTTCTCATCCCT TTG
IFN-γ	F	ACAGCTCTCCGTCCTCGTAT
	R	CCTTAGAGTAGAAAGACCG
IL-1β	F	CTGGTGTGTGACGTTCCC
	R	TGTCGTTGCTTGGTTCTCCT
IL-6	F	CCTCTGGTCTTCTGGAGTAC
	R	GGAAATTGGGGTAGGAAGG
IL-10	F	AGCCACATGCTCCTAGAGC
	R	GCCTGGGGCATCACTTCTAC
Bax	F	CAGGATGCGTCCACCAAGAA
	R	CGTGTCCACGTCAGCAATCA
Bcl-2	F	ACGAGTGGGATGGGGGAGATGTG
	R	GCGGTAGCGGCGGGAGAAGTC
Bcl-x$_L$	F	TCCTGCAAGATTGGCCTGTG
	R	CCCACAGCTGCTCTGTATAGCTTC
BDNF	F	TCAAGTTGGAAGCCTGAATGAATG
	R	CTGATGCTCAGGAACCCAGGA
ER-β	F	TGAGCCACCCAATGTGCTA
	F	AACAGGCTGAGCTCCACAAAG
GAPDH	F	ATGGTGAAGGTCGGTGTGAAC
	R	GCCTTGACTGTGCCGTTGAAT

BDNF, brain-derived neurotrophic factor; COX-2, cyclooxygenase 2; ER-β, estrogen receptor beta; IFN-γ, interferon gamma; IL, interleukin; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor α.

enhanced chemiluminescence detection kit (GenDEPOT, Barker, TX, USA). Band intensity was analyzed using ImageJ 2.1.4.6 software (National Institutes of Health, Bethesda, MD, USA).

2.10. Statistical analysis

All statistical data were analyzed using GraphPad Prism 5.0 (GraphPad Software). All values in the figures are expressed as mean ± standard error of the mean. Each experiment was performed in triplicate. Statistical comparison was performed using one-way analysis of variance and Bonferroni's multiple comparison test. A p value of < 0.05 was considered statistically significant.

3. Results

3.1. ILTHS induced proinflammatory responses

Because excessive exposure to high temperature induces various abnormal behaviors and triggers pathophysiological responses in tested animals [16], we first measured physical parameters such as body and organ weights to assess the severity of damage induced by ILTHS (data not shown). Although ILTHS significantly lowered liver weight, other organs did not show any significant change in weight [20]. To further assess whether ILTHS exhibits degradative effect on brain physiology, we examined neuroinflammatory responses. Because oxidative stress and its association with the inflammatory network contribute to the pathogenesis of neurodegenerative diseases [12,16], we first evaluated the effect of heat stress on the brain by quantitative PCR (qPCR). ILTHS (HC group) caused significant upregulation of the proinflammatory mediators cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) at the mRNA level in the HC group (Fig. 1A and B). To confirm the protein expression of these mediators, Western blotting was performed. Consistently, ILTHS
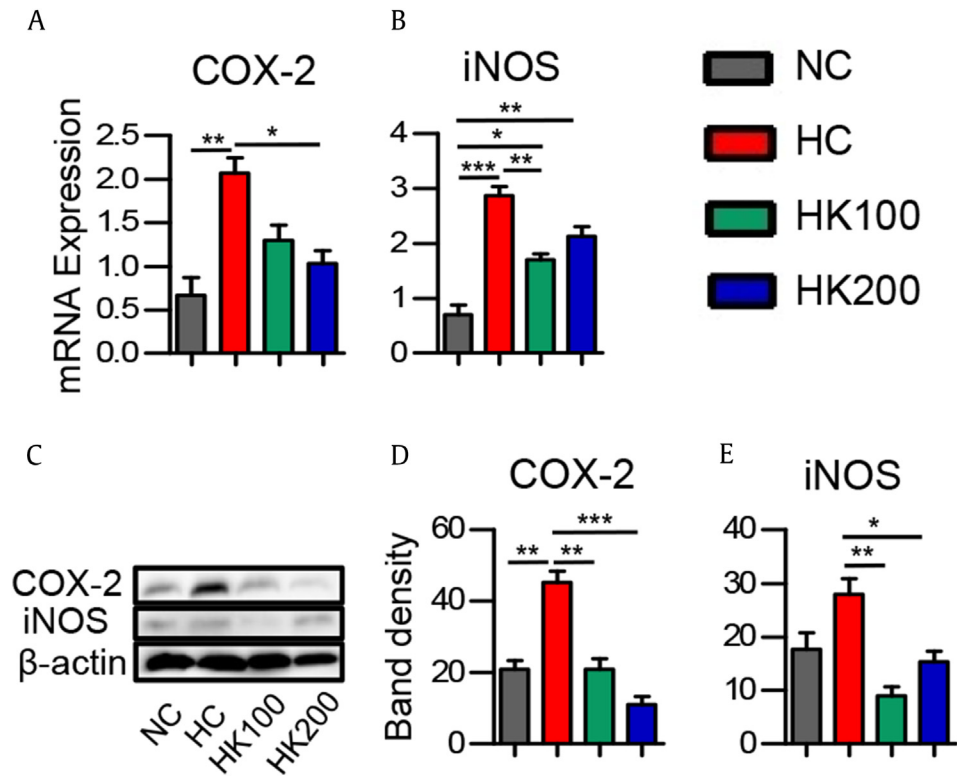
Fig. 1. KRG suppressed the induction of COX-2 and iNOS expression in heat stress−stimulated rat brains. (A, B) To determine the mRNA expression of COX-2 and iNOS, qPCR analysis was performed. (C−E) To determine protein expression of COX-2 and iNOS, Western blotting analysis was performed. (A and B) GAPDH was used as internal controls for qPCR analysis. (D and E) β-actin was used as internal controls for Western blotting analysis. Comparison among groups was evaluated by mean ± SEM for (n = 4/group). Statistical comparison was performed using one-way ANOVA and Bonferroni's multiple comparison test. A *p value < 0.05 was considered as significant. ** indicates the significance of p value. ** indicated less significant whereas *** indicates more significant.
ANOVA, analysis of variance; COX-2, cyclooxygenase 2; HC, heat-stressed control; HK100, heat-stressed plus KRG 100 mg/kg; HK200, heat-stressed plus KRG 200 mg/kg; iNOS, inducible nitric oxide synthase; KRG, Korean Red Ginseng; NC, normothermic control; qPCR, quantitative polymerase chain reaction; SEM, standard error of the mean.

