

Association of increased heat shock protein 70 levels in the lymphocyte with high risk of adverse pregnancy outcomes in early pregnancy: a nested case-control study

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Abstract Studies suggest that heat shock proteins (Hsps), Hsp70 in particular, may play a role in embryogenesis and reproduction. As the first trimester is the critical period of human fetal development, we tested whether there is an association between Hsp70 expression in lymphocytes and adverse pregnancy outcomes (APOs) during that period. We measured lymphocyte Hsp70 levels by immunoblot in 55 pregnant women with APOs and 110 well-matched controls selected from 778 pregnant women in a nested case-control study. Multivariate logistic regression models were used to investigate the association between lymphocyte Hsp70 levels and risk of developing APOs. Our data showed that Hsp70 levels in women with APOs, especially those younger than 29 years old, were significantly higher than controls (193 vs 135 units, $P < 0.001$) and that the elevated Hsp70 levels were associated with a significantly increased risk of APOs (adjusted OR = 1.014; 95% CI = 1.008–1.020, $P < 0.001$). Our results also showed that the sensitivity, specificity, and positive and negative predictive values were 78%, 60%, 50%, and 85%, respectively, among these pregnant women. Adjusted ORs and 95% CI for the association between a Hsp70 value > 153 IOD and APOs were statistically significant (OR = 8.78, 95% CI = 2.79–27.64, $P < 0.001$). These results suggest that Hsp70 may play a role in the etiology of APOs. However, the underlying mechanisms for the elevation of Hsp70 in women with APOs and whether Hsp70 can be applied as a clinical indicator of APOs warrant further investigations.

INTRODUCTION

Adverse pregnancy outcomes (APOs) are a group of common obstetric diseases, including abortions, dead fetus, dead delivery, preterm delivery, abnormalities, and intra-uterine growth restriction, all of which are far more frequent in the developing world (Kramer 2003). Although

APOs are important for both mother and infant, their etiology has long remained obscure. Many studies have recently been conducted in an effort to clarify risk factors for APOs. Thus, APOs have been reported to be associated with maternal psychological, social, physiological, pathological, and environmental stresses, such as smoking, alcohol use, and infection (Gibbs 2001; Wadhwa et al 2001; Cogswell et al 2003; Silbergeld and Patrick 2005). It is well known that these risk factors can induce the synthesis of a group of highly conserved proteins, called heat shock proteins (Hsps). Hsps function as intracellular mo-

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lecular chaperones by participating in folding and by facilitating synthesis, assembly, and intracellular trafficking of proteins (Hightower 1991; Bukau and Horwich 1998). Hsps, particularly Hsp70, are rapidly and abundantly up-regulated to protect cells, organs, and living organisms from damage in response to an array of stresses, including hyperthermia, inflammation, infection, chemicals such as ethanol, and exposure to numerous xenobiotics (Welch 1992; Currie et al 1993; Wu et al 1996; Plumier et al 1997; Benjamin and McMillan 1998; Beck et al 2000; Xiao et al 2002, 2003; Mehta et al 2005). Hsp70 has also been found as an extracellular protein either expressed at the cell surface or free in plasma, where it can influence the immune system as suggested by numerous reports on the presence of autoantibodies to these proteins (reviewed in Tanguay and Wu 2006; Wu and Tanguay 2006).

Interestingly, Hsp70 has been shown to play an important role in cellular differentiation and embryonic development in mammals (Luft and Dix 1999; Christians et al 2003), and recent studies suggested an association between a previous infection and immunity to Hsps and reproductive failure or birth defects (Neuer et al 2000; Child et al 2006). Hsp70 is a potentially quantitative indicator of environmental stress and toxicity in human cells (Delmas et al 1995) and in humans (Xiao et al 2002, 2003); therefore, Hsp70 has served as biomarkers for evaluating disease states (Wright et al 2000; Jin et al 2004b) and damage resulting from exposure to environmental stresses (Xiao et al 2002, 2003). It is thought that human development undergoes a defined but adjustable program, depending on the plasticity of embryonic cell response to physiological and environmental changes, and the first trimester is the critical period of fetal development. Although changes of microenvironment within the mother are believed to influence fetal development and growth through exchanges in placenta and blood flow, few studies have investigated the role of maternal microenvironment changes in fetal development in humans, and no study has investigated the role of Hsps in APOs.

