

Original Article

Increased Anti-HSP60 and Anti-HSP70 Antibodies in Women with Unexplained Recurrent Pregnancy Loss

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Vascular dysfunction has been reported in women with recurrent pregnancy loss (RPL). We investigated the severity of vascular dysfunction in non-pregnant women with RPL and its correlation with anti-heat shock protein (HSP) antibodies that are known to induce arteriosclerosis. We measured the serum anti-HSP60 antibodies, anti-HSP70 antibodies, and anti-phospholipid antibodies (APA) in 68 women with RPL and 29 healthy controls. Among the women with RPL, 14 had a diagnosis of antiphospholipid syndrome (APS), and in the remaining 54, the causes for RPL were unexplained. Compared to the controls, the brachial-ankle pulse wave velocity (baPWV), carotid augmentation index (cAI), and uterine artery pulsatility index (PI) were all significantly higher in the women with both APS and unexplained RPL. Compared to the controls, the anti-HSP60 antibody levels were significantly higher in the APA-positive group of women with unexplained RPL, and the anti-HSP70 antibody levels were significantly higher in APS and APA-positive group of women with unexplained RPL. However, the anti-HSP60 and anti-HSP70 antibody levels did not correlate with the values of baPWV or cAI. Our results demonstrated anti-HSP60 and anti-HSP70 antibodies are increased in women with unexplained RPL. Further studies are needed to elucidate the roles of anti-HSP antibodies and their pathophysiology in unexplained RPL.

Key words: recurrent pregnancy loss, autoantibody, heat shock protein, pulse wave velocity, arterial stiffness

Recurrent pregnancy loss (RPL) is defined as the occurrence of more than two failed clinical pregnancies [1]. Two consecutive miscarriages occur in <5% of women, and three or more consecutive miscarriages occur in approx. 1% [2]. The causes of RPL are diverse and include uterine malformations, immune disorders such as antiphospholipid antibody syndrome (APS) and systemic lupus erythematosus (SLE), endocrine disorders such as thyroid dysfunction and diabetes, coagulopathies, infection, and parental or fetal

chromosomal abnormalities. However, approx. 50% of RPL cases remain unexplained [3]. Although it is assumed that abnormal embryonic karyotype is involved in many unexplained RPL cases [4], the causes of the unexplained RPL remain to be established.

We reported uterine circulatory failure with vascular dysfunction in women with RPL [5-8]. Vascular dysfunction in the uterine arteries is thought to play an important role in causing miscarriages, fetal growth restriction, and fetal death [8]. The evidence that uterine artery vascular resistance at the early stage of preg-

Received October 26, 2016; accepted December 7, 2016.

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Conflict of Interest Disclosures: No potential conflict of interest relevant to this article was reported.

nancy is significantly higher among women with RPL compared to healthy controls is indicative of an association between the uterine artery blood flow and the outcomes of pregnancy [5,9]. It is also known that women with RPL have higher uterine artery vascular resistance even when not pregnant [6,10]. We showed that the uterine artery pulsatility index (PI) is especially high in women with APS [6], which is diagnosed by measuring anti-cardiolipin (CL) antibodies, lupus anticoagulant (LAC) and anti-cardiolipin β 2 glycoprotein I (CL β 2GPI) antibodies [11]. Women with APS are known to experience coagulation abnormalities and vascular dysfunction [12,13]. In addition, a subset of non-pregnant women with unexplained RPL demonstrate relatively high uterine artery vascular resistance [7,14], implying a relationship between vascular dysfunction and pregnancy loss. Adrenomedullin, which increases in order to compensate for vascular dysfunction, is present at higher levels in women with RPL, especially in those with APS [6].

An evaluation of arterial stiffness in women with RPL by using volume-plethysmography demonstrated increased values of brachial-ankle pulse wave velocity (baPWV) and the carotid augmentation index (cAI) [8]. Although increased baPWV values were expected in women with APS, their presence in women with unexplained RPL indicates the progression of subclinical arteriosclerosis.

Arteriosclerosis including subclinical arterial stiffness and advanced atherosclerosis is a multifactorial process [15,16], and its clinical manifestation is thought to involve an inflammatory response. The levels of anti-heat shock protein (HSP) antibodies are known to be elevated in patients with atherosclerosis, with numerous studies reporting an association of anti-HSP60 and anti-HSP70 antibodies in particular with arteriosclerosis [17-19]. HSPs are expressed in response to exposure to stress factors such as fever, infection, and hypoxemia [20-22]. HSPs function as molecular chaperones to repair degenerated proteins and are present in many prokaryotic and eukaryotic species, and their amino-acid sequence and steric structure are highly conserved across species [23].

