

Heat Shock Protein 72 Expression in the Right Ventricle of Patients Undergoing Congenital Cardiac Surgery

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While heat shock protein (HSP) 72 is known as a stress protein, there have been no reports of HSP 72 expression in patients who have undergone surgery for congenital heart disease. Fourteen patients (7 males and 7 females) who had undergone surgery for congenital heart disease were studied. The ages of the patients ranged from 2 months to 43 years old (mean 6.5 ± 10.8 years old; median 3.0 years old). The diagnoses were Tetralogy of Fallot in seven, pulmonary atresia with ventricular septal defect (VSD) in three, complex anomalies in three, and VSD in one patient. Histological study and HSP analysis using Western blots and immunostaining with anti-HSP 72 monoclonal antibody were performed for right ventricular muscle samples resected during the surgery. The histological findings showed hypertrophic changes of ventricular cardiomyocytes in all samples studied. Western blots detected HSP 72 expression of various degrees in all specimens. Immunostaining using monoclonal antibody against HSP 72 showed that the protein was present in the nuclei and cytoplasm of cardiomyocytes. In conclusion, although it is difficult to determine the cause of the "stress" that triggers HSP 72 expression in cardiomyocytes, low O₂ saturation and pressure overload might act as a "stress", and the only common factor that induced HSP 72 in every sample was hypertrophy.

Key words: heat shock protein 72 (HSP 72), human, heart, congenital cardiac surgery, hypertrophy

Heat shock protein 72 (HSP 72) has been thought to protect the heart from stresses such as ischemia, ischemia-reperfusion injury, metabolic disorder, and other stressful events including cardiac surgery (1-4). Generally many potential functions of HSP against cell injury have been revealed, including the suppression of pro-inflammatory cytokines, the reduction of oxidative burst, NO-induced protection, the prevention of apoptosis, the repair of ion channels, collagen synthesis and fibrosis, and the modulation of immune-mediated injury (5). Many studies have indicated that HSP 72 expression in hearts prevents cardiomyocytes from becoming necrotic and maintains normal cardiac function well after ischemia/ischemia-reperfusion injury (1-13). Additionally, HSP 72 expression during and/or after cardiac surgery has been observed, along with its protective effects and correlation with diseases and cardiac function (4, 6, 14-19). Although techniques in cardiac surgery have made great progress, there remain very severe congenital heart disease cases. There have been some reports of HSP 72 expression during cardiac surgery for human adult cases (14-19), but none have described HSP 72 expression in cause of human congenital heart disease. Our study reflects the importance of investigating the factors involved in HSP 72 expression in these patients.

Materials and Methods

Patients and sampling. A total of 14 patients who had undergone congenital cardiac surgery were studied; Western blotting was performed in 11 patients and immunostaining in 3. The clinical diagnoses are

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Table 1 Patient diagnoses and sampling

Case No.	Age	Sex	BW (kg)	Diagnoses	Sample (g)	HI	Histology	W/I
1	3y	F	13.5	TOF	1.00	7.41	Yes	W
2	7y	M	18.8	PS, s/p TOF	0.85	4.52	No	W
3	2m	M	3.2	DORV (Taussig-Bing)	0.29	9.06	No	W
4	8y	F	19.0	Criss-Cross heart, TGA, SAS	0.12	0.63	No	W
5	1y	M	11.0	DIRV, DORV, SAS, PS	0.26	2.36	Yes	W
6	7y	F	19.0	PA, VSD	0.57	3.00	Yes	W
7	2y	F	13.0	TOF	0.32	2.46	Yes	W
8	3y	M	9.6	PA, VSD	0.24	2.50	No	W
9	43y	M	48.0	PS, s/p TOF	0.98	2.04	Yes	W
10	2y	M	9.0	PA, VSD	0.34	3.78	Yes	W
11	7y	F	22.9	VSD	0.56	2.45	Yes	W
12	3y	F	12.6	TOF, PAPVD				I
13	1y	M	8.8	TOF, s/p m-BT shunt				I
14	3y	F	8.5	TOF, PLSVC				I

BW, body weight; HI, hypertrophy index = sample weight (g)/BW (kg) \times 100, W/I, for Western blot analysis/for immunostaining; TOF, tetralogy of Fallot; PS, pulmonary stenosis; s/p, status of post-; DORV, double outlet right ventricle; TGA, transposition of the great arteries; SAS, subaortic stenosis; DILV, double inlet left ventricle; PA, pulmonary atresia; VSD, ventricular septum defect; PAPVD, partial anomaly of pulmonary vein drainage; m-BT, modified BT; PLSVC, patent of left supra vena cava.

