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Research article

Pectinase-treated *Panax ginseng* protects against chronic intermittent heat stress-induced testicular damage by modulating hormonal and spermatogenesis-related molecular expression in rats

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ABSTRACT

Background: Elevated testicular temperature disrupts spermatogenesis and causes infertility. In the present study, the protective effect of enzymatically biotransformed *Panax ginseng* Meyer by pectinase (GINST) against chronic intermittent heat stress-induced testicular damage in rats was investigated.
Methods: Male Sprague–Dawley rats (4 wk old, 60–70 g) were divided into four groups: normal control (NC), heat-stress control (HC), heat-stress plus GINST-100 mg/kg (HG100), and heat-stress plus GINST-200 mg/kg (HG200) treatment groups. Each dose of GINST (100 mg/kg and 200 mg/kg) was mixed separately with a regular pellet diet and was administered orally for 24 wk. For inducing heat stress, rats in the NC group were maintained at 25°C, whereas rats in the HC, HG100, and HG200 groups were exposed to 32 ± 1°C for 2 h daily for 6 mo. At week 25, the testes and serum from each animal were analyzed for various parameters.
Results: Significant ($p < 0.01$) changes in the sperm kinematic values and blood chemistry panels were observed in the HC group. Furthermore, spermatogenesis-related molecules, sex hormone receptors, and selected antioxidant enzyme expression levels were also altered in the HC group compared to those in the NC group. GINST (HS100 and HS200) administration significantly ($p < 0.05$) restored these changes when compared with the HC group. For most of the parameters tested, the HG200 group exhibited potent effects compared with those exhibited by the HG100 group.
Conclusion: GINST may be categorized as an important medicinal herb and a potential therapeutic for the treatment of male subfertility or infertility caused by hyperthermia.

1. Introduction

Body temperature regulation is critical to ensure optimal functioning of various organs depending on the molecular and cellular mechanisms associated with each particular process [1,2]. Elevated testicular temperature disrupts spermatogenesis and causes infertility [3,4]. Extensive preclinical studies in rats, mice, and monkeys indicate that mild testicular heat stress (43°C for 15 min) induces a massive decrease in sperm production along with oxidative damage by activation of diverse signaling pathways. The adverse effect of hyperthermia on normal adult testis in humans is well known [5,6]. Elevated temperatures are becoming more prevalent because of modern lifestyle and hazards related to occupation. These factors in particular may influence sperm production and contribute to male infertility [7,8].

Testicular heat stress involves oxidative damage to the germ cells, Leydig cells, and most likely, the Sertoli cells [9]. The production of free radicals and reactive oxygen species (ROS) including the superoxide anion and hydrogen peroxide can induce positive changes in sperm function such as hyperactivation, capacitation, and acrosome reaction [10,11]. However, overproduction of ROS can be detrimental to sperm and may lead to male infertility.

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Spermatozoa are highly sensitive to ROS-induced damage [12]. As both spermatogenesis and Leydig cell steroidogenesis are vulnerable to oxidative stress, peroxidative damage is regarded as the most important cause of impaired testicular function. Increased body temperature negatively alters metabolic function, thereby enhancing the formation of ROS, leading to severe oxidative stress [13]. Oxidative stress occurs following a disturbance in the balance between the production of ROS and antioxidant defense systems [14]. Therefore, in order to ensure sperm quality it is important to prevent whole body heat stress.

Several strategies have been reported to ameliorate local heating to the scrotum in experimental animals [2,15]. However, none of these strategies have been suitable for clinical practice. Much interest has been centered on the role of naturally occurring medicinal herbs for the control and management of male sexual dysfunctions [16–18]. Ginseng (*Panax ginseng* Meyer, Araliaceae), which is considered the thousand-year-old phytomedicine, has been used to treat various diseases in Asia and in other countries [19,20]. It was suggested that *P. ginseng* has potent effects on sexual function and could relieve erectile dysfunction [18], senile testicular dysfunction [21], and dioxin-induced testicular damage [22]. Pectinase-treated *P. ginseng* (GINST), an enzymatic biotransformed *P. ginseng*, exhibits increased bioefficiency of ginseng extracts/products and shows strong pharmacological effects including ameliorating testicular problems [20]. It was well reported that the biotransformation of ginsenosides with the treatment of various enzymes including pectinase and rapidase proved to be beneficial for conversion of ginsenoside into products with increased bioefficiency of ginseng extracts [23]. Reports also revealed that the enzyme pectinase transforms ginsenosides into Compound K (CK), Rg3, Rg5, Rk1, Rh1, F2, and Rg2 providing greater bioefficiency [24,25]. However, the protective effect and mechanisms of GINST remain unknown in the context of alleviative action against heat stress. In this study, we investigated the protective effects of GINST against heat stress-induced testicular damage and further explored the underlying mechanisms involved.

2. Materials and methods

2.1. GINST preparation and high-performance liquid chromatography analysis

Korean Red ginseng was harvested in the fall, around September to October, when its roots are 6 yr old. Growth conditions include cultivation in soil with rich humus in a wide open area, and with good drainage. The harvested Korean Red ginseng was steamed, dried, and stored under room temperature with vacuum packing. The dried ginseng (1 kg) was extracted with 5 L of 50% ethanol at 85°C. To remove the alcohol, the extract was evaporated at 35–40°C in a vacuum until its volume is halved to obtain a dark brown, viscous solution. The extract was subsequently dissolved in water containing 2.4% pectinase (Sigma-Aldrich, Inc., St. Louis, MO, USA) and was incubated at 55°C for 24 h [26]. The GINST extract was then concentrated *in vacuo*, and the ginsenosides in the extract were analyzed using high-performance liquid chromatography (HPLC) (Agilent, Santa Clara, CA, USA) using a diode array detector. An HS C18 (25 cm × 4.6 mm, i.d., 5 μm; SUPELCO, St. Louis, MO, USA) was used for all separations at room temperature. The binary gradient elution system consisted of water (Solvent A) and acetonitrile (Solvent B). The separation was achieved using the following gradient program: 0–10 min (20% B), 40 min (32% B), 48 min (42% B), 60 min (45% B), 78 min (75% B), and 80 min (100% B). The column temperature was kept constant at room temperature; the flow rate was 1.6 mL/min.

2.2. Experimental animals

Male Sprague–Dawley rats (40 total, 4 wk old, 60–70 g) were purchased from Samtako Bio Korea, Inc. (Osan, Korea) and were acclimated to the animal facility for 1 wk prior to the experiment. They were provided with a standard pellet diet and were kept at a constant temperature (23 ± 2°C) and relative humidity (55 ± 5%) on a 12/12-h light/dark cycle with access to food and water *ad libitum*. The rats were maintained in the Regional Innovation Center Experimental Animal Facility, Konkuk University, Korea, in accordance with the Institutional Animal Care and Use Committee Guidelines. The study was approved by the Animal Ethics Committee (Permission No: KU12052) in accordance with Article 14 of the Korean Experimental Animal Protection Law.

