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Research Article

Korean Red Ginseng (*Panax ginseng* Meyer) with enriched Rg3 ameliorates chronic intermittent heat stress–induced testicular damage in rats *via* multifunctional approach

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ABSTRACT

Background: *Panax ginseng* Meyer, known as Korean Red Ginseng (KRG), is one of the important age-old traditional herbs used in boosting *libido* and improving male fertility. In this study, the effects of Rg3-enriched KRG extract (KGC04P) on heat stress–induced testicular damage in experimental rats was evaluated.

Methods: Male rats (Sprague-Dawley) were divided into four groups (n = 10): normal control (NC), heat-stressed control (HC), heat-stressed plus KGC04P-100 mg/kg (HK100), and heat-stressed plus KGC04P-200 mg/kg (HK200) groups. Starting 1 week prior to heat stress, animals were administered orally with KGC04P (100 and 200 mg/kg) mixed with a regular pellet diet and continued for 25 weeks. Heat stress was induced to HC, HK100, and HK200 groups by intermittently exposing the animals to high temperatures (32 ± 1°C, 2 h/day). After 6 months, animals were euthanized under general anesthesia with carbon dioxide and evaluated for various parameters in serum and testicular tissue by using Western blotting, biochemical kits, and reverse transcription-polymerase chain reaction.

Results: Significant ($p < 0.05$) alterations in several parameters, such as body/organ weight, sperm kinematics, and lipid metabolism marker levels, in the serum and testis of rats were observed. Further, the expression of testicular antioxidant enzymes, inflammatory cytokines, sex hormonal receptors, and spermatogenesis-related genes were also affected significantly ($p < 0.05$) in the heat-stressed group. However, KGC04P prevented the heat stress–induced changes in rats significantly ($p < 0.05$) at both concentrations.

Conclusion: KGC04P attenuated heat stress–induced testicular damage by a multifunctional approach and can be developed as an excellent therapeutic agent for hyperthermia-mediated male infertility.

1. Introduction

The incidence of reproductive malignancies particularly in males has been constantly increasing and represents a serious health concern [1]. It is estimated that 10–15% of all couples have experienced an infertility problem [1,2]. According to a meta-analysis in 61 studies performed between 1940 and 1990, men showed nearly 50% decrease in their sperm count and viability [3]. Importantly, evidence suggests that male reproductive dysfunction is directly linked to environmental factors, affecting semen quantity and quality significantly [4,5]. This might be due to changes in lifestyle conditions such as inactivity leading to obesity, mental stress, smoking, continuous exposure to hazardous toxic agents (heavy metals, chemotherapy, pesticide residues, high temperature, and air pollution), and chronic diseases such as diabetes, cancer, and hyperhomocysteinemia. These factors alone or collectively might show an effect on sperm production contributing to overall male subfertility and infertility [6].

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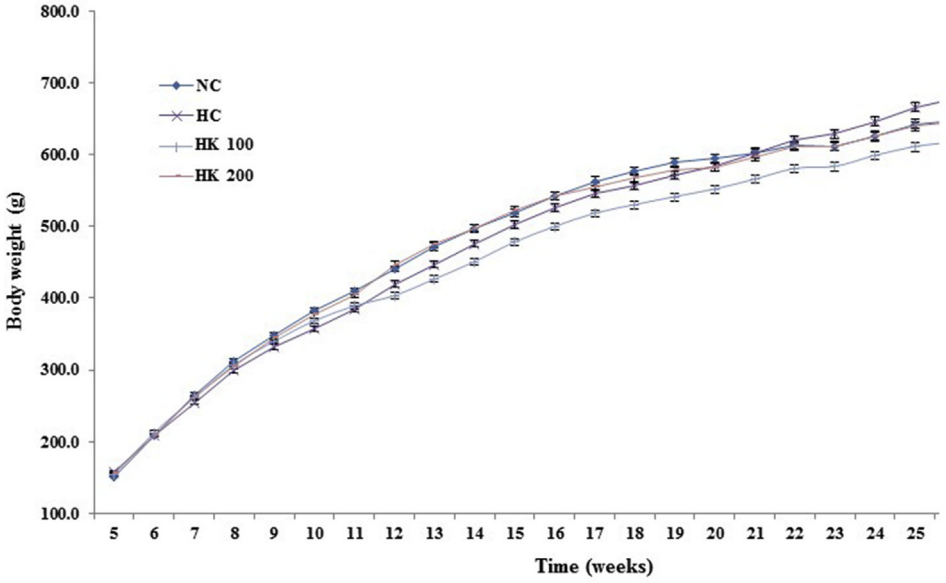

Fig. 1. Influence of KGC04P on body weight in heat-stress rats. Body weight changes during the course of treatments in NC, HC, HK100, and HK200 groups were shown (n = 10). KGC04P, Rg3 enriched Korean Red Ginseng–water extract; NC, normal control; HC, heat-stress control; HK100, heat-stress plus KGC04P 100 mg/kg b.w.; HK200, heat-stress plus KGC04P 200 mg/kg b.w.