upregulated COX-2 and iNOS protein expression (Fig. 1C, D, E). These results indicated that ILTHS induced inflammatory responses.

3.2. KRG suppressed the expression of the proinflammatory mediators COX-2 and iNOS

To investigate whether KRG treatment modulates inflammatory responses in the ILTHS model, we examined the mRNA expression of COX-2 and iNOS. We found that KRG significantly diminished the expression of these genes, suggesting that KRG exerted neuro-inflammatory activity *in vivo* (Fig. 1A and B). To confirm decreased protein expression of COX-2 and iNOS, Western blotting analyses were performed. Consistently, KRG significantly decreased the levels of these proteins (Fig. 1C−E), suggesting that KRG inhibited ILTHS-induced COX-2 and iNOS expression in the brain.

### 3.3. KRG attenuated neuroinflammation and cell death

The severity index of ILTHS in the rat model was used to investigate pathologic changes in neuronal cells caused by elevated levels of inflammatory mediators in rat brain tissue. Thus, we examined the histological features and apoptotic levels in the hippocampus (Fig. 2A−B). In the HC group, significant damage in brain tissue was observed through H&E staining, including inflammatory cell infiltration, crypt architecture, muscle thickening, immune cell infiltration, goblet cell depletion, and loss of tissue

architecture compared with the control group (Fig. 2A). Similar to the control group, KRG-WE significantly reduced and restored tissue damage in a dose-dependent manner (Fig. 2A). To evaluate whether histological damage to the brain is associated with apoptotic neuronal cell death, TUNEL assay was performed (Fig. 2B). The ILTHS model showed reduced neuronal cell viability and enhanced hippocampal neuronal apoptosis, as indicated by the increased number of TUNEL-positive neurons in the hippocampus. KRG reduced hippocampal neuronal loss and neuronal apoptosis in a dose-dependent manner. Moreover, our results showed that TUNEL-positive cells in rat hippocampal tissues were significantly increased in the ILTHS-induced model as compared with KRG-treated groups (https://www.spandidos-publications.com/mmr/18/3/3437 Fig. 2B).