2.3. Experimental design

Rats were divided into four groups: normal control (NC), heat-stress control (HC), heat-stress plus GINST-100 mg/kg (HG100), and heat-stress plus GINST-200 mg/kg (HG200) treatment groups. The administration of GINST was done based on our previous study [27]. Breifly, Each dose of GINST (100 mg/kg and 200 mg/kg) was mixed evenly with sterilized standard diet powder and administered orally after pelletization for 24 wk. The experimental pellet diet was stored in a cool, dry area, and no contamination was found throughout the course of administration. The GINST dose (100 mg/kg and 200 mg/kg b.w.) was based on our previously reported study [26]. It was adjusted every 2 wk based on body weight and the daily dietary intake. For inducing heat stress, rats in the NC group were maintained at 25°C, whereas rats in the HC, HG100, and HG200 groups were exposed intermittently to high temperatures for long periods: 32 ± 1°C, 2 h/d, for 6 mo. At week 25, all animals were fasted for 24 h with access to water *ad libitum* and were euthanized under general anesthesia with carbon dioxide. The testes were excised, washed in ice cold saline, and cleaned of the adhering fat and connective tissues. A 10% testicular tissue homogenate was prepared in Tris-hydrochloride buffer (0.1M, pH 7.4) and was centrifuged (489 × *g* for 10 min at 4°C) to pellet the cell debris. The clear supernatant was used for the subsequent assays.

2.4. Measurement of serum biochemical parameters

Blood was taken from the abdominal vein and collected in an SST gel & clot activator tube (Becton and Dickinson, Franklin Lakes, NJ, USA). The serum was separated by centrifugation at 1,500*g* for 10 min at room temperature. Serum biochemical parameters such as serum glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), albumin concentration (ALB), serum total cholesterol (T-CHO), low-density lipoprotein-cholesterol (LDL-C), triglyceride (TG), and glucose (GLU) were analyzed using their respective commercially available kits (Diagnostic Product Corporation, Los Angeles, CA, USA).

2.5. Measurement of sperm kinematic values

Sperm samples were extracted from the left caudal epididymis by cutting it with scissors; one drop of caudal fluid was immediately placed in a culture dish containing 5 mL Hank's balanced salt solution prewarmed to 37°C and supplemented with 10 mg/mL BSA (bovine serum albumin). After incubation for 5 min at 37°C, an aliquot of the suspension was collected with a micropipette and diluted to contain 40 ± 10 sperm under the defined microscopic field (×100 magnification). The suspension (10 μL) was then added to a 2X-CEL slide (depth: 80 μm, thickness: 0.15 mm; Hamilton Thorne Res., Beverley, MA, USA) that had been prewarmed in a CO_2

incubator (Sanyo Electric Co., Osaka, Japan) at 37°C. Sperm motility was recorded using a computer-assisted sperm analyzer (CASA; Hamilton Thorne Res.) with a ×4 objective lens and a charge-coupled device camera. At least 200 sperm in each sample were monitored for motility pattern analysis.

2.6. Western blot analysis

Equal amounts of testis protein from each sample were separated via 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and were transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Each membrane was incubated for 1 h in Tris-buffered saline containing 0.1% Tween-20 and 5% skimmed milk to block nonspecific antibody binding. The membranes were subsequently incubated with specific primary antibodies (1:2,000 dilution; Santa Cruz Biotech, Santa Cruz, CA, USA). Beta-actin was used as an internal control. Each protein was detected using horseradish peroxidase-conjugated secondary antibodies and a chemiluminescence detection system (GE Healthcare Life Sciences, Little Chalfont, UK).

2.7. RNA isolation and real-time reverse transcription polymerase chain reaction

For the *in vivo* analysis, total RNA was extracted from testicular tissue by using the RNA-Bee reagent according to the manufacturer's instructions, and the RNA (1 μg) was reverse-transcribed following the procedures described previously [26]. The primers used are listed in Table 1. Polymerase chain reaction (PCR) was performed for 30 cycles at 95°C for 40 s, 56°C for 40 s, and 72°C for 40 s. After amplification, the PCR products were separated using electrophoresis on a 2.0% agarose gel containing ethidium bromide, and the bands were visualized with ultraviolet fluorescence. The intensity of the bands was analyzed using the ImageJ software package (version 1.410; National Institutes of Health, Bethesda, MA, USA).

2.8. Statistical analysis

The data are expressed as mean ± standard deviation. Significance was analyzed using Student *t* test for comparisons between two groups and analysis of variance for multiple comparisons, using the GraphPad Prism version 4.0 (Graph Pad Software, San Diego, CA, USA). A p value < 0.05 was considered statistically significant.

3. Results

3.1. HPLC fingerprint analysis of GINST

The HPLC chromatogram of GINST is shown in Fig. 1A. We previously observed that during pectinase treatment, the major saponin peaks including ginsenosides Rg1, Rg2 R, Rb1, Rb2, and Rd in *P. ginseng* extract were substantially reduced [26]. In agreement to our previous data, the peaks related to the ginsenosides in GINST were reduced in the batch used for the present study. Furthermore, increased peak with higher concentrations of CK was observed. The ginsenoside content of GINST determined on a dry weight basis was as follows: Rg1 (46.72 mg/g), Re (18.66 mg/g), Rf (62.18 mg/g), Rh1 (33.68 mg/g), stereoisomer Rg2 S (10.02 mg/g), stereoisomer Rg2 R (10.34 mg/g), Rc (53.49), Rb1 (18.99 mg/g), Rb2 (79.98 mg/g), Rd (103.77 mg/g), Rg3 S (21.65 mg/g), Rg3 R (28.85 mg/g), CK (280.48 mg/g), stereoisomers Rh2 S (35.68 mg/g), and Rh2 R (53.94 mg/g).

Table 1
Primers used in the study

Peroxiredoxin (PRx) 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 (GST) 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 (GPx) 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 (AR)	Forward: 5′-CTG GAC TAC CTG GAT CTC TA-3′ Reverse: 5′-CCT GGG CTG TAG TTT TAT TG-3′
Follicle-stimulating hormone receptor (FSHR)	Forward: 5′-GGA CTG AGT TTT GAA AGT GT-3′ Reverse: 5′-TTC CAT AAC TGG GTT CAT CA-3′
Luteinizing hormone receptor (LHR)	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 (GAPDH)	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 (CREB-1)	Forward: 5′-ACT GGC TTG GCA CAA CCA GA-3′ Reverse: 5′-GGC AGA AGT CTC TTC ATG ATT-3′

3.2. Effect of GINST on body weight increments and organ weight in heat-stress rats

As shown in Fig. 1B, minor differences in body weight increment between groups were observed, but these variances were not statistically significant. The final body weight at week 24 for the NC group was 624.8 ± 46.5 g, 646.3 ± 59.3 g for HC, 598.3 ± 49.6 g for HG100, and 610.4 ± 36.8 g for HG200. All animals survived the experimental period, and no abnormal behavior was observed in the NC, HC alone, and/or HG100- and HG200-treated groups during the course of the study.