Although HSPs are present in human vascular endothelial cells even under non-stress conditions, their expression on the cell surface increases with exposure to stress [16]. Since human HSPs share similarities with bacterial HSPs, they can be recognized as antigens by

antigen-presenting cells, resulting in the production of anti-HSP antibodies. The subsequent immune response results in failure or the activation of the endothelial cells, causing arteriosclerosis [15-19].

In recent years, the toxicity of anti-HSP60 and anti-HSP70 antibodies on vascular endothelial cells was confirmed in both *in vitro* and *in vivo* studies [24-27], suggesting that the presence of anti-HSP60 and anti-HSP70 antibodies could be correlated with arteriosclerosis. Vascular dysfunction by elevated levels of anti-HSP60 and anti-HSP70 antibodies may be a novel cause of RPL. Many women with unexplained RPL test positive for several anti-phospholipid antibodies (APA) including anti-phosphatidylethanolamine (PE), anti-phosphatidylserine (PS), and anti-prothrombin (PT) antibodies [28,29], which are not within the APS criteria [11]. However, the positive ratio of anti-HSP60 and anti-HSP70 antibodies in women with RPL, especially those with unexplained RPL, is not known.

Here we assessed the presence of anti-HSP60 and anti-HSP70 antibodies in women with unexplained RPL, and we examined the correlation of these antibodies' levels with vascular dysfunction.

Materials and Methods

Subjects. Ninety-seven non-pregnant women who visited the outpatient clinic of Okayama University Hospital between 2006 and 2010 were enrolled in this study. The enrolled women consisted of 68 women with a history of loss of 2 or more pregnancies, forming the RPL group, and 29 healthy women with a normal obstetric history and no more than one miscarriage as controls. Women with uterine malformations, SLE, history of smoking, endocrine disorders such as thyroid dysfunction and diabetes, coagulopathies and chromosomal abnormalities in couples were excluded from the study. No subjects had signs of infection when they had aborted spontaneously.

The research protocol was approved by the Research Ethics Committee of the Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and Okayama University Hospital (No. 1911, 1794). All subjects underwent blood tests, an ultrasound examination and a pulse wave analysis after providing informed consent.

Biochemical analysis. Blood samples were collected in the morning following overnight fasting

during menstruation. Total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglyceride were analyzed by the absorbance measurement method with an automatic biochemical analyzer (JCA-BM 2250; JEOL, Tokyo, Japan). Glucose was measured using a glucose analysis apparatus (GA08 III; A&T, Kanagawa, Japan). Insulin was measured using a fully automated enzyme immunoassay apparatus (AIA-2000; Tosoh, Tokyo, Japan). Insulin resistance was assessed based on the levels of insulin in fasting blood and the homeostasis model assessment-R (HOMA-R) index: glucose (mmol/L) \times insulin (mIU/L)/22.5 [30].

We used an enzyme-linked immunosorbent assay (ELISA) (SRL, Tokyo, Japan) to measure the APA levels including anti-CL IgG antibody, anti-CL IgM antibody, anti-CL β 2GPI antibodies, anti-PE IgG antibody, anti-PE IgM antibody, anti-PS IgG antibody, anti-PS IgM antibody, and anti-PT antibodies. We performed the dilute Russell viper venom test to analyze the levels of LAC (BML, Tokyo, Japan). The measurements of anti-CL IgG antibody, anti-CL IgM antibody, LAC, and anti-CL β 2GPI antibodies were conducted twice with a minimum interval of 12 weeks. APS was diagnosed by using the Sapporo criteria [11]. Anti-HSP60 and anti-HSP70 antibodies were measured by using their respective ELISA kits (EKS-650, EKS-750; Enzo Life Sciences, Farmingdale, NY, USA) at a serum dilution of 1 : 1000.

