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Original Article

Role of Hsp-70 responses in cold acclimation of HUVEC-12 cells

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Abstract: Background: Endothelial recovery is a central feature of tissues after frostbite injuries. Thermo tolerance plays an important role in protecting cells against injury after frozen and thawing. The present study aimed to quantitatively assess the injury of human umbilical vein endothelial cells HUVEC-12 after repeated low temperature. Material and method: Pretreatments (HUVEC-12) cells were repeatedly exposed to cold (1°C/min decrement to -20°C). Their proliferation, death, apoptosis, and protein and mRNA expressions of HSP70 were determined. Results: Endothelial cells after repeated cold exposures were more resistant to apoptosis and necrosis than normal cells. The expressions of HSP70 in cells after repeated cold exposures were significantly higher than in normal HUVEC-12 cells ($P < 0.05$). Conclusion: Cold acclimation may induce the expression of HSP-70 which plays a protective role in the temperature tolerance.

Keywords: Temperature, thawing, heat shock protein 70, human umbilical vein endothelial cells-12

Introduction

The mechanism of frostbite injuries remains unclear and thus there are no effective strategies for the therapy of frostbite injuries [1]. Endothelial cells are located at the blood-intima interface, where they interact directly with the complements of blood after freeze-thaw injury [2]. Therefore, they are the most important cells affected by freeze-thaw injuries [2, 3], and the pathophysiology of endothelial cells is important for a variety of vascular conditions including coagulation and hemostasis resulting from clinical frostbite injury. Several *in vitro* studies on endothelial preservation have been performed using cell culture models [4, 5].

The 70-kD heat shock proteins (HSP70s) are a subset of highly conserved molecular chaperons involved in the responses to freeze-thaw stress. They play diverse roles in the transportation, folding, unfolding, assembly and disassembly of multi-structured units as well as the degradation of misfolded or aggregated proteins under stress conditions. Cold acclimation has been regarded as one of the most important strategy for the prevention of frostbite injury. Prior studies have shown that rapid thawing decreases the risk for cell damage due to recrystallization during thawing [6, 7]. However, few studies have been conducted to investigate the role of HSP70 expression in the protective effects of cold acclimation.

This study aimed to quantitatively detect HSP70 expression in endothelial cells under four thawing conditions after the same freezing process, and explore the role of HSP70 in the protection against frostbite injury.

Materials and methods

HUVEC-12 cell culture

Human umbilical vein endothelial cells (HUVEC-12) were seeded into flasks and maintained in DMEM (Gibco, USA) supplemented with 10% fetal calf serum (FCS) and antibiotics. When cell confluence reached 70-80%, cells were pas-