The weights of seven organs (the liver, spleen, kidney, testis, heart, epididymis, and adrenal gland) were measured in the NC, HC, HG100, and HG200 groups (data not shown). No pathological changes were observed in any of the organs in all treatment groups. Only two organs (kidney and epididymis) showed a significant ($p < 0.05$) reduction in the HC group compared with the NC group (Table 2). However, treatment with GINST at 200 mg/kg attenuated this decrease significantly ($p < 0.05$). Other organ weights were maintained at constant levels.

3.3. Effect of GINST on serum biochemical parameters in heat-stress rats

With regard to hepatic markers, although GPT and ALB levels were unaltered in all groups, GOT levels increased significantly ($p < 0.05$, Table 3) in the HC group compared to those in the NC group (from 77.3 ± 14.6 to 105.5 ± 23.5). Treatment with 200 mg/kg

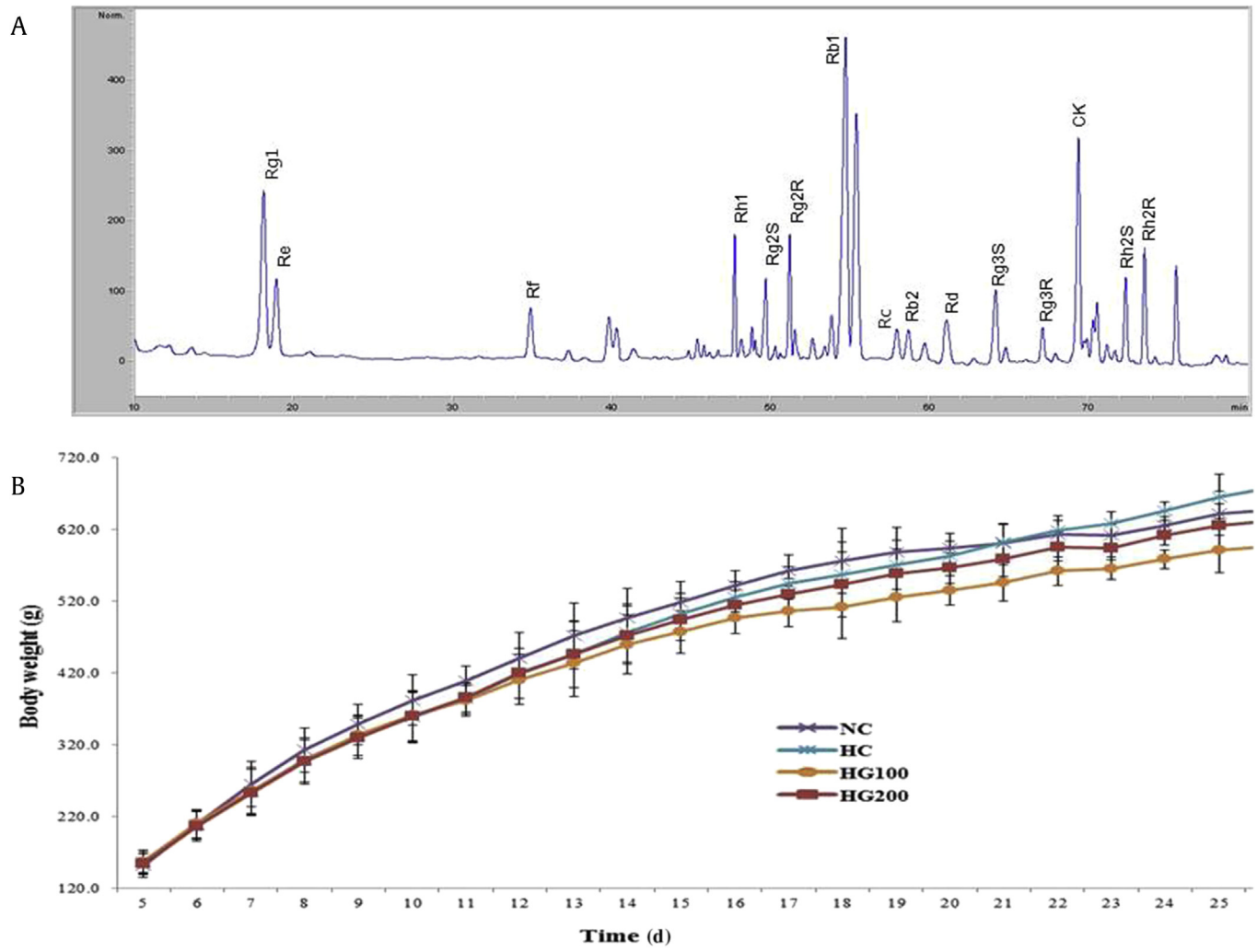

Fig. 1. HPLC fingerprint analysis of GINST and effect of GINST on the body weight increment in heat-stress rats. (A) HPLC chromatogram. (B) The overall body weight of NC, HC, HG100, and HG200 groups from weeks 1 to 25 are shown. Each point represents the mean ($n = 10$). b.w., body weight; GINST, pectinase-treated *Panax ginseng* extract; NC, normal control; HC, heat-stress control; HG100, heat-stress and received GINST 100 mg/kg b.w.; HG200, heat-stress and received GINST 200 mg/kg b.w; HPLC, high-performance liquid chromatography.

GINST reduced the increased levels of GOT significantly ($p < 0.05$) to near-normal levels (78.4 ± 10.2). In the lipid profile, T-CHO and TG levels were decreased significantly ($p < 0.05$ at 100 mg/kg and $p < 0.01$ at 200 mg/kg) in the HC group compared with those in the NC group, respectively. However, LDL-C levels were not altered in all groups tested. GLU levels were also decreased significantly ($p < 0.05$) in the HC group compared with those in the NC group (from 161.5 ± 10.5 to 140.9 ± 15.5). However, treatment of heat-stress rats with 200 mg/kg GINST significantly ($p < 0.05$) attenuated this decrease (164.8 ± 27.4).

