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• *BRIEF REPORTS* •

Oral administration of geranylgeranylacetone plus local somatothermal stimulation: A simple, effective, safe and operable preconditioning combination for conferring tolerance against ischemia-reperfusion injury in rat livers

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Abstract

AIM: To explore a simple, effective, safe and operable pretreatment for conferring tolerance against ischemia-reperfusion (I-R) injury in rat livers.

METHODS: Forty-five rats were divided into five groups (each group n = 9). Group C: control group; group G: geranylgeranylacetone (GGA) was administered without heat stress; group S: local heat stress alone; group WG: GGA plus whole-body heat stress; group SG: GGA administration plus local heat stress. After completion of the I-R procedure, the ischemic-reperfused liver lobes in five groups were resected and tested for heat shock protein (HSP70) by RT-PCR, Western blotting analysis and immunohistochemical staining. The blood samples were collected for ALT and AST measurement at the end of occlusion of blood supply, 30 min after reperfusion, 24, 48, 72 h after surgery from the inferior vena cava. Survival was monitored for 1 wk.

RESULTS: The production of HSP70 after I-R injury increased, the liver enzyme levels after reperfusion decreased rapidly, and the survival rates increased in groups C-SG.

CONCLUSION: The combination of GGA plus local somatothermal stimulation is a simple, effective, safe and operable pretreatment to induce HSP70 in patients with liver tumor and cirrhosis before hepatectomy and in donors before harvesting graft for liver transplantation.

Key words: Ischemia-reperfusion injury; Heat shock protein; Geranylgeranylacetone; Local somatothermal stimulation

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INTRODUCTION

Ischemia-reperfusion (I-R) injury is the main cause of hepatic damage induced by temporary clamping of the hepatoduodenal ligament during liver resection (Pringle maneuver), and severe complications of liver transplantation such as primary graft nonfunction or malfunction are also the consequence of reperfusion injury[1,2]. Furthermore, even moderate reperfusion damage that does not severely affect the graft can impair long-term hepatic recovery and enhances the patient's susceptibility to infections and multiple organ failure[1]. Though improvements in liver preservation and surgical techniques have been achieved, hepatic ischemia/reperfusion remains an important clinical problem. Furthermore, because of the shortage of organs for transplantation, the use of steatotic livers, which have a lower tolerance to hypoxia and are more susceptible to reoxygenation damage, greatly increase the risks related to reperfusion injury[3,4]. Therefore, it is clinically significant to find a simple, effective, safe and operable preconditioning combination before liver surgery to protect liver against I-R injury.

Hepatic preconditioning can be induced by transient I-R[5-9], short-term hyperthermia[10,11], oxidative stress[12], or ANP infusion[13,14]. All these stimuli improve postischemic sinusoidal perfusion, leukocyte infiltration, bile production, and aminotransferase release 48 h after their application to liver. Among these stimuli, hyperthermic preconditioning has been proved to be a potential clinical application measurement. After heat shock preconditioning, organisms may produce endogenous cytoprotective substances which can ameliorate I-R injury in subsequent hepatectomy or liver transplantation.

The cytoprotective action of heat shock preconditioning is closely related to the endogenous cytoprotective protein, heat shock protein (HSP), which has been proved to play an important role in protection against I-R injury[16,17]. In

the HSP family, inducible form of HSP70 is the main cytoprotective protein[18,19]. Research on how to apply the cytoprotective action of HSP70 during liver surgery by certain heat shock preconditioning form is scarce. Therefore, how to find out a clinically feasible heat shock stress form remains to be elucidated.

Generally, heat shock stress includes two types: whole-body heat and local heat. By heating the whole body in warm water or on electronic pad, body temperature must be above 41 ℃-the critical temperature[11,20] to activate heat shock stress of viscera where the inducible form of HSP70 is induced to play the cytoprotective action[18,21]. Local somatothermal stimulation (LSTS), a kind of local heat stress on designated skin area, is speculated to be the consequence of a somatovisceral reflex through a heat-sensitive neural release of NO. It is well known that NO is a strong inducer of gene expression of HSP[22-24], and it is interesting that LSTS also has the same critical temperature 41 ℃. Chiu *et al*.[26], have applied LSTS on the right seventh intercostal nerve territory[25] and left median nerve territory[27] respectively (they are regarded in Chinese medicine as sites to cure hepatobiliary and cardiovascular diseases), and found that HSP70 gene expression increases in liver and heart, and I-R injury in liver and heart was also relieved. Thus, for the two types of heat stress, this study aimed to estimate which type is more simple, effective, safe and operable for clinical application.