In recent years, the influence of genital heat stress was proved to be an important hazard to diminish male fertility [7,8]. Early experimental evidence by Fukui and Moore & Quick indicated that scrotal temperature plays a major role in testicular function [7,8], which should be within 2–8°C below the body temperature. This condition in general is maintained and controlled by scrotum, pampiniform plexus, and muscles serving as a cooling system [9]. Higher temperatures increase the testicular metabolism without a corresponding increase in blood supply and might result in local hypoxia damaging the testicular tissue [10,11]. Despite such a negative impact of heat on spermatogenesis, research on the mechanism of heat-induced testicular alterations and approaches to attenuate heat-induced testicular damage is limited.

Panax ginseng Meyer, particularly steamed and dried Korean Red Ginseng (KRG), is an age-old health-promoting herbal medicine well studied to treat various diseases in Asian countries. Pharmacologically, KRG is known to be effective in various diseases such as diabetes, cancers, Alzheimer's, hypertension, acquired immune deficiency syndrome and reproductive problems [12,13]. Further, KRG has been traditionally considered beneficial in boosting libido and treating infertility in men. Earlier studies from our laboratory showed that KRG protected against testicular damage induced by doxorubicin and aging [12]. Further KRG improved spermatogenesis in experimental models of testicular dysfunction [12,14]. In view of the above literature, we evaluated the effects of KRG extract and its possible mode of action against testicular damage induced by chronic intermittent heat stress in rats. Parameters including the protein and mRNA expression of selected antioxidative enzymes, spermatogenesis-related molecules, sperm kinematics, sex hormone receptors, and inflammatory cytokines were evaluated.

2. Materials and methods

2.1. Korean Red Ginseng extract preparation

KRG extract was generously supplied by Korea Ginseng Corporation (KGC), South Korea. The extraction procedures were performed as previously described with minor modification [15]. Briefly, tap water washed 6-year-old *P. ginseng* root was steamed and dried before subjecting to hot water and aqueous ethanol (80%) extraction, and filtered. The filtrates were pooled, concentrated under reduced pressure, and lyophilized. The resultant extract obtained was a ginsenoside Rg3-enriched brown powder (KGC04P), confirmed by using ultra-performance liquid chromatography equipped with photodiode array at 203 nm [16]. The contents in KGC04P used in this study were as follows (mg/g): Rg1 (0.38), Re (0.55), Rf (1.26), Rh1(1.43), Rg2s (2.73), Rb1 (5.77), Rc (1.93), Rb2(1.81), Rd (0.92), Rg3s (8.38), and Rg3r (2.66).

2.2. Animals and experiment design

Male Sprague Dawley rats (40; 4-weeks-old, 60–70 g) were purchased from Samtako Bio Korea, Inc. (Osan, Korea) and were acclimated for one week in our specific pathogen free lab at the Regional Innovation Center Experimental Animal Facility, Konkuk University, South Korea. Constant temperature (23 ± 2°C) with relative humidity (55 ± 5%) on a 12 h light/dark cycle was maintained supplying with food and water *ad libitum*. The rats were maintained in compliance with the Institutional Animal Care and Use Committee Guidelines and was approved by the Animal Ethics Committee (Permission No: KU12052), South Korea.

Animals were divided into four groups (n = 10), namely normal group (NC); heat-stressed alone group (HC); heat-stressed plus KGC04P-100 mg/kg group (HK100); and heat-stressed plus KGC04P–200 mg/kg group (HK200). Starting 1 week prior to heat stress, animals were administered orally with KGC04P (100 and 200 mg/kg) mixed with a regular pellet diet and continued for 25 weeks. The KGC04P dose (100 and 200 mg/kg) was based on our previous study [17] and was adjusted every 2 weeks by taking the body weight increase and the daily dietary intake into account. Except for NC group maintained at 25°C, heat stress was induced to other three groups by intermittently exposing the animals to high temperature (32 ± 1°C, 2 h/day). After 6 months, all animals were fasted for 24 h with access to water *ad libitum* and then euthanized under general anesthesia with carbon dioxide (CO_2). The testes were excised, and the samples were prepared for biochemical experiments as described in our previous report [17].

