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—Original Article—

Angelica keiskei (Ashitaba) powder and its functional compound xanthoangelol prevent heat stress-induced impairment in sperm density and quality in mouse testes

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Abstract. Recently, gradual decline in human sperm production has become a serious worldwide concern because it leads to increased rates of infertility. Endocrine disrupters, lifestyle changes, and varicocele, all of which elevate testicular temperature, are thought to be the main causes of this decline. The present study aimed to determine whether the dietary phytochemicals *Angelica keiskei* (Ashitaba) powder (57.5 mg/kg) and its functional component, xanthoangelol (3 mg/kg), can prevent heat stress-induced impairment in sperm density and quality in mice. Sperm parameters were analyzed 28 days after mice exposure to heat. Supplementation with Ashitaba powder completely prevented heat-induced impairment in sperm parameters, including densities of motile sperms and progressive sperms (> 25 μm/sec), and amplitude of lateral head displacement. Xanthoangelol did not exert a complete protective effect; nevertheless, it significantly prevented heat stress-induced reduction in most parameters. Both Ashitaba powder and xanthoangelol elevated the expression of the widely expressed heat shock proteins (HSPs) *Hspa1a* and *Hsp40* and the antioxidant enzyme *glutathione synthase* in non-stressed testes. Ashitaba powder significantly prevented heat stress-induced reduction in the expression of *Hspa1l* and *Hspa2*, which are highly expressed in the testes and critical for fertility. Our results showed that Ashitaba powder and xanthoangelol protected testicular cells from heat stress, probably by elevating the levels of antioxidant enzymes and HSPs. Supplementation with dietary functional phytochemicals may help prevent heat stress-induced male infertility.

Key words: *Angelika keiskei* (Ashitaba), Antioxidant enzymes, Heat shock protein, Heat stress, Male infertility

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Accumulating evidence indicates that the prevalence of human infertility has increased over the past few decades [1–3]. Approximately 15% of couples are infertile. Male infertility is solely responsible in 20% of such cases, while it contributes to another 30%–40% of these cases.

Temperature-sensitive mammalian spermatogenesis occurs in the testes at a temperature lower than that of the rest of the body [4]. Therefore, maintaining testicular temperature within a physiological range is critical for normal spermatogenesis. Numerous studies have found that sperm production in men is gradually declining [1–3]. In addition to endocrine disrupters, shifts in lifestyles, such as driving a vehicle for prolonged periods and wearing tight-fitting underwear, are possible risk factors because they increase scrotal temperature [1–3, 5, 6]. In addition, the prevalence of varicocele among males ranges from 5%–20%, and this is also associated with infertility due to impaired spermatogenesis, low sperm production, and decreased sperm quality [7, 8]. The pathogenesis of varicocele is also related to increased scrotal temperature. Thus, heat stress in the testes is a major cause of male infertility and linked to excessive production of reactive oxygen species (ROS) and induction of apoptosis of spermatozoa in the testes [8–10]. Reduced reproductive capacity due to heat stress is a serious problem for not only humans but also livestock [11–14]. Summer heat stress considerably decreases livestock fertility, which is referred to as summer sterility. Until now, heat stress-induced disorders of the testes have been treated surgically (for varicocele) or by *in vitro* fertilization. However, the effects of dietary phytochemicals, such as polyphenols, on heat stress-induced impairment of sperm density and quality have not been reported.

The cellular expression of heat shock proteins (HSPs) minimizes the harmful effects of various types of stressors, including heat stress, physical stress, and viral infection. These proteins comprise of several families, including Hsp90 (Hspc), Hsp70 (Hspa), and Hsp27. The Hsp70 proteins Hspa2 and Hspa1l are mainly and highly expressed in the testes and crucial for male fertility [15–18]. In fact, *Hspa2*- and *Hspa1l*-knockout mice show defects in spermatogenesis [15, 16]. In contrast to the expression of most HSPs, that of *Hspa2* and *Hspa1l* is decreased by heat stress [17]. Hspa1a and Hsp40 are widely distributed in internal organs, including the testes. Hspa1a widely contributes to minimizing heat-stress damage, whereas Hsp40 is essential for the cellular functions of the Hsp70 family. The effects of dietary phytochemicals on the expression of these HSPs in the testes have not been reported.

The edible herb *Angelica keiskei* (Ashitaba) is indigenous to Japan, and it contains two chalcones, xanthoangelol (XA) and 4-hydroxyder-

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ricin (4HD), as major functional polyphenolic compounds that exert antihypertensive, anti-obesity, antidiabetic, and other beneficial effects [19, 20]. However, whether these compounds could be useful to improve stress-induced testicular dysfunction has not been examined.

The present study aimed to first determine whether the dietary phytochemicals Ashitaba powder (AP) and XA can prevent heat stress-induced impairment in sperm density and quality parameters, including percentage of progressive sperms (> 25 μm/sec), as well as histological damage of the testis, and then elucidate the molecular mechanisms involved. We also assessed the expression of antioxidant enzymes and HSPs that may be involved in protecting the testes against heat stress. Heat stress in our experiments was single and transient, as opposed to the long-lasting heat stress caused by varicocele and changed lifestyles. However, we consider our experimental condition as a good model for finding dietary phytochemicals that can protect the testes from heat stress.