### 3.4. KRG suppressed the expression of proinflammatory cytokines

Because there is a close association between circulating inflammatory mediators and ROS [16], we investigated whether KRG pretreatment is effective against ILTHS. We analyzed the mRNA expression of proinflammatory cytokines and antiinflammatory cytokines and found a significant increase in the mRNA expression of TNF-α, IFN-γ, interleukin (IL)-1β, and IL-6 in the HC group as compared with the control group (Fig. 3). Conversely, KRG pretreatment significantly attenuated the upregulation of proinflammatory cytokines in the HC group and nonsignificantly increased the IL-10 mRNA level (p value > 0.05) (Fig. 3). These
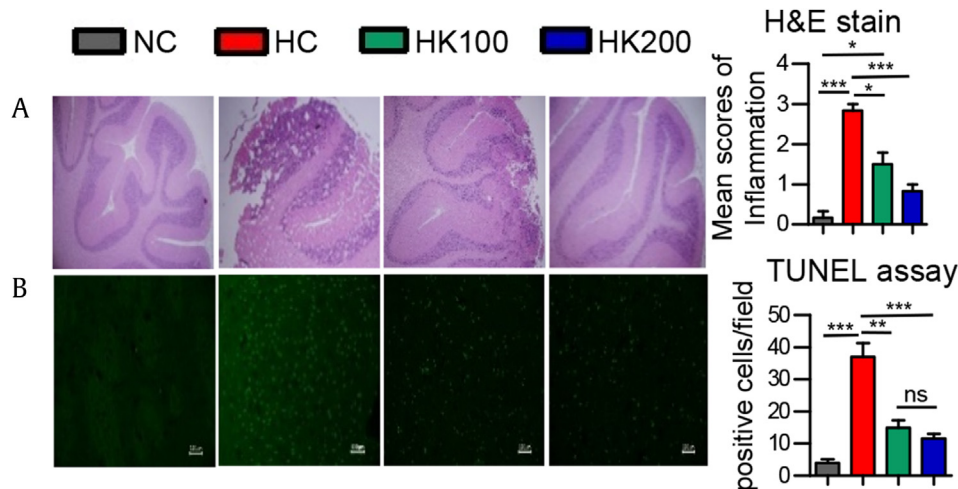
Fig. 2. KRG decreased the severity of inflammation and cell death in the ILTHS-induced model. (A) Hematoxylin and eosin (H&E) staining was performed to assess the expression of inflammatory cell infiltration in the hippocampus. Bar graph represents the difference in disease severity, expressed as the histopathological score among different groups (n = 4 mice/group). All images were obtained at 4 × (upper) magnification. (B) Representative photomicrographs (10 × magnification) and bar diagram show increased TUNEL reactivity. Bar diagram represents apoptotic index of neurons (%). A *p value < 0.05 was considered as significant. ** indicates the significance of p value. ** indicates less significant whereas *** indicates more significant and ns represents non-significant.

HC, heat-stressed control; HK100, heat-stressed plus KRG 100 mg/kg; HK200, heat-stressed plus KRG 200 mg/kg; ILTHS, intermittent long-term heat stress; KRG, Korean Red Ginseng; NC, normothermic control; TUNEL, terminal deoxynucleotidyl transferase—mediated dUTP nick-end labeling.
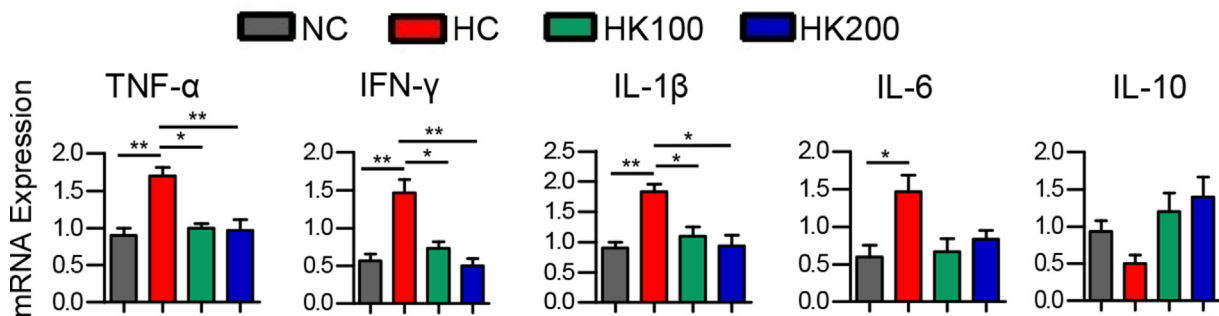
Fig. 3. KRG reduced ILTHS-induced proinflammatory cytokine mRNA levels. Hippocampus samples were collected and subjected to RNA extraction for qPCR. The mRNA expression of TNF-α, IFN-γ, IL-1β, IL-6, and IL-10 was analyzed using GAPDH as an internal control. Data are expressed as mean ± SEM. A p value of < 0.05 was considered statistically significant, as analyzed by using one-way ANOVA and Bonferroni's multiple comparison test. A *p value < 0.05 was considered as significant. * indicates the significance of p value. * indicates less significant whereas ** indicates more significant. In overall, increased significant trend follows as *** > ** > * .

ANOVA, analysis of variance; HC, heat-stressed control; HK100, heat-stressed plus KRG 100 mg/kg; HK200, heat-stressed plus KRG 200 mg/kg; IFN-γ, interferon gamma; IL, interleukin; ILTHS, intermittent long-term heat stress; KRG, Korean Red Ginseng; NC, normothermic control; qPCR, quantitative polymerase chain reaction; SEM, standard error of the mean; TNF-α, tumor necrosis factor α.

findings clearly showed that KRG attenuated ILTHS-induced neuroinflammation, suggesting that KRG could serve as a potential antiinflammatory agent.