During cardiac surgery, hypertrophied ventricular muscle which caused right ventricular (RV) outlet stenosis was resected. No. 1-11 and No. 12-14 samples were studied by Western blots or immunostaining, respectively. Seven samples were studied by histology, which showed hypertrophy of cardiomyocytes in all samples.

described in Table 1. The ages of the patients ranged from 2 months to 43 years old (mean 6.5 ± 10.8 years old; median 3.0 years old). Pre-operative studies included hemoglobin (Hb), hematocrit (Hct), O_2 saturation (SAO_2), the pulmonary/systemic flow ratio (Q_p/Q_s), and the pressure gradient (PG) between the right ventricle (RV) and the pulmonary artery (or the ascending aorta) by catheterization when applicable.

During cardiopulmonary bypass, the hypertrophic muscle in RV causing RV outflow stenosis was resected via the pulmonary artery (or the ascending aorta) and the right atrium. Resection of the hypertrophied muscle was performed soon after the aortic cross-clamp with cold (4 °C) crystalloid cardioplegia. We calculated the hypertrophy index (HI) as follows: $HI = \text{sample weight (g)}/\text{body weight (kg)} \times 100$. HI is not always an absolute indicator of hypertrophy, but HI can relatively reflect the degree of hypertrophy.

Eleven muscle samples for Western blots were frozen in liquid nitrogen and stored at -80°C until analysis, and 7 of these 11 samples were analyzed histologically. In addition, 3 samples were analyzed by immunostaining.

Western blot analysis. The 11 frozen muscle samples used for Western blotting were homogenized,

200 mg in 1 ml of 100 mM phosphate-buffered saline (PBS, pH 7.4), and centrifuged at 11,000 rpm for 60 min. Protein concentrations were determined with a commercial assay kit (BCA Protein Assay Reagent Kit, Pierce, Rockford, IL, USA). Five microliters of each protein sample were boiled in the same volume of the sample buffer (0.5 M Tris-HCl, pH 6.8, 50% glycerol, 10% [w/v] sodium dodecyl sulfate [SDS], β -mercaptoethanol, 0.1% [w/v] bromophenol blue). SDS polyacrylamide gel electrophoresis (PAGE) was performed at a constant voltage of 150 volts for 1 h, using 2 gels (Ready Gel[®]; Bio-Rad Laboratories, Richmond, CA, USA) at the same time. A 50 μg protein sample was loaded into each lane, and, where appropriate, 1 μg of recombinant human HSP 72 (SPP-755, StressGen Biotechnologies Corp., Victoria, BC, Canada) and the molecular size marker (161-0324, Bio-Rad Laboratories, CA, USA) were co-electrophoresed.

After electrophoresis, the proteins were transferred to 2 polyvinylidene difluoride (PVDF) membranes (0.2 μm thickness; Bio-Rad Laboratories) as described by Tobin *et al.* (20), by the use of a Bio-Rad miniprotein II gel transfer system. The proteins were transferred to the PVDF membrane at a constant voltage of 90 volts for

1 h.

