Effect of *Panax ginseng* and *Acanthopanax senticosus* on the Expression of HSPs during Heat Stress

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1. Introduction

Heat stress is accompanied by high body temperatures during physical exertion, which typically results in exhaustion or collapse and is accompanied by evidence of tissue and organ damage(Gardner JW, 2001). The effects of heat stress on cellular function include inhibitionof DNA synthesis, transcription, RNA processing, and translation. Heat stress can also cause inhibition of progression through the cell cycle, denaturation and misaggregation of proteins, increased degradation of proteins through both proteasomal and lysosomal pathways, disruption of cytoskeletal components, alterations in metabolism that lead to a net reduction in cellular ATP, and changes in membrane permeability that lead to an increase in intracellular Na^+, H^+, and Ca^{2+}(Kuhl and Rensing, 2000; Lindquist, 1986). Biologically, the ability to survive and adapt to heat stress appears to be a fundamental requirement of cellular life, and heat shock proteins(HSPs) are involved in heat responses(Lindquist, 1986; Parsell and Lindquist, 1993). Among the heat-inducible genes, those encoding the HSP 27 kDa, HSP 70 kDa and HSP 90 kDa have been the most extensively studied(Buckley and Hofmann, 2002; Dietz and Somero, 1992; Maloyan and Horowitz, 2002; Maloyan et al., 1999). These proteins confer cytoprotection by chaperoning the correct folding of other proteins or the degradation of abnormal proteins, or, by facilitating the molecular signaling of cytoprotective pathways(Morimoto and Santoro, 1998). These actions are achieved by activating the trimerization and nuclear translocation of cytoplasmic heat shock factor-1(HSF-1) to enable binding with the heat shock elements(HSE) and consequent transcription of the HSP genes(Asea, 2007).

Data from the National Center for Environmental Health

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show that 3,442 deaths in the United States were attributed to excessive heat from 1999 to 2003(2006). The incidence of such deaths may increase with global warming and the predicted worldwide increase in the frequency and intensity of heat waves(Easterling et al., 2000; Nakai et al., 1999; Rooney et al., 1998; Sartor et al., 1995). In Korea, *samgyetang*(a mixture of ginseng soup and chicken) has traditionally been eaten to protect health and prevent exhaustion due to heat. *Samgyetang* contains chicken, ginseng or other medicinal herbs such as *Astragalus senticosus* and *A. membranaceus*. *P. ginseng* and *A. senticosus* have been used for over 2000years in Asian countries to enhance stamina and immune function, and it has been suggested that these products exert pharmacological activities in the cardiovascular, endocrine, immune, and central nervous systems(Attele et al., 1999; Brekhman and Kirillov, 1969). Saponins from *P. ginseng* and *A. senticosus* have a four-ring, steroid-like structure with sugar moieties attached. Approximately 30 different types of saponins have been isolated from these herbs. In addition, non-ginsenoside constituents of ginseng are also known to exert pharmacological effects(Odashima et al., 1985; Tsang et al., 1985).

Several studies have examined the pharmacological effects of *P. ginseng* and *A. senticosus*; however, their protective effects against heat-stress have not been investigated to date. Therefore, in this study, we examined the protective effect of *P. ginseng* and *A. senticosus* against changes in the expression of HSP27, HSP70 and HSP90 in heat-stressed rats.

2. Materials and methods

2.1. Preparation of the sample extract

For this experiment, 4 and 5 year old cultivated P. ginseng was collected from Anseong, Korea in 2007. In addition, *A. senticosus* was collected from Korea in 2007. Five-hundred g of Korean *P. ginseng*, 500g of *A. senticosus* and a mixture of 500g of Korean *P. ginseng* + 500g of *A. senticosus* were macerated with 20 times distilled water and then refluxed 2 times for 3 hours each in a water bath. The extracts were then filtered through filter paper(Whatman) and concentrated using a vacuum evaporator.

2.2. Component analysis of the concentrate

Acidic polysaccharides from each concentrate were analyzed by the carbazole-sulfuric acid method(Bitter and Muir, 1962) using β -D-galacturonic acid as a reference standard. The total phenolics in each concentrate were analyzed using the Folin-ciocalteu method(Ainsworth and Gillespie, 2007). The ginsenoside composition of the concentrate was analyzed by HPLC(Jasco International Co. Ltd, Japan) using a μ -Bondapak C18 column(10㎛, 3.9×300 cm, Waters). The mobile phase used for HPLC was a mixture of acetonitrile(HPLC Grade, Sigma, U.S.A.) and distilled water(HPLC Grade, J.T. Baker, U.S.A.). During elution, the content of acetonitrile was sequentially decreased from 80% to 68%, 57%, 20% and then 0%. The operating temperature was set to room temperature, and the flow rate was 1.0 ml/min. The chromatogram was detected at 203nm using a uv/vis detector.

