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Oxidative Stress in Patients With Acute Coxsackie Virus Myocarditis

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XIE Bo[*,1], ZHOU Jun-Fu[#], LU Qun[#], LI Chong-Jian[#], AND CHEN Peng[#]

Huzhou Municipal Central Hospital, 106 Hongqi Road, Huzhou, 313000, Zhejiang, China; # The Second Affiliated Hospital, Medical College of Zhejiang University, 88 Jiefang Road, Hangzhou 310009, Zhejiang Province, China

Objective To study the state of oxidative stress in patients with acute coxsackie virus myocarditis (ACM), and to investigate the pathological chain reactions of a series of free radicals and oxidative and lipoperoxidative damages in their bodies. **Methods** Eighty ACM patients and 80 healthy adult volunteers (HAV) were enrolled in a case-control study, in which concentrations of nitric oxide (NO) in plasma, lipoperoxides (LPO) in plasma and LPO in erythrocytes (RBC), vitamin C (VC), vitamin E (VE) and β-carotene (β-CAR) in plasma as well as activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) in RBC were determined by using spectrophotometric assays. **Results** Compared with the average values (AV) of the above biochemical parameters (BP) in the HAV group, the AV of NO in plasma, and LPO in plasma and RBC in the ACM group were significantly increased (P=0.0001), while the AV of VC, VE, β-CAR, SOD, CAT and GSH-Px in the ACM group were significantly decreased (P=0.0001). The values of the above BP were used to estimate the relative risk ratio (RR) between the ACM group and the HAV group; the RR and its 95 % confidence interval were 12.467 (5.745~27.051), 4.333 (2.126~8.834), 6.517 (3.225~13.618), 3.310 (1.598~6.858), 31.000 (12.611~76.201), 4.663 (2.228~9.759), 11.769 (5.440~25.462), 3.043 (1.486~6.229) and 6.594 (3.045~14.281) respectively, and their P levels ranged from 0.002 to 0.0001. The results were as follows: D = 22.143 - 0.017SOD + 0.008NO + 0.244LPO in RBC, Eigenvalue = 13.659, Canonical correlation = 0.965, Wilks' λ = 0.068, χ^2 = 420.212, P = 0.0001. The correct rate of discrimination to the ACM group and to the HAV group was 87.5% and 95.0 %, respectively, and 91.3 % of originally grouped cases was correctly classified. **Conclusion** The findings in this study suggested that the oxidative stress in bodies of ACM patients was severely aggravated, and marked high oxidative constituents and low antioxidants and antioxidases in the human body might increase the relative risk of inducing acute coxsackie virus myocarditis, and measuring the values of NO in plasma, SOD and LPO in RBC might increase the correct rates of discriminatory analysis of the ACM.

Key words: Oxidative stress; Coxsackie virus; Myocarditis; Nitric oxide; Antioxidants; Antioxidases; Lipid peroxide; Free radicals

[1]Correspondence should to be addressed to Associate Prof. Xie Bo, Huzhou Municipal Central Hospital, 106 Hongqi Road, Huzhou, 313000, Zhejiang, China. Tel:86-572-2117518; E-mail: xwystced@hotmail.com.

[2]Biography of the first author:Xie Bo, M. D., Associate Professor, male, born on 1963-01-23, graduated form Zhejiang Medical University, having 19 papers published. Studying directions: Clinical cardiology.

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INTRODUCTION

Myocarditis is a common cardiac disease. Some authors reported that activities of superoxide dismutase and catalase in blood of patients and animals with viral myocarditis can produce some changes, and reactions of free radicals in their bodies are aggravated[1-4]. However, up to now, there are neither reports on changes of nitric oxide (NO) and other free radicals (FRs) in patients with acute coxsackie virus myocarditis (ACM), nor reports about relationship between oxidative and lipoperoxidative damages and the disease. To investigate oxidative stress in ACM patients' bodies, in a case-control study, the levels of some oxidative constituents were measured such as NO and LPO in plasma and in erythrocytes (RBC) of 80 ACM patients and 80 healthy adult volunteers (HAV). At the same time the levels of some antioxidants were determined such as vitamin C (VC), vitamin E (VE) and β-carotene (β-CAR) in their plasma as well as the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) in their RBC. The differences between the average values (AV) of the above biochemical parameters (BP) in the ACM group and in the HAV group were compared, and the values of the above BP were used to estimate the relative risk ratio (RR) between the ACM group and the HAV group. Additionally, the above BP was used to make the stepwise discriminatory analysis for 80 ACM patients and 80 HAV.