Because human lymphocytes are frequently used as the surrogate tissue to investigate association between protein expression in studies of diseases and cellular response to environmental stresses (Bonassi and Au 2002), we therefore hypothesized that the lymphocyte Hsp70 levels in early pregnancy women might be associated with APOs, and this was tested in a prospective nested case-control study.

MATERIALS AND METHODS

Subjects

The study population comprised 778 pregnant women in Wuhan between 1 May and 31 December 2003 who went

to Hubei Hospital of Maternal Health Care for routine checkup in the first trimester and consented to participate in this prospective study. These women had been pregnant for a period of 4 to 16 weeks. The routine checkup included questionnaire, full physical examination (eg, weight, height, signs, blood pressure, heart rate, oral temperature, electrocardiogram, and B echogram), and laboratory tests (ie, blood and urine tests, infection test including toxoplasma, rubellavirus, cytomegalovirus, herpes simplex virus, hepatitis B antigen, and their antibodies). The detailed questionnaire consisted of 50 items including age of both pregnant woman and her husband, ethnicity, educational and marital status, active or/and passive smoking (prenatal active and/or passive tobacco smoke exposure), alcohol consumption, living and working environments, and medical history (ie, previous infection and diseases and maternal psychological status). All participants were followed up for pregnancy outcomes that were diagnosed by Hubei Hospital of Maternal Health Care. There were 74 women with APOs by the end of October 2004. After exclusion of pregnant women without blood samples, there were 55 women with APOs, including 19 natural abortions, 14 induced abortions, 11 dead fetuses, 1 dead delivery, 4 preterm deliveries, 5 abnormalities (1 fetal defect and 4 malformations), and 1 intrauterine growth restriction, who are referred to as the case group. All induced abortions were for medical reason because of infections (5), fever of unknown origins (4), injury (1), radiation exposure (1), and contraindication for pregnant women (1). Each case was matched to 2 control subjects who were normal pregnant women without APOs according to age (least 3 years) and gestation period (1 week). The Ethics Committees of Tongji Medical College approved the study and all subjects provided their written, informed consent.

Blood sampling

To isolate lymphocytes, venous blood (~5 ml) was drawn from each subject after overnight fasting. The collected blood was placed into a heparinized tube for isolating the lymphocytes using Ficoll-Hypaque (Biochemical Reagent Co, Shanghai, China) as previously described (Xiao et al 2002). The collected lymphocytes were washed twice with phosphate-buffered saline (PBS) and counted. The number of lymphocytes was adjusted to 5000/ μ L with PBS. Two hundred microliters of lymphocytes in PBS was centrifuged at room temperature and the buffer solution removed as soon as possible. Lymphocytes were stored at -80°C for further analysis.

Detection of Hsp70

Hsp70 levels in lymphocytes were determined as previously described (Xiao et al 2002) with minor modifica-

Table 1 Basic characteristics of patients (cases) and controls

Parameters	All subjects (778)	Controls (n = 110)	Cases (n = 55)	P-value
Age (mean ± SD, y)	26.7 ± 3.1	26.5 ± 3.0	26.9 ± 4.1	0.488 ^a
Gestation weeks (mean ± SD, wk)	10.3 ± 3.5	8.7 ± 3.2	8.6 ± 3.2	0.866 ^a
Active smoking (N, %)				
No	763	109 (99.1%)	52 (94.5%)	0.095 ^b
Yes	15	1 (0%)	3 (3.6%)	
Passive smoking (any contact with smokers) (N, %)				
No	487	92 (83.6%)	29 (52.7%)	0.001 ^b
Yes	291	18 (16.4%)	26 (47.3%)	
Alcohol consumption (N, %)				
No	720	99 (90.0%)	47 (85.5%)	0.283 ^b
Once in a while	58	11 (10.0%)	8 (12.7%)	
Self-reported infection history (N, %)				
No	450	74 (67.3%)	31 (56.4%)	0.170 ^b
Yes	328	36 (32.7%)	24 (43.6%)	
Virus infection (N, %)				
No	730	100 (90.9%)	46 (83.6%)	0.177 ^b
Yes	48	10 (9.1%)	9 (16.4%)	

^a Independent samples Student's *t*-test.