2.3. Animals and experimental design

For this study, 4 week-old male Sprague-Dawley rats(Weight; 218.9 ± 19.1) were purchased from Japan SLC, Inc.(Shizuoka, Japan). The rats were maintained in an air-conditioned room(23±3℃; relative humidity 50-55%) under a 12-h light/dark cycle and allowed ad libitum access to tap water and a basal diet(AIN 76A, Diets, USA). The experiment was performed in accordance with the guidelines for animal experimentation of the Faculty of Agriculture, CHA institute.(Gyeonggi-Do, Korea). After a one week adaptation period, the rats were assigned to 5 groups comprised of eight animals per group using a randomized complete block design. One group was housed at room temperature(23±3℃; relative

Table 1. Animals & Experimental Designs

Group 1) (=8)	For 4 weeks	For 2 weeks (37℃, 5hr/day)
CR	Room Temp. AIN 76A diet	Room Temp. AIN 76A diet
HS	Room Temp. AIN 76A diet	Heat Environment. AIN 76A diet
GD	Room Temp. AIN 76A diet + GS ext.	Heat Environment. AIN 76A diet + GS ext.
FD	Room Temp. AIN 76A diet + FG ext.	Heat Environment. AIN 76A diet + FG ext.
FGS	Room Temp. AIN 76A diet + GS + FG ext.	Heat Environment. AIN 76A diet + GS + FG ext.

[1] CR: Control room temperature, HS: heat stress, GD: *A. senticosus extract*, FD: *P. ginseng extract*, FGS: mixture of P. *ginseng C. A. Meyer and A. senticosus extracts*

humidity 50-55%) and provided with a basal diet(AIN 76A) for 6 weeks. Another group was provided with a basal diet(AIN 76A) for 4 weeks and then subjected to heat stress(temperature, 37~38℃) while receiving a basal diet (AIN 76A) for 2 weeks(HS). The remaining groups were provided with *P. ginseng*(FD), *A. senticosus*(GD) or a mixture of *P. ginseng* and *A. senticosus*(FGS) for 4 weeks at room temperature. And then the groups were subjected to heat stress and administrated either *P. ginseng*(FD), *A. senticosus*(GD) or a mixture of *P. ginseng* and *A. senticosus*(FGS) for 2 weeks. *P. ginseng*(FD) and *A. senticosus*(GD) or a mixture of *P. ginseng* and *A. senticosus(*FGS) concentrate dissolved in 1ml water were administrated orally. The animals and experimental design are described in detail in table 1. After the treatment period, the rat livers were excised, weighed, and cut into small pieces that were frozen in RNA*later*(Ambion, Austin, TX, USA) and stored at -80℃ until analysis.

2.4. Total RNA isolation and reverse transcription polymerase chain reaction(RT-PCR)

The total RNA from three rats per dose group was extracted using TRIzol reagent(Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA samples with an O.D. 260/280 ratio higher than 2.0 were used for reverse transcription polymerase chain reaction.

RT-PCR was performed using a Taq DNA polymerase kit(Solgent, DaeJeon, Korea) and a PCR DNA Thermal Cycler(Corbett, San Francisco, CA, USA). First-strand cDNA was synthesized from 2㎕ of the total RNA using the Maxime RT PreMix(Intron Biotechnology, Corp.) in a 20㎕ reaction mixture. cDNA reactions were conducted under the following conditions: one cycle at 45℃ for 60min using activated HotStarTaq DNA Polymerase that had been heated

Table 2. Primer sequences used for RT-PCR and Real-Time PCR analysis

Gene	Primer sequence (5' to 3')	RT-PCR TM (℃)	Real-Time PCR TM (℃)	Product size (bp)
β-actin	F: 5'-CTATGTTGCCCTAGACTTCG-3' R: 5'- ACTTCATGATGGAATTGAATG-3'	61	58	230
HSP27	F: 5'- GAGGAGCTCACAGTGAAGAC-3' R: 5'- CTGATTGTGTGACTGCTTTG-3'	63	60	211
HSP70	F: 5'- CTGAGAAAGAGGAGTTCGTG-3' R: 5'- GTCTGTCTCTAGCCAACACC-3'	61	60	206
HSP90	F: 5'- ACGACAAAGCTGTCAAAGAC-3' R: 5'- AACAAGAACTGCAGCAATG-3'	63	63	178

at 95℃ for 15min. PCR of the first-strand cDNA was then conducted under the following conditions : 35 cycles of 30 sec at 94℃, 30 sec at 50-68℃(depending on the gene), and 1 min at 72℃. This was followed by a final extension at 72℃ for 10 min. All primers and annealing temperatures used in this study are shown in table 2. The RT-PCR products were then subjected to electrophoresis on 1.5% agarose gels. β -actin was used as a housekeeping gene for quantitative PCR to ensure that equal amounts of reverse-transcribed cDNA were used in the PCR reaction.

