〈Regular Article〉

Reactive oxygen metabolites as a biomarker of congenital heart disease in children

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ABSTRACT Brain natriuretic peptide (BNP), as a hematological biomarker, has been widely used in congenital heart disease (CHD). However, its sensitivity and specificity vary depending on age and pathological condition. In the present study, we assessed whether reactive oxygen metabolites (ROMs) and biological antioxidant potential (BAP), as oxidative stress indicators, could be new biomarkers in CHD.

Forty-two patients diagnosed with CHD were enrolled in this study. The levels of ROMs, BAP, BNP, cardiac muscle creatinine kinase, and heart-type fatty acid-binding protein were measured using the findings of echocardiography. The ROM and BNP levels were significantly higher than the standard reference levels. The estimated Qp/Qs correlated mildly with BNP and ROM levels. The medication caused a significant decrease in BNP and ROM levels. The optimal decision, Qp/Qs greater than 1.5, estimated from receiver operator characteristic (ROC) curves was 371 U.CARR (58% sensitivity, 90% specificity) for ROMs, and that for BNP was 28.4 pg/ml (97% sensitivity 45% specificity). Direct comparison of ROMs and BNP did not show significantly different area under the curve values.

ROM levels can be a new biomarker for oxidative stress evaluation in children with CHD at almost the same sensitivity as the previous biomarkers, and an effective indicator when combined with other biomarkers and indicators.

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Key words：Reactive oxygen metabolites, Biological antioxidant potential, Biomarkers, Congenital heart disease

INTRODUCTION

The frequency of congenital heart disease (CHD) in children is about 1%, including hemodynamic abnormalities caused by impaired sites in the developmental stage of the heart in the fetal period and various diseases caused thereby. Concerning the severity of the disease, there is a wide range from those that naturally heal to those that require surgical intervention in the early postnatal period. Precise and accurate medical treatment or preoperative/

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postoperative management influences the prognosis. In order to assess the medical condition in children with CHD, brain natriuretic peptide (BNP), a hematological biomarker of heart failure[1], as well as cardiac muscle creatinine kinase (CK-MB) and heart-type fatty acid-binding protein (H-FABP), markers of associated myocardial damage, are widely used. These markers reflect the volume and pressure overload of the ventricles and atriums as well as myocardial damage. However, their sensitivity and specificity vary depending on the age and pathological condition. Especially in the case of BNP, newborns are likely to have high values and many false positives and negatives due to other pathological conditions[2, 3]. Furthermore, there is a certain time lag at the clinical site before it reflects hemodynamics, and the amount of blood collected is about 5 ml, which is very large for low-birth-weight newborns and infants. Therefore, there is a need for the discovery of alternative new biomarkers.

Oxidative stress occurs when there is a disruption in the balance between the production and elimination of reactive oxygen species (ROS). Oxidative stress is associated with various cardiovascular diseases, including myocardial infarction and hypertension in adults[4-9], and Kawasaki disease in children[10-13]. The association between oxidative stress and vascular or myocardial disorders has been clarified; however, there is no report of an association between heart failure due to CHD in children caused by oxidative stress. On the other hand, reactive oxygen metabolites (ROMs) and biological antioxidant potential (BAP) have been identified as oxidative stress biomarkers with high reproducibility and stability[14].

We hypothesized that ROMs and BAP for oxidative stress evaluation could serve as new biomarkers in children with CHD. We aimed to investigate the correlation between the change in oxidative stress levels and the severity of CHD, further assessing the equivalence or superiority of ROMs and BAP compared to previous biomarkers, BNP, CK-MB, and H-FABP.

MATERIALS AND METHODS

Patient Population

We enrolled children (0-15 years) with CHD, who were diagnosed at the Kawasaki Medical School between September 2016 and March 2019. All patients were diagnosed using echocardiography by a skilled pediatric cardiologist. Patient characteristics, blood test results, echocardiographic findings, and clinical course were reviewed from the medical records. The patients with basal diseases other than CHD or history of cardiac surgery were excluded. The procedures followed were in accordance with the "Declaration of Helsinki" and the ethical standards of the responsible institutional committee on human experimentation. The study was approved by the Kawasaki Medical School Hospital Institutional Ethics Review Board (Reference Number: 2476). The written informed consent was obtained from the study subjects (or their parents).

