

Lymphocyte Hydrogen Sulfide Production Predicts Coronary Artery Lesions in Children with Kawasaki Disease: A Preliminary, Single-Center Study

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ABSTRACT

To identify whether lymphocyte hydrogen sulfide production is a potential biomarker for predicting coronary artery lesions (CAL) in children with Kawasaki disease (KD). Eighty-six children with KD, 33 normal children and 43 children with fever from June 2016 to January 2019 in Shaanxi Provincial People's Hospital were enrolled. Of 86 KD patients, 16 patients exhibited CAL. Lymphocyte hydrogen sulfide production was significantly greater in KD patients (13.7 ± 2.7) nmol/min/ 10^8 lymphocytes than in the controls (9.26 ± 3.33) nmol/min/ 10^8 lymphocytes and the fever group (8.21 ± 2.77) nmol/min/ 10^8 lymphocytes. The lymphocyte hydrogen sulfide production was greater in CAL patients than the non-CAL patients [(16.24 ± 1.81) vs. (13.12 ± 2.58) , $p < 0.001$]. Receiver operating characteristic curve indicated when the lymphocyte hydrogen sulfide production was >15.285 nmol/min/ 10^8 lymphocytes, the sensitivity and specificity for predicting CAL at convalescence were 87.5% and 82.9%, respectively. Lymphocyte hydrogen sulfide production in the acute period is a potentially useful biomarker for predicting CAL in KD children.

KEYWORDS: hydrogen sulfide, Kawasaki disease, lymphocyte, inflammation

INTRODUCTION

Kawasaki disease (KD) is an acute systemic vasculitis of unknown etiology that commonly develops in children, and is a leading cause of acquired heart disease among children in developed countries [1]. In fact, in most developed countries, KD has surpassed acute rheumatic fever as the leading cause of acquired heart disease in children [2]. Treatment with high-dose intravenous immunoglobulin and aspirin effectively resolves

inflammation and reduces the occurrence of coronary artery lesions (CAL) in KD patients [3]. However, approximately 10–20% of KD patients exhibit persistent or recurrent fever after treatment with intravenous immunoglobulin and aspirin, and these patients appear to have a high risk of developing CAL [4]. Therefore, it is vital to predict CAL in patients with KD. Accordingly, recent research has focused on the identification of useful biomarkers of CAL.

It has been reported that several biomarker are associated with CAL [5–7], however, their predictive value is not satisfactory. Sun *et al.* reported that plasma hydrogen sulfide is a biomarker for predicting CAL in children with KD [8]; however, there was no explanation where the plasma hydrogen sulfide comes from. Since the KD was an acute systemic vasculitis disease, we attempted to identify whether there are any differences in lymphocyte hydrogen sulfide production abnormalities during the pathogenesis of KD, by examining this value between children with and without KD and between KD cases with and without CAL.

METHODS

Subjects

We enrolled a total of 162 subjects, including 86 children with KD, 33 normal children and 43 children with fever from the inpatient pediatric department of Shaanxi Provincial People's Hospital from June 2016 to January 2019. All KD patients received intravenous immunoglobulin at 1 g/kg/day for 2 days and oral aspirin at 30–50 mg/kg/day. The aspirin dose was reduced to 3–5 mg/kg/day for 8 weeks at 3 days after a normal temperature value was recorded. Seventy patients without coronary dilation or coronary aneurysms were assigned to the non-CAL subgroup, whereas 16 patients with coronary dilation and coronary aneurysm were assigned to the CAL subgroup. Thirty-three healthy children served as the control group; the healthy children exhibited normal findings on medical history taking, physical examination and laboratory tests. The fever groups were diagnosed without KD. All the children were informed about the purpose of the research and agreed to provide relevant research information. Written informed consent were obtained from their parents, next of kin, or guardians. The authors assert that all procedures contributing to this work complied with the ethical standards of the relevant national guidelines on human experimentation and with the Helsinki Declaration on 1975, as revised in 2008, and approved by Shaanxi Provincial People's Hospital Ethics Committee. All procedures contributing to this work comply with the ethical standards expressed in relevant national guidelines. The specific flow chart was show in Fig. 1.

General, clinical and laboratory data

Age and gender were recorded from direct interviews with the patients and their guardians. Data on general biomarkers, including white blood cell count, red blood cell count, hemoglobin level, blood platelet level and C-reactive protein level, were collected from the medical records.

