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**The Free Oxygen Radicals Test (FORT) to assess circulating oxidative stress in patients
with acute myocardial infarction**

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ABSTRACT

Background and aim: Reactive oxygen species (ROS) play an important role in the pathogenesis of many diseases including cardiovascular diseases. Several methods have been developed for the direct or indirect measurement of oxygen free radical and its by-products. The current study was designed to validate the new Free Oxygen Radicals Test (FORT) and to investigate the potential relationships between ROS and clinical or biological factors in male patients with acute myocardial infarction (AMI).

Methods: We analysed FORT values in samples from 66 patients with AMI.

Results: FORT values ranged from 324 to 1198 FORT units, with a median value of 581 (494-754) FORT units. In univariate analysis, FORT values were positively related only to LVEF <40% ($p=0.005$), levels of CRP ($r=0.438$, $p<0.001$) and peak CK ($r=0.274$, $p=0.028$). Multiple linear regression analysis showed that CRP ($p=0.023$), LVEF <40% ($p<0.001$) and the presence of diabetes ($p=0.039$) were independent predictors of serum FORT values. This statistical model can explain 45% of the variance in FORT values ($R^2=0.45$).

Conclusions: The FORT is a simple tool to assess circulating ROS in routine clinical practice. Oxidative conditions such as inflammation and diabetes are the major determinants of FORT values in patients with AMI.

Keywords: Reactive oxygen species, FORT, Myocardial infarction.

CONDENSED ABSTRACT

The current work was designed to study the FORT, which is a new method to determine levels of circulating ROS, and to investigate the potential relationships between ROS and clinical or biological factors in male patients with acute myocardial infarction (AMI). FORT values ranged from 324 to 1198 FORT units, with a median value of 581 (494-754) FORT units. In univariate analysis, FORT values were influenced by LVEF <40% ($p=0.005$), levels of CRP ($r=0.438$, $p<0.001$) and peak CK ($r=0.274$, $p=0.028$). Multiple linear regression analysis showed that CRP ($p=0.023$), LVEF <40% ($p<0.001$) and the presence of diabetes ($p=0.039$) were independent predictors of serum FORT levels ($R^2=0.45$).

ABBREVIATIONS LIST

ACE: ANGIOTENSIN-CONVERTING ENZYME

AMI: ACUTE MYOCARDIAL INFARCTION

BMI: BODY MASS INDEX

CRP: C-REACTIVE PROTEIN

EPR: ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

FORT: FREE OXYGEN RADICALS TEST

GRACE: GLOBAL REGISTRY OF ACUTE CORONARY EVENT

HDL: HIGH DENSITY LIPOPROTEIN

LDL: LOW DENSITY LIPOPROTEIN

LVEF: LEFT VENTRICULAR EJECTION FRACTION

NON-STEMI: NON-ST SEGMENT ELEVATION MYOCARDIAL INFARCTION

NT-proBNP: n-terminal pro-b-type natriuretic peptide

ORAC: OXYGEN RADICAL ABSORBANCE CAPACITY

RICO : observatoire des Infarctus de Côte-d'Or

ROS: REACTIVE OXYGEN SPECIES

STEMI: ST SEGMENT ELEVATION MYOCARDIAL INFARCTION

WBC: WHITE BLOOD CELL COUNT

INTRODUCTION

It has been suggested that reactive oxygen species (ROS) are involved in several pathological processes underlying cardiovascular diseases, particularly in the development of ischemia-induced myocardial reperfusion injury. When the balance between the cellular sources of ROS and the defence system is disturbed, ROS may react with cellular components like phospholipids and proteins. These reactions lead to lipid peroxidation and the oxidation of thiol groups resulting in the impaired function of both the cell membranes and various cellular proteins that influence responses to cardiac cell injury. Such responses include cell death, apoptosis and inflammation.^{1,2} Therefore, understanding the role of oxidative stress in the development and initiation of atherothrombotic complications remains paramount. Direct measurement of ROS is very difficult because many are present in low concentrations and have short half-lives. In general, the action of ROS is assessed indirectly by studying the effects of substances involved in the production or elimination of these species. Methods to analyse these substances include chemical assays using direct chemiluminescence and either direct or spin-trap electron paramagnetic resonance spectroscopy (EPR). A number of reviews have described these methods, their logistical constraints, and their use in the measurement of ROS.^{3,4} The Free Oxygen Radicals Test (FORT), (FORM[®], CR 2000, Callegari, Italy) was designed as a point-of-care system using freshly-collected heparinized whole blood.⁵ Strong correlations between the FORT index and other indices of inflammation or oxidative stress have been reported.^{6,7} The current study was designed to test in our clinical conditions the potential relationships between the ROS and clinical or biological factors in male patients with acute myocardial infarction (AMI).