Table 1
Nucleotide sequence of primers employed in this study

Peroxiredoxin 4	Forward: 5′-CTG ACT GAC TAT CGT GGG AAA TAC T-3′ Reverse: 5′-GAT CTG GGA TTA TTG TTT CAC TAC C-3′
Glutathione S-transferase m5	Forward: 5′-TAT GCT CCT GGA GTT TAC TGA TAC C-3′ Reverse: 5′-AGA CGT CAT AAG TGA GAA AAT CCA C-3′
Glutathione peroxidase 4	Forward: 5′-GCA AAA CCG ACG TAA ACT ACA CT-3′ Reverse: 5′-CGT TCT TAT CAA TGA GAA ACT TGG T-3′
Inhibin-α	Forward: 5′-AGG AAG GCC TCT TCA CTT ATG TAT T-3′ Reverse: 5′-CTC TTG GAA GGA GAT ATT GAG AGC-3′
Androgen receptor	Forward:5′-CTG GAC TAC CTG GAT CTC TA-3′ Reverse:5′-CCT GGG CTG TAG TTT TAT TG-3′
Follicle-stimulating hormone receptor	Forward:5′-GGA CTG AGT TTT GAA AGT GT-3′ Reverse:5′-TTC CAT AAC TGG GTT CAT CA-3′
Luteinizing hormone receptor	Forward:5′-CTA TCT CCC TGT CAA AGT AA-3′ Reverse:5′-TTT GTA CTT CTT CAA ATC CA-3′
Nectin-2	Forward:5′-AGT GAC CTG GCT CAG AGT CA-3′ Reverse:5′-TAG GTA CCA GTT GTC ATC AT-3′
Glyceraldehyde-3-phosphate dehydrogenase	Forward:5′-AAC TTT GGC ATT GTG GAA GGG C-3′ Reverse:5′-ACA CAT TGG GGG TAG GAA CAC G-3′
cAMP responsive element binding protein-1	Forward: 5′-ACT GGC TTG GCA CAA CCA GA-3′ Reverse: 5′- GGC AGA AGT CTC TTC ATG ATT-3′

cAMP, cyclic adenosine monophosphate.

2.3. Measurement of serum biochemical parameters

Serum biochemical parameters such as glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, albumin, total cholesterol (T-CHO), low-density lipoprotein cholesterol (LDL-C), triglyceride, and glucose were measured with the blood collected from the abdominal vein into SST® gel and clot activator tubes (Becton and Dickinson, Franklin Lakes, New Jersey). The measurement of serum biochemical parameters was analyzed using commercially available kits (Diagnostic Product Corporation, Los Angeles, USA) as described in our previous report [18].

2.4. Measurement of sperm kinematic values

The protocol for sperm sample extraction and analysis was performed as described earlier [18]. Computer-assisted sperm analyzer (CASA, Hamilton Thorne Res., Massachusetts, USA) with a 4× objective lens with a charge-coupled device camera was used for recording sperm motility. For evaluating motility pattern, each sample contained not less than 200 sperms.

2.5. Western blotting and reverse transcription-polymerase chain reaction

The protocol for the measurement of protein and mRNA expression level was followed as described previously [17]. The intensity of the bands was analyzed and justified using the ImageJ software package (version 1.410; National Institutes of Health, Bethesda, MA, USA). The nucleotide sequence of primers employed in this study was shown in Table 1.

2.6. Statistical analysis

The data are expressed as the mean ± standard error of the mean (S.E.M), and statistical analysis was carried out by Student *t* test for comparisons between two groups and analysis of variance for multiple comparisons, using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of KGC04P on body weight increments and organ weight in heat-stressed rats

The final body weight for the NC group at 6 months was 647.3 ± 62.57 g (Fig. 1). Body weight increases in the HC, HK100, and HK200 groups demonstrated slightly different patterns from that of NC, but these changes were not statistically significant. The final body weight at 6 months was 678.89 ± 74.78, 618.10 ± 37.75, and 646.43 ± 40.47 g for HC, HK100, and HK200 groups, respectively. All animals survived the entire experimental period, and no abnormal behavior was observed in any group. Further, organ weights such as liver, spleen, kidney, testis, heart, epididymis, and adrenal gland were assessed for each group, and no pathological changes in any tested group were observed (Table 2). However, only two organs, kidney and epididymis, weighed significantly less in the HC group compared with those in the NC group ($p < 0.05$). Increased kidney and epididymis weights were observed in rats that received KGC04P when compared to the HC group, but the values were statistically significant only in HK200 treated group. Overall, the body and organ weights were not markedly influenced by KGC04P treatment compared to the untreated HC group.

3.2. Effect of KRG on sperm kinematics in heat-stressed rats

The normal motile sperm percentage shown in the NC group was 91.67 ± 6.29%. However, the motile sperm percentage was

Table 2
Effect of KGC04P on organ weight in heat-stressed rats

Organ group	Liver	Spleen	Kidney	Testis	Heart	Epididymis	Adrenal gland
NC	14.28 ± 1.97	0.75 ± 0.12	3.24 ± 0.12	3.41 ± 0.12	1.68 ± 0.19	0.69 ± 0.04	0.07 ± 0.08
HC	13.73 ± 2.10	0.77 ± 0.06	3.09 ± 0.10*	3.35 ± 0.02	1.70 ± 0.19	0.66 ± 0.02*	0.07 ± 0.01
HK100	12.98 ± 1.13	0.79 ± 0.11	2.97 ± 0.10	3.43 ± 0.19	1.73 ± 0.14	0.66 ± 0.07	0.06 ± 0.01
HK200	13.72 ± 0.70	0.76 ± 0.06	3.20 ± 0.18**	3.45 ± 0.12	1.75 ± 0.21	0.69 ± 0.06**	0.07 ± 0.01