Besides, in view of the induction of HSP70, the use of pharmacologic inducers might be different. Although most chemicals that are able to induce HSP expression are cellular toxins[28-30], geranylgeranylacetone (GGA, teprenone or Selbex; Eisai Co., Ltd, Tokyo, Japan) is a commercially available medication used to treat gastric ulcer, which has been used in clinic in Japan for decades. Hirakawa *et al*.[31], reported that GGA has the potential to activate HSP70 gene expression in gastric mucosal cells in response to ethanol stress or water-immersion stress. In addition, the cytoprotective effect of GGA has been reported in liver[32,33] and small intestine[34]. Mizushima *et al*.[35], reported that pretreatment with low concentrations of ethanol, as well as GGA-induced HSPs in cultured guinea pig gastric mucosal cells, can prevent DNA fragmentation[35], but further investigation is necessary to know whether ethanol is efficacious in liver pretreatment because the liver has a strong activity of alcohol dehydrogenase. Bimoclomol is a new nontoxic compound, introduced by Vígh *et al*.[36] in 1997, which facilitates the formation of chaperone molecules by inducing or amplifying expression of heat shock genes. It was reported that four- to fivefold HSP70 gene is transcribed by the simultaneous application of bimoclomol and heat. The potency of GGA to enhance HSP70 induction under heat shock stress is as high as that of bimoclomol[21]. In this sense, pharmacologic modulation of heat shock preconditioning by GGA may have a clinical priority because its nontoxicity to human subjects has been proven during the long-term clinical use.

Therefore, the other aim of this study was to explore whether there is any difference in the induction of HSP70 in liver between administration of GGA and heat shock stress, and whether there is an additive effect if GGA is used in combination with heat shock stress.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 250-300 g were obtained from Animal Center of the Fudan University, Shanghai, China. They were housed in chip-bedded cages at room temperature (24±1 ℃) in a 12-h light/dark cycle. Rats were permitted free access to water and standard rat chow. The study was approved by the Committee on Experimental Animals of Second Military Medical University.

Grouping

Forty-five rats were divided into five groups (each group n = 9). Group C: experiment for I-R injury (as a control); group G: GGA was administered 2 h before experiments for I-R injury (administering GGA without heat stress); group S: LSTS was performed 12 h before experiment for I-R injury (local heat stress alone); group WG: GGA was administrated 2 h before whole-body heat shock exposure, and experiment for I-R injury was performed 48 h after whole-body heat shock exposure (GGA plus whole-body heat stress); group SG: GGA was administered 2 h before LSTS, and experiment for I-R injury was performed 12 h after LSTS (GGA administration plus local heat stress).

Extraction of mRNA and reverse transcriptase polymerase chain reaction

Total RNA was isolated from rat liver using a modified single-step guanidinium thiocyanate method (Tri Reagent, T-9424, Sigma Chemical Co., St. Louis, MO, USA)[37]. Reverse transcription (RT) was performed using a reverse transcription kit. One microgram of rat tissue RNA was used as a template and RT-generated cDNA encoding Hsp70 and β-microglobulin (β-MG internal control) were amplified using PCR. The primers used in this study were Hsp70 (354 bp), sense: 5'-AAC-GTG-CTG-CGG-ATC-ATC-AA-3', anti-sense: 5'-CGT-GAT-GGA-CGT-GTA-GAA-GT-3', and rat-β-MG (100 bp), sense: 5'-AAT-ATC-GAG-ATG-TCA-GAT-CTG-TC-3', anti-sense: 5'-GCT-TCA-ATG-AGT-GTT-TTG-ATC-AG-3'. The possible contamination of any PCR component was excluded by performing a PCR with these components in the absence of RT products in each set of experiments (negative control). Twenty microliters of RT-PCR products was checked by 2% agarose gel electrophoresis containing 0.2 mg/mL ethidium bromide, followed by photography under ultraviolet transillumination.