^b Two-sided χ^2 test.

tions. Briefly, the lymphocytes were mixed with 1× sodium dodecyl sulfate (SDS) sample buffer and boiled at 100°C for 10 minutes, and equal numbers of cells from each subject were loaded onto each lane of SDS-polyacrylamide gel. After electrophoresis, proteins were transferred electrophoretically to nitrocellulose membranes, and the membranes were then saturated with blocking buffer (PBS containing 5% skim milk powder) for 1 hour at 37°C. The rabbit anti-human Hsp70 antibody specific for the inducible Hsp71 (#799 in Tanguay et al 1993) at a dilution 1:1000 in PBS containing 5% skim milk powder was incubated with the nitrocellulose membranes at 37°C for 1 hour with gentle agitation. After washing the membranes 6 times (10 minutes each time) with 200 μ L PBS-0.05% Tween 80, horseradish peroxidase labeled goat anti-rabbit IgG in blocking buffer (1:1000) was incubated with nitrocellulose membranes at 37°C for another 1 hour. Membranes were washed 4 times with 200 μ L PBS-0.05% Tween 80. The presence of Hsp70 on nitrocellulose membranes was revealed using DAB (3,3-diaminobenzidine tetrahydrochloride) buffer for 3–5 minutes. The relative signal intensity was quantified by densitometry with Gel pro3.0 image software (Media Cybernetics, Silver Spring, MD, USA) on an IBM-compatible personal computer, and the integrated optical density (IOD) was used to present the relative levels of Hsp70. The status of pregnancy outcome was blind to the experimenters.

Statistical analyses

All data were entered into a computerized database. Further analysis was carried out by using the statistical analysis software SPSS 12.0 package (SPSS Inc, Chicago, IL, USA). Measurements of continuous data were analyzed

by univariate analysis of variance and independent sample Student *t*-tests. Qualitative data were computed by the Pearson χ^2 contingency tables. Crude and adjusted odds ratios (ORs) with 95% confidence intervals (CI) were computed by multivariate logistic regression analysis to estimate the magnitude of associations between APOs risk and the Hsp70 levels. Statistical inference was based on the significance of $P < 0.05$.

RESULTS

Baseline characteristics of cases and controls

The characteristics of cases and controls are summarized in Table 1. Cases and controls were well matched for age and pregnancy weeks. There were no significant differences in the frequencies of active smoking, drinking, self-reported infection history, and virus infection among cases and controls, although the rates of self-reported infection history or virus infection were slightly higher in the case than in the control group (Table 1). The passive smoking was also significantly more common in cases than in controls ($P = 0.001$).

Lymphocyte Hsp70 levels in cases and controls

Figure 1 shows a graphic presentation of lymphocyte Hsp70 values for all individuals of both cases and controls. There was a large variation among Hsp70 levels in women of both groups, and the lymphocyte Hsp70 levels were decreased as age increased, especially in the cases group (Table 2). Table 2 lists the mean IOD of the lymphocyte Hsp70 levels of both cases and controls. The Hsp70 levels for all the cases were significantly higher

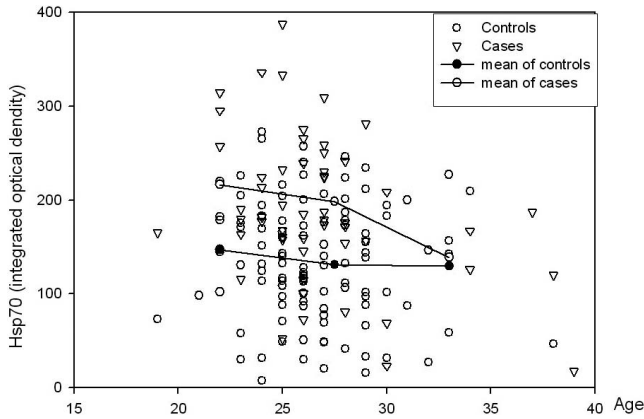


Fig 1. Lymphocyte Hsp70 levels in cases and controls of different age-groups of pregnant women. Lymphocytes were isolated from 165 subjects aged between 19 and 39 years, and Hsp70 levels were determined by Western blot. The levels of Hsp70 are expressed in integrated optical density (IOD).