Data are expressed as mean ± S.E.M (n = 10). Statistical analysis was carried out by Student *t* test and one-way ANOVA using GraphPad Prism version 4.0
* $p < 0.01$ compared with the NC group.
** $p < 0.05$ compared with the HC group.
ANOVA, analysis of variance; b.w., body weight; KGC04P, Korean Red Ginseng–water extract; HC, heat-stressed control; HK100, heat-stressed and received KGC04P 100 mg/kg b.w.; HK200, heat-stressed and received KGC04P 200 mg/kg b.w.; NC, normal control; S.E.M, standard error of the mean.

decreased to 58.33 ± 2.68% ($p < 0.01$) in the HC group. On the other hand, the percentage of motile sperm in the HK100 and HK200 groups were significantly higher, at 81.00 ± 5.69% and 85.10 ± 7.46%, respectively ($p < 0.01$), when compared to that in the HC group. The percentage of sperm with progressive movement in the NC group was 31.93 ± 3.81%, which decreased to 18.89 ± 8.27% ($p < 0.01$) in the HC group. The percentage of sperm with progressive motility in the HK100 and HK200 groups increased significantly to 30.57 ± 9.61% and 33.10 ± 6.89% ($p < 0.01$), when compared to that in the HC group. Other sperm parameters were not significantly altered by HC, HK100, or HK200 compared to the NC group (Table 3).

3.3. Effect of KGC04P on serum biochemical parameters in heat-stressed rats

There was a significant decrease in T-CHO and LDL-C levels in the HC group when compared to those in the NC group ($p < 0.05$) (Table 4). However, KGC04P treatment at both doses did not influence the lipid metabolite profiles.

3.4. Effect of KGC04P on the expression of antioxidant enzymes in heat-stressed rat testis

The protein expression levels of glutathione peroxidase 4 (GPx4), glutathione S-transferase mu 5 (GSTm5), and peroxiredoxin 4 (PRx4) in the HC group were downregulated when compared to those in the NC group (Fig. 2A); quantification revealed a significant suppression in the protein levels of GPx4 and PRx4. Although we observed a decrease in GSTm5 expression, the level was not significant. However, treatment with KGC04P at both doses significantly ($p < 0.05$) prevented the downregulation of the protein expression levels of these antioxidant enzymes. A similar pattern was observed in the mRNA expression of these antioxidant enzymes (Fig. 2B).

3.5. Effect of KGC04P on spermatogenesis-related factors in heat-stressed rat testis

The testicular mRNA and protein expression levels of inhibin-α were downregulated in the HC group compared to the NC group (Figs. 3A, 3B). Quantification of the data revealed that the protein and mRNA expression levels of inhibin-α were significantly ($p < 0.05$) decreased in the HC group compared to the NC group and were prevented by KGC04P treatment at both doses ($p < 0.05$). However, no changes were observed in nectin-2 and CREB-1 expression in any treatment group (Figs. 3A, 3B).

Table 4
Effect of KGC04P on serum biochemical parameters in heat-stressed rats.

Group	GLU (mg/dL)	T-CHO (mg/dL)	TG (mg/dL)	LDL-C (mg/dL)
NC	156.8 ± 22.7	92.4 ± 12.9	30.2 ± 14.1	46.7 ± 7.1
HC	169.0 ± 28.3	83.5 ± 9.9[#]	41 ± 12.4	44.2 ± 3.8[#]
HK100	153.0 ± 6.0	76.6 ± 15.0	38.7 ± 4.7	44.7 ± 8.1
HK200	158.0 ± 10.4	84.6 ± 8.5	43.0 ± 12.0	43.0 ± 7.9

Data are expressed as mean ± S.E.M. (n = 10).
[#] $p < 0.05$ compared with the NC group by Student t test and ANOVA using GraphPad Prism version 4.0.
ANOVA, analysis of variance; b.w., body weight; GLU, glucose; T-CHO, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HC, heat-stressed control; HK100, heat-stressed and received KGC04P 100 mg/kg b.w.; HK200, heat-stressed and received KGC04P 200 mg/kg b.w.

3.6. Effect of KGC04P on the expression of sex hormone receptors in heat-stressed rat testis

Heat stress induced a significant ($p < 0.05$) suppression in the mRNA and protein levels of sex hormone receptors such as the androgen receptor, luteinizing hormone receptor and follicle-stimulating hormone receptor compared to the NC group (Fig. 3). Interestingly, these changes were significantly ($p < 0.05$) prevented by KGC04P treatment when compared to the HC group (Figs. 4A, 4B).