MATERIALS AND METHODS

Study Design

The design of case-control study was used in this study. In this design the principles of random sampling, control, replication and equilibrium as well as management factors, experimental effects and subjects were taken into account in order to obtain a comparatively objective and correct research conclusion[5].

Subjects

ACM. Eighty patients with acute coxsackie virus myocarditis were randomly sampled from 182 ACM patients, whose diagnosis was confirmed by blood serum virological immunoassay in Huzhou Municipal Central Hospital, and the Second Affiliated Hospital, Medical College of Zhejiang University and whose selection was made according to their inclusion and exclusion criteria[6-11], with "Select Cases — Random Sample of Cases" in "SPSS 10.0 for Windows". Their ages were 15~52 (34.1 ± 9.7) years old, 46 cases were male and 34 female. They were all confirmed by Coxsackie virus B infections, and ST-segment and T-wave abnormalities were found on their electrocardiogram; myocardial involvement was associated with symptoms of fatigue, dyspnea, palpitations, precordial discomfort, tachycardia and temperature elevation, and the first heart sound was muffled, but they had no specific complaints referable to the cardiovascular system[5,10]. All the patients were within normal ranges in their routine blood, urine and feces tests and radiographs, and disorders associated with brain, lung, liver, kidney and other organs were excluded, and diseases such as hypertension, chronic bronchitis, autoimmune disease, diabetes, atherosclerosis and tumors were also excluded.

HAV. Eighty healthy adult volunteers were randomly sampled from 400 HAV, whose diagnosis was confirmed by the Second Affiliated Hospital, Medical College of Zhejiang University, with "Select Cases—Random Sample of Cases" in "SPSS 10.0 for Windows".

Their ages were 15~50 (34.3 ± 9.5) years old, 45 were male and 35 female.

No significance was found between the average values of the age in the ACM group and in the HAV group by t test ($t = 0.090$, $P = 0.928$), and between the sexual proportions in the ACM group and the HAV group by χ^2 test ($\chi^2 = 0.025$, $P = 0.873$).

All the volunteers were within normal ranges in their routine blood, urine and feces tests, radiographs and electrocardiogram. Disorders associated with heart, brain, lung, liver, kidney and other organs were excluded, and diseases such as hypertension, chronic bronchitis, autoimmune disease, diabetes, atherosclerosis and tumors were also excluded.

All the subjects had never been exposed to radiation or toxic chemicals. They had not taken any antioxidant supplements such as vitamin C, vitamin E, β-carotene, ginkgo biloba, theo- polyphenols or other similar substances before they were enrolled in this study a month ago.

Methods

Fasting venous blood samples were collected in the morning for all the subjects and heparin sodium was added as anticoagulant, the separated plasma and erythrocytes were stored at -50℃ immediately.

The methods used in the determination of biochemical substances are outlined below, with additional details and references provided[12,13]. The colorimetry of thiobarbituric acid reactive substances (TBARS) was used to determine the content of LPO in plasma, and its concentration was expressed as μmol/L. The colorimetry of TBARS was used to determine the content of LPO in RBC, and its concentration was expressed as nmol/g·Hb. The coloration of d-naphthylamine was used to determine the NO concentration in plasma and it was expressed as nmol/L. The ferrozine coloration was used to determine the concentrations of VC and VE in plasma and they were expressed as μmol/L. The plasma β-CAR content was extracted with a mixture of ethyl alcohol and petroleum ether, and was assayed by colorimetry, and the β-CAR concentration was expressed as μmol/L. The spectrophotometry of inhibiting pyrogallol auto-oxidation was used to determine the activity of SOD in RBC, and its activity was indicated as U/g·Hb. The spectrophotometry of coloration of hydrogen peroxide and acetic acid-potassium bichromate was used to determine the activity of CAT in RBC, and the CAT activity was indicated as K/g · Hb. The improved Hafeman's spectrophotometry was used to determine the activity of GSH-Px in RBC, and its activity was expressed as U/mg · Hb.