Materials and Methods

Animals

Self-breeding male CD-1 mice (8 weeks old) were housed under a 12-h light/dark cycle in a temperature- and humidity-controlled room with free access to food and water. The mice were fed only MF powder (Oriental Yeast, Tokyo, Japan) or MF powder containing AP (57.5 mg/kg) or XA (3 mg/kg) using Roden CAFE (Oriental Yeast) for 7 days. For heat stress experiment, supplementation with AP or XA was continued after heat exposure until the mice were killed for analyses. The body weight and food intake of the mice were measured every other day to give the same amount of the phytochemicals per kg body weight of each mouse. The diet was changed every other day. The administered dose of AP (57.5 mg/kg) was decided based on our preliminary experiments, which used increased protein expression of the antioxidant enzyme HO-1 as index. AP (57.5 mg) contains approximately 3 mg XA. AP was obtained from Japan Bio Science Laboratory, Osaka, Japan, whereas XA was purified from AP in our laboratory as follows. XA was extracted from AP with ethyl acetate, and subjected to chromatography using silica gel 60 (Merck) followed by Cosmosil 75C18-OPN (Nacalai Tesque, Kyoto, Japan). Next, the syrup was crystallized with methanol. The study was conducted in accordance with the University of Tsukuba guidelines for animal care, handling, and termination (approval no. 18344), which conforms to international and Japanese guidelines for animal care and welfare.

Heat stress

Mice were anesthetized and subjected to a single heat stress at 41°C for 15 min for investigating the effect of phytochemicals on sperm parameters and the mRNA expression of several proteins, and at 42°C for 20 min for histological evaluation and detection of DNA ladder and lipid peroxide. The lower body of each animal, including the hind legs, tail, and scrotum, was immersed in a water bath. Thereafter, the mice were dried and returned to their cages. Control mice were anesthetized and left at room temperature.

Histological evaluation

Mice were killed by cervical dislocation 48 h after heat shock. Resected testes were immersion-fixed in Bouin's fluid (ScyTek Laboratory, Logan, UT, USA) at 4°C for 4 h, and then stored in 70% (v/v) ethanol. Testicular tissues were processed into paraffin wax, sectioned, and stained with hematoxylin-eosin. Abnormal seminiferous tubules were defined as being depleted of spermatocytes and spermatids.

DNA extraction and apoptotic ladder detection

Mice were killed by cervical dislocation 48 h after heat stress. Testicular DNA was extracted using Isogenome DNA extraction kits (NIPPON GENE, Tokyo, Japan), and fragmented DNA was electrophoretically separated on a 2.0% agarose gel. Separated DNA was stained with Gelred™ nucleic acid gel (Biotium, Fremont, CA, USA).

Assessment of sperm parameters

Mice were killed 28 days after heat stress. The cauda epididymis was retrieved from euthanized mice and placed in 1 ml of warm sperm buffer (2.2 mM HEPES, pH 7.4; 1 1.2 mM $MgCl_2$; 100 mM NaCl; 4.7 mM KCl; 4.8 mM lactic acid Ca; 5.5 mM glucose; 20 mM sodium bicarbonate; and 88 mM pyruvic acid). Spermatozoa were released from the epididymis by slicing with an 18-gauge needle and then placed in the buffer at 37°C for 15 min. After sperm motility recovered, sperm suspensions were separated by centrifugation. Sperm parameters in supernatants were analyzed using a sperm motility analysis system (DITECT, Tokyo, Japan).

Measurement of thiobarbituric acid reactive substance (TBARS)

Mice were killed 30 min after heat stress. Lipid peroxides were extracted by homogenizing testes on ice with RIPA buffer (50 mM Tris-HCl, pH 7.6; 10% glycerol; 1% Triton-X100; 0.2 mM PMSF; 150 mM NaF; 1 mM EDTA; 1 mM Na_3VO_4; 3 μg/ml antipain; 10 μg/ml leupeptin; and 10 μg/ml aprotinin). The homogenate was then separated by centrifugation at 4,200 rpm for 10 min at 4°C, and the supernatant was collected. Concentrations of TBARS in supernatants were determined using a TBARS assay kit (Cayman Chemical, Ann Arbor, MI, USA).

RNA preparation and quantitative real-time RT-PCR

Mice were killed at 1 h after heat stress for analyzing heat shock factor 1 (HSF1) and 2 (HSF2), and at 6 h after heat exposure for analyzing Hspa1l and Hspa2. Total RNA was extracted from the testes using Isogen II (NIPPON GENE) and reverse-transcribed into cDNA using ReverTra (TOYOBO, Osaka, Japan). Quantitative real-time RT-PCR was performed using a KAPA SYBR Fast qPCR kit (Kapa Biosystems, Wilmington, MA, USA) and 7300 real-time PCR (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. The measured value of mRNA expression was normalized to that of *18S rRNA*. The primers used are listed in Table 1.