Antibody and Western blot analysis for hsp70 expression

Mouse monoclonal antibody against inducible HSP70 was purchased commercially (HSP 70 K-20: sc-1060, Santa Cruz Biotechnology, Inc.). Liver tissue was snap-frozen *in situ* using stainless steel tongs precooled in liquid nitrogen. Tissue homogenates from rat liver were obtained in the presence of protease inhibitors on ice, and the homogenate protein was assayed by the Bradford method[38]. After protein concentrations were measured, samples were applied to a 10% polyacrylamide gel with 0.1% sodium dodecylsulfate.

The gel-resolved proteins were transferred to polyvinylidene difluoride membranes (Marsh Biomedical Products Inc., Rochester, NY, USA). The blots were incubated with primary antibody (monoclonal K-20: sc-1060) and anti-mouse immunoglobulin horseradish peroxidase-linked secondary antibody (NA 931; Amersham International, Buckinghamshire, UK). They then reacted with enhanced chemiluminescence detection reagent (Amersham). The bands were recorded on X-ray film (Hyper film ECL, Amersham). Intensity of the blots was then measured densitometrically using a GS-700 imaging densitometer. To standardize the blotting as well as the visualization of each film, 0.005 μg of HSP70 (SPP-755, StressGen Biotechnologies Corp.) was simultaneously electrophoresed on each gel as an external standard. Anti-β-actin (monoclonal: clone AC-74, Sigma Inc.) was used as an internal control.

Immunohistochemical staining

Tissue samples fixed overnight in Bouin's solution[39] were immersed first in 50% ethanol for 24 h and then in 70% ethanol for 24 h for their dehydration and the removal of picric acid. Specimens were then embedded in paraffin and cut into 3.5-μm-thick sections. The slightly modified avidin-biotin complex method was used for immunohistochemical staining with a monoclonal antibody (1.0 μg/mL) against the inducible form of HSP70 (monoclonal K-20: sc-1 060.12) normal rabbit serum (diluted to 1:75) as an inhibitor of nonspecific binding of the secondary antibody. Biotin-labeled rabbit anti-mouse IgG serum (diluted to 1:300) as a secondary antibody and avidin-biotin-alkaline phosphatase complex (diluted to 1:100) were purchased from Dako (Kyoto, Japan). The substrate for alkaline phosphatase (black) was obtained from Vector Laboratories (Burlingame, CA, USA).

Administration of GGA

After overnight fasting, each rat in groups G, WG and SG was given 8 mL/kg of an emulsion composed of 200 mg/kg GGA in a 5% gum arabic solution (vehicle). The dose was administered intragastrically through a metal tube. In the groups not receiving GGA (groups C and S), only the vehicle was administered in a similar fashion. GGA used in this study was a generous gift from Eisai Co., Ltd (Tokyo, Japan). We chose a dose of 200 mg/kg, in which abundant HSP70 was produced with whole-body heat shock stress at 41-42 ℃ [21].

Whole-body heat shock exposure

Rats were anesthetized with intramuscular injections of atropine sulfate (0.05 mg/kg) and ketamine (50 mg/kg) 2 h after the GGA administration. They were then immersed in a warm water bath to elevate their rectal temperature to 41-42 ℃ for 15 min. Rectal temperature was measured by a digital thermometer. The timing of 2 h between GGA administration and heat shock treatment was decided according to the reports of *in vivo* metabolism of GGA in rats by Nishizawa *et al*.[40,41]. They showed that tissue level of GGA in the liver reaches plateau 2 h after oral administration in rats. Subsequent experiments for I-R injury were performed 48 h after whole-body heat shock exposure[19].

Local heat stress by local somatothermal stimulation

Local heat stress was confined to LSTS on a designated skin area but no direct contact with the skin surface. LSTS could induce relaxation of the sphincter of Oddi in cats and rabbits and as well as internal sphincter in rabbits[42,43]. In brief, LSTS was achieved by application of a heat generator 0.5 cm on and above the right seventh intercostal nerve territory, just at the junction of right midclavicular line and the seventh intercostal space (Figure 1, under light anesthesia with atropine sulfate, 0.05 mg/kg and ketamine 50 mg/kg, im). The skin temperature at the heating point fluctuating above and below the critical point 41 ℃ was obtained by intermittently turning the switch of the heat generator on and off, namely 4 min on and 5 min off for three courses. Usually, it took 27 min to compete one dose of LSTS. Subsequent experiment for I-R injury of the liver was performed 12 h after the preconditioned LSTS[25].