Evaluation of arterial stiffness. We assessed arterial stiffness with a pulse wave analysis by using a volume-plethysmographic apparatus (Form/ABI, Omron Colin, Tokyo, Japan) [31-33]. The measurements were made with the subject in a supine position after a 5-min rest. A blood pressure cuff was applied on each upper arm and each ankle, and electrocardiogram (ECG) leads were applied to each wrist. A microphone was placed on the left edge of the rib to facilitate the collection of cardiac sounds. The ECG and phonocardiogram measurements were taken simultaneously, along with the blood pressure measurement from the 4 limbs.

The ankle-brachial index (ABI) is expressed as the ratio of the maximum blood pressures measured at the ankles and the upper arms. An ABI $<$ 1.0 in either leg was considered abnormal, suggesting peripheral arterial disease; progressively lower ABI values were considered to indicate more severe obstruction. Arteriosclerosis obliterans (ASO) was defined as an ABI \leq 0.9.

The baPWV was used as a substitute for aortic PWV, as there is a strong correlation between the two. Since the baPWV of the upper right arm correlated strongly with the baPWV of the left arm ($r=0.96$, $p<0.0001$), we used the right arm baPWV values for the subsequent analysis.

The cAI was measured by placing a multi-element applanation tonometry sensor on the common carotid artery and calculated based on the central artery waveform impacted by the reflected wave from the peripheral artery. Pulse waves over a 30-sec period were recorded and stored. The validity and reliability of this tonometry sensor have been reported [34].

Pulsed Doppler ultrasonography of the uterine arteries. The uterine artery PI was measured during the midluteal phase of the menstrual cycle with a 5.0-MHz transvaginal probe (SSD-3500[®], Aloka, Tokyo, Japan) [5, 7].

Statistical analyses. The Mann-Whitney *U*-test was used to compare differences between the groups. The Pearson product-moment correlation coefficient was used to examine the correlations between the different variables. All analyses were performed using the software package IBM SPSS Statistics, ver. 20. Significance was set at $p<0.05$.

Results

Clinical features and laboratory data. Fourteen subjects with RPL were diagnosed as having APS by using the Sapporo criteria (Table 1). Fifty-four subjects did not satisfy the Sapporo criteria and were classified as having unexplained RPL. Among the 54 women with unexplained RPL, 32 tested positive for at least one APA including anti-CL antibodies (immunoglobulins G and/or M), LAC, anti-CL β 2GPI antibodies, anti-PE antibodies (immunoglobulins G and/or M), anti-PS antibodies (immunoglobulins G and/or M), and/or anti-PT antibodies. The remaining 22 subjects tested negative for all of these APAs.

We divided the entire RPL group into the APS group and the unexplained RPL group. The unexplained RPL group was further divided into the APA-positive group and the APA-negative group. There were no significant differences in age, height, body weight, or body mass index (BMI) among the different groups.

The subjects' total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, glucose, and insulin

levels in fasting blood samples were all within the normal range. There were no significant differences in these values among the groups.

Arterial stiffness. None of the subjects were found to have high blood pressure values (Table 2). While systolic blood pressure was higher in the APS group compared to the control group, diastolic blood pressure was not significantly different among the groups. The ABI, which we used as an index of arterial occlusion, was not significantly different among the groups. However, baPWV, which we used as an indi-

cator of arterial stiffness, was significantly higher in the women with RPL compared to the control group. In addition, all of the subgroups of women with RPL had significantly higher values of baPWV compared to the control group.

The cAI, which we used as another index of arterial stiffness, was significantly higher in the women with RPL compared to the control group. In addition, all of the subgroups of women with RPL had significantly higher cAI values compared to the control group. Similarly, the uterine artery PI values were also signifi-