Measurement of Blood Parameters, and ROM and BAP Levels

We performed blood tests in our clinic as needed for each visit. These were performed to measure levels of plasma, BNP (reference value: < 18.4 pg/ml), CK-MB, and H-FABP as previous biomarkers. The blood samples were stored at -80℃ until analysis.

The principle of the ROM and BAP test has been described previously[15, 16]. Levels of both ROMs and BAP were measured using a Free Radical Elective Evaluator (FREE Carrio Duo®; Wismerll Co Ltd, Tokyo, Japan). The ROM test was based on the ability of transition metals to catalyze the formation of free radicals trapped by alchilamine in the presence of peroxides. The alchilamine reacted to form a colored radical; the concentration was

measured using spectrophotometry (505 nm). The results were expressed in conventional units called U.CARR (Carrtelli units), such that 1 U.CARR corresponded to 0.8 mg/l H_2O_2. Also, the BAP test was based on the ability of a colored solution, containing a source of ferric irons bound to a unique chromogenic substrate, to decolorize when ferric irons were reduced to ferrous iron when a reducing/antioxidant source was added. The concentration was then measured using spectrophotometry (505 nm). The standard reference levels of ROMs and BAP were 250 to 300 U.CARR and over 2,200 μM, respectively.

Evaluation of CHD

All children underwent echocardiography using Aplio™ 300 (CANON Medical Systems Co Ltd, Tochigi, Japan), to assess the morphology, volume, and pressure load on the ventricles and atria. Measurement sites to evaluate the medical condition and severity varied for each disease. However, the standard measurement sites used were left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), and right ventricular end-diastolic diameter (RVEDD). Each percentage was calculated according to standard values[17].

The estimated ratio of the pulmonary to systemic blood flow (Qp/Qs) was defined by using 2-D echocardiography[18] and spectral Doppler measurements in patients who had intra-cardiac shunts.

Qp = right ventricular outflow tract (RVOT) subvalvular velocity time integral (VTI) × π × $(RVOT/2)^2$

Qs = left ventricular outflow tract (LVOT) subvalvular velocity time integral (VTI) × π × $(LVOT/2)^2$

The estimated ratio of the right ventricular pressure (RVP) to left ventricular pressure (LVP) was defined by the interventricular septal (IVS) curvature index from the short-axis view using 2-D echocardiography measurements in patients who had pulmonary hypertension.

Data Collection

Collected data included patient demographics, diagnosis of CHD, blood test results, echocardiography findings, and the clinical course. Clinical data were collected at each visit as needed. All children underwent echocardiography under the supervision of a pediatric cardiologist. Levels of both ROMs and BAP were measured in triplicates, and the median was adopted. If there had been any clinical intervention, medication, or surgical repair, the post-intervention data were defined after the patient was stabilized.

Statistical Analysis

Continuous variables were expressed as mean, standard deviation, median and range depending on the distribution of each variable. Comparisons of continuous data, measured at the baseline for the study groups, were analyzed using the Wilcoxon signed-rank test. Correlation analysis was performed using the Spearman's rank correlation. The diagnostic efficiency of biomarkers was compared using ROC curve analysis with a comparison of area under the curve (AUC). A P-value of < 0.05 was considered significant. Statistical analyses were performed using the SPSS statistical software, version 25.0 (SPSS Inc, Chicago, Illinois, USA).

RESULTS

Patient Characteristics

Forty-two patients (13 males), diagnosed with CHD, were enrolled in this study between September 2016 and March 2019. The median age was 1.3 years (range 10 days-5.2 years), and weight was 9.4 kg (range 2.9-17.2 kg). Most patients, except one, had acyanotic CHD, 16 (38%) had an

atrium septal defect (ASD), and 15 (36%) had a ventricular septal defect (VSD). During the study, 10 (24%) were introduced to medication, 4 (10%) underwent surgical intervention (Table 1).