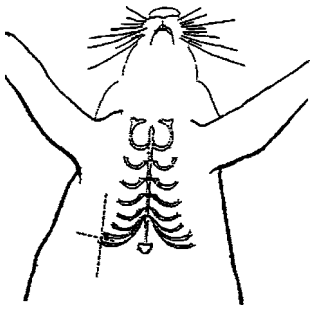

Figure 1 The heating site of LSTS.

Animal model and parameters for liver ischemia-reperfusion injury

The I-R injury rat model was established as described previously[44,45]. In brief, male Sprague-Dawley rats were anesthetized with diethyl ether (1.25 g/kg, ip), and the trachea was cannulated for artificial respiration with a ventilator. The liver was exposed through an upper midline incision, and one clamp for dog's ear was used to clamp the left branches of hepatic hilum and to allow occlusion of the blood supply to either the median or the left lobes (left branch). For I-R injury study, ischemia of left of the median lobes was maintained for 60 min, followed by reperfusion of the left/median lobes with occlusion of the right branches of hepatic hilum for another 30 min. After completion of the reperfusion procedure, the initial ischemia-reperfused left lobes were resected and tested for HSP70 by PT-PCR, Western blotting analysis and immunohistochemical staining. All rats were given 10 mL/kg of 5% glucose solution subcutaneously immediately after completion of the surgery, and all rats were allowed free access to 10% glucose solution after they recovered from the anesthesia. The blood samples were collected for ALT and AST measurement at 60 min after occlusion of the blood supply to the left branch; 30 min after reperfusion to the left branch: 24, 48, and 72 h after surgery from the vena caudalis. Signs of distress and survival were monitored for 1 wk.

Statistical analysis

The data were expressed as mean±SD. SPSS for Windows, version 11.0, was used with *t*-test, LSD, and Wilcoxon. $P<0.05$ was considered statistically significant.

RESULTS

Figure 2 shows the expression of HSP70 mDNA detected by RT-PCR and production of protein HSP70 detected by Western blotting in the five groups after I-R injury. Liver tissues in groups C and G exhibited the most pallid bands of HSP70 compared to that in the other three groups ($P<0.05$). Liver tissue in groups S and WG showed comparative bands of HSP 70 ($P>0.05$), while liver tissue in group SG showed the higher bands compared to that in groups C, G, and S ($P<0.05$). Generally, the production of HSP70 after I-R injury increased in groups C-SG.

Figure 3 shows immunohistochemical staining of HSP70 in liver tissue from groups C to SG. The accumulation of HSP70 in groups C, G, and S was significantly less than