Echocardiography

Echocardiography was performed for all KD children prior to the initial treatment and during the convalescent period. Two-dimensional color Doppler echocardiography was performed by a cardiologist who was blinded to the clinical history. Routine chamber sizing in standard mode was used, and the wall thickness in systole and diastole, as well as coronary diameter, were measured. The aortic root, left atrial, left ventricular end-systolic and left ventricular end-diastolic dimensions, along with the coronary diameter, were all measured using echocardiography.

Diagnostic criteria for KD

KD was diagnosed based on the 2004 American Heart Association/American Academy of Pediatrics guidelines [9]. Complete KD was diagnosed when subjects had at least 5 of the following 6 principal clinical signs: (i) fever persisting for ≥ 5 days; (ii) bilateral conjunctival congestion; (iii) changes to the lips and oral cavity; (iv) polymorphous exanthema; (v) changes to the peripheral extremities; and (vi) acute non-purulent cervical lymphadenopathy. Incomplete KD was defined as having ≤ 4 principal signs, with or without the presence of cardiac lesions [10].

Diagnostic criteria for coronary artery lesions

Echocardiography was used to assess CAL; it was performed prior to the initial treatment and was repeated at 1, 2 and 4 weeks after the initial treatment. CAL was defined as one of the following conditions: (i) internal lumen diameter of >2.5 mm in children younger than 3 years old, >3 mm in children 3–9 years and >3.5 mm in children 9–14 years; (ii) internal diameter of a segment measuring ≥ 1.5 times that of an adjacent segment; or (iii) lumen was clearly irregular [11].

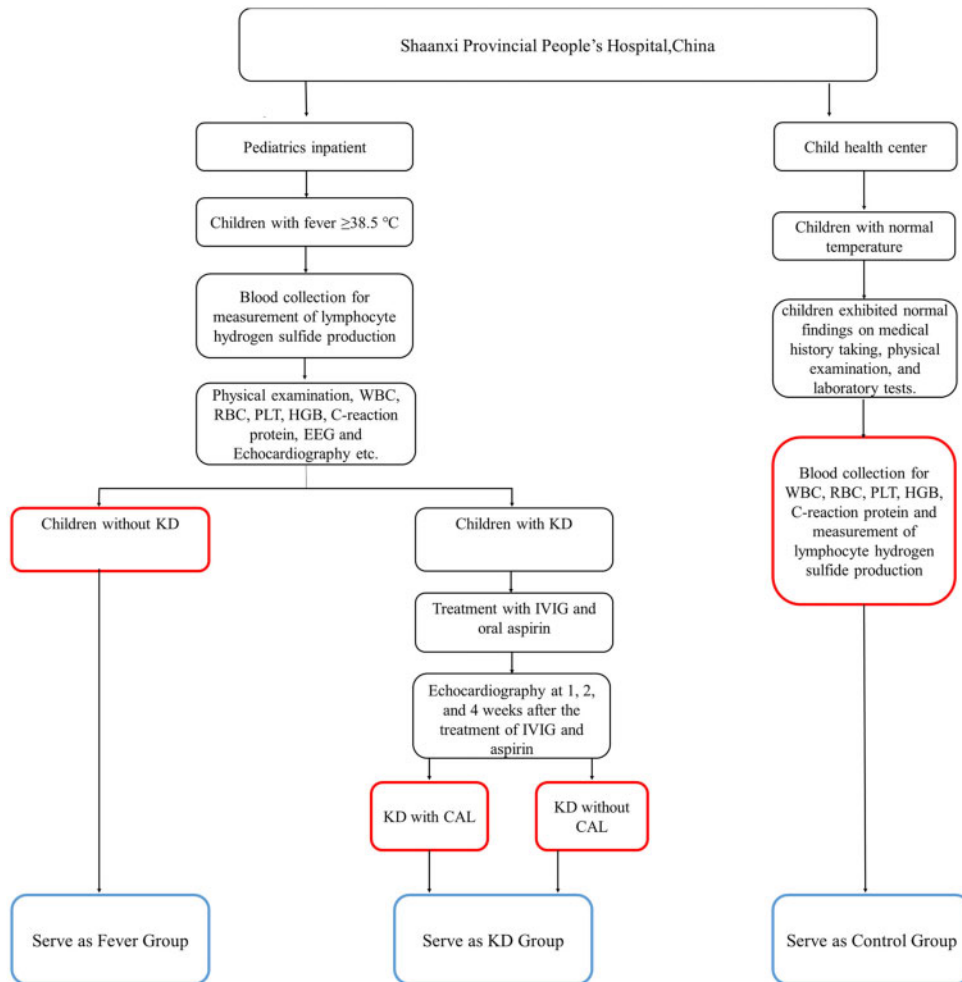


Fig. 1. A flow chat to present the experimental process. KD: Kawasaki disease; WBC: white blood cell; RBC: red blood cell; PLT: blood platelet; HGB: hemoglobin; CAL: coronary artery lesions.