than in the controls (193 vs 135 IOD, $P < 0.001$) (Table 2). Further analyses by stratification of age showed that there was a significantly higher level of Hsp70 in the age <25 (216 vs 147 IOD) and the 25–29 case group (198 vs 131 IOD) ($P = 0.001$ and $P < 0.001$) but not in the >29 case group (139 vs 130, $P = 0.902$) compared with the corresponding control group.

Association of Hsp70 levels with adverse pregnancy outcomes

Because there were many risk factors that may influence pregnant outcomes, multiple logistic regression analysis was performed. As shown in Table 3, there was a significantly positive association of APOs with passive smok-

Table 3 Association of adverse pregnancy outcomes with risk factors by multiple logistic regression analysis

	Adjusted OR ^a	95.0% CI ^b for OR		P-values
		Lower	Upper	
Hsp70	1.017	1.010	1.025	0.000
Passive smoking	5.732	2.358	13.934	0.000
Virus infection	1.894	0.698	5.141	0.210
Mood				0.267
Depression	0.943	0.082	10.861	0.963
Irritability	1.567	0.679	3.618	0.292
Nervousness	3.145	0.871	11.363	0.080

^a Adjusted odds ratio (OR), which adjusted for age, occupation, and education.
^b CI, confidence interval.

ing and Hsp70 after adjustment for age, occupation, and educational status ($P < 0.001$). Further analysis showed that there was a significant positive correlation of lymphocyte Hsp70 levels in all early pregnant women with APOs (OR: 1.0014, 95% CI: 1.008–1.0020, $P < 0.001$) (Table 2). In addition, there was a significant correlations of lymphocyte Hsp70 levels in early pregnant women with APOs in the age <25 and 25–29 case groups ($P = 0.05$ and $P < 0.001$) but not in the >29 case group ($P > 0.05$) compared with the corresponding controls (Table 2).

Table 4 presents lymphocyte Hsp70 levels by different types of APOs and their associations. As shown in this table, there were significantly positive correlations of increased Hsp70 with natural abortion ($P < 0.001$), induced abortion ($P = 0.002$), and dead fetus ($P = 0.002$). However, only borderline positive correlations of increased Hsp70 were observed in the combined group of other conditions, including 1 dead delivery, 4 preterm delivery, 5 abnormalities, and 1 intrauterine growth restriction ($P = 0.064$).

Table 2 Lymphocyte Hsp70 levels and their association with adverse pregnancy in women of different age-groups

	Age-groups (y)			
	<25	25–29	>29	Total
Controls				
N	26	70	14	110
Hsp70 level (mean \pm SD, IOD) ^a	147 \pm 69	131 \pm 59	130 \pm 70	135 \pm 62
Cases				
N	13	33	9	55
Hsp70 level (mean \pm SD, IOD)	216 \pm 66	198 \pm 75	139 \pm 99	193 \pm 80
P-values ^b	0.001	<0.001	0.902	<0.001
Crude OR (CI) ^c	1.016 (1.003–1.030)	1.016 (1.008–1.024)	1.002 (0.991–1.021)	1.012 (1.007–1.018)
P-values	0.014	<0.001	0.782	<0.001
Adjusted OR (CI) ^d	1.107 (1.002–1.033)	1.018 (1.009–1.027)	1.001 (0.989–1.013)	1.014 (1.008–1.020)
P-values	0.031	<0.001	0.853	<0.001
Adjusted OR (CI) ^e	1.019 (1.003–1.035)	1.017 (1.008–1.025)	1.001 (0.990–1.012)	1.013 (1.007–1.018)
P-values	0.018	<0.001	0.858	<0.001

^a IOD, integrated optical density.
^b Independent samples Student's *t*-test.
^c CI, confidence interval.
^d Adjusted odds ratio (OR), which adjusted for passive smoking, self-report infection history, and virus infection.
^e Adjusted OR, which adjusted for passive smoking.