Statistical analyses

All results are expressed as means ± standard error (SE). The statistical significance of differences in values between two groups was analyzed using the unpaired *t*-test or Wilcoxon rank-sum test. All

Table 1. Primer sequences used for amplification using real-time PCR

Gene name	Forward primer	Reverse primer
Heme oxygenase-1 (HO-1)	5'-CCTTCAAGGCCTCAGACAAA-3'	5'-GAGCCTGAATCGAGCAGAAC-3'
Glutathione synthase (GSS)	5'-GTGAATGGGGCATACGTCA-3'	5'-CAAAGCAGGCCATAGACAGG-3'
Heat shock protein family A member 1-A (Hspa1a)	5'-AGCCCACGTGCAATACACAA-3'	5'-TGGCCTTGAGGACTGTCATT-3'
Heat shock protein family A member 1-like (Hspa1l)	5'-TCCCGTCCATTAAAGTAGTCCTG-3'	5'-ACACGTCCATCACTAGAGCAC-3'
Heat shock protein family A member 2 (Hspa2)	5'-TGAGACGCTCGGTGTCAGT-3'	5'-GCGTGGGGGTATTCCAACAT-3'
Dnaj heat shock protein family member B1 (Hsp40)	5'-CACCGAAGAACTCAGCAAACA-3'	5'-TTCGACCGCTATGGAGAGGAA-3'
Heat shock transcription factor 1 (Hsf1)	5'-GCCTCCCCAGGCAGGAGCATA-3'	5'-AGGGCTCGCCTCCAGTACCC-3'
Heat shock transcription factor 2 (Hsf2)	5'-GCACTACTTTTCGGAAGCCA -3'	5'-CATCACCTGGAGTCAGAATGGA-3'
18S ribosomal RNA	5'-CCATCCAATCGGTAGTAGCG-3'	5'-GTAACCCGTTGAACCCCATT-3'

other data were calculated using the Dunnett or Holm test. $P < 0.05$ was considered statistically significant.

Results

Ashitaba powder protected against heat stress-induced histological damage and germ cell apoptosis in the testes

We compared testis sections between the control and heat-stressed mice treated with or without AP to determine the protective effects of AP on heat-stressed testes. The seminiferous tubules of mice at 48 h after heat exposure contained fewer spermatogenic cells, shorter seminiferous epithelium, and wider, empty lumen (Fig. 1A) than those of the control mice (Fig. 1B). These findings are in close agreement with those of previous studies [10, 21–23]. Supplementation with AP suppressed this histological damage. AP suppressed the reduction in the seminiferous epithelium and cells of the spermatogenic series (Fig. 1C). The obviously increased ratio of abnormal seminiferous tubules after heat stress was significantly suppressed in the mice supplemented with AP (Fig. 1D). We further examined internucleosomal DNA fragmentation in the testes because heat stress induces the loss of germ cells in the seminiferous tubules via apoptosis [9, 10, 21, 23]. Figure 1E shows that AP suppressed DNA fragmentation in response to heat stress. These findings suggested that dietary supplementation with AP can prevent heat stress-induced testicular dysfunction.

The protective effect of AP on the ratio of abnormal seminiferous tubules was significant but partial, although its suppressive effect on DNA fragmentation appeared to be complete. We consider that this difference may be caused by the detection sensitivity of DNA fragments or related to the report [24] indicating that autophagy as well as apoptosis is involved in heat stress-induced reduction of germ cells in the seminiferous tubules; the effect of AP on suppressing autophagy of germ cells may be slightly weak.

Ashitaba powder completely protected against heat stress-induced reduction in sperm density and quality

To evaluate the protective effect of AP on sperms, sperm parameters were analyzed on day 28 after heat treatment. Table 2 shows that heat stress significantly reduced the density of total sperms, motile sperms, and progressive sperms, as well as the ratio of motile sperms and average path velocity (VAP) ($P < 0.05$). Dietary AP significantly and completely prevented reductions in all these parameters, excluding progressive sperm straight-line velocity (VSL). These results showed that stress-induced impairment in sperm density and quality can be completely avoided by supplementation with AP.

Heat stress was converted to oxidative stress and AP supplementation raised the mRNA expression of antioxidant enzymes

Several studies have shown that heat stress produces ROS in the testes [8–10, 25]. Concentration of peroxidized lipid was significantly elevated in the testes exposed to heat compared with that in the control testes (Fig. 2A), suggesting that heat stress was converted to oxidative stress in the testes. We therefore investigated whether the expression of antioxidant enzymes is increased by AP. Figure 2B shows that AP increased the mRNA expression of glutathione synthase *(GSS)* and heme oxygenase-1 *(HO-1)*. Thus, AP may reduce heat stress-induced generation of ROS by increasing *GSS* and *HO-1* expression.