3.5. KRG suppressed ILTHS-induced lipid peroxidation

Oxidative stress markers have long been described in the pathophysiology of stress models. MDA is an end product of ROS-induced peroxidation and widely used as an oxidative stress biomarker [28]. Thus, we evaluated the effect of ILTHS on the MDA level. ILTHS increased the MDA level, whereas KRG treatment repressed the MDA level back to the control level (Fig. 4A), suggesting that KRG possessed antioxidant activity.

To further confirm that activated inflammatory cells in the brain break down myelin proteins and generate high levels of oxidative stress [29], MPO activity was determined. ILTHS significantly elevated MPO activity, whereas HK100 reversed this effect (Fig. 4B). These results showed that ILTHS elevated MPO and MDA levels, indicating that ILTHS increased ROS production in the brain, whereas KRG, an antioxidant, ameliorated these effects.

3.6. KRG protected the brain from ILTHS-induced apoptosis

Next, we examined whether ILTHS induces apoptosis via oxidative damage. The mRNA levels of proapoptotic (Bax) and antiapoptotic (Bcl-2 and Bcl-x_L) genes [30] were determined by qPCR, and ILTHS increased Bax expression but did not affect Bcl-2 and Bcl-x_L expression at the mRNA level compared with the normal control (Fig. 5A–C). To confirm this finding, Western blotting was performed. ILTHS did not affect Bax and caspase-3 protein levels but significantly decreased Bcl-2 and increased Bcl-x_L expressions compared with the normal control (Fig. 5D–H). KRG significantly downregulated mRNA expression of the Bax gene but upregulated the mRNA level of Bcl-2 and Bcl-x_L (Fig. 5A–C). To further confirm these findings, the levels of the corresponding proteins were determined by Western blotting. Consistently, KRG decreased the Bax protein level but increased Bcl-2 and Bcl-x_L protein levels (Fig. 5D–G). KRG also nonsignificantly decreased the caspase-3 level (Fig. 5H). These results indicated that KRG has a protective ability against cell death and promotes cell survival during heat stress.
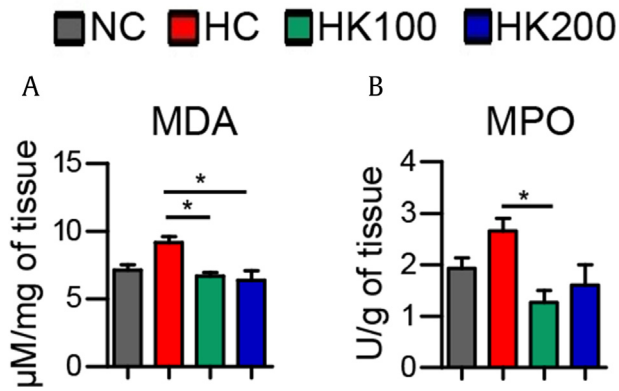
Fig. 4. KRG showed antioxidative effect in the heat-exposed brain. (A) To determine the extent of oxidized substances, an MDA assay was performed. (B) Neutrophil infiltration was quantified by measuring MPO activity. Each experiment was carried out in triplicate. Data are expressed as mean ± SEM, and comparison among groups was evaluated (*p value < 0.05).

HC, heat-stressed control; HK100, heat-stressed plus KRG 100 mg/kg; HK200, heat-stressed plus KRG 200 mg/kg; KRG, Korean Red Ginseng; NC, normothermic control; MDA, malondialdehyde; MPO, myeloperoxidase; SEM, standard error of the mean.

### 3.7. KRG induced BDNF and ER-β expression and cell survival

Because BDNF is a critical regulator involved in the pathophysiology of major depressive disorders and it plays a fundamental role in the development, differentiation, and survival of neuronal cells [31], we addressed whether ILTHS modulates BDNF expression. The results of qPCR analysis showed that ILTHS did not affect BDNF

expression (Fig. 6A). Consistently, the results of Western blotting analysis showed that ILTHS did not significantly affect BDNF expression (Fig. 6C and D). However, KRG upregulated BDNF expression at both the mRNA and protein levels (Fig. 6A, C, D), suggesting that KRG upregulated BDNF under ILTHS conditions.

Several studies have suggested that estrogen and their ligands strongly influence brain functions to regulate various bodily functions at the hormonal level [32]. In addition, *in vitro* oxidative stress and *in vivo* immobilization stress downregulate ER-β [19,22]. However, it remains unclear whether ILTHS modulates ER-β. Moreover, the neuroprotective role of KRG in modulating ER-β against thermal stress is not well understood. We found that ILTHS downregulated ER-β expression, whereas KRG upregulated ER-β expression (Fig. 6B, C, E), suggesting that KRG inhibited apoptosis and antagonized oxidative stress in the brain via BDNF and ER-β upregulation.