In determination of the above biochemical substances, major analytical reagents, such as Vitamin C, Vitamin E, beta-carotene, 5, 6-diphenyl-3-(2-pyridyl)-1, 2, 4-triazinedisulfonic acid disodium salt, Cu/Zn-superoxide dismutase, catalase, α-naphthylamine, 1, 2, 3-trihydroxybenzene, 1, 1, 3, 3-tetraetho xypropane, 2-thiobarbituric acid, were purchased from Sigma Chemical Company®, USA; and the other analytical-grade reagents were produced in China, the fresh four-distilled-water was prepared with a quartz glass distilling apparatus, the main analytical instruments were Hewlett Packard 8453- spectrophotometer, USA, and UV-754-spectrophotometer, 721-spectrophotometer, and others.

In determination of the above biochemical substances, standardization of experiment, e. g. the same lot number of each reagent, the quality control, the identic lab assistant and the identical analytical apparatus, was used for each experiment in order to control and decrease error and bias, and to insure veracity.

All data were statistically analyzed with SPSS/10.0 for Windows and Statistica/6.0

for Windows statistic software. The parameters in this study were all normally distributed, and expressed as mean plus or minus standard deviation ($x \pm s$) and 95% confidence interval (95% CI). Hypothesis testing methods included independent-samples t test, χ^2 test, estimation of relative risk ratio, stepwise discriminant analysis, and so on. In the statistic analysis, the level of hypothesis testing (α) was $\leqslant 0.05$ in order to avoid false positives, and the power of hypothesis testing (*power*) was $\geqslant 0.80$ to avoid false negatives[15].

RESULTS

Comparison Between the AV ($\bar{x} \pm s$) of BP in ACM Group and in HAV Group

Compared with the AV of BP in the HAV group, the AV of NO in plasma, LPO in plasma and RBC in ACM group were significantly increased, and the AV of VC, VE and β-CAR in plasma as well as the AV of SOD, CAT and GSH-Px in RBC in ACM group were significantly decreased (Table 1).

The 95% CI of the AV of BP in ACM Group and in HAV Group

The lower limits of the 95% CI of the AV of NO in plasma, LPO in plasma and RBC in ACM group were greater than the upper limits of the 95% CI in the corresponding AV in HAV group. The upper limits of the 95% CI in the AV of VC, VE and β-CAR in plasma as well as SOD, CAT and GSH-Px in RBC in ACM group were less than the lower limits of the 95% CI in the corresponding AV in HAV group (Table 1).

Estimation of the Relative Risk Ratio (RR) of BP Values Between ACM Group and HAV Group, and the 95% CI of RR

Supposing the values of NO in plasma, and the LPO in plasma and RBC in ACM group $\geqslant (\bar{x} + s)$ of the values of the corresponding biochemical parameters in HAV group to be the reference criterion of the positive numbers of the ACM, and the values of VC, VE and β-CAR in plasma as well as SOD, CAT and GSH-Px in RBC in ACM group $\leqslant (\bar{x} - s)$ of the corresponding biochemical parameters in HAV group to be the reference criterion of the positive numbers of the ACM, the relative risk ratio (RR) between ACM group and HAV group was 3.043~31.000, and the 95% CI of RR was 1.486~6.229 to 12.611~76.201 (Table 2).

Discriminant Analysis of BP of ACM Patients and HAV

The above biochemical parameter in ACM patients and in the HAV used to make independent discriminant analysis suggested that the correct rate of discrimination to the ACM patients and HAV was 67.5%~83.8% and 63.8%~80.0%, respectively (Table 3).