The PVDF membranes were then blocked in PBS containing 1% (wt/vol) non-fat dry milk and 0.1% (vol/vol) Tween-20 overnight at 4 °C. After washing the membranes in Tris-buffered saline containing Tween-20 (TTBS: 10 mM Tris, pH 7.5; 150 mM NaCl; 0.05% Tween-20), one membrane was incubated for 1 h at 37 °C with the anti-HSP 72 monoclonal antibody (RPN 1197, Amersham Corp., Arlington Heights, IL, USA), used at a 1:500 dilution in RPMI 1640 medium containing 10% fetal calf serum (FCS). The other membrane was incubated for 1 h at 37 °C with 1 µg/ml of irrelevant IgG1 monoclonal mouse antibody in RPMI 1640 medium supplemented with 10% FCS. After washing, the membranes were placed in the blocking solution for 15 min at room temperature. After another washing, they were then placed in a solution of secondary antibody (goat-anti-mouse-immunoglobulin G conjugated to alkaline phosphate (AP), 115-035-071, Bio-Rad Laboratories) consisting of a 1:3,000 dilution in RPMI 1640 medium containing 10% FCS and incubated for 30 min at 37 °C. Following additional washing, the blots were developed with the AP Color Development reagent (Bio-Rad Laboratories). After development, the blots were dried, photographed, and scanned. HSP 72 was quantified by the use of a computerized densitometer (NIH Image 1.60/ppc, Macintosh). The samples were analyzed 3 times by Western blotting, and the data was obtained using their average.

Immunostaining. Mouse monoclonal antibody against HSP 72 (Amersham Corp.) was used for the immunostaining of 3 RV muscle samples (Table 1). Single immunostaining was performed using the avidin-biotin peroxidase reaction. Frozen sections (2-3 µm thick) were fixed in acetone for 10 min at room temperature with anti-HSP 72 (IgG1); in addition, other sections were stained using an irrelevant IgG1 monoclonal mouse antibody as controls. Monoclonal biotinylated goat antibodies against mouse IgG1 were then applied. After these reactions, streptavidin-biotinylated peroxidase complex was added. These reagents were obtained from ScyTek Laboratories (UT, USA). As a substrate for the peroxidase reaction, 3,3'-Diaminobenzidine tetrahydrochloride (Wako, Tokyo, Japan) was used. The sections were faintly counterstained with hematoxylin.

Study limitations. Since no normal human heart should ever be resected for experimental purposes, no "normal" heart samples were taken in this study.

Informed consent was not obtained because all samples were resected not for experiments but for surgical reasons; as a result, only the hypertrophied muscles causing RV outlet stenosis were resected. Therefore, HSP 72 expression in the "normal" human heart was not determined.

Statistical analysis. All values were expressed as the mean \pm 1 SD, with $P < 0.05$ considered as significant. HSP 72 expression and HI or pre-operative data were statistically analyzed by multiple regression analysis.

Results

The resected RV muscle sample weight ranged from 0.12 to 1.00 g (mean 0.50 ± 0.31 g). Mean HI were 3.6 ± 2.4 (0.63-9.06). The histological findings showed hypertrophic changes of cardiomyocytes in all 7 samples examined (Table 1). Cardiomyocytes were hypertrophied (mildly ~ moderately), and their nuclei were enlarged. Partial contraction bands were seen in the cardiomyocytes, but no degeneration or necrosis was observed. There was no pathological malalignment of muscle fibers. These findings were identical in all samples studied. In some specimens, interstitial fibrotic change was observed.

Western blotting revealed HSP 72 expression in all 11 specimens tested (Fig. 1, Table 2). HSP 72 expression was at similar levels in all samples, except that the No. 4. HSP 72 band in No. 4 was thin, which implied a smaller amount of HSP 72. Repeated experiments confirmed these.

Immunostaining detected the expression of HSP 72 in the cardiomyocytes of all ($n = 3$) RV specimens: nuclei and cytoplasm were stained as shown in Fig. 2. That is, nuclei were stained with a granular pattern, and the cytoplasm was stained with a granular and funicular pattern. These findings indicate that high levels of HSP 72 were present in all cardiomyocytes tested.