Ashitaba powder elevated the mRNA expression of HSPs and heat shock factors, preventing the heat stress-induced reduction in their expression

The expression of Hspa1l and Hspa2, which are mainly and highly expressed in the testes, is known to decrease in the testes exposed to heat stress [17]. Therefore, dietary components that increase the expression of these HSPs should protect the testes against heat stress. HSF1 and HSF2 play a critical role in the regulation of Hspa1l and Hspa2 expression [26]. Supplementation with AP increased *Hspa1l* and *Hsf1* in addition to *Hspa1a* and *Hsp40* in the mice not exposed to heat stress (Fig. 3A). Hspa1a and Hsp40 are ubiquitously expressed and Hsp40 is an essential HSP for the function of the HSP70 (Hspa) family. Moreover, AP significantly prevented the heat stress-induced reduction in *Hspa1l*, *Hspa2*, and *Hsf2* expression (Fig. 3B and C). These data suggested that AP protected testicular cells from heat stress by increasing the expression of these HSPs and their transcription factors, HSF1 and HSF2.

Xanthoangelol protected against heat stress-induced histological damage and increased GSS and HSP mRNA expression

Similar to the results of AP (Fig. 1A–C), XA suppressed heat stress-induced severe reduction in cells of the spermatogenic series, shortening of the seminiferous epithelium, and widening of the empty lumen in the seminiferous tubules (Fig. 4A–C). The obvious increase in the ratio of abnormal seminiferous tubules after heat stress was

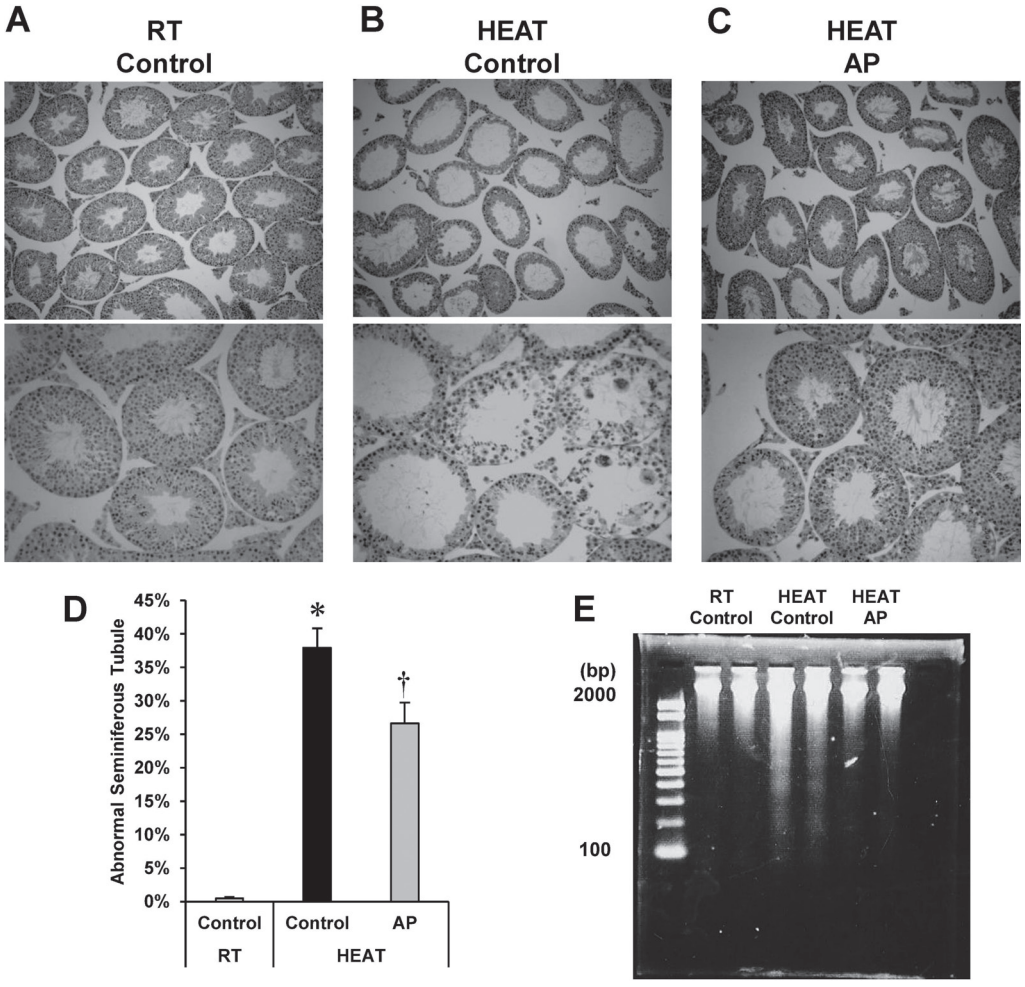

Fig. 1. Ashitaba protected male germ cells against heat stress. Male mice were supplemented with or without AP (57.5 mg/kg) for 7 days and then heat-stressed at 42°C for 20 min. (A–C) Hematoxylin-eosin-stained testicular sections at 48 h after hyperthermia (A, RT-Control; B, HEAT-Control; C, HEAT-AP). Upper panels, original magnification ×100; lower panels, × 200. (D) Ratio of abnormal seminiferous to total tubules. Data are presented as means ± SE (n = 6–7), and groups were compared using the Holm tests. * $P < 0.05$ *vs*. RT-Control, † $P < 0.05$ *vs*. HEAT-Control group. (E) DNA extracted from the testis 48 h after exposure to heat stress was fractionated on a 2.0% agarose gel and stained with SYBR Green.