3.4. Effect of GINST on sperm kinematics in heat-stress rats

The ratio of motile sperm in the NC group was 91.67 ± 6.29%, and the ratio in the HC group decreased to 58.33 ± 20.68% ($p < 0.01$, Table 4). The motile sperm ratio in the HG100 and HG200

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

Group	Organ						
	Liver	Spleen	Kidney	Testis	Heart	Epididymis	Adrenal gland
NC	14.28 ± 1.97	0.75 ± 0.13	3.25 ± 0.12	3.41 ± 0.12	1.67 ± 0.19	0.75 ± 0.041	0.08 ± 0.01
HC	13.73 ± 2.11	0.78 ± 0.07	3.07 ± 0.10$^{\#}$	3.36 ± 0.03	1.70 ± 0.19	0.62 ± 0.03$^{\#}$	0.08 ± 0.01
HG100	13.89 ± 1.16	0.80 ± 0.11	3.09 ± 0.12	3.49 ± 0.11	1.73 ± 0.14	0.64 ± 0.05	0.08 ± 0.01
HG200	14.73 ± 1.89	0.79 ± 0.01	3.22 ± 0.09*	3.52 ± 0.09	1.71 ± 0.01	0.72 ± 0.08*	0.08 ± 0.01

Data are expressed as mean ± SEM ($n = 10$). 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; GINST, pectinase-treated *Panax ginseng* extract; HC, heat-stressed control; HG100, heat-stressed and received GINST 100 mg/kg b.w.; HG200, heat-stressed and received GINST 200 mg/kg b.w.; NC, normal control; SEM, standard error of the mean.

Table. 3
Effect of GINST on serum biochemical parameters in heat-stressed rats

Group	Serum biochemical parameters (mg/dL)						
	GOT	γ-GPT	ALB	T-CHO	TG	LDL-C	GLU
NC	77.3 ± 14.6	26.5 ± 5.8	2.7 ± 0.1	89.3 ± 20.5	70.5 ± 29.7	6.0 ± 2.2	161.5 ± 10.5
HC	105.5 ± 23.5$^{\#\#}$	22.5 ± 2.6	2.7 ± 0.2	66.3 ± 8.2$^{\#\#}$	39.8 ±9.9$^{\#}$	4.5 ± 1.2	140.9 ± 15.5$^{\#}$
HG100	96.3 ± 35.8*	23.0 ± 3.9	2.7 ± 0.1	74.1 ± 10.1*	43.9 ± 20.1*	4.8 ± 1.8	150.6 ± 16.1*
HG200	78.4 ± 10.2**	21.7 ± 2.4	2.8 ± 0.2	86.4 ± 8.8**	62.5 ± 15.5**	5.2 ± 2.5	164.8 ± 27.4**

Data are expressed as mean ± SEM ($n = 10$).
$^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ compared with the NC group; * $p < 0.05$ and ** $p < 0.01$ compared with the HC group by Student t test and one-way ANOVA using GraphPad Prism version 4.0.
ALB, albumin concentration; ANOVA, analysis of variance; b.w., body weight; GINST, pectinase-treated *Panax ginseng* extract; GLU, glucose; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; HC, heat-stressed control; HG100, heat-stressed and received GINST 100 mg/kg b.w.; HG200, heat-stressed and received GINST 200 mg/kg b.w.; LDL-C, low density lipoprotein-cholesterol; NC, normal control; SEM, standard error of the mean; T-CHO, total cholesterol; TG = triglyceride.

groups increased significantly to 81.40 ± 10.40% ($p < 0.05$) and 90.40 ± 9.73% ($p < 0.01$), respectively, when compared with that in the HC group. The ratio of sperm with straightforward movement in the NC group was 31.90 ± 13.81%, and the ratio in the HC group decreased markedly to 18.89 ± 8.27% ($p < 0.01$). The ratio of sperm with straightforward movement in the HG100 and HG200 groups was significantly higher, 26.29 ± 16.02% ($p < 0.05$) and 35.90 ± 9.40% ($p < 0.01$), respectively, when compared with the HC group.

3.5. Effect of GINST on the expression of antioxidant enzymes in heat-stress rat testis

The effect of GINST on the mRNA expression levels of the antioxidant enzymes PRx4, GPx4, and GSTm5 in heat-stress rat testis is shown in Fig. 2. The HC group showed a significant ($p < 0.01$) decrease in GPx4, GSTm5, and PRx4 mRNA expression level compared with those in the NC group (Fig. 2A). However, treatment of heat-stress rats with GINST significantly ($p < 0.01$) and dose-dependently attenuated the decrease at both 100 mg/kg and 200 mg/kg as revealed by the PCR product band intensities (Figs. 2B and 2C). Interestingly, GSTm5 expression was considerably affected in the HC group ($p < 0.001$) when compared with the NC group, and GINST treatment at both doses inhibited this decrease significantly ($p < 0.05$ and $p < 0.01$ at 100 mg/kg and 200 mg/kg doses, respectively). In agreement with the mRNA data, the protein expression levels of GPx4, GSTm5, and PRx4 also significantly ($p < 0.05$) decreased in the HC group (Fig. 3A). Band intensities revealed that treatment of heat-stress rats with GINST significantly attenuated the decrease at both 100 mg/kg and 200 mg/kg doses (Figs. 3B and 3C).

3.6. Effect of GINST on the expression levels of spermatogenesis-related molecules in heat-stress rat testis

The testicular mRNA expression levels of cAMP responsive element binding protein 1 (CREB-1), nectin-2, and inhibin-α, which are known to be the key proteins related to spermatogenesis, decreased in the HC group compared with those in the NC group (Fig. 4A). Densitometric analysis revealed that the decrease in the testicular mRNA expression level of CREB-1 in the HC group was more pronounced and was reduced by more than 2-fold that of nectin-2 and inhibin-α (Figs. 4B–4D). However, treatment with GINST (100 mg/kg and 200 mg/kg) significantly ($p < 0.05$) ameliorated this decrease in heat-stress rat testis. GINST at both 100 mg/kg and 200 mg/kg doses showed similar effects on attenuating the changes observed in the HC group.

Similarly, the testicular protein levels of nectin-2, CREB-1, and inhibin-α were significantly ($p < 0.01$) decreased in the HC group compared with the NC group and were significantly ($p < 0.05$) ameliorated by treatment with GINST at both 100 mg/kg and 200 mg/kg doses in heat-stress rat groups (Fig. 5). In agreement with the mRNA data, the protein expression level of CREB-1 was substantially suppressed (3-fold) in the heat-stress rat group when compared with the protein expression of nectin-2 (Figs. 5B and 5C). The results suggest that GINST (100 mg/kg and 200 mg/kg) exhibited its effects at both protein and transcriptional levels in ameliorating the changes observed in spermatogenesis-related molecules in heat stressed rats.