Table 4 Lymphocyte Hsp70 levels in different types of adverse pregnancy outcomes (APOs) and their association with APOs

	APOs				
	Natural abortion	Induced abortion	Dead fetus	Other	Normal
No.	19	14	11	11	110
Hsp70 level (mean \pm SD, IOD) ^a	202 \pm 78	195 \pm 94	204 \pm 68	162 \pm 76	135 \pm 62
Crude OR (CI)	1.015 (1.007–1.024)	1.013 (1.004–1.022)	1.018 (1.007–1.030)	1.007 (0.997–1.017)	1.00 ^b
<i>P</i> -values ^c	<0.001	0.004	0.002	0.179	—
Adjusted OR (CI) ^d	1.017 (1.008–1.027)	1.020 (1.007–1.033)	1.022 (1.008–1.037)	1.012 (0.999–1.024)	1.00 ^b
<i>P</i> -values	<0.001	0.002	0.002	0.064	—

^a IOD, integrated optical density.

^b Reference group.

^c Independent samples Student's *t*-test.

^d Adjusted odds ratio (OR), which adjusted for age, passive smoking, self-report infection history, and virus infection.

Table 5 Logistic regression analysis of Hsp70 level in cases and controls

Variables	Control	Case	Crude OR (CI)	Adjusted OR (CI) ^a	<i>P</i> -value
Hsp70, <i>N</i> (%)					
Hsp70 level <153 IOD	66 (60.0)	12 (21.8)	1.00 ^b	1.00 ^b	—
Hsp70 level >153 IOD	44 (40.0)	43 (78.2)	6.68 (2.74–16.2)	8.78 (2.79–27.64)	<0.001

^a Adjusted odds ratio (OR), which adjusted for age, passive smoking, self-report infection history, and virus infection.

^b Reference group.

We then plotted a received operating characteristic (ROC) curve and established the cutoff value for IOD of Hsp70 levels and its predicted value of Hsp70 was 153. The sensitivity of the predicted value was 78%, the specificity was 60%, the positive predictive value (PPV) was 50%, and the negative predictive value (NPV) was 85% when this value (ie, Hsp70 = 153) was used to predict APOs among pregnant women. On the basis of this value, cases and controls were divided into 2 subgroups, and the numbers and percentage of each subgroup are listed in Table 5. This table also shows the crude and adjusted ORs and 95% CI for the associations of Hsp70 with APOs before (OR = 6.68, 95% CI = 2.74–16.28, *P* < 0.001) or after (OR = 8.78, 95% CI = 2.79–27.64, *P* < 0.001) adjustment for age, passive smoking, infection history, and virus infection in multivariate logistic regression analysis.

DISCUSSION

Human fetus development is a defined program that can be influenced by the plasticity of embryonic cells response to physiological and environmental changes within the maternal body through full pregnancy. The key period of this process is the first trimester of pregnancy for extra-embryonic development and organogenesis. Many psychosocial, physiological, pathological, and environmental factors contribute to APOs, such as age, maternal stress, exposure to heavy metals, organic solvents, alcohol, ionizing radiation, and infection (Gardella and Hill 2000; Wadhwa et al 2001). It is very difficult, if not impossible, to fully investigate and understand these multiple and complicated risk factors of APOs. The features of the

stress response (eg, low expression of Hsps under normal conditions and their fast overexpression after a wide range of stresses) and importance of Hsps (ie, cytoprotection, cellular differentiation, and mammalian embryonic development) suggested that these proteins could be used to evaluate the status of pregnancy. Human lymphocytes are often used as the common surrogate cells, and the microenvironment within the mother's body may influence fetal development and growth. However, the possible association of lymphocyte Hsp70 in early pregnant women with APOs is still unknown.