Table 2. Protective effects of Ashitaba powder on sperm density and quality in mice exposed to scrotal heat

	RT Control	HEAT Control	HEAT AP
Sperm density (× 10^6/ml)	13.7 ± 1.1	8.2 ± 1.4 [a]	15.2 ± 1.6 [b]
Motility (%)	71.1 ± 0.9	54.1 ± 6.5 [a]	72.1 ± 2.5 [b]
Motile sperm density (× 10^6/ml)	9.8 ± 0.9	5.0 ± 1.3 [a]	11.0 ± 1.3 [b]
Progressive VSL (%)	41.7 ± 2.5	26.7 ± 7.3	48.3 ± 3.3
Progressive VCL (%)	66.7 ± 1.5	45.0 ± 8.2	69.0 ± 2.6 [b]
Progressive VAP (%)	50.3 ± 2.4	33.8 ± 7.6 [a]	56.5 ± 3.2 [b]
ALH (μm)	4.4 ± 0.2	3.4 ± 0.4	4.6 ± 0.2 [b]
Progressive sperm density (× 10^6/ml)	5.7 ± 0.6	2.9 ± 1.0 [a]	7.4 ± 1.0 [b]

Male mice were supplemented with AP (57.5 mg/kg) for 7 days, heat-stressed, then continued to be treated for 28 days. Sperms collected from the epididymis were subsequently analyzed. Results are presented as means ± SE (n = 6 or 9). Progressive VSL (%), VCL (%), and VAP (%) indicate the ratio of progressive sperm (> 25 μm/sec) density to total sperm density. ALH, amplitude of lateral head displacement; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity. [a] $P < 0.05$ *vs*. RT-Control; [b] $P < 0.05$ *vs*. HEAT-Control.

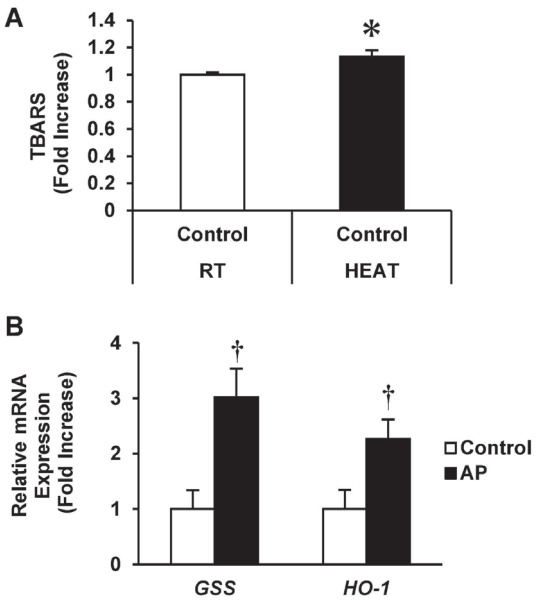

Fig. 2. Effects of AP supplementation on lipid peroxidation and antioxidant enzyme expression with or without heat stress. (A) Male mice were exposed to heat stress at 42°C for 20 min, and TBARS was detected 30 min later. Results are presented as means ± SE (n = 6). * $P < 0.05$ *vs*. RT-Control (unpaired *t*-test). (B) Non-heat-stressed mice were supplemented with 57.5 mg/kg of AP for 7 days. Thereafter, the mRNA expression of antioxidant enzymes was evaluated by qRT-PCR. Results are presented as means ± SE (n = 6). † $P < 0.05$ *vs*. Control (unpaired *t*-test).