3.7. Effect of KGC04P on the inflammatory cytokine mRNA levels in heat-stressed rat testis

The mRNA expression of inflammatory cytokines such as cyclooxygenase-2, interleukin-6, interleukin-1β, and tumor necrosis factor-α increased in the HC group compared to the NC group (Fig. 5A). Quantification revealed that KGC04P administration to heat-stressed rats significantly prevented the increase in expression of inflammatory cytokines when compared to the HC group (Fig. 5B).

4. Discussion

Increased body temperature adversely affects the testicular tissue, altering spermatogenesis and leading to infertility [19–23]. Previous studies revealed that exposure to heat at a particular temperature (43°C for 15 min) caused damage to many types of germ cells in experimental animals [24,25]. Studies on various animal and human tissues have also demonstrated that hyperthermia can adversely affect the physiology of cells by disrupting transcription and altering oxidative metabolism [26–29]. It is well

Table 3
Effect of KGC04P on sperm kinematics in heat-stressed rats

Average group	Motile (%)	Progressive (%)	VAP (mm/s)	VSL (mm/s)	VCL (mm/s)	STR (%)	LIN (%)	Elongation (%)
NC	91.67 ± 6.29	31.93 ± 3.81	32.87 ± 6.39	23.08 ± 3.74	76.00 ± 4.41	69.47 ± 5.41	39.33 ± 5.61	95.47 ± 3.62
HC	58.33 ± 2.68[*]	18.89 ± 8.27[*]	30.11 ± 6.43	21.78 ± 8.78	74.38 ± 2.01	73.33 ± 7.48	40.89 ± 5.16	97.89 ± 2.52
HK100	81.00 ± 5.69	30.57 ± 9.61[**]	34.86 ± 1.39	25.41 ± 1.46	80.30 ± 9.44	72.71 ± 7.13	42.29 ± 7.72	98.29 ± 1.60
HK200	85.10 ± 7.46[**]	33.10 ± 6.89[**]	34.20 ± 7.20	23.85 ± 5.89	82.76 ± 4.02	69.60 ± 4.81	38.00 ± 4.42	95.60 ± 2.07[*]

Data are expressed as mean S.E.M., (n = 10). Statistical analysis was carried out by Student t test and one-way ANOVA using GraphPad Prism version 4.0
[*] $p < 0.01$ compared with NC group.
[**] $p < 0.05$ compared with the HC group.
ANOVA, analysis of variance; b.w., body weight; KGC04P, Korean Red Ginseng–water extract; HC, heat-stressed control; HK100, heat-stressed and received KGC04P 100 mg/kg b.w.; HK200, heat-stressed and received KGC04P 200 mg/kg b.w.; LIN, linearity; NC, normal control; S.E.M., standard error of the mean; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity.