Table 2 also shows the results of the pre-operative examinations. HSP 72 levels (determined as a proportion of total protein loaded) ranged from 0.024 to 0.403 (mean 0.30 ± 0.11). The mean pre-operative data were as follows; $\text{SaO}_2 = 86.1 \pm 9.3$ (%), $\text{PG} = 56.6 \pm 33.7$ (mmHg), $\text{Qp/Qs} = 1.38 \pm 0.66$, $\text{Hb} = 16.2 \pm 2.6$ (g/dl), and $\text{Hct} = 48.5 \pm 8.4$ (%). Although HSP 72 was detected in all samples tested, the sample obtained from Patient No. 4 demonstrated an HSP level of less than 1/10 and an HI level of less than 1/3, respectively, of the

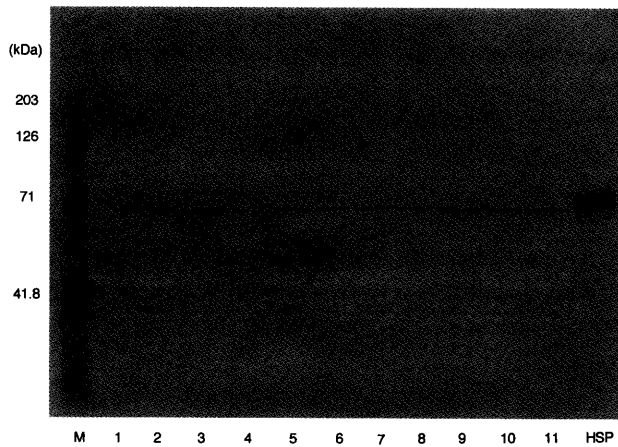


Fig. 1 Western blot analysis for heart muscle samples.

M, Marker lane; HSP, 1 μ g of human recombinant HSP 72. A total of 50 μ g of protein was loaded in each sample lane (No. 1-11). This is a representation of the Western blot analysis, which was carried out 3 times.

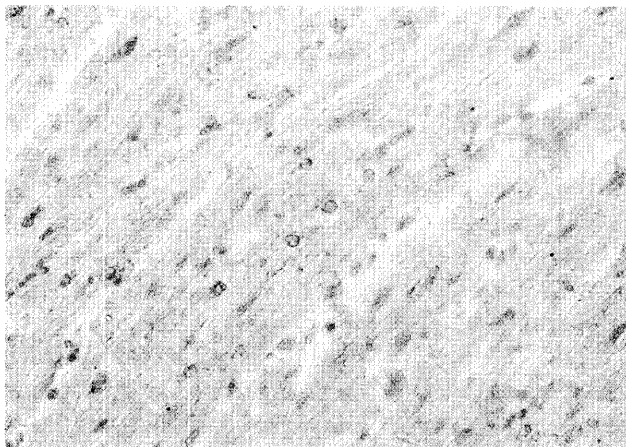
Each lane number coincides with the case number. Western blots detected various degree of HSP 72 expression in all samples (detail in Table 2).

Table 2 HSP 72 expression and pre-operative data

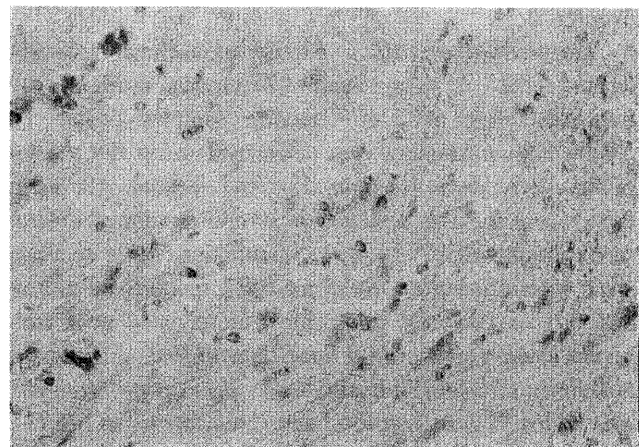
Case No.	HSP 72 (%)	SaO ₂ (%)	PG (mmHg)	Qp/Qs	Hb (g/dl)	Hct (%)
1	0.300	94	78	1.25	12.6	38.0
2	0.391	97	85	1.00	-	-
3	0.248	76	0	-	16.2	48.9
4	0.024	83	25	1.40	15.5	44.1
5	0.354	71	5	-	20.8	65.1
6	0.265	74	-	0.77	17.7	53.1
7	0.401	94	74	1.80	13.8	39.8
8	0.403	78	-	1.18	20.3	60.1
9	0.240	94	70	2.10	-	-
10	0.370	83	85	1.05	17.2	52.1
11	0.354	98	5	1.27	13.4	40.8
12	-	92	75	1.60	14.3	41.9
13	-	-	55	-	15.0	45.5
14	-	86	64	0.40	17.0	52.4