4. Discussion

There is substantial evidence that under inflammatory conditions, the thermoregulatory ability of the hypothalamus is compromised, thereby raising body temperature and disrupting the blood−brain barrier [33]. Ginseng has long been widely used as a folk and traditional medicine to treat multiple diseases, including depression and neurodegenerative disorders [34]. In the present study, we examined that 6 months of ILTHS-induced oxidative stress and inflammation resulted in apoptosis via downregulation of BDNF and ER-β, whereas KRG alleviated inflammatory disorders, suggesting that KRG could be a therapeutic agent for preventing oxidative stress and neurodegenerative diseases.
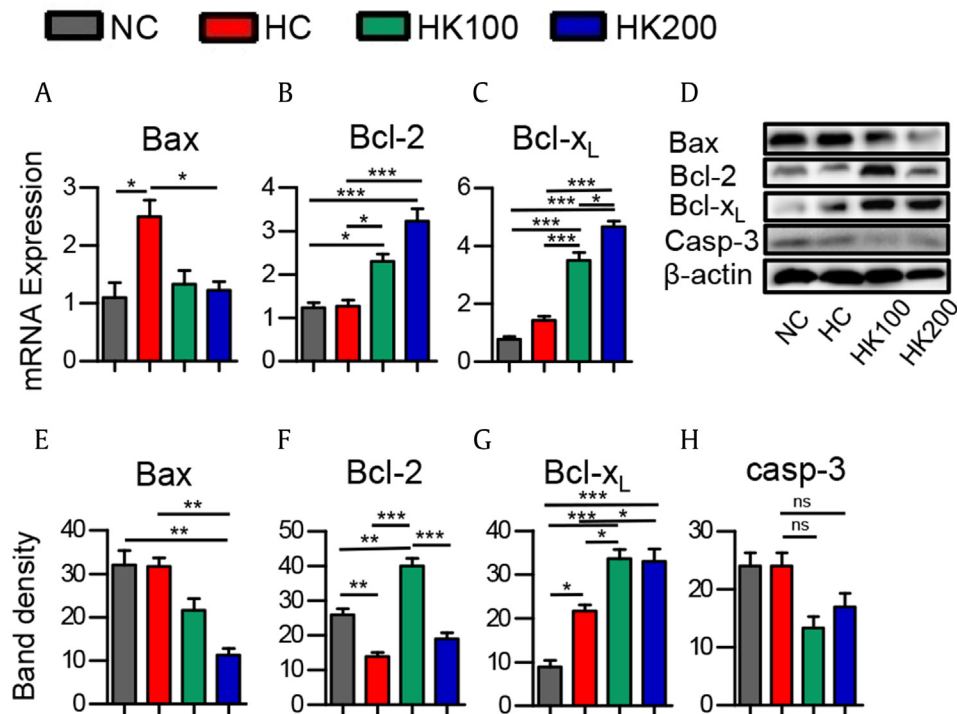
Fig. 5. KRG increased the expression of cell survival and apoptotic genes in the heat stress−induced rat brain. (A-C) The mRNA expressions of Bax, Bcl₂ and Bcl-xᴸ were determined by qPCR analysis. (D-H) The protein expressions of Bax, Bcl₂, Bcl-xᴸ and caspase-3 were determined by western blot analyses. (A−C) GAPDH was used as an internal control for qPCR analysis. (D-H) β-actin was used as an internal control for Western blotting analyses. Values are mean ± SEM. Statistical comparison among groups was performed using one-way ANOVA and Bonferroni's multiple comparison test (*p value < 0.05). A *p value < 0.05 was considered as significant. ** indicates the significance of p value. ** indicates less significant whereas *** indicates more significant and ns represents non-significant.