Measurement of lymphocyte hydrogen sulfide production

The lymphocyte hydrogen sulfide production were detect immediately after the blood was collect. Blood was collected via venipuncture into a tube with heparin. In brief, 1 ml of blood was mixed with 1 ml 0.1 mol/l phosphate buffered saline, and the mixture is added to lymphocyte separation liquid. The mix liquid was then centrifuged at 2000 r/min for 15 min at 4°C, and the second layer of lymphocytes is collected. The lymphocytes are washed twice with 0.1 mol/l phosphate buffered saline and centrifuged at 4000 g for 10 min at 4°C. The cells are counted, and the lymphocytes are then stored in the

refrigerator until the assay is performed. A total of 1×10^8 lymphocytes are lysed in 900 μ l of ice-cold Tris-HCl (50 mmol/l, pH 7.4), and are subjected to ultrasound cracking for 15 s. Lymphocyte hydrogen sulfide production is then measured using human hydrogen sulfide ELISA kits (Mlbio, Shanghai, China), and hydrogen sulfide production is expressed as unit nmol/min/ 10^8 lymphocytes.

Statistical analysis

Data are analyzed using SPSS 19.0 software (SPSS Inc, Chicago, IL). Normally distributed data are presented as $\bar{X} \pm S$ and were assessed by using *F* tests among groups; continuous data that were non-normally

distributed are expressed as median (inter-quartile range) and were analyzed by using non-parametric tests. Categorical data are presented as frequency and were compared by using the χ^2 test. Receiver operating characteristic curve analysis was used to evaluate lymphocyte hydrogen sulfide production for predicting CAL at convalescence. A p value of <0.05 was considered statistically significant.

RESULTS

Demographic characteristics and bio-marker values among KD patients, the control group and the fever group

The KD group included 50 boys and 36 girls. The fever time, white blood cell count, red blood cell count; hemoglobin level, blood platelet, C-reactive protein level and lymphocyte hydrogen sulfide production significantly differed among the three groups. The lymphocyte hydrogen sulfide production was significantly greater in the KD patients ($p < 0.001$, Table 1).

Comparison of laboratory data and lymphocyte hydrogen sulfide production between the CAL and non-CAL patients

No significant difference was observed in age; white blood cell count, red blood cell and blood platelet counts; and hemoglobin, C-reactive protein level

between the CAL and non-CAL patients. However, fever time, lymphocyte hydrogen sulfide production was significantly greater in CAL patients than in the non-CAL patients ($p < 0.05$, Table 2).

Predictive value of lymphocyte hydrogen sulfide production for CAL at convalescence in KD patients

Receiver operating characteristic curve indicated that the area under the curve was 84.6% (95% confidence interval, 75.1–94.2%; $p < 0.001$), which suggests that lymphocyte hydrogen sulfide production has a strong ability to predict CAL in patients with KD at convalescence. In fact, when lymphocyte hydrogen sulfide production was >15.28 nmol/min/ 10^8 lymphocytes, the sensitivity and specificity for predicting CAL at convalescence were 87.5% and 82.9%, respectively (Fig. 2).

DISCUSSION

KD is an acute, self-limited and systemic vasculitis that predominantly affects children younger than 5 years, and is the leading cause of acquired heart disease in children in developed countries [12]. Although combined intravenous immunoglobulin and aspirin are commonly used for KD treatment, and can effectively decrease the incidence of CAL, high rates of CAL have been reported in recent years

Table 1. Demographic characteristics and bio-markers among KD patients, fever group and control group