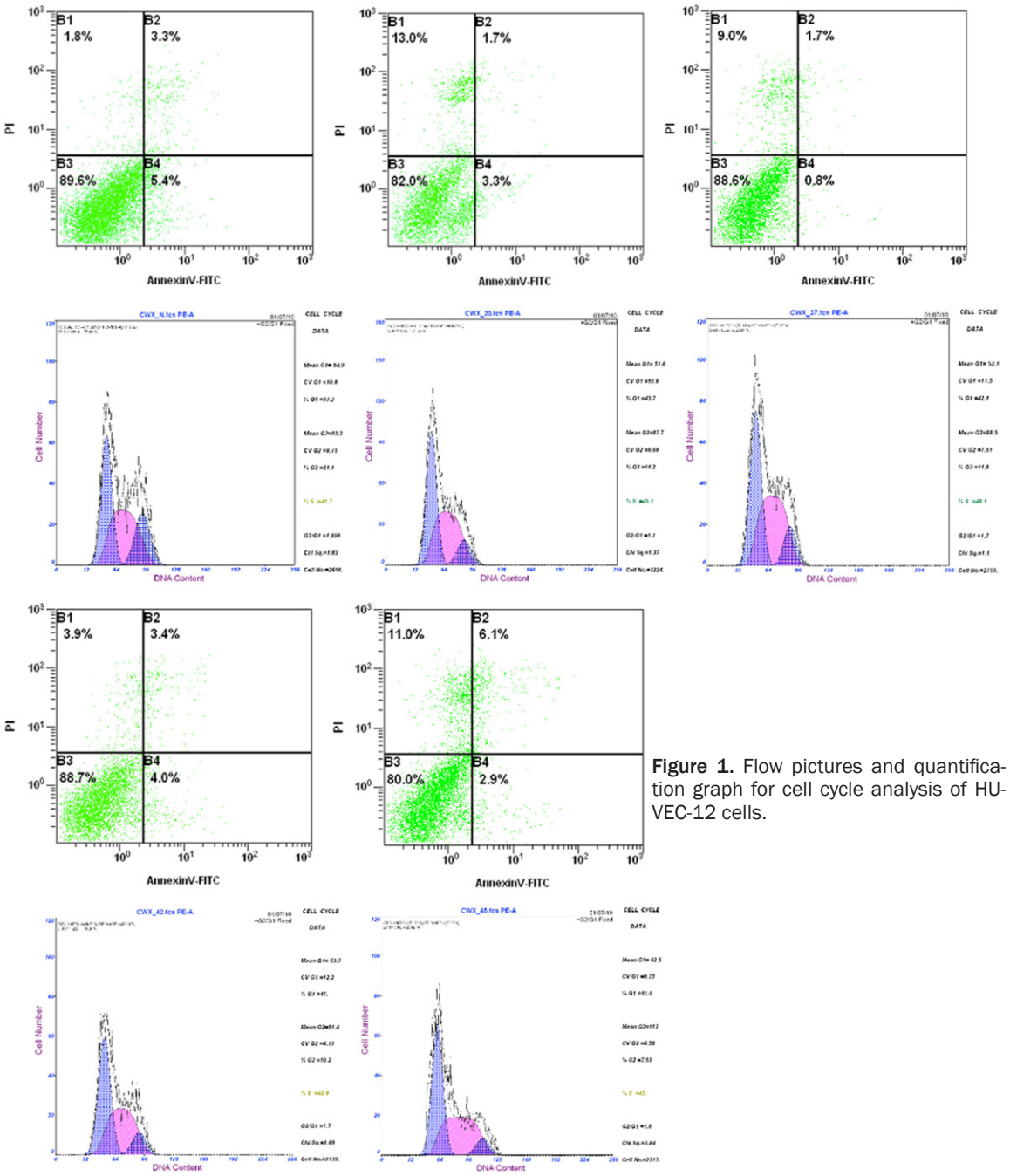

Figure 1. Flow pictures and quantification graph for cell cycle analysis of HUVEC-12 cells.

saged, and cells of 4th to 6th generation were used for experiments. Cells were divided into 5 groups: normal control group, 20°C thawing group, 37°C thawing group, 42°C thawing group and 45°C thawing group.

Cell morphology

Cell morphology and proliferation after freeze-thaw stress were evaluated under an OLYMPUS IX 71 phase contrast microscope (OLYMPUS, Japan) equipped with a digital camera at 100×. The number of attached cells was counted in 4 randomly selected fields for each sample.

Freezing-thawing of cells

Freezing-thawing was performed as described previously [5]. Briefly, flasks were kept in Cryo 1°C freezing containers (Nalgene, USA) that allow temperature to drop at a rate of 1.0 ± 0.3°C/min in a -80°C refrigerator. Cells were

Table 1. Primer for GAPDH and HSP70

Gene	Primer	Sequence
Human HSP70	Forward	5'-CTGGGAAGCCTTGGGACAAC-3'
	Reverse	5'GGATAACGGCTAGCCTGAGGAG-3'
Human GAPDH	Forward	5'-GCACCGTCAAGGCTGAGAAC-3'
	Reverse	5'-TGGTGAAG ACGCCAGTGGA-3'

then thawed in thermostat-controlled water-bathes at different temperatures. The freezing-thawing temperatures were recorded with a temperature system designed by ourselves [5].

Detection of apoptotic cells by flow cytometry

Cells were harvested and apoptotic cells were determined by flow cytometry with FACS Calibur (BD Biosciences, USA). In brief, cells were washed twice with cold PBS and re-suspended in 1× binding buffer at 10^6 cells/mL. Then, 100 μl of cell suspension (10^5 cells) was then transferred into a 5-mL tube, followed by addition of 5 μL of FITC Annexin V (BD Biosciences, USA) and 5 μL of propidium iodide (PI). The tube was gently vortexed and incubated at 4°C in dark for 15 min. A total of 400 μL of 1× binding buffer were added to each tube. Flow cytometry was performed within 1 h.