SaO₂, in room air, arterial SaO₂ was studied by catheter examination. PG, pressure gradient between the RV and pulmonary artery (or ascending aorta); Qp/Qs, the pulmonary/systemic flow ratio; Hb, hemoglobin; Hct, hematocrit.

Various levels of HSP 72 were revealed in all samples. HSP 72 levels are shown using the average of 3 Western blotting analyses, and were determined as a proportion of the total protein loaded.



A



B

Fig. 2 Immunostaining using monoclonal anti-HSP 72 antibody.

A: The sample was stained using mouse monoclonal anti-HSP 72 antibody.

The nucleus and cytoplasm of cardiomyocytes were stained. This is a representation and was obtained from case No. 13. (Original magnification \times 200.)

B: The same sample was also stained using an irrelevant IgG1 monoclonal mouse antibody. No region was stained. (Original magnification \times 200.)

other samples. All patients except No. 4 and 11 had either low SaO₂ (< 80%) or high PG (> 50 mmHg) between the RV and the pulmonary artery (or the ascending aorta). Naturally high Hb and Hct were recognized in low-SaO₂ (cyanotic) patients. Statistically, the HSP 72 levels were not related to the patients' diagnoses, age, HI, or any other pre-operative data.

Discussion

HSP 72 expression can be induced by hyperthermia (21), ischemia (7, 8, 22-28), metabolic stress (26), pressure overload (27), drugs (6-8), congestive heart failure (29), *etc* (5, 28, 30, 31). HSP is expected to play a role in protecting the cardiomyocytes (1-4). There have been some reports of HSP 72 in human hearts during and/or after cardiac surgery (14-19). Demidov *et al.* have studied HSP 72 expression in patients who have undergone coronary artery bypass grafting before and after cardiopulmonary bypass. The induction of HSP 72 was observed in 40% of all patients and correlated with the endurance of cardiopulmonary bypass and with disease duration (16). Knowlton *et al.* have studied HSP 27, 60, 72, 90, and HSC 70 in the hearts of transplantation donors (normal = control), dilated cardiomyopathy (DCM) patients, and ischemic cardiomyopathy (IHD) patients. HSP 27 levels were increased almost twofold in DCM compared to normal hearts, and were significantly greater than in IHD hearts. The levels of HSP 60 were doubled in both DCM and IHD hearts. In contrast, HSP 72, HSC 70, and HSP 90 levels were not significantly changed (19).

Our data showed HSP 72 expression in all heart samples, making this the first report of HSP 72 expression in human congenital cases; HSP 72 was detected by Western blots in 11 patients and by immunostaining in 3. It has been reported that HSP 72 becomes localized within the nucleus (32) and around the nucleolus (33) when nucleolar function appears to be compromised (34). During subsequent recovery, the nucleoli regain their normal morphology and HSP 72 moves back to the cytoplasm. In human cases, it has been reported that HSP 70 is primarily located in the cytoplasm and nucleus/nucleolus of cardiomyocytes (17). In our study, HSP 72 was detected in both the nuclei and the cytoplasm in all 3 immunostained samples.

We therefore had 3 questions: 1) Was HSP 72 expression induced by cardiac surgery or anesthesia? 2) Is HSP

72 expression in the patients' hearts related to preexisting pathologic conditions and pharmacologic intervention, or did it simply represent an inherently high expression level of HSP 72? 3) Is the degree of HSP 72 expression related to age, cardiac morphology, pre-operative cardiac conditions, *etc.*?