2.5. Real-Time RT-PCR

Quantitative analysis of the RT-PCR results was conducted to determine if the expression of several genes was increased or decreased by treatment with *P. ginseng* and *A. senticosus* and their related genes were then quantified by Real-Time RT-PCR. To confirm the results, quantitative Real-Time RT-PCR with SYBR Green PCR master mix(Applied Qiagen, Foster City, CA, USA) was conducted using the Roter-Gene 3000 Sequence Detection System(Applied Corbett Research). The Real-Time RT-PCR reaction was conducted according to the SYBR green PCR master mix protocol using the same oligonucleotide primers that were used for the RT-PCR(Table 2). The reaction conditions for the quantitative Real-Time-PCR were as follows: initial denaturation at 95℃ for 15 minutes, followed by 40cycles of denaturation at 95℃ for 10s, annealing at 60~63℃ for 15s, extension at 75℃ for 15s, after which the samples were melted by increasing the temperature from 72℃ to 95℃. The relative expression levels of HSP27, HSP70 and HSP90 were then calculated using the comparative C_t method, which generated relative HSP27, HSP70 and HSP90 mRNA levels that were adjusted for the β -actin endogenous control mRNA. The data points for each animal represent the average of triplicate measurements.

2.6. Statistical Analysis

The levels of HSP family mRNA in the room temperature group vs. those of the heat stress group were determined using tissues taken from a subset of experimental subjects(n=3) and then compared via a one-tailed paired samples t-test. The effects of *P. ginseng* and *A. senticosus* extracts on HSP27, HSP70 and HSP90 mRNA levels in the liver were assessed between-subjects by one-way ANOVA(8.01, SAS program). Post-hoc comparisons were conducted using Tukey's Honestly Significant Difference test. Differences were considered to be statistically when the *P* values were less than 0.05.

3. Results

3.1. Components of *A. senticosus* and *P. gi-nseng* extracts

The components of the *A. senticosus* and *P. ginseng* extracts are summarized in Table 3. *A. senticosus* extract(2.89mg) contained 0.48% acidic polysaccharide and 0.58% phenol. *P. ginseng*(28.35mg) contained 6.31% acidic polysaccharide, 0.93% phenol and 3.54% ginsenosides including Rb1 and Rg1. *P. ginseng* + *A. senticosus* extract(31.23mg) contained 10.7% acidic polysaccharide, 2.2% phenol and 5.6% ginsenosides including Rb1 and Rg1.

Table 3. Bioactive components of ginseng and *Acanthopanax senticosus* (%)

Sample	Yield	A.P.[1)]	Total phenolics	Crude ginsenosides[2)]
Acanthopanax senticosus	7.7	0.48	0.58	–
Panax ginseng C. A. Meyer	56.7	6.31	0.93	3.54
Panax ginseng C. A. Meyer + Acanthopanax senticosus	35.7	10.7	2.2	5.6

[1)] Acidic polysaccharides

[2)] Typical active components of Korean ginseng

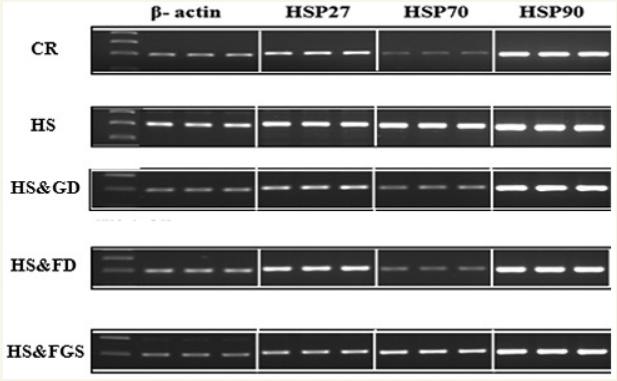

Fig 1. **RT-PCR.** Expression of HSPs in control room temperature and heat stressed rats, as well as in rats that were subjected to heat stress administrated ginseng and medicinal herbs.