3.7. Effect of GINST on the expression of sex hormone receptors in heat-stress rat testis

The mRNA expression levels of the sex hormone receptors (SHRs)—androgen receptor (AR), luteinizing hormone receptor (LHR), and follicle stimulating hormone receptor (FSHR)—significantly ($p < 0.01$) decreased in the HC group compared with those in the NC group (Fig. 6A). Densitometric analysis showed that GINST treatment at the indicated doses (100 mg/kg and 200 mg/kg) significantly ($p < 0.05$ and $p < 0.01$, respectively) ameliorated this decrease (Figs. 6B–6D). Similarly, in agreement with the mRNA data, the testicular protein expression levels of AR, LHR, and FSHR significantly ($p < 0.01$) decreased in the HC group compared with those in the NC group (Fig. 7A). AR and FSHR substantially

Table. 4
Effect of GINST on sperm kinematics in heat-stressed rats

Group	Average							
	Motile (%)	Progressive (%)	VAP (mm/s)	VSL (mm/s)	VCL (mm/s)	STR (%)	LIN (%)	Elongation
NC	91.67 ± 6.29	31.93 ± 13.81	32.87 ± 6.39	23.08 ± 3.74	76.00 ± 14.41	69.47 ± 5.41	39.33 ± 5.61	95.47 ± 3.62
HC	58.33 ± 20.68$^{\#}$	18.89 ± 8.27$^{\#}$	30.11 ± 6.43	21.78 ± 8.78	74.38 ± 12.01	73.33 ± 7.48	40.89 ± 5.16	97.89 ± 2.52
HG100	71.14 ± 15.94*	26.29 ± 16.02*	35.04 ± 5.21	24.64 ± 3.90	80.50 ± 7.93	69.70 ± 2.71	38.90 ± 3.03	92.70 ± 3.53
HG200	90.40 ± 9.73**	35.90 ± 9.40**	34.94 ± 8.19	23.94 ± 7.17	81.54 ± 16.81	68.86 ± 5.98	39.43 ± 7.55	96.00 ± 4.36

Data are expressed as mean ± SEM ($n = 10$). 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; * $p < 0.05$ and ** $p < 0.01$ compared with the HC group.
ANOVA, analysis of variance; b.w., body weight; GINST, pectinase-treated *Panax ginseng* extract HC, heat-stressed control; HG100, heat-stressed and received GINST 100 mg/kg b.w.; HG200, heat-stressed and received GINST 200 mg/kg b.w.; LIN, linearity; NC, normal control; SEM, standard error of the mean; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity.

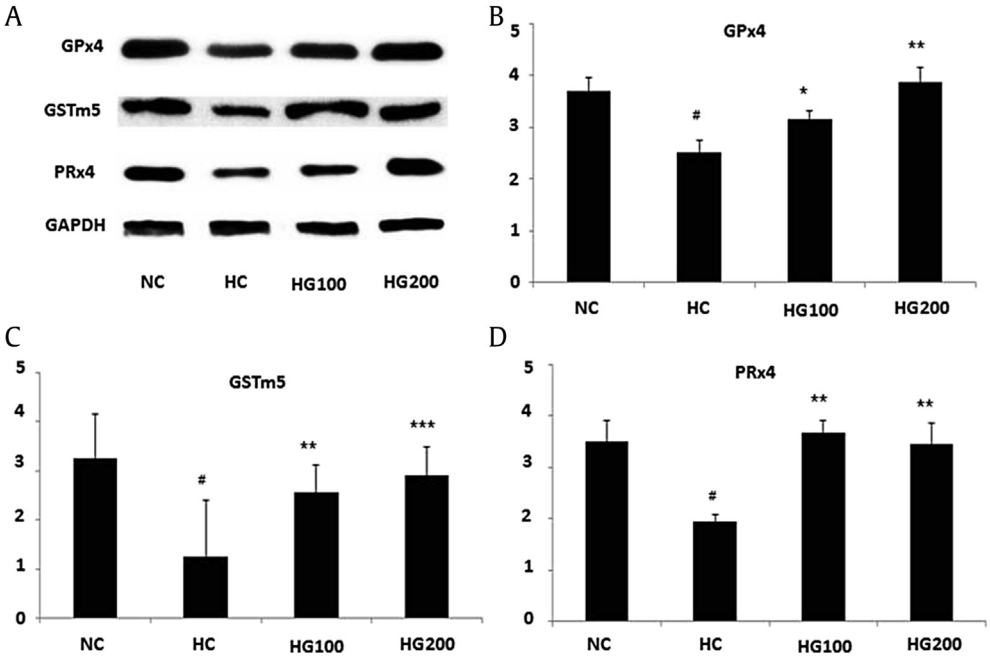

Fig. 2. Effect of GINST on mRNA expression of antioxidant enzymes in heat-stress rat testis. (A) The mRNA expression of GPx4, GSTmu5, and PRx4 in testicular tissue is shown. The internal control used was GAPDH. (B–D) The polymerase chain reaction band intensity of GPx4, GSTmu5, and PRx4, respectively, normalized to that of GAPDH is shown. Data are mean ± SD ($n = 10$). $^{\#}p < 0.05$ compared with the NC group; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the HC alone group by Student *t* test and one-way ANOVA using GraphPad Prism version 4.0. ANOVA, analysis of variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GINST, pectinase-treated *Panax ginseng* extract; HC, heat-stress control; NC, normal control; SD, standard deviation.

decreased compared with LHR (Figs. 7B–7D). This decrease was significantly ($p < 0.05$) inhibited in the HG100 and HG200 groups.

4. Discussion

One possible mechanism affecting the function of testis is the disturbance of prooxidant and antioxidant balance by generation of ROS [28]. Testicular hyperthermia above the normal range causes impaired spermatogenesis due to heat-related oxidative stress [29,30]. Hence, oxidative stress is a major factor in the etiology of male infertility. However, the intrinsic molecular events following heat stress are not well documented.

Ginsenosides in *P. ginseng* are regarded as the active components responsible for various pharmacological effects [31].