	Control group	Fever group	KD patients	F/H/ χ^2	p
Cases	33	43	86		
Gender (M/F)	17/16	24/19	50/36	0.482	0.807
Age (month)	25.33 \pm 9.97	25.48 \pm 11.36	21.47 \pm 10.99	2.64	0.074
Fever time (day)	0	2.91 \pm 1.44	4.52 \pm 2.55	61.213	<0.001
WBC ($\times 10^9/l$)	8.10 \pm 3.07	9.91 \pm 4.12	14.45 \pm 7.45*	16.72	<0.001
RBC ($\times 10^{12}/l$)	4.66 \pm 0.57	4.56 \pm 0.23	4.18 \pm 0.40*	22.242	<0.001
HGB (g/l)	128.0 \pm 13.17	121.86 \pm 7.56	111.63 \pm 11.32*	31.225	<0.001
PLT ($\times 10^9/l$)	304.56 \pm 95.03	249.9 \pm 53.73	341.22 \pm 117.99	11.974	<0.001
CRP (mg/l)	3 (2, 4)	5.69 (4, 10.11)	37.07 (16.4, 63.4)*	29.793	<0.001
Lymphocytes H ₂ S production (nmol/min/ 10^8 lymphocytes)	9.2 \pm 3.33	8.21 \pm 2.77	13.7 \pm 2.70*	63.021	<0.001

* $p < 0.05$ when compared with the other two groups. KD: Kawasaki disease; WBC: white blood cell; RBC: red blood cell; HGB: hemoglobin; PLT: blood platelet; CRP: C-reactive protein.

Table 2. Comparisons of laboratory data and lymphocytes H₂S production between CAL patients and non-CAL patients with KD

	CAL patients	Non-CAL patients	$t/H/\chi^2$	p
Cases	16	70		
Gender (M/F)	8/8	42/28	0.535	0.464
Age (month)	21.37 ± 13.18	21.50 ± 10.54	0.41	0.968
Fever time (day)	5.69 ± 3.56	4.26 ± 2.20	-2.065	0.042
WBC ($\times 10^9/l$)	15.44 ± 4.95	14.23 ± 7.92	-0.582	0.562
RBC ($\times 10^{12}/l$)	4.19 ± 0.22	4.18 ± 0.43	-0.123	0.903
HGB (g/l)	111.75 ± 6.46	111.61 ± 12.19	-0.062	0.951
PLT ($\times 10^9/l$)	350 ± 144.40	339.21 ± 112.23	-0.28	0.782
CRP (mg/l)	43.27 (31, 65.67)	33.74 (11.54, 63.45)	-0.803	0.424
Lymphocytes H ₂ S production (nmol/min/ 10^8 lymphocytes)	16.24 ± 1.81	13.12 ± 2.58	-5.68	<0.001

KD: Kawasaki disease; WBC: white blood cell; RBC: red blood cell; HGB: hemoglobin; PLT: blood platelet; CRP: C-reactive protein; CAL: coronary artery lesions.

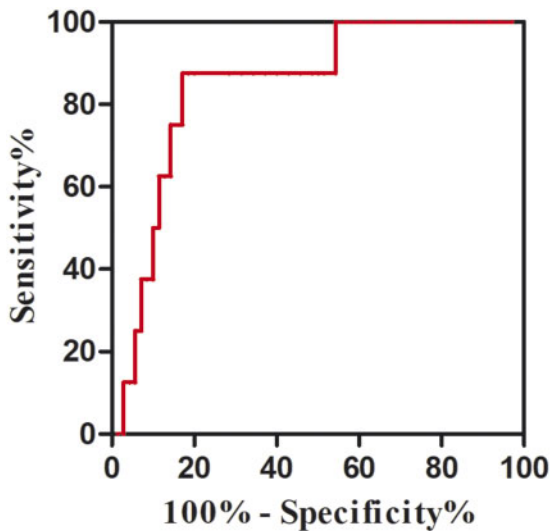


Fig. 2. Receiver operating characteristic curve analysis of the predictive value of lymphocyte hydrogen sulfide production for coronary artery lesions in patients with Kawasaki disease at convalescence. The area under the curve was 84.6% (95% confidence interval, 75.1–94.2%), indicating that lymphocyte hydrogen sulfide production was a strong predictor of coronary artery lesions in patients with Kawasaki disease at convalescence.

[4, 7, 13, 14]. Studies have shown that blood platelet count, neutrophil count, platelet hematocrit, platelet distribution width, mean platelet volume, erythrocyte

sedimentation rate, cardiac troponin I, endothelin-1, serum albumin and hemoglobin values are associated with CAL; however, their predictive ability remains unclear [14].