Detection of cell cycle by flow cytometry

After freezing-thawing, cells were harvested, re-suspend in 1× binding buffer at 5 × 10^6 cells/mL, and fixed in 70% ethanol at 4°C overnight. These cells were centrifuged and washed once with PBS. Then, 500 μl of PI staining solution (50 μg/mL PI and 50 μg/mL RNaseA) were added, and the proportions of cells in S, G_2-M, and G_1-G_0 phases were determined by flow cytometry with FACS Calibur (BD Biosciences, USA) on the basis of fluorescence-activated cell sorting (FACS) analysis (**Figure 1**).

Detection of HSP70 protein expression by Western blot assay

Whole cell extracts were prepared from cells as described by Ismail et al [8]. Protein samples (20 μg) were then separated on 10% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore, USA) using a semi-dry transfer apparatus (Bio-Rad, USA). Membranes were blocked for 1 h in 10% non-fat milk in PBS containing 0.3% Tween 20. The expressions of both constitutive and inducible forms of HSP70 were detected using the mouse anti-HSP70 monoclonal antibodies (1:350; Zhongshan Goldenbridge, China). Horseradish-peroxidase (HRP) conjugated rabbit anti-mouse immunoglobulin (1:3000, Boster, China) was used as a secondary antibody. Quantitative analysis of chemiluminescence signals was done using a cooled charge-coupled camera (Alpha Innotech Corporation, USA).

Detection of mRNA expression of HSP70 by RT-PCR

Total RNAs were extracted from tissues and cells using Trizol Reagent (Invitrogen, USA) according to the manufacturer's instructions. cDNA synthesis was performed using PrimeScript RT kit (TaKaRa, China). Real-time PCR was done by using the SYBR Premix Ex Taq kit (TaKaRa, China) with 20-μL mixture. Primers for HSP70 and glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) are listed in **Table 1**.

PCR was performed using the Bio-Rad IQ5 Real-Time System (Bio-Rad, USA) as follows: denaturation for 30 sec at 95°C, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. Then, a melting curve analysis was performed to assess the specificity of amplified PCR products. PCR products were measured by the cycle (Ct) value at which specific fluorescence became detectable. The Ct value was used for kinetic analysis and was proportional to the initial copies of target gene in the sample. The real time fluorescence of SYBR Green® I (Molecular Probes) bound to double-stranded DNA was detected using the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad, USA).

Statistical analysis

Data are presented as mean ± standard deviations. Comparisons were done with repeated measurement test and paired *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

Temperature curve of freezing-thawing process

Freezing process is roughly classified into slow freezing with a temperature decrease at 0.02-0.2°C/min, commercial freezing with a temper-