Initial Biomarker Data and Echocardiography Findings

Initial biomarker data collected at the first visit are summarized in Table 2. The ROM (411 ± 71 U.CARR) and BNP (36.3 ± 80.3 pg/ml) levels were significantly higher than the standard reference levels. Alternatively, the BAP, CK-MB, and H-FABP levels were almost within the normal range. Initial findings of echocardiography are summarized in Table 3. All patients had LVEF over 70%. All diagnoses, except valvular pulmonary stenosis (PS), were evaluated using mean estimated Qp/Qs over 1.5.

Table 1. Demographics of all patients included in the study.

	All patients (n = 42)
Age (median)	1.3 years (10 days - 5.2 years)
Males	13 (30%)
Body weight (median: kg)	9.4 (2.9 - 17.2)
Diagnosis	
ASD	16 (38%)
(+PS)	(4)
VSD	15 (36%)
(+ASD)	(3)
Valvular PS	6 (14%)
PDA	4 (10%)
CAVSD	1 (2%)
Clinical intervention	
Introduced Medication	10 (24%)
Diuretics	10
Underwent Surgical repair	4 (10%)
Follow-up	
Times (median)	3 (1-13)
Duration (median : days)	553 (1-1029)

VSD, ventricular septal defect; ASD, atrium septal defect; PDA, patent ductus arteriosus; PS, pulmonary stenosis; CAVSD, complete atrium ventricular septal defect.

Table 2. Initial level of biomarkers in subjects.

		ROMs (U.CARR)	BAP (μM)	BNP (pg/mL)	CK-MB (ng/mL)	H-FABP (ng/mL)
Total (mean ± SD)		411 ± 71	2604 ± 192	36.3 ± 80.3	29.3 ± 19.0	5.0 ± 4.0
Diagnosis						
ASD		427 ± 63	2632 ± 184	17.2 ± 17.2	23.5 ± 6.8	3.7 ± 1.9
	(+PS)	417 ± 71	2739 ± 142	12.9 ± 12.6	26.5 ± 3.2	5.3 ± 2.0
VSD		386 ± 80	2537 ± 229	46.3 ± 94.3	30.2 ± 8.5	5.4 ± 6.2
	(+ASD)	419	2459	341.9	17	2.3
Valvular PS		401 ± 52	2648 ± 128	16.1 ± 8.1	30.3 ± 5.1	6.1 ± 2.6
PDA		431 ± 78	2618 ± 135	27.3 ± 26.8	22.3 ± 4.1	7.4 ± 1.6
CAVSD		582	2587	396	15	2.3

ROMs, reactive oxygen metabolites; BAP, biological antioxidant potential, BNP, brain natriuretic peptide; CK-MB, cardiac muscle creatinine kinase; H-FABP, heart-type fatty acid-binding protein; SD, standard deviation; VSD, ventricular septal defect; ASD, atrium septal defect; PDA, patent ductus arteriosus; PS, pulmonary stenosis; CAVSD, complete atrium ventricular septal defect.

Table 3. Initial data of echocardiogram in subjects.

		LVEDD (% of normal)	LVEF (%)	RVEDD (% of normal)	Estimated Qp/Qs	Estimate RVP/LVP
Diagnosis						
ASD		101 ± 5.4	71 ± 5.3	193 ± 41.9	1.63 ± 0.45	0.35 ± 0.08
	(+PS)	97 ± 2.2	72 ± 7.5	212 ± 29.1	1.75 ± 0.05	0.44 ± 0.04
VSD		137 ± 17.1	71 ± 4.3	124 ± 20.9	1.58 ± 0.75	0.37 ± 0.13
	(+ASD)	158 ± 17.1	71 ± 3.3	161 ± 5.3	2.52 ± 1.19	0.57 ± 0.15
Valvular PS		104 ± 4.2	74 ± 5.8	110 ± 2.2	1	0.36 ± 0.04
PDA		136 ± 13.8	72 ± 4.5	105 ± 8.7	1.68 ± 0.77	0.41 ± 0.19
CAVSD		170	79	250	3.8	0.80

LVEDD, left ventricle end-diastolic diameter; RVEDD, right ventricle end-diastolic diameter; Qp/Qs, pulmonary to systemic blood flow ratio; RVP, right ventricle pressure; LVP, left ventricle pressure; VSD, ventricular septal defect; ASD, atrium septal defect; PDA, patent ductus arteriosus; PS, pulmonary stenosis; CAVSD, complete atrium ventricular septal defect.