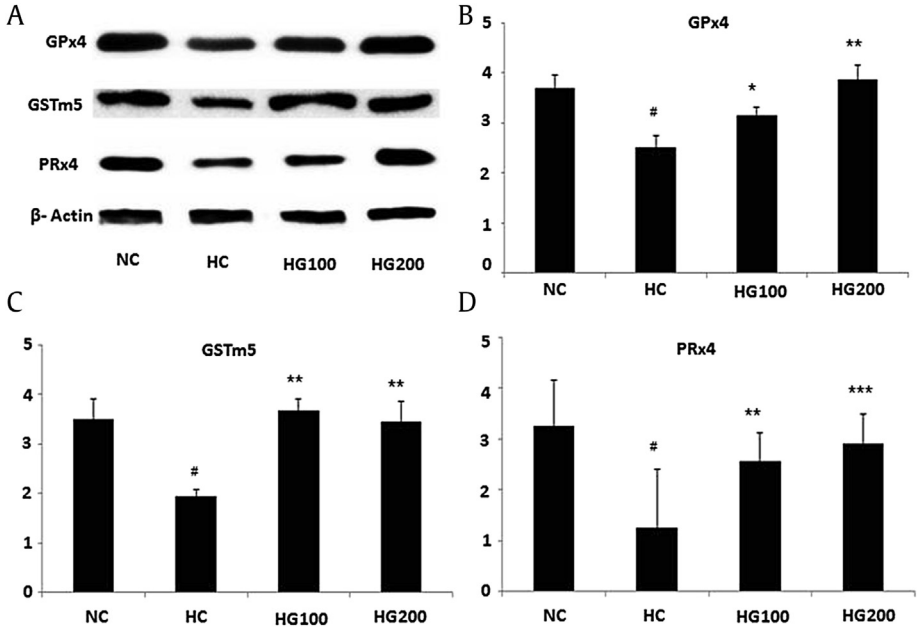

Fig. 3. Effect of GINST on protein expression of antioxidant enzymes in heat-stressed rat testis. (A) Protein expression of GPx4, GSTmu5, and PRx4 in testicular tissue was analyzed using Western blot analysis. The internal control used was β-actin. (B–D) Quantification of GPx4, GSTmu5, and PRx4, respectively, from three independent experiments. Data are expressed as mean ± SD ($n = 10$). 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. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the HC group. ANOVA, analysis of variance; b.w., body weight; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GINST, pectinase-treated *Panax ginseng* extract; HC, heat-stress control; HG100, heat-stress and received GINST 100 mg/kg b.w.; HG200, heat-stress and received GINST 200 mg/kg b.w.; NC, normal control; SD, standard deviation.

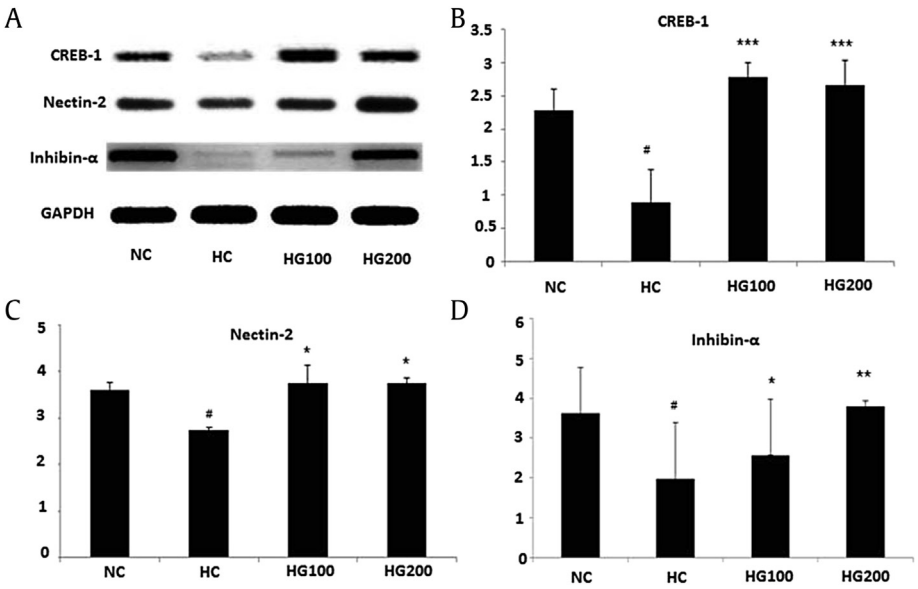

Fig. 4. Effect of GINST on mRNA expression levels of spermatogenesis-related molecules in heat-stress rat testis. (A) The mRNA expression of CREB-1, nectin-2, and inhibin-α in testicular tissue was shown. The internal control used was GAPDH. (B–D) The polymerase chain reaction band intensity of CREB-1, nectin-2, and inhibin-α, respectively, normalized to that of GAPDH is shown. Data are expressed as mean ± SD ($n = 10$). Statistical analysis was carried out by Student's t test and one-way ANOVA using GraphPad Prism version 4.0. $^{\#}$ $p < 0.01$ compared with the NC group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the HC group. ANOVA, analysis of variance; b.w., body weight; CREB-1, cAMP responsive element binding protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GINST, pectinase-treated *Panax ginseng* extract; HC, heat-stress control; HG100, heat-stress and received GINST 100 mg/kg b.w.; HG200, heat-stress and received GINST 200 mg/kg b.w.; NC, normal control; SD, standard deviation.

Ginsenosides are enzymatically biotransformed in the human intestine forming potent by-products to increase their efficiency [32]. Pectinase, commonly produced by lactic acid bacteria in the intestine [24], produces large amounts of CK by biotransforming ginsenosides such as Rb1, Rb2, and Rc. Pectinase-treated extracts of *P. ginseng* produce CK as well as several other forms of ginsenosides, including Rg3, Rg5, Rk1, Rh1, F2, and Rg2 [25]. These biotransformed compounds possess various biological activities. GINST has potent antioxidant effects and increases the protein expression level of enzymatic and nonenzymatic antioxidants in rats [27]. In addition, GINST rescued testicular impairment in aged rats via regulation of the oxidative defense systems [26]. We confirmed that administration of GINST could ameliorate testicular damage in intermittently heat-stress rats.

Intermittent heating stress to rats induced a marked testicular abnormality as determined by serum biochemical and molecular