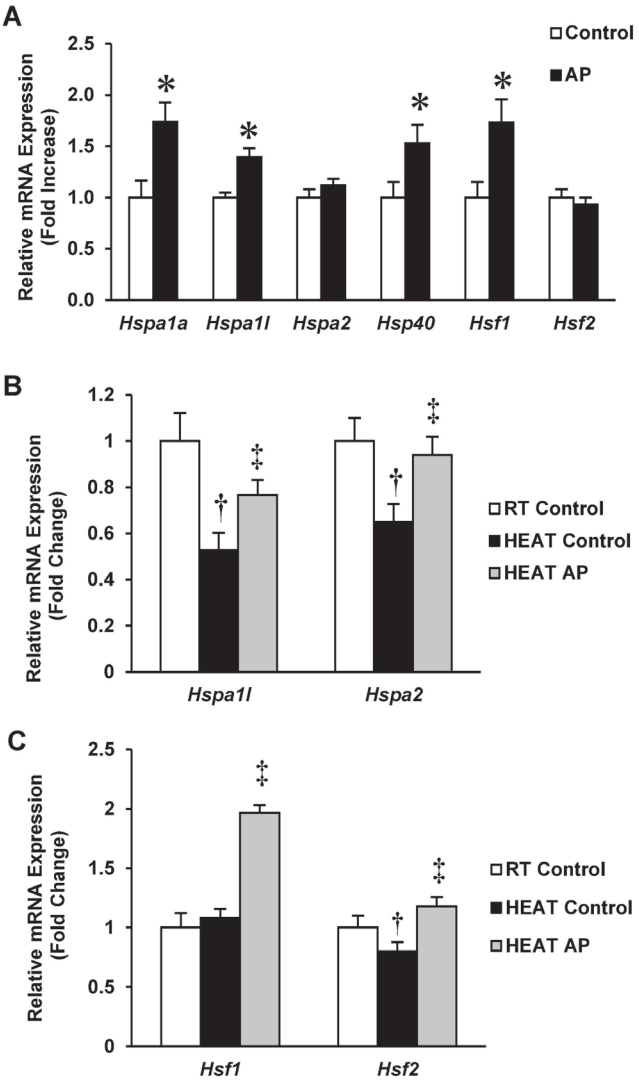

Fig. 3. Supplementation with AP changed the expression of testicular HSPs and HSFs. (A) Non-heat-stressed mice were treated with or without 57.5 mg/kg AP for 7 days. Thereafter, HSP mRNA expression was evaluated by qRT-PCR. Results are presented as means ± SE (n = 6). * $P < 0.05$ *vs*. Control (unpaired *t*-test). (B–C) Mice were treated with or without 57.5 mg/kg AP for 7 days and then exposed to heat stress. The testes were collected at 6 h (B) and 1 h (C) after exposure to heat, and HSP and HSF mRNA expression was evaluated by qRT-PCR. Control mice (RT Control) were kept for another 1 or 6 h without stress. Results are presented as means ± SE (n = 5–6). † $P < 0.05$ *vs*. RT-Control (Holm test); ‡ $P < 0.05$ *vs*. HEAT-Control (Holm test).

significantly suppressed by XA (Fig. 4D). Supplementation with XA significantly increased the expression of *GSS*, but not of *HO-1* (Fig. 4E). Similarly, it tended to increase the expression of *Hspa1a* ($P = 0.08$) and *Hsp40* ($P = 0.08$), but not of *Hspa1l* and *Hspa2* (Fig. 4F). These data suggested that supplementation with XA can prevent heat stress-induced dysfunction of the testes.

Xanthoangelol protected against heat stress-induced reduction in sperm density and quality

We evaluated the protective effects of XA on the number and quality of sperms in mice exposed to heat. Most of the sperm parameters were significantly decreased after exposure to heat (Table 3). Supplementation with XA significantly prevented reductions in sperm motility, progressive sperm density, and progressive sperm velocity (VSL, VCL, and VAP) ($P < 0.05$). Although XA (3 mg/kg), unlike AP, did not completely improve these sperm parameters under our experimental conditions, these data showed that XA can prevent heat stress-induced impairment in sperm density and quality.

Discussion

The present study is the first to suggest that dietary phytochemicals may improve heat stress-induced male infertility via increased HSP expression. The main findings were as follows. First, AP supplementation completely prevented heat stress-induced impairment in sperm parameters, including densities of motile sperms and progressive sperms. Second, XA, a functional component of AP, at 3 mg/kg significantly prevented most of heat stress-induced reductions in sperm parameters, but not completely. Third, both AP and XA elevated the expression of the ubiquitous *Hspa1a* and *Hsp40* genes, as well as that of the antioxidant enzyme *GSS* in non-stressed testes. Fourth, AP significantly prevented heat stress-induced reduction in the expression of not only *Hspa1l* and *Hspa2*, which are highly expressed in normal testes and critical for fertility, but also the transcription regulator