ANOVA, analysis of variance, HC, heat-stressed control; HK100, heat-stressed plus KRG 100 mg/kg; HK200, heat-stressed plus KRG 200 mg/kg; KRG, Korean Red Ginseng; NC, normothermic control; qPCR, quantitative polymerase chain reaction; SEM, standard error of the mean.
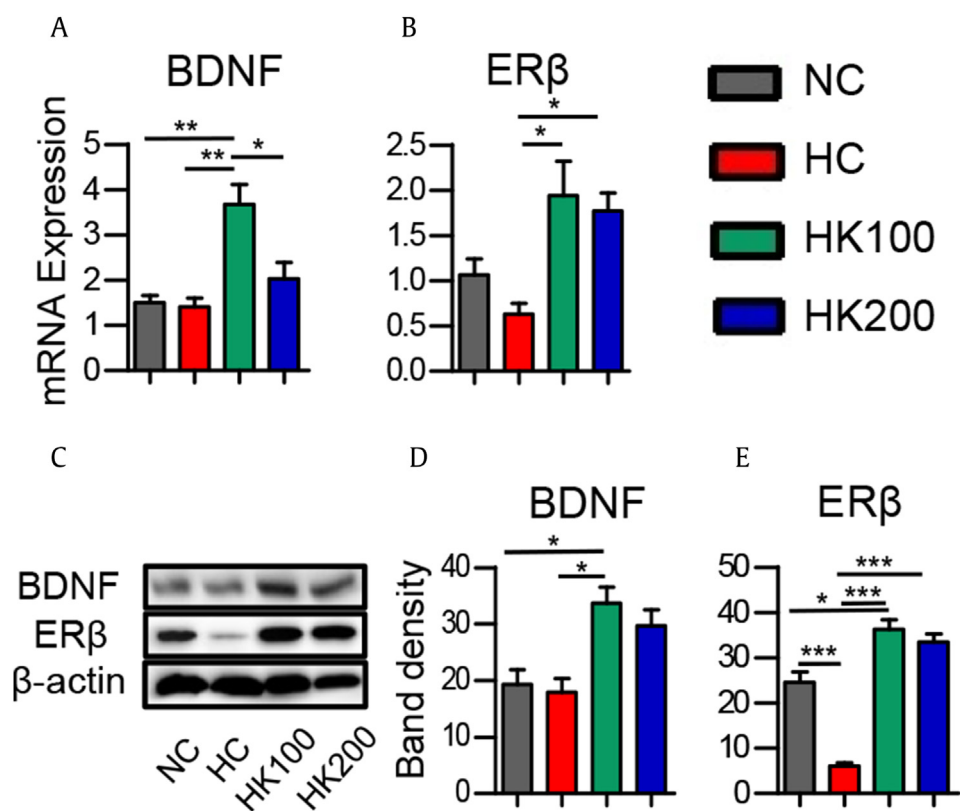
Fig. 6. KRG increased cell survival by upregulating ER-β and BDNF expression. (A and B) The mRNA expression of ER-β and BDNF was determined by qPCR analyses. (C−E) The protein expression of ER-β and BDNF was determined by Western blotting analyses. Values of the bar graph are presented as mean ± SEM (n = 4/group). Statistical comparison was performed using one-way ANOVA and Bonferroni's multiple comparison test (*p value < 0.05). A *p value < 0.05 was considered as significant. ** indicates the significance of p value. ** indicates less significant whereas *** indicates more significant and ns represents non-significant.

ANOVA, analysis of variance, HC, heat-stressed control; HK100, heat-stressed plus KRG 100 mg/kg; HK200, heat-stressed plus KRG 200 mg/kg; BDNF, brain-derived neurotrophic factor; ER-β, estrogen receptor beta; KRG, Korean Red Ginseng; NC, normothermic control; qPCR, quantitative polymerase chain reaction; SEM, standard error of the mean.

In the brain, stress stimulates glucocorticoid secretion, resulting in activation of nuclear factor-κB and subsequent induction of COX-2 and iNOS, marked indicators of the inflammatory reaction that leads to secondary injury and ultimately neurological damage [13,14]. Our study showed that KRG had a potent ameliorative effect on elevated COX-2 and iNOS expression, suggesting that KRG was a promising therapeutic candidate for relieving inflammatory diseases. Increased expression of COX-2 in the brain cortex was observed after a short-term exposure (4−6 h) to restraint stress in rats [14] and forced swim stress in mice [35]. KRG pretreatment also lowered COX-2 expression in an immobilization stress model [22]. Similarly, administration of PG extract for 14 days inhibited COX-2 and iNOS expression in the amygdala after chronic restraint stress treatment [36]. Cortex sections and hippocampal regions stained with H&E at the site of injury displayed neuronal loss, signs of swollen cells, and strong monocyte infiltration [25]. Consistent with these findings, our data showed several apoptotic neurons with cell gaps and debris (Fig. 2A). To further investigate that cell death and neuronal apoptosis in hippocampal tissues are associated with development of neurodegeneration during brain dysfunction [37], TUNEL assay was performed (https://www.spandidos-publications.com/mmr/18/3/3437 Fig. 2B). KRG-WE significantly alleviated apoptosis symptoms and histological damage to brain tissue in a dose-dependent manner (https://www.spandidos-publications.com/mmr/18/3/3437 Fig. 2).

Mounting evidence indicates that stress-related disorder is associated with activation and alteration in microglia function, possibly through subsequent release of proinflammatory cytokines

[38]. Release of proinflammatory cytokines is mediated by neurons, astrocytes, microglia, and endothelial cells, and it can cause neuronal and glial cell death [38]. Microglia promotes proinflammatory responses through excessive TNF-α, IL-1β, iNOS, and ROS production [23], contributing to neural network dysfunction [13,14]. Previous studies have also confirmed that ginsenoside Rg3 effectively suppresses proinflammatory cytokines in lipopolysaccharide (LPS)- or amyloid β−stimulated BV-2 microglial cells, ultimately attenuating inflammatory response [39,40]. Ginsenoside Rh1 suppressed the production of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, and inflammatory enzymes, such as iNOS and COX-2, but increases the expression of the antiinflammatory IL-10 in LPS-stimulated microglia [41]. Consistent with these findings, our results showed that KRG significantly inhibited microglial activation and decreases proinflammatory cytokine production (TNF-α, IFN-γ, IL-1β, and IL-6), but increases the production of the antiinflammatory cytokine IL-10 in the ILTHS-stimulated brain (Fig. 3). Ginsenoside Rb1 was shown to increase neuron cell survival [42] and delay neuronal death because of ischemic damage [43], as well as decrease TNF-α and IL-6 expression, inhibit NF-κB activation, and modulate microglial activation after brain ischemia [44]. Pretreatment with ginsenoside Rg1 activates the phospholipase C signaling pathway and modulates the expression of iNOS, COX-2, TNF-α, IL-1β, and NF-κB in LPS-induced BV-2 microglial cells [45]. Taken together, these results indicate that ginseng has anti-neuroinflammatory effects by reducing microglia activation.