The results from this prospective nested case-control study showed that there was a significant increase in lymphocyte Hsp70 levels in early pregnant women with APOs compared with the well-matched controls. An age-related decrease in Hsp70 levels was observed as previously reported (Rao et al 1999; Jin et al 2004a). Our data showed that early pregnant women with lymphocyte Hsp70 levels >153 IOD have about 9-fold higher risk to develop APOs than those with lymphocyte Hsp70 levels <153 IOD, particularly in age-group 25–29. Therefore, age is a very important factor to investigate the role of Hsp70 as a biomarker in the evaluation of diseases and stress status.

It is well known that Hsps are expressed at low levels under normal and physiological conditions and that they rise to higher levels under stressful or pathological conditions. An unusual high Hsp70 level may indicate an overwhelming injury caused by pathological changes in the target tissues. For example, there are increased Hsp70 levels in lymphocytes of patients with heat-induced illness, even after recovery (Xiao et al 2003). Hsp70 levels

in lymphocytes may represent a response to the continuous presence of harmful stimuli and/or an excess of damage that cannot be repaired as shown in the late stage of cerebral infarction (Jin et al 2004b). The cytoprotective role of Hsps is well documented for cultured cells, animals, transgenic animals, and humans. As indicated by our data, increased Hsp70 in lymphocytes of early pregnancy women may reflect a high level of stress in their early pregnancy in that APOs were more frequent in the group with higher levels of lymphocyte Hsp70.

Our data appear to be consistent with published data. For example, the results from Fukushima et al (2005) suggested that serum Hsp70 levels were particularly high in preterm delivery cases. In contrast, Child et al (2006) reported that there were nonsignificantly higher serum Hsp70 levels in 16-week gestational women who delivered babies with birth defects compared with well-matched controls and suggested that serum Hsp70 could be released from peripheral blood mononuclear cells (Hunter-Lavin et al 2004). However, the presence of antibodies against Hsp60 and Hsp70 in blood as well as Hsp60- and Hsp70-antibody complexes in the placenta has prognostic and diagnostic significance for subsequent birth outcomes (Shah et al 1998; Ziegert et al 1999). For example, antibodies against Hsp70 were significantly higher in women bearing fetus with birth defects (Child et al 2006). Indeed, our data showed an association between elevated lymphocyte Hsp70 levels and APOs. Further, it is reported that antibodies against Hsp70 are a marker of a prior increase in the levels of Hsp70 caused by stress that resulted in damage to the cells (Child et al 2006; Wu and Tanguay 2006). Unfortunately, we did not have data on antibodies against Hsp70 in the present study.

Furthermore, Hsps have also been shown to play a role in immunoreactions (Pockley 2003), in vitro fertilization and embryo transfer (Witkin et al 1994), and spontaneous abortion (Kligman et al 1998). The systematic analysis from Shah et al (1998) suggested that there were well-defined temporal and spatial patterns of Hsps expression in human placentas. Several investigations suggested that the expression of Hsps as a landmark of placenta stress response might have prognostic values (Ziegert et al 1999; Christians et al 2003). For example, one study found that the expression Hsp70 and Hsp90 increases in the chorionic villi of first trimester obtained from missed miscarriages compared to full-term placentas (Sotiriou et al 2004), and another study found that the expression of Hsp70 was also increased in tissues obtained from missed miscarriages compared with the controls (Hempstock et al 2003). However, the relationship between maternal lymphocyte Hsp70 and embryonic Hsp70 of early pregnant women remains unknown.

In the present study, we established the Hsp70 cutoff

value at 153 IOD by the ROC curve. Our results showed that there seems to be a close correlation between Hsp70 and APOs: the pregnant women with elevated Hsp70 levels had a higher risk for APOs. The results also showed that the specificity was 60%, the sensitivity was 78%, the PPV was 50%, and the NPV was 85%. However, the level of Hsp70 alone was insufficient to predict APOs. Therefore, it is necessary to further investigate other possible predictors for APOs.

In summary, our present findings indicate that lymphocytic Hsp70 levels were associated with APOs. The mechanisms by which Hsp70 may play a role in the etiology of APOs and whether it can be applied as a clinical indicator of APOs warrant further investigation.

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