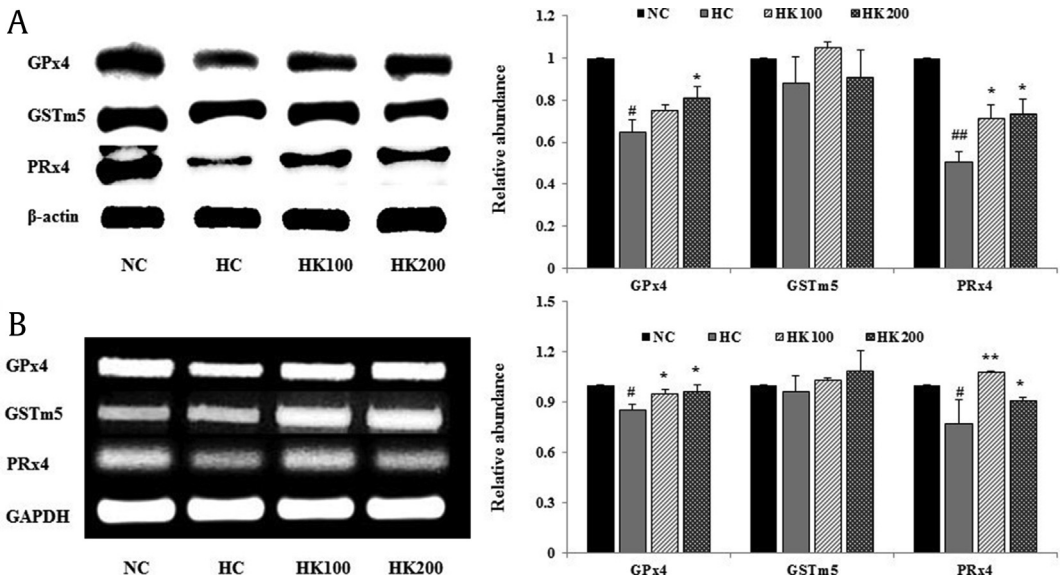

Fig. 2. Influence of KGC04P on antioxidant enzymes, glutathione peroxidase 4 (GPx4), glutathione S-transferase mu 5 (GSTm5), and peroxiredoxin 4 (PRx4) in heat-stress rats. (A) Western blotting analysis and band intensities (right panel) in testicular tissue with β-actin as internal control were shown. (B) RT-PCR analysis and band intensities (right panel) in testicular tissue with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were shown. Data are represented as mean ± standard error of mean (S.E.M.; n = 10). $^{\#}p < 0.05$ compared with the NC group and $^{*}p < 0.05$, compared with the HC alone group by Student *t* test and one-way ANOVA using GraphPad Prism version 4.0.
ANOVA, analysis of variance; KGC04P, Rg3 enriched Korean Red Ginseng–water extract; NC, normal control; HC, heat-stress control; HK100, heat-stress plus KGC04P 100 mg/kg b.w.; HK200, heat-stress plus KGC04P 200 mg/kg b.w; RT-PCR, reverse transcription-polymerase chain reaction.

known that heat stress in male damage both spermatogenesis and the testicular endocrine function, leading to sterility conditions [24,25,30].

In the past few years, researchers have been actively engaged in linking occupations with prolonged heat exposure and a decrease in testicular function [31]. Reports also showed that complementary and alternative therapies, including supplementation with antioxidants, can protect testicular cells from heat stress [25,26]. Attempts have been made using various agents with different mechanisms such as zinc, ascorbic acid, GnRH agonist, and anti-androgen to attenuate the negative effects of heat stress on male fertility. However, many of them were shown to be clinically inefficient [27,30,32–35].

There is a growing interest in the role of naturally occurring medicinal plants with numerous traditional claims for the control and management of various diseases. Here, we explored the effect

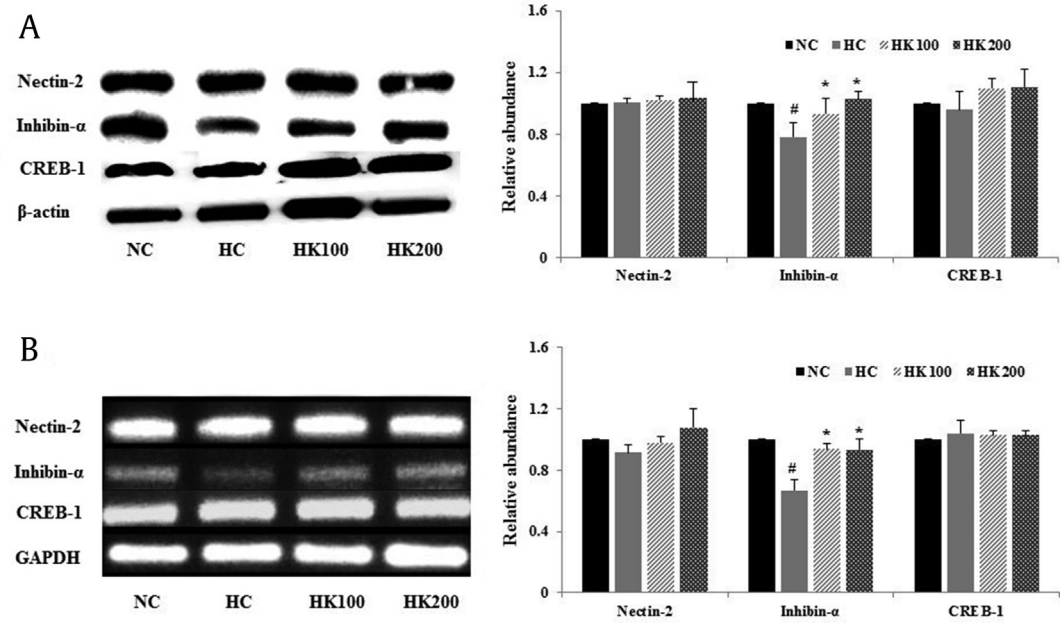

Fig. 3. Spermatogenesis-related biomarker expression (nectin-2, CREB-1, and inhibin-α) **in heat-stress rats treated with KGC04P extract**. (A) Western blot analysis and band intensities (right panel) in testicular tissue were shown from three independent experiments with β-actin as internal control. (B) The mRNA expression and band intensities (right panel) in testicular tissue were shown with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control. Data are represented as mean ± standard error of mean (S.E.M.; n = 10). $^{\#}p < 0.05$ compared with the NC group and $^{*}p < 0.05$ compared with the HC alone group by Student *t* test and one-way ANOVA using GraphPad Prism version 4.0.
ANOVA, analysis of variance; KGC04P, Rg3 enriched Korean Red Ginseng–water extract; NC, normal control; HC, heat-stress control; HK100, heat-stress plus KGC04P 100 mg/kg b.w.; HK200, heat-stress plus KGC04P 200 mg/kg b.w.