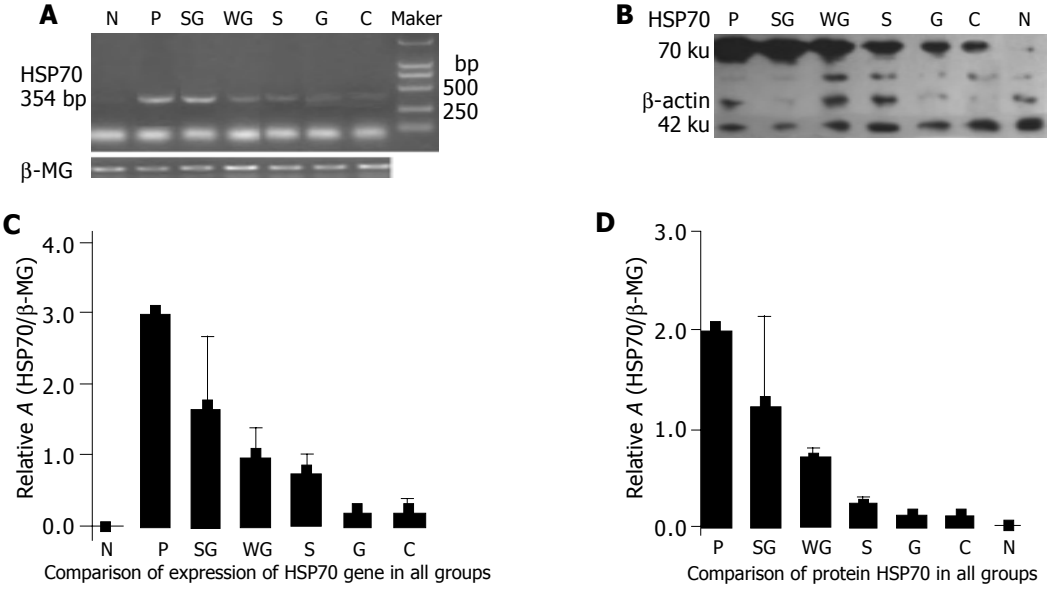

Figure 2 Detection of HSP70. **A**, **B**, The expression of HSP70 gene detected by RT-PCR is shown. **C**, **D**, The production of HSP70 protein detected by Westem blotting is shown. P, positive control. N, nagetive control. SG WG G S C N, group name. The mean difference is significant compared with group G and C at the 0.05 level. The mean difference is significant compared with group S at the 0.05 level.

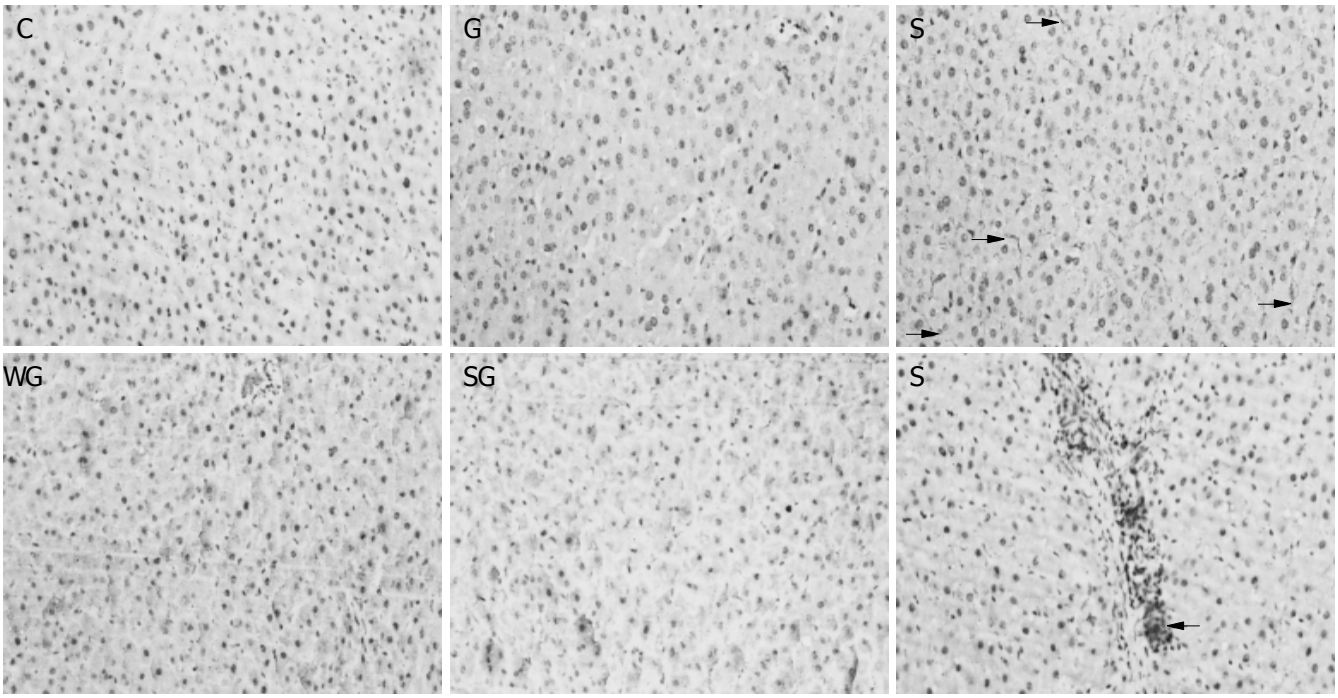

Figure 3 Immunohistochemical staining of HSP70 in liver tissue in group C,G, S,WG and SG HSP70-positive cells in group S were located in the hepatic sinusoid (→) and periportal areas (←).