Stepwise Discriminant Analysis of BP of 80 ACM Patients and 80 HAV

Let the values of the NO in plasma, and the LPO in plasma and RBC, and VC, VE and β-CAR in plasma as well as SOD, CAT and GSH-Px in RBC in ACM group, be expressed as "Result 1", and let the BP in HAV group be expressed as "Result 2", let BP be expressed as X^1, X^2, X^3, X^4, X^5, X^6, X^7, X^8 and X^9 respectively, the findings of the stepwise discriminant analysis were as follows: its unstandardized equation was $D = 22.143 + 0.008\ X^1 + 0.244$

TABLE 1

Comparison Between the AV ($\bar{x} \pm s$) of BP in ACM Group and in HAV Group

Group	n	Oxidative Substances			Antioxidative Substances					
		Plasma		RBC	Plasma			RBC		
		NO	LPO	LPO	VC	VE	β-CAR	SOD	CAT	GSH-Px
		(nmol/L)	(μmol/L)	(nmol/g · Hb)	(μmol/L)	(μmol/L)	(μmol/L)	(U/g · Hb)	(K/g · Hb)	(U/mg · Hb)
ACM	80	516.9 ± 128.6	12.56 ± 1.82	37.52 ± 6.71	44.72 ± 11.13	19.48 ± 4.57	1.36 ± 0.35	1871 ± 144	245.1 ± 61.5	21.68 ± 5.79
		(488.3~545.6)	(12.15~12.96)	(36.03~39.01)	(44.24~47.20)	(18.46~20.50)	(1.28~1.44)	(1839~1903)	(231.4~258.8)	(20.40~22.97)
HAV	80	350.1 ± 102.4	10.85 ± 1.57	28.22 ± 5.96	54.96 ± 13.68	26.36 ± 4.38	1.74 ± 0.43	2088 ± 149	303.9 ± 75.6	28.58 ± 7.11
		(327.3~372.9)	(10.50~11.20)	(26.90~29.55)	(51.92~58.01)	(25.38~27.33)	(1.64~1.83)	(2055~2122)	(287.0~320.7)	(27.00~30.16)
t^a		9.075	6.356	9.265	5.196	9.721	6.001	9.382	5.394	6.727
P		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

[a]Independent-samples t test; figures in parentheses are 95% CI.

TABLE 2

Estimate of the Relative Risk Ratio (RR) Between ACM Group and HAV Group, and the 95% CI of RR

Group	*n*	Plasma				RBC		Plasma						RBC					
		NO		LPO		LPO		VC		VE		β-CAR		SOD		CAT		GSH-Px	
		+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
ACM	80	55	25	40	40	51	29	33	47	62	18	38	42	54	26	33	47	41	39
HAV	80	12	68	15	65	17	63	14	66	8	72	13	67	12	68	15	65	11	69
Relative Risk Ratio (*RR*)		12.467		4.333		6.517		3.310		31.000		4.663		11.769		3.043		6.594	
(95 % CI)		(5.745~27.051)		(2.126~8.834)		(3.225~13.168)		(1.598~6.858)		(12.611~76.201)		(2.228~9.759)		(5.440~25.462)		(1.486~6.229)		(3.045~14.281)	
x^2		47.479		17.316		29.565		10.876		74.057		17.989		45.493		9.643		25.641	
P		0.0001		0.0001		0.0001		0.0010		0.0001		0.0001		0.0001		0.0019		0.0001	

Note. "+" indicated "number of cases" that the values of NO in plasma, and LPO in plasma and RBC in ACM group were $\geqslant (\bar{x} + s)$ of the values of the corresponding BP in HAV group, and that the values of VC, VE and β-CAR in plasma as well as SOD, CAT and GSH-Px in RBC in ACM group were $\leqslant (\bar{x} - s)$ of the corresponding BP in HAV group; while "-" expressed opposite those.