Correlation Between Biomarkers and the Severity of Load in Echocardiography

The scatter plots show the correlation between biomarkers and estimated Qp/Qs in echocardiography (Fig. 1). The estimated Qp/Qs was correlated mildly with BNP ($r = 0.372$, $p < 0.01$) and ROM ($r = 0.304$, $p < 0.01$) levels, whereas, it was not correlated with BAP ($r = 0.119$, $p = 0.156$) levels.

Change in Biomarkers in Clinical Interventions

Medication caused a significant decrease in BNP levels from 69.2 (median: 28.9-396.1) to 43.2 pg/ml (median: 14.7-242) ($p < 0.01$), and also in ROM levels from 424 (median: 350-562) to 360 U.CARR (median: 282-436) ($p < 0.01$). Surgical repair caused a significant decrease in BNP levels from 106.4 (median: 48.5-242) to 13.1 pg/ml (median: 8.1-18) ($p < 0.01$). There was no significant change in ROM levels from 443 (median: 424-562) to 319 U.CARR (median: 269-371), but it tended to decrease. In contrast, there were no significant changes in BAP levels after both clinical interventions (Fig. 2).

Diagnostic Efficiency of BNP and ROMs

One of the surgical indications in left-right shunt disease was defined as Qp/Qs greater than 1.5 in echocardiography findings. The optimal decision threshold, estimated from ROC curves was 371 U.CARR (58% sensitivity, 90% specificity) for ROMs and 28.4 pg/ml (97% sensitivity 45% specificity) for BNP. Direct comparison of ROMs and BNP gave AUCs of 0.804 (95% CI: 0.711-0.896) and 0.700 (95% CI: 0.579-0.820), respectively. The difference between AUC for ROMs and BNP was not significant ($p = 0.205$). The ROC curves are shown in Fig. 3.

DISCUSSION

This study examined the ROMs and BAP levels for oxidative stress evaluation in children with CHD and explored their potential as new biomarkers. We clarified that the levels of ROMs were almost equivalent to those of BNPs.

In this study, all patients except one had acyanotic CHD, among which 86% had left-right shunt disease. Initial findings of echocardiography suggested volume overload in ASD, VSD, PDA, and CAVSD, also pressure overload in valvular PS as the primary etiologies. ROM levels are high even though ASD and valvular PS are generally less likely to raise BNP levels compared to the degree of cardiac load[19]. In VSD, PDA, and CAVSD, the ROM and BNP levels were high due to volume overload, and in valvular PS, they were high due to pressure overload. In contrast, BAP was within the

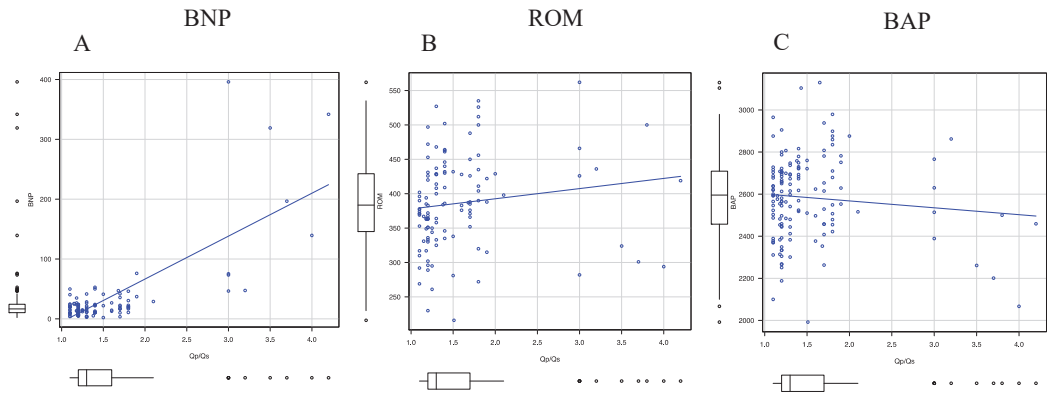

Fig. 1. Correlation between biomarkers and Qp/Qs obtained using the Pearson's correlation analysis. BNP, brain natriuretic peptide; ROMs, reactive oxygen metabolites; BAP, biological antioxidant potential.