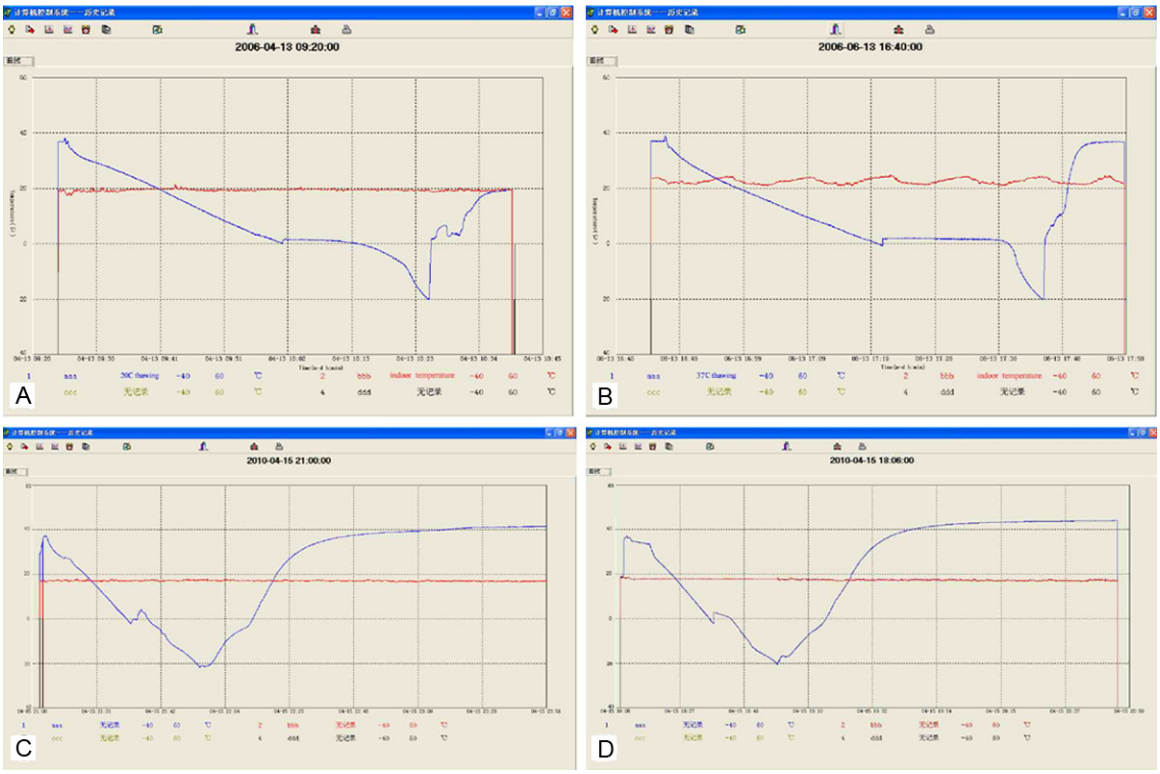

Figure 2. Temperature changes during freezing-thawing. The flask temperature was decreased to -20°C and then thawing was done at 20°C, 37°C, 42°C, and 45°C, independently. Red line: room temperature; blue line: flask temperature.

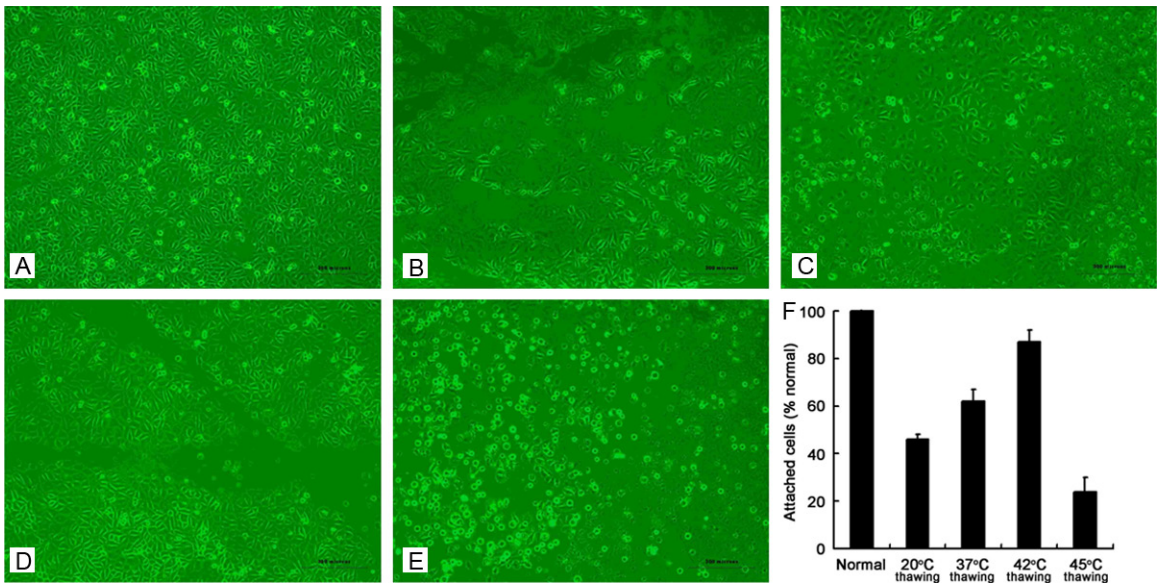

Figure 3. Morphological changes in cells at different thawing temperatures. A: normal HUVEC-12 cells; B: cells undergoing thawing at 20°C; C: cells undergoing thawing at 37°C; D: cells undergoing thawing at 42°C; E: cells undergoing thawing at 45°C. Scale bar: 300 mm; F. Number of attached cells (% normal) after thawing.