Table 1 Clinical features and Laboratory data of the study subjects

	RPL					
	Control (n = 29)	Total (n = 68)	APS (n = 14)	Total (n = 54)	Unexplained	
					APA positive (n = 32)	APA negative (n = 22)
Age (Years)	30.6 ± 7.5	33.7 ± 4.6	33.8 ± 5.5	33.7 ± 4.4	33.4 ± 4.5	34.2 ± 4.4
Pregnancy loss	0 [0–1]	3 [2–5]	4 [2–5]	3 [2–5]	2 [2–5]	3 [2–5]
Height (cm)	158.3 ± 5.1	156.8 ± 4.9	157.4 ± 6.9	156.7 ± 4.4	157.1 ± 4.4	156.0 ± 4.4
Body weight (kg)	55.2 ± 9.6	52.4 ± 5.3	53.9 ± 5.2	52.0 ± 5.3	52.7 ± 5.1	50.9 ± 5.4
BMI (kg/m ²)	21.9 ± 3.4	21.3 ± 2.4	21.9 ± 2.8	21.2 ± 2.3	21.4 ± 2.2	21.0 ± 2.5
Total cholesterol (mg/dL)	196.1 ± 32.9	189.3 ± 27.7	193.8 ± 38.2	188.1 ± 24.5	184.7 ± 22.2	192.9 ± 27.3
HDL-C (mg/dL)	71.4 ± 16.3	70.4 ± 13.3	70.2 ± 17.7	70.5 ± 12.3	69.8 ± 11.7	71.4 ± 13.3
LDL-C (mg/dL)	109.5 ± 29.5	105.7 ± 24.6	108.9 ± 36.6	105.0 ± 21.3	103.1 ± 20.9	107.5 ± 22.0
Triglycerides (mg/dL)	60.9 ± 27.1	65.6 ± 32.0	82.4 ± 51.4	61.8 ± 24.8	61.1 ± 21.9	62.6 ± 28.8
Glucose (mg/dL)	90.5 ± 8.2	90.1 ± 6.1	88.4 ± 7.6	90.6 ± 5.6	91.4 ± 5.8	89.4 ± 5.1
Insulin (μU/ml)	4.5 ± 2.1	4.7 ± 2.5	5.4 ± 3.3	4.6 ± 2.3	4.7 ± 2.7	4.4 ± 1.6
HOMA-R	1.0 ± 0.5	1.1 ± 0.6	1.3 ± 0.8	1.1 ± 0.6	1.2 ± 0.8	0.9 ± 0.4

Values are expressed as mean ± S.D or median [range].

RPL, recurrent pregnancy loss; APS, anti-phospholipid syndrome; APA, anti-phospholipid antibody; BMI, body mass index; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; HOMA-R, homeostatic model analysis ratio.

Table 2 Arterial stiffness evaluated by volume-plethysmography and endovaginal ultrasonography

	RPL					
	Control (n = 29)	Total (n = 68)	APS (n = 14)	Total (n = 54)	Unexplained	
					APA positive (n = 32)	APA negative (n = 22)
Systolic BP (mmHg)	106.1 ± 7.4	109.8 ± 9.1	113.5 ± 9.8*	108.8 ± 8.8	110.2 ± 9.5	106.8 ± 7.5
Diastolic BP (mmHg)	63.8 ± 7.3	66.8 ± 7.7	67.9 ± 8.9	66.5 ± 7.4	67.7 ± 8.2	64.7 ± 5.9
ABI	1.05 ± 0.06	1.07 ± 0.07	1.05 ± 0.07	1.07 ± 0.08	1.06 ± 0.08	1.09 ± 0.06
baPWV (cm/sec)	1,075 ± 102	1,170 ± 117**	1,220 ± 160**	1,157 ± 103**	1,159 ± 113**	1,154 ± 87.4*
cAI (%)	−7.8 ± 13.8	3.6 ± 14.2**	1.8 ± 12.1*	4.1 ± 14.8**	1.6 ± 14.6**	7.8 ± 14.6**
Uterine artery PI	2.30 ± 0.33	3.07 ± 1.1**	3.21 ± 1.2*	2.99 ± 1.1*	2.87 ± 0.9*	3.20 ± 1.4*

Values are expressed as mean ± S.D. Statistically significant when compared to the control group, * $p < 0.05$, ** $p < 0.01$.

RPL, recurrent pregnancy loss; APS, anti-phospholipid syndrome; APA, anti-phospholipid antibody; BP, blood pressure; ABI, ankle-brachial index; baPWV, brachial-ankle pulse wave velocity; cAI, carotid augmentation index; PI, pulsatility index.

cantly higher in all of the subgroups of women with RPL compared to the control group.

Anti-HSP antibodies. The anti-HSP60 antibody levels in the APS group were not significantly different from the levels in the control group (Fig. 1). These levels were significantly higher in the entire unexplained RPL group compared to the control group. Among the subgroups in the unexplained RPL group, the APA-positive group had higher anti-HSP60 antibody levels compared to the control group, whereas the APA-negative group did not show any significant difference in anti-HSP60 antibody levels.