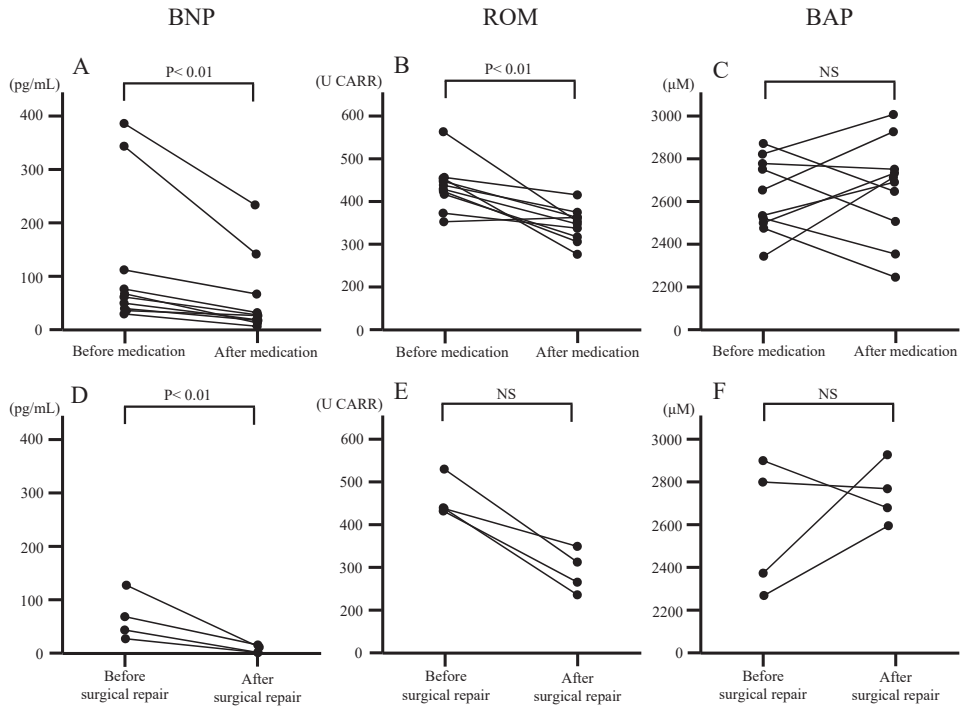

Fig. 2. Change in biomarkers before and after clinical interventions.
BNP, brain natriuretic peptide; ROMs, reactive oxygen metabolites; BAP, biological antioxidant potential.

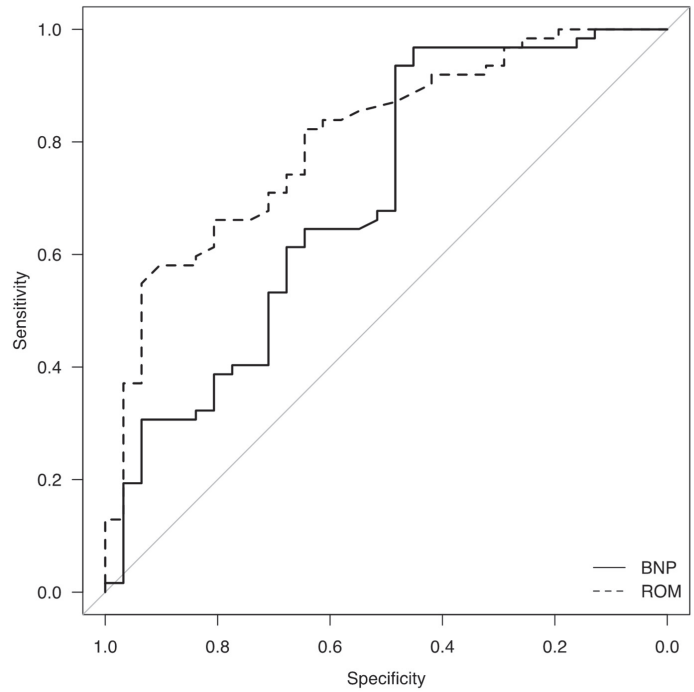

Fig. 3. Test results for the difference in AUC value between BNP and ROMs.
BNP, brain natriuretic peptide; ROMs, reactive oxygen metabolites; AUC, area under the curve.