ature decrease at 0.2-0.83°C/min, and rapid freezing with a temperature decrease at > 0.83°C/min [9]. In this study, samples were rapidly frozen with a temperature decrease at

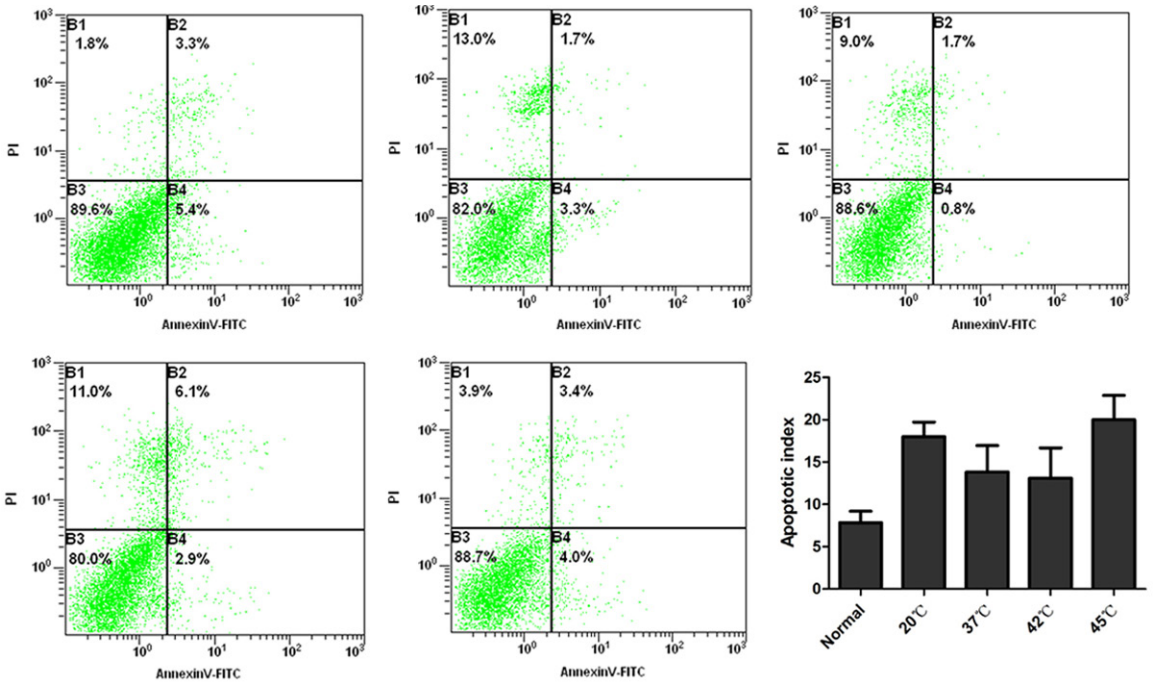

Figure 4. Apoptotic HUVEC-12 cells after thawing at different temperatures (flow cytometry). HUVEC-12 cells were thawed at different temperatures, stained with annexin-V-FITC and PI, and subjected to flow cytometry to detect apoptotic and necrotic cells. B1: necrotic cells; B2 + B4: apoptotic cells; B3: viable cells.

1.0 ± 0.3°C/min. Cells were pre-warmed at 37°C overnight before freezing. Temperature decreased in a linear fashion at the beginning, and then remained unchanged during the cocrystallization, followed by a sudden temperature drop to the super cooling point (**Figure 2**).

The thawing was performed at different temperature (**Figure 2**). After the temperature of flasks reached -20°C, the Cryo 1°C freezing containers were put into thermostat-controlled water-bathes at 20°C, 37°C, 42°C, and 45°C, respectively. The flasks were incubated at 37°C in a humidified atmosphere with 5% CO_2 and 95% air after thawing.