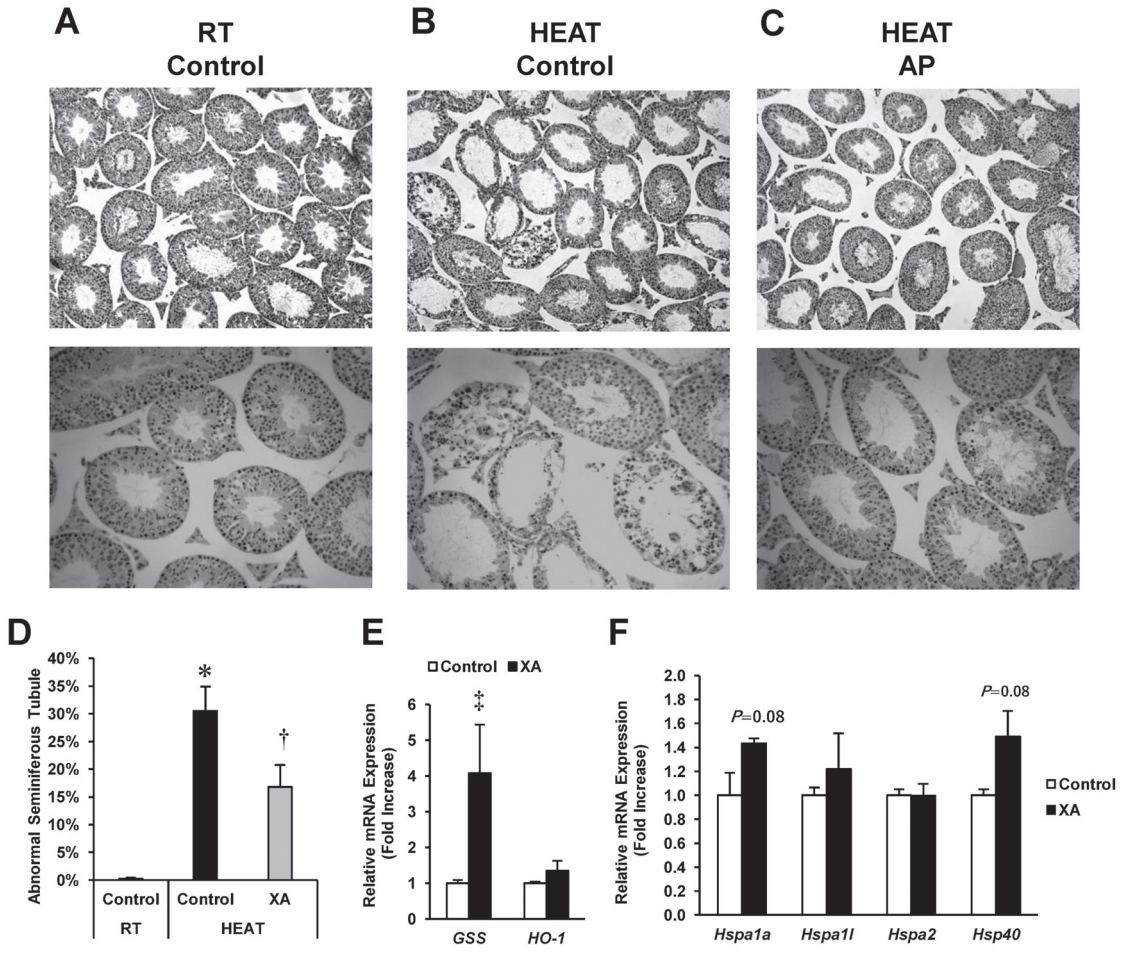

Fig. 4. Xanthoangelol protected sperm cells against heat stress accompanied by altered expression of antioxidant enzymes and HSPs. (A–C) Testicular sections stained with hematoxylin-eosin at 48 h after heat stress. A, RT-Control; B, HEAT-Control; C, HEAT-XA. Upper panels, original magnification × 100; lower panels, × 200. (D) Numbers of abnormal seminiferous tubules among total tubules. Results are presented as means ± SE (n = 6–7). * $P < 0.05$ *vs*. RT-Control (Holm-test). † $P < 0.05$ *vs*. HEAT-Control (Holm-test). (E–F) Non-heat-stressed mice were treated with 3 mg/kg XA for 7 days and mRNA expression of antioxidant enzymes was evaluated by qRT-PCR. Results are presented as means ± SE (n = 5 or 7). Groups were compared using the Wilcoxon rank-sum test for GSS and unpaired *t*-test for others. ‡ $P < 0.05$ *vs*. Control.

Table 3. Protective effects of xanthoangelol on sperm density and quality in mice exposed to scrotal heat

	RT Control	HEAT Control	HEAT XA
Sperm density (× 10^6/ml)	33.0 ± 3.2	24.4 ± 2.2	26.7 ± 2.5
Motility (%)	79.4 ± 1.4	66.3 ± 3.1 [a]	80.5 ± 2.4 [b]
Motile sperm density (× 10^6/ml)	26.1 ± 2.4	16.5 ± 1.9 [a]	21.8 ± 2.4
Progressive VSL (%)	51.9 ± 1.8	35.3 ± 2.4 [a]	49.4 ± 3.2 [b]
Progressive VCL (%)	75.6 ± 1.2	56.1 ± 3.2 [a]	72.4 ± 3.5 [b]
Progressive VAP (%)	62.0 ± 1.5	42.2 ± 2.7 [a]	58.5 ± 3.9 [b]
ALH (μm)	6.3 ± 0.2	4.8 ± 0.1 [a]	5.1 ± 0.3
Progressive sperm density (× 10^6/ml)	17.1 ± 1.6	8.7 ± 1.0 [a]	13.4 ± 1.7 [b]

Male mice were supplemented with XA (3 mg/kg) for 7 days, heat-stressed, then continued to be treated for 28 days. Sperms collected from the epididymis were subsequently analyzed. Results are presented as means ± SE (n = 8–10). Progressive VSL (%), VCL (%), and VAP (%) indicate the ratio of progressive sperm (> 25 μm/sec) density to total sperm density. ALH, amplitude of lateral head displacement; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity. [a] $P < 0.05$ *vs*. RT-Control; [b] $P < 0.05$ *vs*. HEAT-Control.