TABLE 3

Discriminant Analysis of the Biochemical Parameter of ACM Patients and HAV

Item	Plasma		RBC	Plasma			RBC		
	NO	LPO	LPO	VC	VE	β-CAR	SOD	CAT	GSH-Px
Eigenvalue	0. 521	0. 256	0. 543	0. 171	0. 598	0. 228	0. 557	0. 184	0. 286
Canonical Correlation Coefficient	0. 585	0. 451	0. 593	0. 382	0. 612	0. 431	0. 598	0. 394	0. 472
Wilks' λ	0. 657	0. 796	0. 648	0. 854	0. 626	0. 814	0. 642	0. 844	0. 777
x^2	66. 072	35. 860	68. 344	24. 844	73. 833	32. 341	69. 741	26. 621	39. 671
P	0. 0001	0. 0001	0. 0001	0. 0001	0. 0001	0. 0001	0. 0001	0. 0001	0. 0001
Constant	-3. 728	-6. 890	-5. 179	-3. 998	-5. 121	-3. 938	-13. 528	-3. 982	-3. 876
x	0. 009	0. 589	0. 158	0. 080	0. 223	2. 542	0. 007	0. 015	0. 154
Correct Rate of Discrimination to ACM (%)	72. 5	67. 5	72. 5	71. 3	83. 8	73. 8	76. 3	71. 3	75. 0
Correct Rate of Discrimination to HAV (%)	80. 0	75. 0	73 8	63. 8	76. 3	65. 0	77. 5	63. 8	67. 5

x^3 - 0.017 x^7, eigenvalue = 13.659, canonical correlation coefficient = 0.965, Wilks' λ = 0. 068, x^2 = 420.212, P = 0.0001. The correct rate of discrimination to ACM patients and HAV was 87.5% and 95.0%, and 91.3% of originally grouped cases was correctly classified.

DISCUSSION

Nitric oxide (NO) is a neurotransmitter, and plays a very important role in metabolism in the human body[12-17]. Vitamin C (VC), vitamin E (VE) and β-carotene (β-CAR) are the most important antioxidants in the human body[12-14,16-23], while superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) are the most important antioxidases in the human body[12-14,16-23]. They play an important role in scavenging oxygen free radicals (OFRs), such as superoxide anion radical (HO_2^-), hydxoyl radical ($^{\cdot}OH$), hydroperoxyl radical (HO_2), and other free radicals (FRs) as well as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) excessive in the human body, and they prevent physiological and pathological aggravation of a series of FRs chain reactions induced by excessive O_2^-, thereby protecting biological membranes of cells against oxidative damage and lipoperoxidative damages[12-14,16-23]. The metabolic state and functional status of NO, antioxidants and antioxidases in the human body are closely related with human health[12-23]. If their metabolic states are abnormal, the dynamic balance between the oxidative system and antioxidative system in the human body is affected or destroyed[12-23]. As a consequence, concentration of FRs may unusually increase and a series of FRs chain reactions may be pathologically aggravated in the human body; and this situation may lead to abnormal vital signs, and accelerate senility of human cells, thus inducing various diseases[12-23]. Marked decrease of antioxidant levels and antioxidase activities in the human body may cause metabolic disorders and pathological aggravation of a series of FRs chain reactions, resulting in the oxidative damage and lipoperoxidative damage of DNA, proteins, enzymes and biological membranes, and inducing a variety of diseases related to the abnormal reactions of FRs[12-14,16-24]. Lipoperoxides (LPO) is a product of peroxidation (auto-oxidation) of lipids exposed to oxygen, and lipoperoxidation is a source of free radicals and may be a cause of cancer, inflammatory diseases, atherosclerosis, aging, etc[21]. LPO and its metabolic products, such as malondialdehyde (MDA), conjugated diene (CD) and others, are important poisonous residual products, and significantly increased LPO, MDA and CD in the human body may strongly attack DNA, proteins, enzymes, biological membranes and others, leading to lipoperoxidative damages of biological membranes[12-14,18-23].