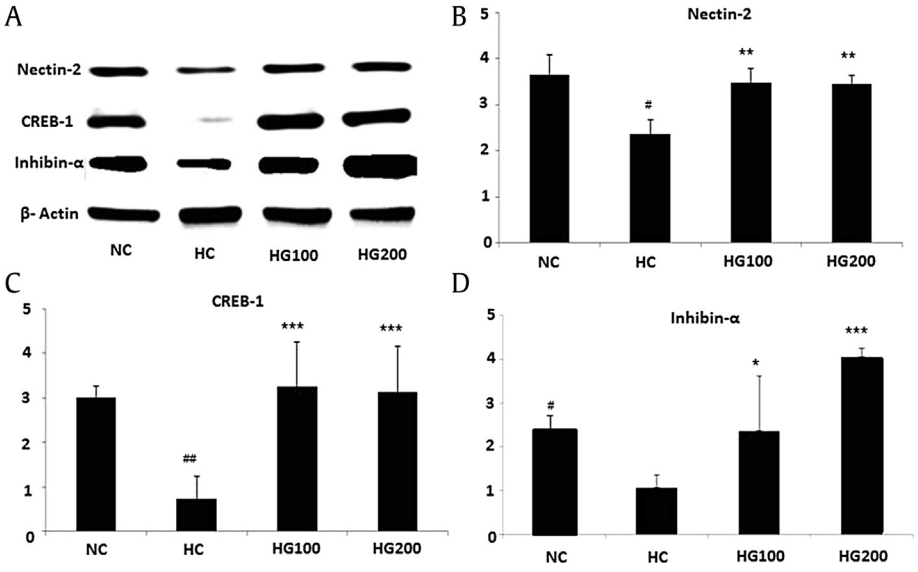

Fig. 5. Effect of GINST on protein expression level of spermatogenesis-related biomarker molecules in heat-stress rat testis. (A) Protein expression of nectin-2, CREB-1, and inhibin-α in testicular tissue was analyzed using Western blot analysis. The internal control used was β-actin. (B–D) Quantification of nectin-2, CREB-1, and inhibin-α, respectively, from three independent experiments. Data are expressed as mean ± SD ($n = 10$). Statistical analysis was carried out by Student t test and one-way ANOVA using GraphPad Prism version 4.0. $^{\#}$ $p < 0.01$ and $^{\#\#}$ $p < 0.01$ compared with the NC group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the HC group. ANOVA, analysis of variance; b.w., body weight; GINST, pectinase-treated *Panax ginseng* extract; HC, heat-stress control; HG100, heat stress and received GINST 100 mg/kg b.w.; HG200, heat-stress and received GINST 200 mg/kg b.w.; NC, normal control; SD, standard deviation.

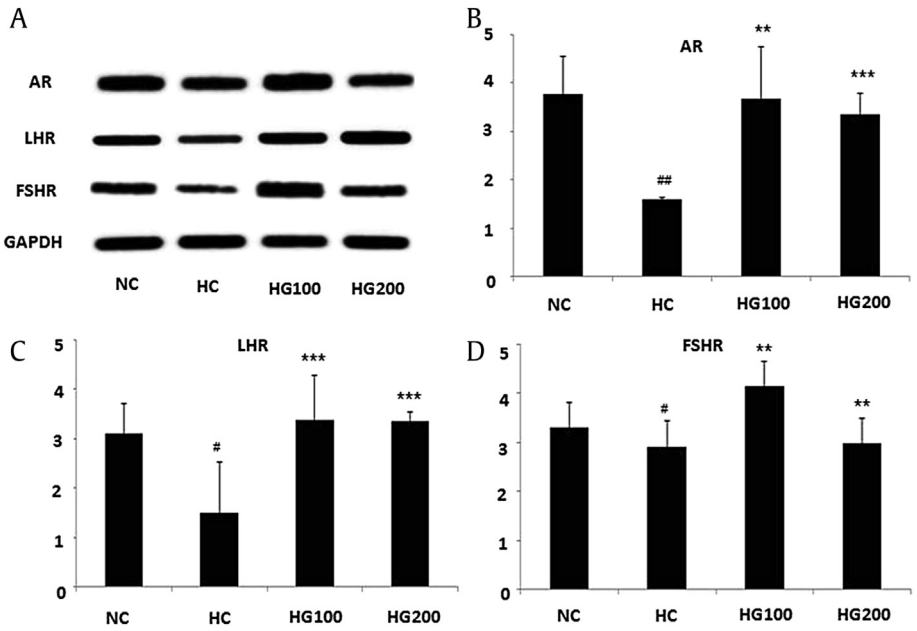

Fig. 6. Effect of GINST on mRNA expression of sex hormone receptors in heat-stress rat testis. (A) The mRNA expression of AR, LHR, and FSHR in testicular tissue is shown. The internal control used was GAPDH. (B–D) The polymerase chain reaction band intensity of AR, LHR, and FSHR, respectively, normalized to that of GAPDH is shown. Data are expressed as mean ± SD ($n = 10$). Statistical analysis was carried out by Student t test and one-way ANOVA using GraphPad Prism version 4.0. $^{\#}$ $p < 0.01$ and $^{\#\#}$ $p < 0.01$ compared with NC group. ** $p < 0.01$, and *** $p < 0.001$ compared with the HC group. ANOVA, analysis of variance; AR, androgen receptor; b.w., body weight; FSHR, follicle stimulating hormone receptor; GINST, pectinase-treated *Panax ginseng* extract; HC, heat-stress control; HG100, heat-stress and received GINST 100 mg/kg b.w.; HG200, heat-stress and received GINST 200 mg/kg b.w.; LHR, luteinizing hormone receptor; NC, normal control; SD, standard deviation.

analyses. The overall and final body and organ weights of all the groups at week 25 after heat stress were not significantly affected. Only the kidney and epididymis showed a reduction in weight in the HS group compared to those in the NC group. Our results are in agreement with earlier reported data indicating that the number of spermatozoa in the epididymis was considerably reduced after heat treatment [15,33,34]. Furthermore, heat stress to rats caused a decrease in kidney weight with both morphological and histological defects [35,36]. However, administration of GINST (200 mg/kg) following heat stress to rats significantly ameliorated these changes. Considering that body and organ weights are determined by anabolic and catabolic processes, it can be argued that GINST was able to counteract some of the effects of hypoxia on metabolic balance in rats.

With regard to serum biochemical parameters, heat-stress groups showed significant increases in the GOT levels compared to those in the NC group. Furthermore, the lipid metabolism-related parameters such as T-CHO, TG, and LDL-C were not significantly altered. However, GLU levels were reduced significantly in the HC group. GINST (200 mg/kg) significantly ameliorated these changes in heat-stress rats. Heat stress has a profound effect on the seminiferous tubules and deteriorates sperm motility, number, and morphology [37,38]. Our sperm kinematic study also revealed a decreased percentage of sperm motility and progression in HC rats compared to those in the NC rats. However, GINST treatment (200 mg/kg) ameliorated this decrease to near-normal levels.

Evidence suggests that peroxidative damage is currently the single most important cause of impaired testicular function, and heat has been regarded as causing pathological consequences in rats resulting in oxidative stress [39–41]. In this study, antioxidative enzymes such as GPx4, PRx4, and GSTm5 were altered by heat stress. The elevation in testicular free radicals in heat-stress rats is supported by a diminution in these important free radical scavenging enzymes [42–44].

Reproductive organs express Prx4 at relatively high levels, and several proteomics studies have shown altered expression of Prx4 under physiological and pathological conditions [45–48]. Furthermore, spermatogenic cells of Prx4-deficient mice are prone to death under normal breeding conditions and are sensitive to heat-induced cell damage [49]. Glutathione-*S*-transferases (GSTs), known to detoxify electrophilic compounds by catalyzing the formation of glutathione conjugates, are also involved in the intracellular transport of a variety of endogenous metabolites and hormones [50]. GST influences the transport, metabolism, and action of steroids and is an important regulator for proliferation and differentiation of germ cells, protecting them against the harmful effects of free radicals [42,44,51,52]. In particular, the GSTmu5 form, which is found in fibrous sheaths, was enriched in the testes and isolated spermatogenic cells [53,54].