Morphological change after freezing and thawing at different temperatures

The cell morphological findings after freezing-thawing treatment are shown in **Figure 3**. Cells in 42°C thawing group had similar morphology (live cell more than 85% of the normal group) and function as in normal control group (**Figure 3A**, **3D**). Rapid thawing was beneficial for cells in maintaining normal morphology as compared to other groups. Thawing at 20°C and 45°C led to cell death (viable cells: 46% and 24% of that in normal control group), and caused cell shrinkage or cytoplasmic loss (**Figure 3B**, **3E**). In 37°C thawing group, the numbers of floating or dead cells was intermediate (viable cells: 62% of that in normal control group).

Flow cytometry

Flow cytometry showed that the number of necrotic cells was the smallest in 42°C thawing group (B1 = 3.9%), followed by 45°C thawing group (B1 = 11.0%) and 20°C thawing group (B1 = 13.0) (**Figure 4**), indicating that the classic treatment of frostbite injury at 42°C is valid. Thawing at 20°C (near the ambient temperature) resulted in high percentage of apoptotic cells (B2 + B4 = 5.0%).

Flow cytometry also demonstrated that normal HUVEC-12 cells had a relatively higher apoptosis index (B2 + B4 = 8.7%). Thawing at a high temperature (45°C) increased the apoptosis index (B2 + B4 = 9.0%), while thawing at lower temperatures (20°C, 37°C, and 42°C) resulted in lower apoptosis index (B2 + B4 = 5.0%, 2.5%, and 7.4%, respectively).

Compared with the untreated (normal) cells, freezing-thawing arrested HUVEC-12 cells in G_0 + G_1 phase. The percentage of cells in G_0 + G_1 phase was 42.1%, 47.4% and 47.0% when cells