Sun *et al.* [8] reported that the plasma hydrogen sulfide concentrations were significantly lower in KD, which could serve as a biomarker for CAL, however, its predicted value was believed to be limited. Previously, our research reported that the lymphocyte hydrogen sulfide production predicts intravenous immunoglobulin resistance in children with Kawasaki disease (Medicine 2018) [15]. Hence, we further investigate whether the lymphocyte hydrogen sulfide production could predict the CAL in KD patients. Just as what we expected, in the present study, we found that lymphocyte hydrogen sulfide production was greater in KD patients; the extent of the increase was larger in CAL patients. Thus, pre-treatment lymphocyte hydrogen sulfide production may serve as a useful predictor of the CAL in children with KD, with concentrations of >15.28 nmol/min/ 10^8 lymphocytes indicating the potential for CAL at convalescence after clinical treatment.

So here comes up a question why the plasma hydrogen sulfide was decreased in KD [8], but the lymphocyte hydrogen sulfide production were increased in KD?

Hydrogen sulfide was long been recognized as a malodorous and highly toxic gas, although recent experimental studies have shown that it is produced

enzymatically in all mammalian species, including man, and exerts a number of critical actions to promote cardiovascular homeostasis and health [16]. Recent pre-clinical studies investigating cardiovascular diseases have demonstrated that the administration of physiological or pharmacological levels of hydrogen sulfide attenuates myocardial injury, protects blood vessels, limits inflammation and regulates blood pressure [17–20]. Hydrogen sulfide could also hinder leukocyte adhesion by inhibiting leukocyte ‘rolling’ and firm adhesion to the endothelium. Hydrogen sulfide has been shown to significantly inhibit the expression of leukocyte adhesion molecules [21–23]. Hence, as Sun *et al.* [8] reported that a decrease in plasma hydrogen sulfide levels may enhance leukocyte adhesion. Coincidentally, in KD patients, initial neutrophil infiltration of the coronary arteries, with necrosis of the arteries beginning at the luminal endothelium, was observed in the first 1–2 weeks of the illness. Hence, a decrease in plasma hydrogen sulfide levels may be associated with leukocyte adhesion or neutrophil infiltration.

In addition, pathological examinations have found that KD patients develop systemic vascular inflammation, moreover, hydrogen sulfide signaling promotes anti-inflammatory actions by preventing tissue edema [21], hence, the plasma hydrogen sulfide levels were lower in KD patients [8], which suggest that decreased plasma hydrogen sulfide maybe related to systemic vascular inflammation.

Several studies have also shown that hydrogen sulfide stimulates endothelial cell proliferation and migration by either further developing current cells or by developing primary endothelial cells [18]. Hydrogen sulfide participates in vascular endothelial growth factor signaling, hence, a decrease in plasma hydrogen sulfide levels [8] may induce a reduction in endothelial cell proliferation and endothelial necrosis.

Several studies have also shown that hydrogen sulfide also affects the vascular tone [20]. Research indicates that the exogenous administration of hydrogen sulfide can cause vasodilation [20]. When the leukocytes infiltrate into the vascular endothelium of coronary arteries and produce an overload of hydrogen sulfide, which consequently induces vasodilatation and increased vascular fragility. Such a

mechanism could explain the development of aortic dilatation and aortic aneurysms. Our study also showed that lymphocyte hydrogen sulfide production increased to a greater extent in patients with CAL, and hence, we believe that lymphocyte hydrogen sulfide production may be related to CAL severity. Thus, lymphocyte hydrogen sulfide production could serve as a useful predictor of CAL in children with KD, with concentrations >15.28 nmol/min/ 10^8 lymphocytes indicating the potential for CAL during follow-up.

The present study had certain limitations. For instance, the number of cases of KD examined was relatively low, the follow-up period was not sufficiently long and the study was conducted at a single center; hence, more studies with larger sample sizes are needed in the future. Nevertheless, this is the first study to focus on lymphocyte hydrogen sulfide production in KD patients, and we found several significant results that could provide a fundamental basis for developing individualized treatments for KD patients.

Based on our findings, we believe that lymphocyte hydrogen sulfide production was a potentially useful biomarker of CAL at convalesce. Moreover, hydrogen sulfide is constantly being secreted and can be easily detected. In addition, only 1 ml of blood is required for detection. Hence, the ease and relatively low cost of detection are advantages of using lymphocyte hydrogen sulfide production as a biomarker for predicting the outcome of coronary artery injury.

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