Similarly, the enzyme GPX is an important cellular protectant against ROS-mediated damage to membrane lipids, proteins, and nucleic acids [55]. Increased intensity of heat treatment in mice downregulated the activity of GPX, implying severe oxidative damage in the testes [56]. Therefore, we evaluated the effect of GINST on the expression levels of these three enzymes (GPX, PRx4, and GSTm5) in heat-stress rats. Consistent with the reported studies, in our current study, intermittent heat stress to rats resulted in a significant decrease in the enzyme expression levels, suggesting a role for oxidative stress in heat-stress-induced rat testes. However, GINST treatment at both doses attenuated the decreased expression at both protein and mRNA levels, indicating that GINST might modulate the enzymatic oxidative defense systems in heat-stress rats.

The spermatogenesis-related proteins such as inhibin-α, nectin-2, and CREB-1 are some of the major transcriptional factors involved in testicular function. Inhibin-α is an important adhesion molecule in Leydig cells that promotes testosterone synthesis, acts as a negative feedback mechanism suppressing follicle-stimulating hormone (FSH) production from the pituitary gland, and is involved

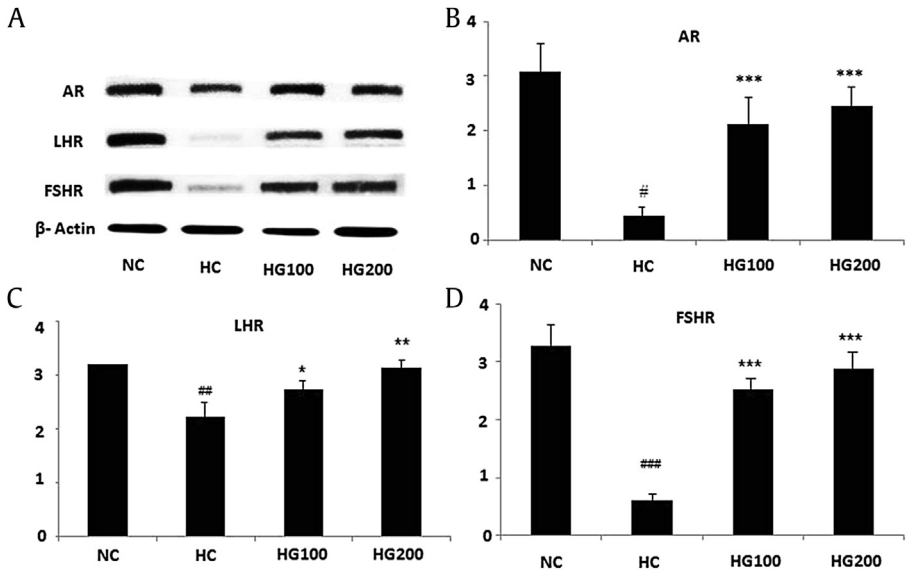

Fig. 7. Effect of GINST on protein expression of sex hormone receptors in heat-stress rat testis. (A) Protein expression of AR, LHR, and FSHR in testicular tissue was analyzed using Western blot analysis. The internal control used was β-actin. (B–D) Quantification of AR, LHR, and FSHR, respectively, from three independent experiments. Data are expressed as mean ± SD ($n = 10$). Statistical analysis was carried out by Student t test and one-way ANOVA using GraphPad Prism version 4.0. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared with the NC group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the HC group. ANOVA, analysis of variance; AR, androgen receptor; b.w., body weight; FSHR, follicle stimulating hormone receptor; GINST, pectinase-treated *Panax ginseng* extract; HC, heat-stress control; HG100, heat-stress and received GINST 100 mg/kg b.w.; HG200, heat-stress and received GINST 200 mg/kg b.w.; LHR, luteinizing hormone receptor; NC, normal control; SD, standard deviation.

in spermatid development during the initial stages of spermatogenesis [57,58]. Decreased inhibin levels were observed in cryptorchid rats and therefore can function as an accurate marker for understanding the function of Sertoli cells and spermatogenesis [59]. Located in the Sertoli germ cell junction, Nectin-2 aids in the development of the matured spermatozoa in the seminiferous epithelium [60]. Disruption of the nectin-2 gene resulted in morphologically aberrant spermatozoa with defects in nuclear and cytoskeletal morphology and mitochondrial localization [61].

CREB-1 is known to play several functional roles in the development of testes and is a major regulatory mechanism during different stages of spermatogenesis [62,63]. Here, heat-stress rats showed altered expression of inhibin-α, nectin-2, and CREB-1 at both the mRNA and protein levels. These findings indicated that heat stress to rats induced oxidative stress, and this might affect the signaling pathways involved in Sertoli function and spermatogenesis. However, GINST significantly reversed the altered mRNA and protein expression, suggesting that GINST might modulate certain key transcription factors affected by heat stress in rats.

Mounting evidence suggest that various stressors generally induce a depression in the hypothalamus–pituitary–testis system, mediated by the activated hypothalamus–pituitary–adrenocortical system, resulting in a fall in plasma sex hormones. This was supported by the suppression of antioxidant enzyme expression and concomitant increase in peroxidative damage [64,65]. Hyperthermia has a detrimental effect on reproduction partly by disrupting the hormone receptors thereby decreasing the release of luteinizing hormone (LH) and FSH from the anterior pituitary gland. Furthermore, heat stress reduces intratesticular testosterone, including androgens and estrogens [42]. The development and differentiation of sperm are maintained through the function of Sertoli cells and are mediated by the action of some key hormones such as FSH and testosterone [66]. Therefore, we evaluated the effects of GINST on the SHR expression in heat-stress rats. Our present study showed a significant reduction in the serum SHR expression including FSHR, LHR, and AR. However, GINST attenuated the reduced expression levels significantly. These results provide a clear evidence that GINST might play a crucial role in regulating the serum SHR molecules responsible for sperm production and functioning. In our present study, HPLC analysis of GINST showed higher contents of CK. Recent reports have shown that GINST contains large amounts of CK as well as several other ginsenosides [25]. Reports have also suggested that GINST exhibited various pharmacological effects including antioxidant, antidiabetic activity, and enhanced testicular function via the alleviation of oxidative stress in experimental models [67]. Although our data suggest that CK and other biotransformed ginsenosides might be responsible for such effects, future in-depth studies are warranted to evaluate if the activity was rhamnogalacturonans from pectin or a synergistic interaction between the constituents.

In conclusion, intermittent heat stress in rats induced significant damage in testicular and germ cell function. Treatment of heat-stress rats with GINST significantly attenuated these changes by modulating the enzymatic antioxidant systems, SHR levels, and gene expression related to the spermatogenesis pathway. GINST may be categorized as an important medicinal herb and a potential therapeutic for the prevention and treatment of male subfertility or infertility caused by hyperthermia.

Conflicts of interest

The authors declare that no competing financial interests exist.

Acknowledgments

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