Table 1 ALT values at different time points in all groups (mean±SD)

Group	60 min after clamping (n = 9)	30 min after reperfusion (n = 9)	24 h after surgery	48 h after surgery	72 h after surgery
C	365.67±13.34	2 601.00±99.21[a]	3 512.75±110.20 (n = 4)[a]	3 895.00±24.04 (n = 2)[a]	–
G	364.22±8.11	2 527.44±74.97[a]	3 521.33±59.53 (n = 3)[a]	3 820.50±72.64 (n=2)[a]	–
S	369.00±12.69	2 174.56±83.99[c]	3 227.50±104.94 (n = 6)[c]	3 589.20±72.64 (n = 5)[c]	3 193.50±101.12 (n = 2)[c]
WG	363.44±9.07	1 977.33±61.07	2 584.63±194.11 (n = 8)	3 040.83±154.93 (n = 6)	2 793.50±122.15 (n=4)
SG	359.11±10.87	1 723.56±94.33	2 013.78±198.22 (n = 9)	2 496.13±139.96 (n = 8)	1 987.25±95.44 (n=7)

[a]P<0.05 *vs* group S, WG, SG; [c]P<0.05 *vs* group WG and SG LSD.

Table 2 AST values at different time points in all groups (mean±SD)

Group	60 min after clamping (n = 9)	30 min after reperfusion (n = 9)	24 h after surgery	48 h after surgery	72 h after surgery
C	365.00±13.73	2 501.89±77.27[a]	3 509.00±125.68 (n = 4)[a]	3 881.00±55.15 (n = 2)[a]	–
G	363.56±12.86	2 525.67±66.83[a]	3 539.67±156.83 (n = 3)[a]	3 932.5±2.12 (n = 2)[a]	–
S	367.67±12.87	2 130.78±78.61[c]	3 221.5±96.67 (n = 6)[c]	3 595.80±60.44 (n = 5)[c]	3 188.00±63.64 (n = 2)[c]
WG	369.67±7.312	1 983.44±50.80	2 591.5±186.35 (n = 8)	3 060.50±145.10 (n = 6)	2 763.75±127.47 (n = 4)
SG	360.67±9.31	1 729.67±93.54	2 019.33±156.83 (n = 9)	2 482.38±113.96 (n = 8)	1 920.38±91.82 (n = 7)

[a]P<0.05 *vs* group S, WG, SG; [c]P<0.05 *vs* group WG and SG LSD.

that in groups WG and SG, whereas a great deal of parenchymal hepatocytes contained HSP70 preferentially in their nuclei and cytoplasm (Figure 3, WG and SG). These results were consistent with those of Western blot and RT-PCR analysis. HSP70-positive cells in group S were located in the hepatic sinusoid and periportal areas (Figure 3, S, arrow).

Tables 1 and 2 show the changes in serum levels of AST and ALT in the five groups. At the end of a 60-min period of clamping, all these parameters remained at low levels in the five groups, and there were no significant differences in these parameters among the groups. During reperfusion, however, these enzyme levels elevated rapidly. Thirty minutes after reperfusion, the levels of AST and ALT in five groups were significantly higher than before reperfusion. But the serum enzyme levels in groups S and G were significantly higher than those in any of the other three groups (P<0.05). The peak of serum enzyme level occurred at 48 h after surgery in groups S, WG, and SG.

Table 3 shows the survival rates at 24, 48, 72 h and 7 d after surgery in all groups. Within 72 h, all rats in groups C and G died, which may be due to the hepatic failure caused by I-R injury after surgery. While in groups SG and WG, the survival rates on d 7 after surgery were as high as 77.8% (7/9) and 44.4% (4/9) respectively, being significantly higher than that in group S (22.2%, P<0.05).

Table 3 Survived rats in all groups

Group (n = 9)	1 d after surgery	2 d after surgery	3 d after surgery	7 d after surgery
C	4	2	0	0
G	3	2	0	0
S	6	5	2	2
WG	8	6	4	4[a]
SG	9	8	7	7[a]

[a]P<0.05 *vs* group S Wilcoxon.