normal range in all patients. Several studies have reported that myocardial injury is associated with oxidative stress[20–22]. However, in this study, ROM levels were high, even though the echocardiography showed good cardiac function, and CK-MB and H-FABP levels were almost within the normal range. This suggested that a high level of ROMs was due to volume and pressure overload. Pirinccioglu, A.G. *et al*. (2012) have confirmed that the levels of inflammatory cytokines increase in CHD[23]. They may be associated with the level of oxidative stress.

We determined the degree of force to predict the severity of the volume load using the estimated Qp/Qs and various echocardiography measurements in left-right shunt disease. BNP and ROM levels showed equivalent and mild correlation with these measurements; however, BAP was not correlated with them. We presumed that the inaccuracy in the estimated Qp/Qs using echocardiography and not cardiac catheterization resulted in a milder correlation for BNP ($r = 0.372$) than what was expected. Initially, all patients should have undergone cardiac catheterization to calculate accurate Qp/Qs; however, we avoided this because not all patients were surgical candidates. Furthermore, ROMs tended to have more variation than those exhibited by BNP, suggesting that it had a relatively small individual difference.

Further, regarding the change in biomarker levels via clinical interventions, both BNP and ROMs significantly decreased with medication. Since all the medications were diuretics, we assumed that the reduction of cardiac overload affected their decrease. There were few cases of surgical intervention with cardiopulmonary bypass (CPB), but this also decreased BNP and ROM levels. There was no significant increase or decrease in BAP levels. Caputo, M. *et al*. (2014) have reported that CPB leads to reoxygenation injury with significant myocardium damage and triggers a systemic inflammatory response[24]; therefore, we expected a temporary increase in ROMs[25]. However, the hemodynamics were radically improved and stabilized compared to those observed immediately after surgical repair, and it had a more substantial impact; therefore, ROM levels were significantly decreased.

We also compared the diagnostic efficiency of BNP and ROMs as biomarkers with the Qp/Qs ratio, which have been efficiently used for considering surgical indication; as a result, no significant difference was observed. However, the specificity of ROMs was relatively higher than that of BNP; therefore, it may help to determine the indication for surgical intervention.

Our data suggest that ROMs can be a new biomarker for oxidative stress evaluation in children with CHD, as their efficacy was almost equivalent to, but not superior to, previous biomarkers. ROMs are expected to be widely used in clinical settings because the blood volume needed is small, and measurement can be easily performed in a short time. However, since there are some individual variations, we presumed that it might be better to evaluate changes in the values in order to assess the transition of the medical condition of the same patient, rather than using it for diagnosis in terms of the degree of the cardiac load. Additionally, it may be possible to predict the surgical indication using the cut-off value while successfully combining with BNP. Further studies with long-term follow-up are required to verify the efficacy of ROM levels in assessing the medical condition of the same patient and their impact on CHD management strategy. Futhermore, the usefulness in adult congenital heart disease and pediatric arrhythmia may be considered in the future.

This study has several limitations. This is a single-institute research comprising a limited number of children with CHD. There were some variations in the initial levels of ROMs and BAP. The estimated Qp/Qs were not evaluated by catheterization

because some cases evidently did not require surgical interventions. Finally, follow-up data were collected for < 2 years, only three times, which might have introduced a bias into the results.

Nevertheless, these initial results show that ROM levels can be a new biomarker for oxidation stress evaluation in children with CHD at almost the same sensitivity as the previous biomarkers. However, there are some individual variations, and further long-term follow-up is required to determine their efficacy and impact on CHD management.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

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