METHODS

Patient population

The participants in this study were recruited from the observatoire des Infarctus de Côte d'Or (RICO) survey, a French regional survey for AMI, the details of which have been previously published.⁸ In the present study, consecutive male patients admitted to one of the aforementioned centres between 31st December 2008 and 1st March 2009 with acute myocardial infarction (ST-Elevation MI and Non-ST-Elevation MI) within 12 hours after symptom onset were included. MI was diagnosed according to European Society of Cardiology and American College of Cardiology criteria.⁹ Patients under 18 years of age, women, patients with acute infection, acute inflammation, severe renal impairment, or with a prior chronic treatment (or for at least 7 days) with vitamin C, steroids or non-steroid anti-inflammatory drugs, and those with a CRP > 30 mg/l were excluded from the study. Women

were excluded from the study because in women, FORT values vary considerably.¹⁰ Sixty-six patients with AMI were recruited during the study period. This study complied with the Declaration of Helsinki, was approved by the ethics committee of the University Hospital of Dijon, and each patient gave written consent before participation.

Data Collection

The following data were collected; patients' characteristics: age, sex, and cardiovascular risk factors (history of or treated hypertension, know history of diabetes, treated hypercholesterolemia, body mass index (BMI)(weight(kg)/height(m²)), current smoker defined as person who reports active smoking of cigarettes within 3 months prior to this admission, prior MI. Hemodynamic parameters on admission (heart rate, systolic blood pressure) and the presence of clinical heart failure as defined by Killip class > 1 were recorded. Patients were diagnosed with ST-segment elevation myocardial infarction (STEMI) when they had new or presumed new ST-segment elevation > 1 mm seen in any location or new left bundle branch block on the index or subsequent electrocardiogram. Anterior wall location was also recorded. Left ventricular ejection fraction (LVEF) was measured by echocardiography at 3 ± 1 days after admission using the Simpson method and dichotomized at 40% for more clinical relevance.¹¹ The Global Registry of Acute Coronary Event (GRACE) risk score was calculated with the following admission variables: age, heart rate, serum creatinine, systolic blood pressure, Killip class, cardiac arrest, ST-segment deviation and cardiac markers (http://www.outcomes-umassmed.org/grace/acs_risk.cfm).¹² The duration of ischemia was the time between symptom onset and reperfusion. Chronic medications before admission (Aspirin, β-Blocker, ACE inhibitors, statin) and the number of percutaneous coronary interventions during the in-hospital period were recorded.

Laboratory analysis

At the time of admission, 10 mL of blood were drawn from the cubital vein into additive-free (serum) containers. Samples were allowed to clot at room temperature for 30 minutes and centrifuged at 3000 rpm for 10 minutes at room temperature and then divided into aliquots. Samples were stored at -20°C until the time of assay (<6 Months). Median (25th-75th) time from symptom onset to blood sampling was 193 (110-280) min.

ROS assay was determined using the free oxygen radical monitor (FORM) and kit.¹³ The FORM indirectly measures the concentration of hydroperoxides. Briefly, the apparatus uses transition metals to catalyze the formation of free radicals (alkoxyl and peroxy radicals) from hydroperoxides in an acidic environment. The alkoxyl and peroxy radicals extract an

electron from the chromagen, N,N-diethyl-para-phenylendiamine. This leads to the formation of a coloured derivative that absorbs light at 505 nm. This absorption is measured using the FORM analyzer. The ROS concentration is reported in FORT units, where 1 FORT unit corresponds to 0.26 mg/L of H₂O₂. This measurement was made in some 5,000 healthy volunteers to determine normal ranges (Incomat Medizinische Geräte manual, 2002). Because we aimed to determine the effect of storage, and the variability and reproducibility of the FORT in serum samples, FORT baseline values were obtained immediately, and the subsequent values after 1 day, 1 week and after one, two and six months of storage were analyzed in 6 patients. Peripheral white and neutrophil blood cell (WBC) counts were determined. Homocysteine concentrations were determined by chemiluminescence on an Immulite 2000 analyzer (Diagnostic Products Corporation, Los Angeles, USA). Plasma NT-proBNP was determined by ELISA using Elecsys NT-proBNP sandwich immunoassay on Elecsys 2010 (Roche Diagnostics). CRP was measured on Dimension Xpand (Dade Behring) using immunonephelometry assay (analytical range 0.1–16 mg/dL). Glucose concentrations (enzymatic method (glucose oxidase)) and creatinine levels were measured on a Vitros 950 analyzer (Ortho Clinical Diagnostics, Rochester, NY). Total cholesterol, high-density lipoprotein cholesterol (HDL-C) and triglyceride (TG) concentrations were measured on a Dimension analyzer (Dade Behring, Newark, NE). The level of low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula. Peak plasma Creatine Kinase (CK) was assessed by sampling every 8 h during the first 2 days after admission and was expressed as % of values 10 times higher than the upper limit of normal (ULN) (men: 170 UI/L and women: 135 IU/L). The level of troponin Ic were measured using a technique based on colorimetry (Dimension analyzer, Roche). Glycated hemoglobin (HbA1c) was measured using a system of ion exchange HPLC. Baseline serum creatinine clearance was estimated using the Cockcroft-Gault formula.¹⁴