The anti-HSP70 antibody levels were significantly higher in the APS group as well as in the unexplained RPL group compared to the control group (Fig. 2). Among the subgroups in the unexplained RPL group, the APA-positive group had higher anti-HSP70 antibody levels compared to the control group, whereas the APA-negative group did not show any significant difference in these levels.

We analyzed the correlations between the levels of the different antibodies and the various indices of arterial stiffness. There was no significant correlation between anti-PE antibodies, anti-PS antibodies or anti-PT antibodies and the baPWV values or the cAI

values. There was also no significant correlation between the anti-HSP60 antibody levels and the baPWV values or the cAI values (Fig. 3A, B). Similarly, there was no significant correlation between the anti-HSP70 antibody levels and the baPWV values or the cAI values (Fig. 3C, D). No correlation was observed regardless of whether the group analyzed was the entire RPL group or the unexplained RPL group (data not shown).

Discussion

In the present study, the women with unexplained RPL showed high uterine artery resistance and increased systemic arterial stiffness despite their youth and the absence of blood pressure or cholesterol elevation. These results are similar to our earlier findings [8]. In the unexplained RPL group, increased values of baPWV and cAI were observed as well as APS, which involves arteriosclerosis.

A baPWV of 1,400 cm/sec is equivalent to intermediate risk on the Framingham risk score [35], with a 10-20% predicted risk of severe coronary artery disease over the next 10 years. In the present study, the mean baPWV value for the women with RPL was 1,170 cm/sec. However, 4.4% of these women (mean age, 39.0 years) had baPWV values of 1,400 cm/sec or higher. Since improvements in baPWV values are known to result from weight loss, smoking cessation, use of anti-

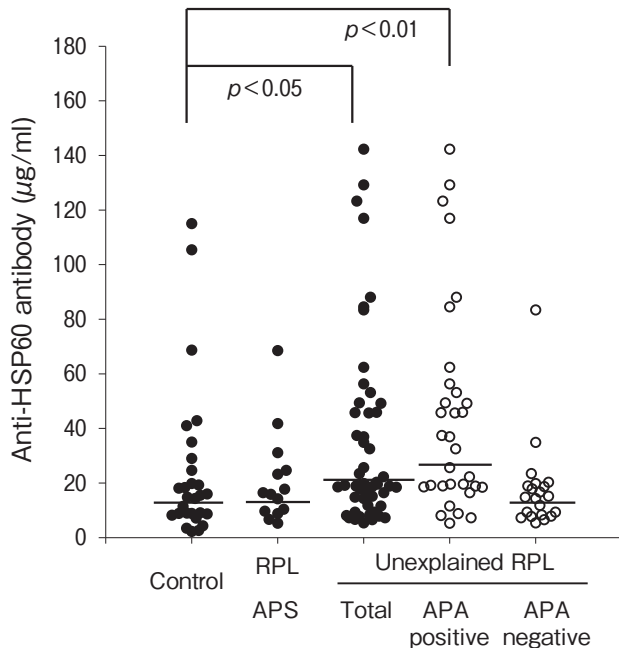


Fig. 1 Anti-HSP60 antibody levels. Bars = the medians. APS, antiphospholipid antibody syndrome; RPL, recurrent pregnancy loss; APA, anti-phospholipid antibody.

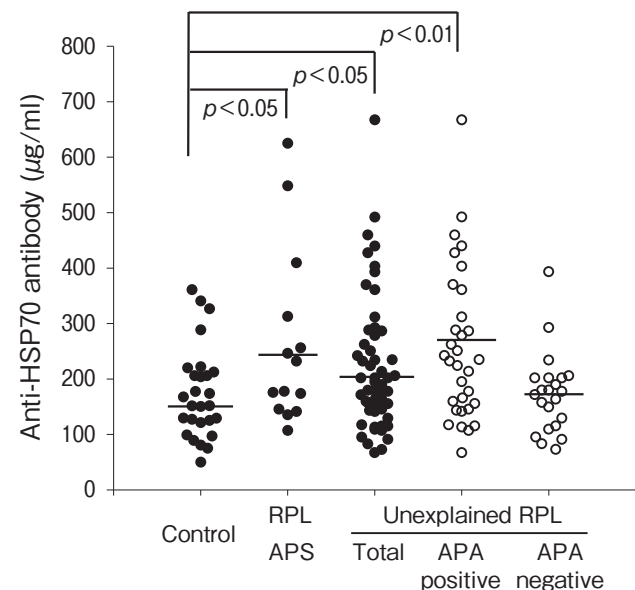


Fig. 2 Anti-HSP70 antibody levels. Bars = the medians.