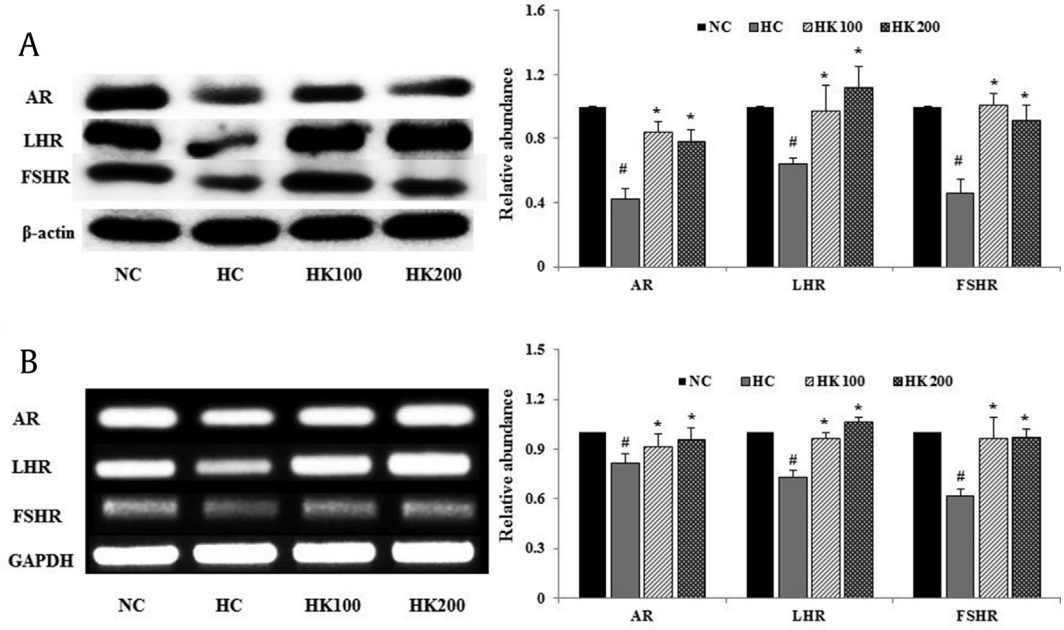

Fig. 4. Sex hormone receptor expression of androgen receptor (AR), luteinizing hormone receptor (LHR) and follicle stimulation hormone receptor (FSHR), in heat-stress rats treated with KGC04P. (A) Western blot analysis and band intensities (right panel) in testicular tissue from three independent experiments with β-actin as internal control were shown. (B) The mRNA expression and band intensities (right panel) in testicular tissue with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control were shown. Data are represented as mean ± standard error of mean (S.E.M.; n = 10). $^{\#}p < 0.05$ compared with the NC group and $^{*}p < 0.05$, compared with the HC alone group by Student *t* test and one-way ANOVA using GraphPad Prism version 4.0.
ANOVA, analysis of variance; KGC04P, Rg3 enriched Korean Red Ginseng–water extract; NC, normal control; HC, heat-stress control; HK100, heat-stress plus KGC04P 100 mg/kg b.w.; HK200, heat-stress plus KGC04P 200 mg/kg b.w.

of *P. ginseng*, which is considered a promising herb with potential benefits in several ailments, against testicular damage in rats induced by heat stress. The findings of our study confirmed that administration of KGC04P (100 and 200 mg/kg) for 6 months prevented the deleterious effects induced by heat stress in several aspects. With respect to the body weight increments, no significant difference or abnormal behavior between any of the groups was observed. The organ weights such as the heart, spleen, liver, adrenal gland, and testis were also not influenced by heat stress when compared with the normal rats. However, a significant decrease in kidney and epididymis weights in the heat stress–induced groups was observed. In agreement with our data, earlier reports indicated that heat stress negatively affects the spermatozoa number in the epididymis and produce changes in kidney tissue both in its morphology and physiology [27,35–38]. However, treatment with KGC04P (200 mg/kg) attenuated the decrease in kidney and epididymis weight observed in HC group.

Further, the sperm kinematics parameters of motility and progressiveness were significantly affected in HC group, which showed a similar tendency to earlier reported studies [27]. Treatment with KGC04P at both doses prevented the decreased sperm motility and progressiveness significantly ($p < 0.05$) when compared with the HC group. Our results were in agreement with a previous clinical study indicating that *P. ginseng* improved sperm viability, concentration, and motility in male infertility patients [39]. Overall our data indicated that KRG protects testicular damage by improving the decreased sperm concentration and number.