Under oxidative stress, polyunsaturated fatty acids can undergo lipid peroxidation to form complex cytotoxic reactive aldehyde

species that may impair protection against ROS [7]. This was observed in our model, in which KRG repressed the induction of MDA and MPO levels (Fig. 4). Consistent with our data, pretreatment with KRG (100 mg/kg) also decreases MDA and MPO levels in immobilized stressed mice [21]. Certain levels of ROS, RNS, nitric oxide, and hydrogen peroxide can alter antioxidant enzyme capacity, leading to the development of depressive disorders [46]. The antioxidant activities of red ginseng are linked to the downregulation of the ROS-stimulated mitogen-activated protein kinase and Akt pathways [18]. Thus, to reduce the risk of oxidative damage, increased antioxidant intake is an attractive strategy to prevent the production of proinflammatory mediators and free radicals and to delay the onset of neurodegenerative diseases [35]. In the present study, we suggested that KRG might exert numerous protective antioxidant defense mechanisms to reduce and prevent radical formation [47]. To confirm that ILTHS aggravates neuronal dysfunction and neuronal death, we evaluated the efficacy of KRG using apoptotic markers. Bax, a member of the Bcl-2 family of proteins, has been shown to promote and accelerate apoptosis, whereas other members of the family, including Bcl-x$_L$ and Bcl-2, repress cell death and promote cell survival [48]. Moreover, the Bcl-2 gene protects against cell death by reducing heat stress−induced oxidative stress [29,30]. The protein expression of Bcl-2 is associated with inflammation and lipid peroxidation and a critical regulator of both apoptosis and autophagy [48]. Our findings suggested that KRG promoted cell survival because it downregulated the expression of a proapoptotic gene (Bax) and upregulated the expression of antiapoptotic genes (Bcl-2 and Bcl-x$_L$) (Fig. 5). Consistent with this, treatment with KRG represses apoptosis by upregulating Bcl-2 expression and downregulating caspase-3 activation in brain cells [18,21,22]. Our data are in consistent with those of a previous study [49], in which *Panax notoginseng* saccharides exhibit antiapoptotic and neuroprotective effects by preventing brain injury in rats after cerebral ischemia.

BDNF and its interaction are considered important oxidative markers in diagnosing symptoms related to neuronal insult and injury [50]. In addition, BDNF generally promotes neuronal survival and has a role in protecting the brain from neurodegenerative disorders [17]. This notion strongly supports our observation that KRG promoted neuronal cell survival by activating BDNF release. PG extract at 150 mg/kg significantly increased the mRNA level of BDNF in the amygdala of chronic restraint stress−induced mice [35]. Similarly, ER-β is regarded as a neuroprotective factor in numerous central nervous system (CNS) animal models [17,21], and its upregulation *in vitro* represses apoptosis by potentiating PI3K/Akt signaling [21]. Moreover, ER-β confers neuroprotection against ischemia and reduces anxiety and depression-like behaviors [17,51]. Accordingly, we found that KRG treatment substantially reduced apoptosis through induction of BDNF and ER-β expression.

Physiological staining results showed severe inflammation (Fig. 2A) and higher cell death (Fig. 2B) in the HC group. In contrast, KRG inhibited inflammation significantly and cell death (Fig. 2A and B), demonstrating antiinflammatory and anti−cell death effect of KRG. Although we observed discrepancy in cell death−related markers between mRNA and protein levels, KRG treatment decreased Bax significantly in both mRNA and protein levels (Fig. 5A and E) but decreased caspase-3 nonsignificantly (Fig. 5H). Consistently, KRG also increased Bcl-2 and Bcl-x$_L$ in both mRNA and protein levels compared with those in the HC group showing anti−cell death effect of KRG.