The findings in the present study showed that in ACM patients' bodies the metabolism of NO presented serious disorders, the dynamic balance between oxidation and antioxidation produced grave imbalance, and the oxidative stress caused pathological aggravation. There might be several interpretations. The cytokines, especially interleukin-I (IL-1) released by inflammatory cells, such as phagocytes like lymphocytes, neutrophilic granulocytes and macrophagocytes, in inflammatory reaction in cardiac muscle tissues and blood, might activate immediately inducible nitric oxide synthase (iNOS), and stimulate the synthesis and /or release of NO, thus producing a large amount of NO[13,15,16]. Excessive NO might inactivate antioxidases by combining with hydrosulfide group (-SH), and might combine with O_2^- to produce superoxide nitroso radical ($ONOO^-$), damaging cell functions and deactivating antioxidases with its extra-strong oxidative ability[12-17]. Moreover, excessive NO might be rapidly oxidized into nitrogen dioxide

(NO_2), and as a strong active catalyst in lipoperoxidation, NO_2 might aggravate lipoperoxidation of polyunsaturated fatty acids (PUFAs)[12-17,19-20]. Excessive OFRs and ROS might also directly attack PUFAs, leading to lipoperoxidation of a large number of PUFAs, and subsequent production of a mass of LPO, MDA and CD, which seriously damage cell functions[12-24]. Additionally, significant decrease in the synthesis or regeneration of GSH-Px decomposing LPO and marked weakness or loss of GSH-Px activity might also result in significant increase of LPO level in ACM patients[12-14,16-20]. Meanwhile, these inflammatory cardiac muscle cells and other organic substances in cardiac muscle tissues also release a large number of OFRs, ROS and other FRs, which provoke pathological aggravation of a series of FRs chain reactions[12-14,16-20].

In general, most antioxidative vitamins such as VC, VE, β-CAR must be acquired from dietary sources because they cannot be synthesized in the human body[12-14,17,22]. Additionally, ACM patients appeared to have poor appetite because of their body temperature elevation, so VC, VE and β-CAR absorbed by their bodies were decreased in quantity, resulting in a significant decrease of antioxidants in their bodies. Nevertheless, poor antioxidative vitamins were difficult to scavenge excessive OFRs, ROS and other FRs and to keep the dynamic balance between oxidation and antioxidation, thus resulting in physiological and pathological aggravation of a series of FRs chain reactions in patients[12-14,17,22]. Under such circumstance ACM patients had to make use of a great quantity of antioxidants and antioxidases in their bodies to scavenge excessive OFRs, ROS and other FRs, so that the dynamic balance between oxidation and antioxidation might be resumed and maintained, and oxidative stress, and damage as well as lipoperoxidative damage might be lessened[12-14,17,22].

The relative risk ratio (RR) between ACM group and HAV group, and the 95% CI of RR in this study showed that RR which ACM would be infected might increase by 3.3~11.5 times when values of NO in plasma, and LPO in plasma and RBC in ACM group were $\geqslant \bar{x} + s$ of the values of corresponding biochemical parameters in HAV group, and that RR which ACM would be infected might increase by 2.3~30 times when the values of VC, VE and β-CAR in plasma as well as SOD, CAT and GSH-Px in RBC in ACM group were $\leqslant \bar{x} - s$ of the corresponding biochemical parameters in HAV group. The findings further suggested that the changes of oxidative constituents, antioxidants and antioxidases in the human body played an important role in the pathogenic mechanism of acute coxsackie virus myocarditis, and that marked high oxidative constituents and low antioxidants and antioxidases were potential main risk factors to induce acute coxsackie virus myocarditis in some degree.

Stepwise discriminant analysis in this study suggested that the correct rate of discrimination to ACM patients and HAV was 87.5% and 95%, respectively, when the levels of NO in plasma, LPO and SOD in RBC were determined simultaneously.

In conclusion, the findings in this study suggest that serious abnormality of oxidative stress in ACM patients, and marked high oxidative constituents and low antioxidants and antioxidases in the human body would increase relative risk of inducing acute coxsackie virus myocarditis. We, therefore, recommend that in treating patients with acute coxsackie virus myocarditis antioxidants, suitable doses of vitamin C, vitamin E, β-carotene and others should be given daily in order to reduce potential oxidative and lipoperoxidative damage[12-14,16-20]. The findings also suggest that determination of the values of NO in plasma, SOD and LPO in RBC could increase the correct rates of discriminant analysis.

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