Heat stress also negatively influenced the serum biochemical parameters such as LDL-C and T-CHO when compared with NC group. It is well-documented that abnormal blood levels of lipids

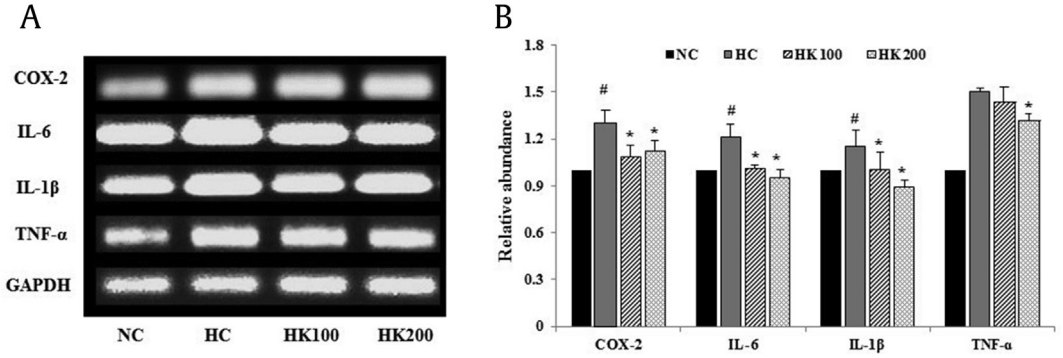

Fig. 5. Influence of KGC04P on the mRNA expression of cyclooxygenase (COX)-2, interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)-α in heat-stress rats. The mRNA expression (A) and band intensities (B) in testicular tissue with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control were shown. Data are represented as mean ± standard error of mean (S.E.M.; n = 10). $^{\#}p < 0.05$ compared with the NC group and $^{*}p < 0.05$, compared with the HC alone group by Student *t* test and one-way ANOVA using GraphPad Prism version 4.0.
ANOVA, analysis of variance; KGC04P, Rg3 enriched Korean Red Ginseng–water extract; NC, normal control; HC, heat-stress control; HK100, heat-stress plus KGC04P 100 mg/kg b.w.; HK200, heat-stress plus KGC04P 200 mg/kg b.w.

including triglycerides, LDL, and cholesterol affect longevity. The condition of hyperlipidemia is known to play a critical role in testicular damage [40]. In the present study, although not significant, increased levels of LDL-C, T-CHO, and triglyceride and decrease in level of HDL-C in the HC group was observed when compared with the NC group. However, these changes were prevented by KGC04P treatment.

The expression of genes related to spermatogenesis such as inhibin-α, nectin-2, and CREB are considered crucial to maintaining normal spermatogenesis, and alterations may affect the production of morphologically abnormal and sterile spermatozoa [41–43]. However, increased testicular temperature causes the testicular germ cells to atrophy and arrests sperm production by altering the transcription factors such as inhibin and nectin related to spermatogenesis [43–45]. In the present study, the inhibin-α expression was decreased in the HC group indicating that heat stress in rats exhibited a deleterious role in genes related to spermatogenesis. KGC04P treatment attenuated this change, supporting the hypothesis that KRG may control the transcription factors and restore the signaling mechanisms involved in spermatogenesis. However, in the present work, nectin-2 and CREB were not influenced by heat stress and needs more detailed study.

Earlier reports revealed that stress altered various oxidative enzymes and might be involved in elevated reactive oxygen species (ROS). Reports have also indicated that heat stress induces mitochondrial degeneration, disturbances in plasma membrane integrity, and increased ROS production in the cytoplasmic and peroxisomal environment in various human cell lines [46,47]. In particular, pachytene spermatocytes and early spermatids are highly vulnerable to ROS induced by heat stress [48,49]. Multifunctional redox enzymes such as GPx4, GSTm5, and PRx4 were involved in spermatogenesis pathway, and any change in the expression of these proteins in testicular cells might cause oxidative damage and cell death [50]. Therefore, we measured the antioxidant enzyme levels in the testis of heat-stressed rats and observed that the protein and mRNA levels of GPx4, GSTm5, and PRx4 were downregulated. However, treatment with KGC04P attenuated these changes significantly ($p < 0.05$). Our results indicated that KGC04P enables normal sperm production by regulating oxidative enzymes in the testis of heat stress–induced rats.

Previous reports have confirmed that pectinase-treated ginseng restored the sex hormone receptors expression level reduced by ROS [51]. In agreement, our present study showed a significant reduction in the sex hormone receptors levels in heat stress-induced groups and treatment with KGC04P significantly ameliorated these changes. Further, our study showed several ginsenosides identified in KGC04P by ultra-performance liquid chromatography analysis (Fig. S1). Some of the ginsenosides, particularly Rg3, were well reported to possess several biological properties [52]. In agreement, the extract in the present study showed high levels of Rg3 (8.83 mg/g), and these results provide more reliable evidence that Rg3-enriched KRG extract may play a role in preventing heat stress–induced changes in sex hormone receptors that affect spermatogenesis.

In conclusion, KGC04P exhibited potent effects in attenuating heat-stress induced testicular damage and might be recommended as a traditional medicine or adjuvant therapy to modern society exposed to occupational testicular hyperthermia. KGC04P can be developed further as a therapeutic agent for the treatment of male subfertility and infertility.

Conflicts of interest

No conflicts of interest exist.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jgr.2018.06.004.

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