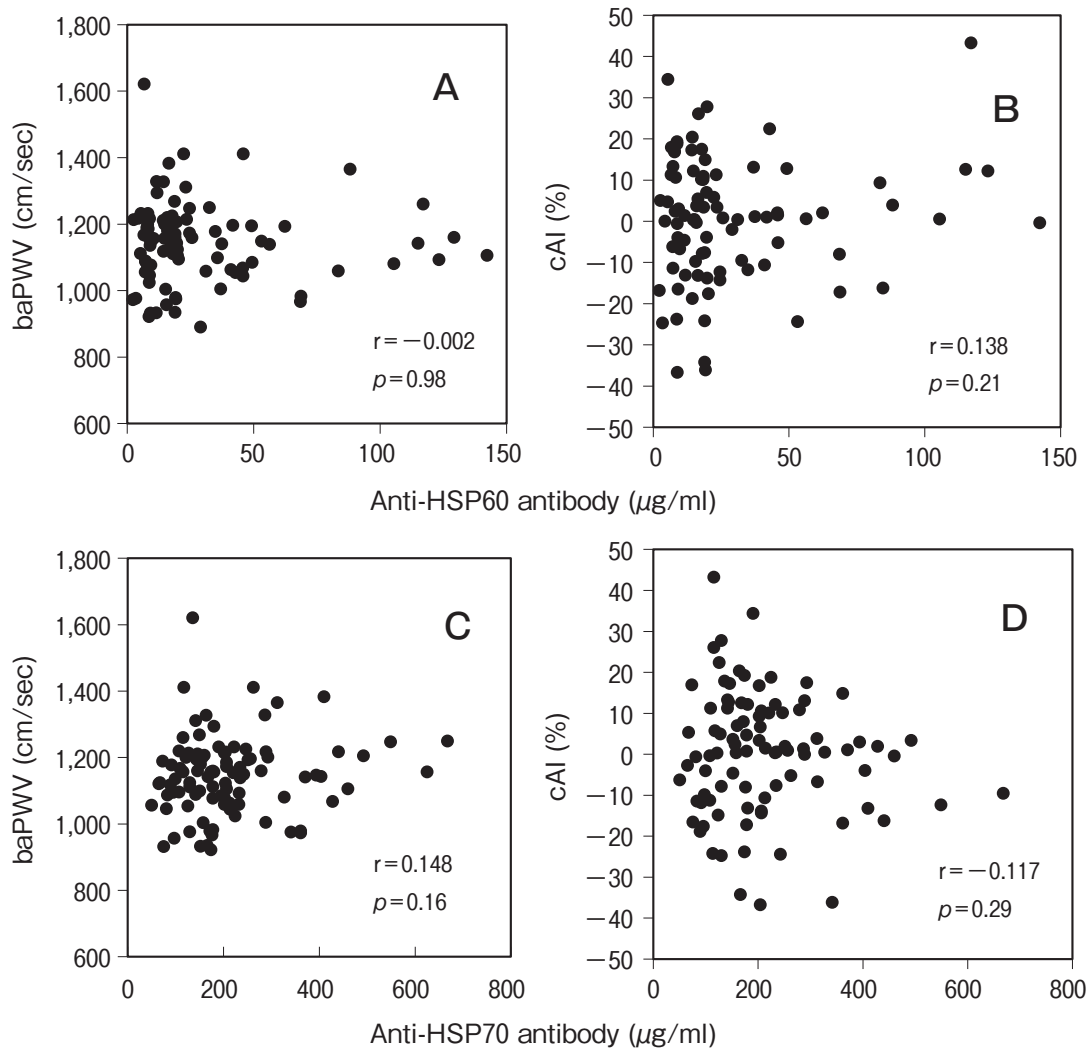


Fig. 3 Correlations of anti-HSP60 antibodies with brachial-ankle pulse wave velocity (baPWV) (A) and carotid augmentation index (cAI) (B). Correlations of anti-HSP70 antibodies with baPWV (C) and cAI (D).

hypertensive drugs and oral hypoglycemic agents, lifestyle interventions such as exercise and proper diet would help prevent severe coronary artery disease.