Statistical Analysis

The results are expressed as median values (25th-75th percentile) for continuous variables or as percentages for qualitative variables. The normality of distribution for each variable was analysed by the Kolmogorov-Smirnov test. The Spearman or the Pearson test was used to perform the correlation between continuous variables and FORT values. For binary variables, the median FORT levels were compared with a Mann-Whitney test. A multiple linear regression analysis was performed to determine the independent factors associated with FORT levels. All variables listed in table 1 were tested in univariate analysis and were

introduced into the multivariate model if the p value < 0.20. All analyses were performed using the Sigmastat Software (Jandel Inc.).

RESULTS

Study population: Among the 66 participants, median age was 60 (50-71). FORT values ranged from 324 to 1198 FORT units, with a median value of 581 (494-754) FORT units. Demographic characteristics, risk factors of the population, treatment on admission and hemodynamic parameters are presented in Table 1. Seventeen percent of the patients were diabetic, and 20% were obese. The biological parameters are summarized in Table 2. The correlation analysis between continuous variables and serum FORT concentrations are described in Tables 1 and 2. Age ($r=0.161$, $p=0.195$), the presence of diabetes ($p=0.102$), a history of MI ($p=0.181$), LVEF <40% ($p=0.005$) and β -blockers before admission ($p=0.053$), ST-Elevation MI ($p=0.058$), levels of CRP ($r=0.438$, $p<0.001$), the neutrophil count ($r=0.203$, $p=0.107$) and CK peak ($r=0.274$, $p=0.028$) were related to FORT values in univariate analysis and introduced into a multiple linear regression analysis. CRP ($p=0.023$), LVEF <40% ($p<0.001$) and the presence of diabetes ($p=0.039$) were independent predictors of FORT values in the serum (Table 3) ($R^2=0.45$).

DISCUSSION

Our study gives valuable insights into the technical feasibility of the FORT in the context of acute MI. The analysis of factors affecting serum levels of FORT showed that CRP, impaired LVEF and the presence of diabetes were independent predictors of high FORT values. Despite the small size of the study population, these variables explain a significant proportion of the variance in FORT values.

The FORT: This simple tool that allows the measurement of circulating ROS may encourage the study of relationships between ROS concentrations and markers of CV diseases. By using a chromophore that is easily oxidized, the FORT provides a non-specific but sensitive and easy measurement of ROS in human samples. First described at the end of the 90s, the FORT is a variant of the d-ROMs test¹⁵ and uses similar methods. Both tests have been used to assess oxidative stress levels in humans.^{6,10,15-20} However, only a few studies are available in the setting of cardiovascular disease. Cesarone et al. reported FORT values of 404 ± 42 IU in patients with peripheral artery disease.¹⁵ To the best of our knowledge, there are no data

about the use of this technique in patients with unstable coronary artery disease. Furthermore, our study is the first that specifically evaluates the relationship between FORT values and biological and clinical parameters in patients with AMI. Our work indicates that FORT values correlate with the presence of diabetes independently of other risk factors. Processes involving oxidative stress may be involved in coronary risk components that are increased in diabetics.²¹ Stranges et al. suggested, in a series of 1700 patients, that increased oxidative stress as measured by the ORAC test in individuals with clinical manifestations of atherosclerosis (AMI) was associated with abnormal glucose metabolism. In this case-control study, after adjustment for confounders, there was no significant difference in levels of oxidative stress measured into the 2 groups of patients (diabetics vs. non-diabetics). Interestingly, levels of oxidative stress were significantly higher only in the group of diabetic patients when compared with non-diabetics, irrespectively of sex or a history of MI.²² Our results also suggest a relationship between high FORT values and impaired LVEF. In experimental and clinical studies, impaired cardiac systolic function seems to be associated with an increase in oxidative stress.²³⁻²⁵