3.2. Effect of *A. senticosus* and *P. ginseng* extracts on heat-stress

Representative RT-PCR images of liver samples obtained from the control room temperature group(CR), heat-stressed group(HS) and the groups that were treated with *P. ginseng* and *A. senticosus* extracts and subjected to heat stress(GD, FD, FGS) are presented in Fig 1. Expression of HSP27, HSP70 and HSP90 was higher in the heat-stressed group than the control room temperature group. However, the expression of these genes in rats that were treated with *P. ginseng* and *A. senticosus* extracts and subjected to heat stress(GD, FD, FGS) were similar to those of the CR group.

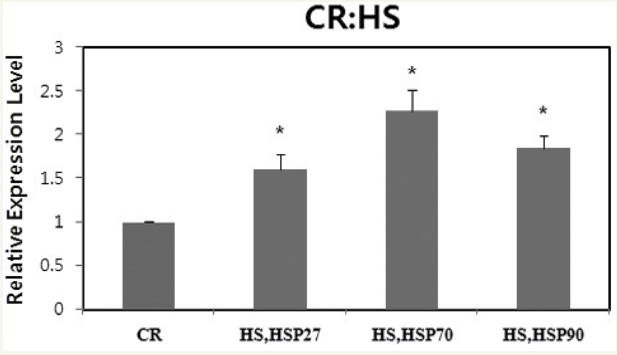

Fig 2.. Gene expression in the liver of rats exposed to heat stress. The relative expression level was increased 1.7~2.5 fold in the heat-induced group when compared to the room temperature group. *$p < 0.05$.

3.3. Expression levels of HSP27, HSP70 and HSP90 in response to heat

The transcript levels of HSP27, HSP70 and HSP90 were assessed by quantitative Real-Time RT-PCR(Fig 2, Fig 3). The levels of HSP27, HSP70 and HSP90 mRNA, which are shown in Fig. 2, were approximately 1.6, 2.27 and 1.85 fold higher in HS group than in CR group*(P<0.05)*.

3.4. Changes in the levels of HSP27, HSP70 and HSP90 in response to heat-stress in rats that were administered *P. ginseng* and *A. senticosus* extracts

As shown in Fig 3A, the HSP27 gene expression levels were approximately 40%, 28% and 44% lower in the GD, FD and FGS groups than in the HS group*(P<0.05)*. As shown in Fig 3B, the HSP70 gene expression levels were approximately 32%, 87% and 15% lower in the GD, FD and FGS groups than in the HS group*(P<0.05)*. Finally, as shown in Fig. 3C, the HSP90 gene expression levels were 28%, 4% and 16% lower in the GD, FD and FGS groups than in the HS group *(P<0.05)*.

4. Discussion

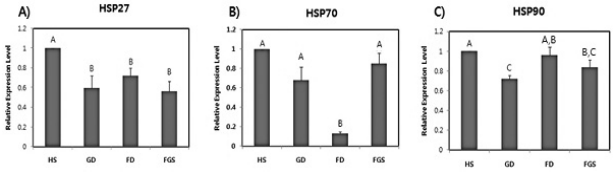

Fig. 3. Effects of *P. ginseng* and *A. senticosus* extracts on heat-stressed rats. A) Expression of HSP27 mRNA in rats was administrated ginseng and medicinal herbs. A significant reduction in the expression of HSP27 was observed in the GD, FD and FGS groups *(P < 0.05)*. B) Expression of HSP70 mRNA in rats that were administrated ginseng and medicinal herbs. A significant reduction in the expression of HSP 70 was observed in the FD group *(P < 0.05)*. C) Expression of HSP90 mRNA in rats that were administrated ginseng and medicinal herbs. A significant reduction in the expression of HSP90 was observed in the GD group *(P < 0.05)*. Statistical differences between groups were determined by one-way ANOVA. Post-hoc comparisons were made using Tukey's Honestly Significant Difference test.

Thermoregulatory failure, exaggeration of the acute-phase response, and alteration of the expression of heat-shock proteins may contribute to the progression from heat stress to heat stroke(Bouchama and Knochel, 2002). Stresses involved in liver regeneration induced by heat, such as a temporary rise in body temperature and ischemia-reperfusion injury could challenge protein homeostasis, thereby inducing an increased flux of non-native proteins(unfolded and misaggregated proteins) in rapidly proliferative hepatocytes of the liver. It is generally accepted that heat stress leads to increased expression of HSPs, and that this increase is closely correlated with the acquisition of thermotolerance. In addition, the increased expression of HSPs during liver regeneration may react to heat stress by repairing protein damage, thereby helping hepatocytes in the liver return to their normal state(Kregel, 2002; Lindquist, 1986; Parsell and Lindquist, 1993). In this study, we focused on the liver because the results of other studies have demonstrated that this organ plays an important role in the ability of an organism to cope with heat stresses such as hyperthermia(Hall et al., 2000a; Hall et al., 2000b; Kregel and Moseley, 1996).