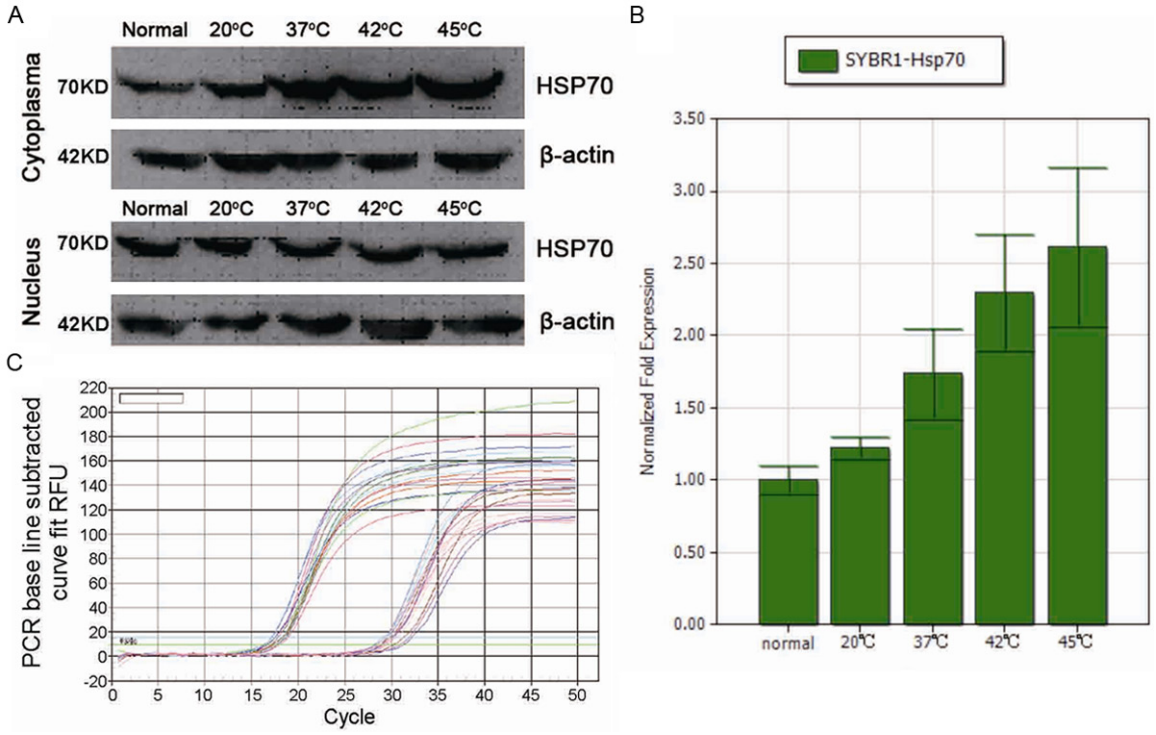

Figure 5. Protein expressions of cytoplasmic and nuclear HSP70 in HUVEC-12 cells after freezing-thawing. Proteins were extracted from HUVEC-12 cells in normal group, 20°C thawing group, 37°C thawing group, 42°C thawing group and 45°C thawing group (6 h later). Equal amount of proteins (10 μg/lane) were loaded into each well. The protein expression of HSP70 was normalized to GAPDH. Data (mean ± SEM) were for 3 independent detections. *P < 0.05.

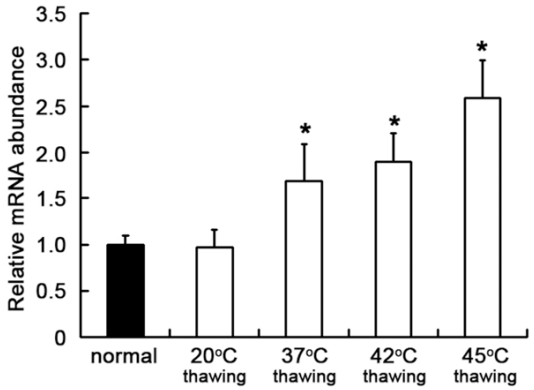

Figure 6. mRNA expression of HSP70 in HUVEC-12 cells after thawing at different temperatures. GAPDH was used as an internal reference for normalization. Data are represented as mean ± SEM. *P < 0.05.

were thawed at 37°C, 45°C and 20°C, respectively. Thawing at 42°C inhibited the proliferation of endothelia cells ($G_0 + G_1 = 43.7\%$).

Protein expression of HSP70

Western blot assay showed that the cytoplasmic HSP70 protein expression markedly elevated as early as 6 h after freezing-thawing (**Figure 5**). The cytoplasmic HSP70 protein expression in normal control group was significantly different from that in 37°C, 42°C and 45°C thawing groups ($P < 0.05$). However, no difference was observed in the cytoplasmic HSP70 protein expression between 20°C thawing group and normal control group. In addition, no difference was observed in the nuclear HSP70 protein expression between normal control group and other groups.

mRNA expression of HSP 70

mRNA expression of HSP70 increased significantly in 37°C, 42°C and 45°C thawing groups when compared with normal control group (**Figure 6**) ($P < 0.05$). However, there was no difference between 20°C thawing group and normal control group ($P > 0.05$).

Discussion

HSP expressions can be induced by a variety of cellular stresses [10-12] and they play protective roles in response to these stresses and subsequent challenges [13]. For examples, in two separate studies, Airaksinen et al [14] and

Zhang et al [15] showed that heat and cold induced HSP70 expression. The disruption of intracellular membranes and organelles is a major cause of freeze-thaw injuries due to the formation of intracellular ice crystals and various stress conditions [16]. The present study focused on the effects of freezing followed by thawing at four different temperature on the HSP70 expression, apoptosis and necrosis of HUVEC-12 cells.

Nollen et al [17] and Krebs et al [18] proposed that appropriate amount of HSP70 in a cell is important for maintenance of normal physiology. Although the amount of Hsp70 produced in a cell correlates with the degree of a stress it is exposed to, the relationship between Hsp70 and cold tolerance is still unclear, because organisms and cells can respond to stresses in many ways. Our study demonstrates that thawing induced a reduction in HSP70 expression in HUVEC-12 cells after freezing. Furthermore, although thawing at 42°C produced the smallest number of necrotic cells and moderate number of apoptotic cells, moderate mRNA and protein expressions of HSP70 were detected. These results suggest that the ideal thawing temperature which may induce HSP70 expression to exert protective effects may be 42°C.

We speculate that HSP70 plays a role in the protection of HUVEC cells against freezing-thawing, and our study provides an important evidence for understanding various freezing-thawing induced damages.

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Disclosure of conflict of interest

None.

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