DISCUSSION

It is still controversial that whether certain stress is necessary to the induction of HSP70 after GGA administration, which is related to the application of GGA in clinic to ameliorate I-R injury. Yamagami *et al*.[21], reported that GGA itself does not directly induce HSP70, but it markedly augments the heat shock response in rat liver, leading to the suppression of I-R injury, suggesting that GGA has only a supplementary action in helping other stressors, such as heat stress, ethanol, or water-immersion stress, to activate the cascade of stress response. Oda *et al*.[46], also agree with this. On the other hand, Fudaba *et al*.[47,48], reported that HSP70 could be detected in liver before I-R, and much more after I-R, after administration of high dose of GGA (400 mg/kg per d, for 3 wk) without any form of stress. It was recently reported that administration of a high dose of GGA (200 mg/kg per day for 4 wk) enhances HSP70 induction in transplanted livers and prevented warm ischemic injury and primary graft nonfunction in recipient rats[49]. Our viewpoint is that may be high dose is effective, but it is not applicable because of the long administration schedule before liver transplantation or hepatectomy. But GGA is slowly absorbed from the gastric mucosa, and the uptake by the rat liver is only 5% of that by the gastric mucosa[40], indicating that administration of normal dose GGA without any stimuli cannot enhance HSP70 induction in rat liver and protect liver against I-R injury, which has been proved by our result in group G (200 mg/kg, once a day before surgery). Therefore, it is necessary to find an operable form of stress for clinical application of GGA. Our results showed that GGA plus whole-body heat (group WG) or LSTS (group SG) could significantly enhance HSP70 induction.

As mentioned above, the whole body must be heated in warm water or on electronic pad above 41 ℃ for a certain period of time, which makes this stress form cumbersome and impractical in some circumstances as in the case of

conscious animals or patients.

Compared to whole-body hyperthermia, LSTS has the following three merits. (1) LSTS has a simple and operable manipulation protocol (see Materials and methods) which can be accepted by patients and performed correctly by doctors or nurses before liver surgery. (2) LSTS is performed intermittently to keep the temperature above and below the critical point 41 ℃, so that the heat-sensitive neural transmission would not cause tolerance and patients would feel better. (3) LSTS is a specific stimulation site and can improve the expression of Hsp70 in designed visceral organs. For example, LSTS on left median nerve skin territories increases myocardial but not hepatic Hsp70 expression[25-27], which is the main difference from whole-body hyperthermia. These merits make LSTS be a potential stress form which can be used in clinic.

However, in order to heat the designated skin area above 41 ℃, LSTS needs certain stimulation intensity which is likely to make patients feel a bit of discomfort. This can be relieved by two methods: lowering the stimulating temperature and reducing the time or intensity of stimulation. The latter method is more likely to limit critical temperature. Yamagami *et al*.[21], reported that GGA can achieve a reduction in the duration of whole-body heat shock exposure, which makes us think about what could happen if GGA is administered before LSTS and whether GGA plus LSTS has an additive effect on the induction of HSP70. This hypothesis has been proved in this study by the facts that group SG exhibited a significant higher production of HSP70 than groups G and S (Figures 2 and 3). Liver-related enzymes in group SG presented better liver function recovery 24-72 h after surgery than those in groups G and S (Tables 1 and 2), which is coincident with the conclusion that HSP70 plays an important role lasting for up to 2-4 d in tolerance to reperfusion injury[19,50]. Survival rates 7 d after surgery in group SG was better than that in groups G and S (Table 3). These facts demonstrate that the combination of GGA plus LSTS pretreatment (group SG) has an addictive effect on the induction of HSP70 and the recovery of liver function compared to GGA (group G) or LSTS (group S) alone. Furthermore, GGA plus LSTS (group SG) is comparable to GGA plus whole-body hyperthermia (group WG), but is more simple, safe and operable as a pretreatment to induce HSP70.

In conclusion, the combination of GGA plus LSTS can be used in patients with liver tumor and cirrhosis before hepatectomy and in donor before harvesting graft for liver transplantation.

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