In the present study, we investigated the administration of *P. ginseng* and *A. senticosus* extracts to determine if they conferred protection against heat to heat-stressed rats. As shown in Fig. 3A, the HSP27 gene expression levels were approximately 40%, 28% and 44% lower in the GD, FD and FGS groups than in the HS group. These findings indicate that treatment with *P. ginseng* and *A. senticosus* may confer protection against heat-stress without over-expression of HSP genes. As shown in Fig. 3B, the largest decrease in the expression of heat-induced HSP70 was 87%, which was observed when the FD group was compared to the HS group. Furthermore, as shown in Fig. 3C, the expression of heat-induced HSP90 was decreased by 28% and 16% in the GD and FGS groups when compared to the HS group. Taken together, these findings indicate that *P. ginseng* and *A. senticosus* may exert a protective effect by attenuating the over-expression of HSP genes that is induced by heat stress. In addition, the differences in the expression levels of HSP27, HSP70 and HSP90 in rats that were administered *P. ginseng* and *A. senticosus* and subjected to heat stress may have occurred due to the different components in *P. ginseng* and *A. senticosus*. Acidic polysaccharides and ginsenoside in *P. ginseng* may exert a protective effect against heat stress. The active constituents found in most ginseng species include ginsenosides, polysaccharides, peptides, polyacetylenic alcohols, and fatty acids(FC, 1992). The ginsenoside, Rg^1, modulates rat adaptive behavior by blockade of temperature-related information into the hypothalamic histamine neurons(Yoshimatsu et al., 1993). Additionally, the ginsenoside, Rb^1, has been shown to inhibit Ca^{2+} accumulation in the liver(Park et al., 1996). Furthermore, the ginsenoside, Rg^3, has been shown to exert significant protective effects against cell DNA damage and apoptosis(Min et al., 2006; Zhang et al., 2008). Moreover, the ginsenoside, Rb^1, has been shown to significantly inhibit apoptosis induced by ischemia-reperfusion in rats, thereby alleviating ischemia-reperfusion injury(Guan et al., 2002). Conversely, various components of *A. senticosus*, which include eleutherosides, chiisanosides, isofraxidin, acanthosides, daucosterine, β -sitosterol, sesamine, and savinine, may exert a protective effect against heat stress(Davydov and Krikorian, 2000).

Based on these findings, there are two possible explanations for the inhibition of the increased expression of HSPs in response to heat stress that was observed in this study. First, administration of *P. ginseng* and *A. senticosus* may exert protective effects against heat stress without HSP over-expression by blocking or interrupting the heat stress-induced mechanism. The heat-regulated activation of the MAP(mitogen-activated protein) kinase suggests that 3 distinct mechanisms are involved in activation of the 3 MAP kinases, ERK, JNK, and p38, and that the activation of these pathways by heat stress is highly specific. Activation of epidermal growth factor(EGF) receptor by heat stress(Lin et

al., 1997) desensitizes the p38 pathway, and the desensitization kinetics correspond exactly to the kinetics of the development of thermotolerance(Dorion et al., 1999; Landry et al., 1991). This activation of p38 and JNK early during heat stress is a mechanism of heat-induced cell death, and the desensitization process may be a mechanism of thermotolerance. Second, we could not exclude the possibility that *P. ginseng* and *A. senticosus* might directly attenuate the action of heat stress at the liver by blocking the heat stress binding site in tissues in the liver.

In conclusion, administration of *P. ginseng* and *A. senticosus* exerted a protective effect in heat-stressed rats. However, treatment with a mixture of *A. senticosus* with *P. ginseng* did not produce the expected synergistic effect. Although the precise mechanism underlying the protective effect of *P. ginseng* and *A. senticosus* is unknown, our data suggest that they play an important role in protecting organisms against heat stress. The most common effects of heat stress are heat stroke, heat exhaustion and exertional heat illness(EHI)(Moran, 2001). EHI is often associated with physical activity while under heat stress, and the incidence of EHI is correlated with a rise in ambient air temperature(Sonna et al., 2004). Therefore, adding *P. ginseng* and *A. senticosus* to foods consumed by manual laborers, military personnel and sports players may protect against EHI. Accordingly, we are currently conducting additional studies to elucidate the precise mechanism(s) by which *P. ginseng* and *A. senticosus* protect rats from heat stress.

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