Heat stress, having caused preventable and lamentable deaths, is hazardous to construction workers in the hot and humid summers around the world. Practically, the ILTHS model can be encountered commonly in many occupational settings. The existence of extreme hot conditions in many work environments may

have a serious negative effect on the health and safety of employees [52]. Such extreme conditions are commonly encountered in many occupational settings such as steel and iron manufacturing, glass factories, mining, textiles, ceramics, food canneries, and outdoor operations [53]. Construction workers are subjected to heat stress not only from outdoor physical work but also in confined spaces, which could be even worse [54]. Although a refined and enhanced heat stress model is now constructed to predict a worker's physiological responses to different meteorological factors, more specific heat stress guidelines can be formulated based on objective and scientific parameters to safeguard workers' health and safety [55]. Moreover, increases in global average temperature cause heat stress−induced disorders by destroying homeostasis. However, no analytical tools on long-term thermal stress in human participants have been established. In this study, as an initiative to simulate thermal stress in the brain, we used the rodent ILTHS model.
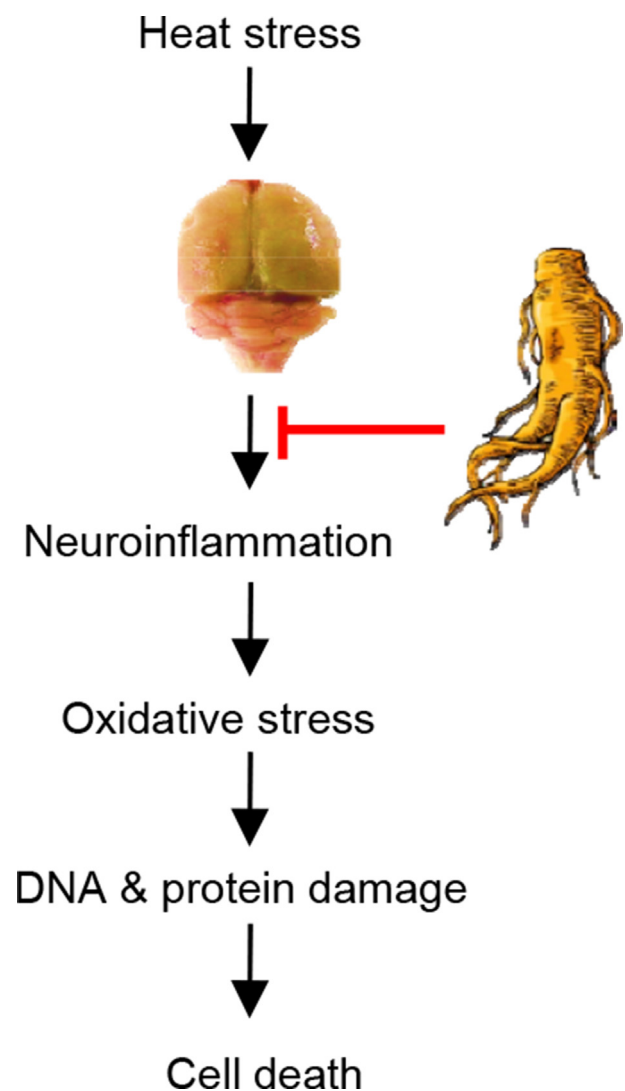
Fig. 7. Protective mechanism of KRG against heat stress in the rat brain. Exposure to thermal stress results in the release of inflammatory mediators and free radicals. This ultimately leads to excess generation of ROS and RNS and may result in damage to various cell components through cytotoxic process of lipid peroxidation and induction of proapoptotic pathways. As a potent antioxidant, KRG reduced DNA damage by upregulating BDNF and ER-β expression levels, preventing neuronal loss, and promoting cell survival possibly by suppressing oxidative impairment.
BDNF, brain-derived neurotrophic factor; ER-β, estrogen receptor beta; KRG, Korean Red Ginseng; RNS, reactive nitrogen species; ROS, reactive oxygen species.

Because 6 months in rodents is equivalent to 18 years in humans [56,57], the ILTHS model would provide enough exposure time for thermal stress analysis.

The results of our experiment using the ILTHS model suggested that hyperthermia occurred owing to the formation of large amounts of peroxides and RNS, which led to oxidative damage in cellular components in the brain, thus exacerbating neuronal impairment. In our model, KRG showed antiinflammatory, antioxidant, antiapoptotic, and immune-stimulatory activities (Fig. 7). The findings of this study strongly supported the preventive and therapeutic potential of long-term KRG administration and showed that it ameliorated heat stress—induced deleterious effects without causing any profound, abnormal effects on brain function. Our data were well supported by related substantial studies performed in rodent models, in which PG did not show any significant effect on body temperature [2,3,6—10]. Therefore, the concept that PG exerts both stimulatory and inhibitory effects on individuals with a tendency for hyperthermia appears to be invalid.

Taken together, the aforementioned findings and emerging evidence suggested that KRG could mediate therapeutic intervention by inhibiting neuroinflammation, delaying the death of vulnerable neurons, and decelerating disease progression by potentiating maximum cell survival responses, thereby preventing neuronal damage and apoptosis in the brain.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

Author contributions

D.-K.R. and H.I. conceived the experiments. H.I., K.-M.C., and M.-S.J. performed the experiments. H.I., S-K.K., P.G., and D.-K.R. analyzed the data. H.I. and D.-K.R. wrote the manuscript. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2019.05.007.

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