Regarding question "1", McGrath *et al.* (14, 15) have reported HSP 72 expression in patients undergoing cardiac operations. In their study, the HSP 72 content remained unchanged in atrial biopsy tissue taken before bypass, after reperfusion, and after bypass. Their data suggest that HSPs are not induced during cardiac surgery. Currie *et al.* have shown that mRNA levels for HSP 71 and HSP 73 are elevated at 1.5 and 3 h, respectively, after the heat shock response elicited by ischemia and appear to return to control levels by 6 h after the ischemic event (9). In contrast, Demidov *et al.* have reported that the induction of HSP 72 during cardiac surgery can be observed in 40% of patients (16). Taggart *et al.* have mentioned that HSP 72 induction during cardiac surgery may be related to myocardial protection methods; an HSP 72 increase was seen in all the coronary artery bypass grafting patients whose hearts were maintained at 34 °C, while no elevation was seen in the aortic valve replacement patients whose hearts were kept at 10 °C (18). In our cases, all heart samples were obtained soon after the aortic cross clamp with cold (4 °C) crystalloid cardioplegia. Therefore, it is not likely that HSP 72 was induced during the cardiac surgery.

As for question "2", Donnelly *et al.* have reported that the "absolute levels" of HSP 72 may be important in conferring protection from ischemic injury in their animal model (10). Amrani *et al.* have mentioned that some critical amount of HSP is necessary to elicit a protective effect and that this "critical value" is more important to define than the mere presence of HSPs (11, 12). Demidov *et al.* have shown that creatine phosphokinase isoenzyme MB (CK-MB) levels are significantly lower in patients with high pre-bypass HSP 72 content, while the average pre-bypass HSP 72 levels make up approximately 0.3% of the total protein. Their data also show that the post-bypass HSP 72 levels are higher in patients with a cardiopulmonary bypass surgery lasting longer than 2 h (16). Knowlton *et al.* have reported the detection of 5.58 µg/mg of HSP 72 in mean in donor hearts (19). According to their report, HSP 72 is induced even in "normal" human hearts. Therefore we hypothesize that the "basal (normal) level" of HSP 72 is produced even in

the normal human heart and that 'stress' can induce the "absolute (critical) level" of HSP 72 for protection. Although there have been no reports regarding congenital cases, it is still unknown whether the HSP 72 levels in our cases were "basal (normal)" or "absolute (critical)".

With respect to the last question, why was HSP 72 expressed to various degrees in our cases? In fact, HSP 72 was produced in all specimens, but its levels were different in each case. Recent data have shown that the HSP 72 content in human adult hearts is influenced by a patient's diagnosis, the surgical methods used, and the patient's status after transplantation (16-19). According to our results, the HSP 72 content and HI in case No. 4 were less than 1/10 and 1/3 of the mean content in other patients, respectively. Pre-operative study revealed that Patient No. 4 did not have low SaO₂ (< 80%) or high PG (> 50 mmHg). On the other hand, even though patient No. 11 did not have a low SaO₂ or high PG, high levels of HSP 72 were detected. As HI of patient No. 11 was approximately 4 times higher than that of No. 4, we can conclude that a significant factor in the high HSP expression of No. 11 was hypertrophy. Delcayre *et al.* have reported that HSP 70 is induced in rat cardiomyocytes that are subjected to a hemodynamic overload (27). On the other hand, it has been reported that hypertrophy does not affect HSP 72 concentrations in rat hearts (29, 35). But the hypertrophy in these cases was possibly of a concentric type, which is different from the eccentric hypertrophy observed in our cases. Additionally, HSP expression in human hearts could be different from that in animals. In our cases, the only common factor to all cases was muscle hypertrophy. Therefore, a low SaO₂ and high PG (pressure overload) might induce HSP 72, and hypertrophy might be the important factor to trigger HSP 72 induction in the congenital hearts.

In conclusion, various degrees of HSP 72 expression were observed in RV cardiomyocytes of all patients who underwent congenital cardiac surgery. It was difficult to determine why HSP 72 expression was seen in these specimens and whether the HSP 72 levels in our cases were "basal (normal)" or "absolute (critical)". Though low SaO₂ and pressure overload might act as a "stress" for cardiomyocytes, myocardial hypertrophy was observed in all the cases of the present study and may be a cause of HSP 72 expression.

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