Hsf2. Fifth, we first showed that phytochemicals can increase the expression of HSPs in the testes. Mice were fed a diet supplemented with AP and XA for 7 days before and 28 days after exposure to heat stress. The elevated expression of HSPs and antioxidant enzymes by AP and XA appeared to suppress the toxic effects of heat stress, including ROS generation.

Elevated ROS in the testes damages germ cells, leading to male infertility [8–10, 27, 28]. Several reports have described the protective effects of antioxidant phytochemicals on sperm density and quality in the testes exposed to stress. For example, supplementation with resveratrol found in red wine and curcumin derived from turmeric protects sperm quality in hyperthyroid rats by changing the expression levels of several antioxidant enzymes [25, 28]. Hyperthyroidism causes male reproductive disorders because increased amount of thyroid hormone induces excess ROS generation in many tissues, including testicular tissues. Resveratrol reverses the decreased expression of catalase and glutathione peroxidase in hyperthyroid rats [28]. Intraperitoneally injected epigallocatechin gallate derived from green tea also protects against ionizing radiation-induced reduction in sperm density and quality [27]. The present study confirmed by measuring TBARS that heat stress was converted to oxidative stress in the testes. The expression of the antioxidant enzyme *GSS* was reduced in heat-stressed testes [29]. Both AP and XA elevated *GSS* expression in the present study, suggesting that increased *GSS* expression was also involved in protecting testicular cells.

One of the mechanisms by which dietary phytochemicals exert antioxidant activity is increased expression of antioxidant enzymes, such as HO-1, catalase, and GSS [30, 31]. The transcription factor Nrf2 binds to the promoter region, called antioxidant responsive element, of these antioxidant enzymes and stimulates their transcription. Several reports suggested that various kinds of chalcones could activate the Nrf2 pathway, although XA and 4HD, major functional chalcones in Ashitaba, have never been investigated [31, 32]. We have recently found that XA protected porcine cultured granulosa cells from the toxicity of H_2O_2 via Nrf2, as indicated by the finding that XA-dependent prevention of granulosa cell apoptosis caused by H_2O_2 was suppressed by Nrf2 siRNA (data not shown). These data suggest that AP and XA may elevate the expression of the antioxidant enzymes HO-1, catalase, and GSS via Nrf2 in the testes.

Although past studies have shown the close relation of increased testicular temperature with impaired spermatogenesis [1–3, 5, 6], whether dietary phytochemicals change HSP expression in the testes has never been investigated. The present study showed that both AP and XA increased the expression of the ubiquitous *Hspa1a* and *Hsp40* genes, suggesting that they are involved in protecting the testes against heat stress. These two HSPs coordinate with each other because Hsp40 is essential for the function of proteins of the HSP70 family, including Hspa1a. Notably, *Hspa1l* expression was increased by AP, but not by XA. In addition, AP reverses heat stress-induced reduction in *Hspa1l* and *Hspa2*, both of which critically contribute to spermatogenesis [15, 16]. This difference in potency on the expression of these HSPs between AP and XA may be associated with the finding that AP protects the testes from heat stress more effectively than XA. We treated male mice with AP (57.5 mg/kg) or XA (3 mg/kg). AP (57.5 mg) contains 3.3 mg of XA and 1.7 mg 4HD, which are the major functional chalcones in Ashitaba. The difference in the ability to increase HSP expression might be due to the ingested dose of XA (3 mg/kg) being too low to exert obvious effects or due to AP containing other functional compounds besides XA that can increase HSP expression.

HSF1 is widely expressed and it activates the transcription of various HSPs. AP increased the expression of *Hsf1,* as well as that of *Hspa1a*, *Hspa1l*, and *Hspa40*. Therefore, AP is considered to increase the expression of these HSPs by elevating the expression of *HSF1*. Trimerization of HSF1 may also be involved in activating the transcription of these HSPs because HSF trimers are known to bind to the promoter region of various HSP genes and activate their transcription [33], although there was no evidence of trimerization in our experiments. Wang *et al.* [26] showed reduced expression of testicular *Hspa1l* in *hsf2*$^{-/-}$ mice and testicular *Hspa1l* and *Hspa2* in *hsf1*$^{-/-}$ *hsf2*$^{-/-}$ mice. Heat stress-induced reduction in testicular *Hsf2* expression was prevented by AP treatment in our experiments. Thus, HSF1 and HSF2 may contribute in preventing heat stress-induced reduction in Hspa1l and Hspa2 expression.

The prevalence of assisted reproductive techniques, including *in vitro* fertilization, has increased over the past few decades. Here, we showed that AP and XA can prevent impaired sperm density and quality caused by testicular heat stress, which is a major cause of male infertility. Supplementation with dietary functional phytochemicals may help prevent heat stress-induced male infertility.

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