The present study is the first to demonstrate higher levels of anti-HSP60 and anti-HSP70 antibodies among the portion of women with unexplained RPL. Although the levels of anti-HSP60 and anti-HSP70 antibodies were significantly higher in the women with unexplained RPL, the APA-positive group was the main contributor to the significant elevation of these antibodies. In the APA-positive group, in which women tested positive for anti-PE, anti-PS, and/or anti-PT antibodies of unknown significance in RPL, anti-HSP60 and anti-

HSP70 autoantibodies may be produced under pathological conditions similar to autoimmune disorders that are not part of the definitive diagnosis of APS.

An *in vivo* experiment involving mice suggested that anti-HSP60 antibodies may contribute to vascular endothelial cell toxicity [24]. In addition, anti-HSP60 antibody adherence to normal vascular endothelial cells may result in the cells' apoptosis [25]. Another study reported the development of aortic atherosclerosis in rats following intravenous injections of anti-HSP70 antibody administered through peripheral vessels [26]. Thus, there is evidence to suggest that increased levels of anti-HSP60 and anti-HSP70 antibodies are associated

with arteriosclerosis. In the present study, however, baPWV and cAI did not correlate with the anti-HSP60 and anti-HSP70 antibody levels in the women with RPL and also in the control group.

For the evaluation of an individual's vascular condition, there are various parameters other than the baPWV and cAI such as flow-mediated vasodilation (FMVD) and carotid artery intima-media thickness (cIMT). A study that examined the relationship between cIMT and anti-HSP antibody reported their positive correlation in healthy individuals [36], but this was a state in which arterial sclerosis had been somewhat advanced. We have found no report examining the relationships between anti-HSP antibody and the baPWV, the cAI and FMVD, which may reflect early-stage vascular dysfunction. The negative impact of anti-HSP antibody on the blood vessels of women with RPL should be monitored over a long term.

It is possible that high levels of anti-HSP60 and anti-HSP70 antibodies, as seen in women with unexplained RPL, may contribute to miscarriage or stillbirth by a mechanism other than vascular dysfunction. HSPs, which are normally present in the placenta of healthy individuals, increase in response to various factors such as oxidative stress, infection and ischemia, as a tissue-protective mechanism. It has been demonstrated that exposing the chorionic villi of rats to ultrasound for 10 and 20 min resulted in an increase in the expression of HSP70 and the inhibition of apoptosis, which in turn was associated with the suppression of tissue damage [37], indicating the placental tissue-protective effect of HSP70.

In addition, the tissue-protective effect of HSP was similarly observed in colonic and oral mucosa [38, 39]. The expression of HSP70 was significantly higher in the chorionic villi of women who spontaneously aborted at 8-13 weeks of gestation compared to women with normal pregnancies at the corresponding gestational week [40]. Similarly, the placentas of women presenting with intrauterine fetal growth restriction also showed higher expressions of HSP27, 60, 70, and 90 in the syncytiotrophoblasts and cytotrophoblasts of avascular villi and villi with thrombi compared to a full-term control group [41]. Anti-HSP antibodies may prevent the ability of HSPs to effectively protect tissues in response to stress.

In addition, *in vitro* studies have confirmed the direct cytotoxicity of anti-HSP70 antibody against tissues that express HSP70 [27]. The presence of anti-

HSP60 and anti-HSP70 antibodies prior to pregnancy may result in direct placental damage if the chorionic cells express a higher antigenic load of HSP60 and HSP70, as is seen with a stressed placenta. This could be another mechanism of anti-HSP antibodies resulting in adverse outcomes of pregnancy, especially in unexplained RPL.

Additionally, in the present study's APA-negative group with relatively low levels of anti-HSP activity, arteriosclerosis was also observed even though women in the group were not considered to have autoimmune disorders. The causes of arteriosclerosis in the APA-negative group are not yet clear. Other factors such as subclinical infection and vascular abnormality may affect arterial conditions resulting in pregnancy loss. However, along with significantly elevated uterine artery resistance, an unexplained cause of arterial disorder might trigger impaired uterine perfusion, resulting in RPL. Further detailed examinations of APA-negative women with RPL may shed light on the causes of unexplained RPL.

Acknowledgments. There are no conflicts of interest to disclose. In addition, the authors have no relationships with companies that may have a financial interest in the information contained in the manuscript.

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