Oxidative stress and CRP: the relationship between the development of atherosclerosis, oxidative stress and inflammation is well documented thanks to various in-vivo studies.^{26,27} However, the relationship between CRP and oxidative stress remains poorly understood in AMI. In a series of obese patients without vascular disease, a strong relationship ($r=0.67$) was found between CRP levels and a urinary metabolite of Oxidized LDL (8-iso PGF 2 α).²⁸ In a population of 391 men, Hulth et al. reported a relationship between levels of oxidized LDL and intima-media thickness, the presence of carotid and/or femoral plaque and levels of CRP and TNF- α .²⁹ Abramson et al.⁶ in a series of 126 healthy adults reported an average FORT value of 136.8 ± 348 IU, and showed a significant relationship between this marker and CRP values ($r=0.29$). In the context of acute coronary syndromes, many studies have demonstrated the prognostic significance of markers of inflammation (CRP) and oxidative stress (MPO, malondialdehyde (MDA)-modified LDL), but no work has analyzed the relationship between the FORT index and markers of inflammation. Our results support the hypothesis of a close relationship between inflammation and this index, since we observed a strong correlation ($r=0.438$, $p<0.001$) between CRP values measured on admission and FORT values. A trend towards a positive relationship between FORT values and the neutrophil count ($r =0.203$, $p =0.107$) was also found, suggesting a link with the inflammatory process.

Study limitations: though our work focuses on a small number of patients, it took place in a context that reflects real world clinical practice. Given the lack of a control group (healthy

subjects, stable coronary artery disease) and the fact that only men were involved, further work needs to be done on this topic. Finally, the clinical interest of the FORT as a prognostic marker remains to be investigated.

CONCLUSIONS

In conclusion, we found a significant positive association between oxidative stress, as measured by the FORT, and CRP in acute MI. Our work suggests the potential interest of the FORT method as a biomarker in patients with acute myocardial infarction. The relationship between this index and factors related to oxidative stress, particularly diabetes and CRP, strengthens the reliability of our results.

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Table 1: Characteristics of the study population and relationship between levels of ROS measured by the FORT and various biological and clinical factors assessed on admission. (n=66). Results are expressed as (n (%)) or median ((25th-75th percentiles). * The p value expresses the correlation coefficient determined by the Spearman rank test for the continuous variables, or by the Mann-Whitney test for dichotomous variables.

	n=66	Median FORT (25 th , 75 th percentiles)	r	p *
Age (years)	60 (50-71)	*	0.161	0.195
BMI (kg/m ²)	26 (23-29)	*	-0.073	0.559
Heart rate (beat/min)	76 (64-90)	*	0.117	0.350
Systolic Blood Pressure (mmHg)	137 (120-163)	*	-0.024	0.853
GRACE Risk Score	120 (93-148)	*	0.114	0.363
Duration of ischemia (min)	183 (110-280)	*	-0.113	0.363
LVEF (%)	59 (50-60)	*	-0.206	0.096
Hypertension	31 (47%)			
Yes (n = 31)		567 (494-802)		0.913
No (n = 35)		582 (506-741)		
Diabetes	11 (17%)			
Yes (n = 11)		720 (558-933)		0.102
No (n = 55)		576 (494-741)		
Dyslipidemia	29 (44%)			
Yes (n = 29)		576 (488-748)		0.727
No (n = 37)		585 (501-764)		
Prior MI	6 (9%)			
Yes (n = 6)		517 (376-638)		0.181
No (n = 60)		583 (499-760)		
Current smoker	35 (53%)			
Yes (n = 35)		585 (496-760)		0.928
No (n = 31)		580 (494-747)		
Obesity	13 (19%)			
Yes (n = 13)		640 (492-764)		0.723
No (n = 53)		580 (494-746)		
LVEF < 40%	5 (8%)			
Yes (n = 5)		810 (796-940)		0.005
No (n = 61)		576 (494-722)		
Aspirin	6 (9%)			
Yes (n = 6)		540 (494-700)		0.662
No (n = 60)		581 (496-762)		
β-Blocker	8 (12%)			
Yes (n = 8)		494 (415-620)		0.053
No (n = 58)		587 (514-764)		
ACE inhibitor	15 (21%)			
Yes (n = 15)		558 (494-629)		0.277
No (n = 51)		589 (506-764)		
Statins	11 (17%)			
Yes (n = 11)		638 (505-837)		0.434
No (n = 55)		576 (494-742)		
Killip >1 on admission	6 (9%)			
Yes (n = 6)		760 (638-1062)		0.034
No (n = 60)		576 (494-737)		
ST-Elevation MI	33 (50%)			
Yes (n = 33)		596 (566-768)		0.058
No (n = 33)		527 (478-746)		
Anterior wall location	17 (26%)			
Yes (n = 17)		596 (517-821)		0.270
No (n = 49)		566 (494-741)		

LVEF: Left Ventricular Ejection Fraction; BMI: Body Mass Index; MI: Myocardial Infarction

Table 2: Biological data of the study population and relationship with ROS levels measured by the FORT determined by the Spearman rank test (n=66). Results are expressed as median ((25th-75th percentiles).

	Median value	Total (n=66)	
		r	p
CRP (mg/l)	3.7 (2.1-8)	0.438	<0.001
Peak Creatinine kinase (UI/l)	637 (222-1457)	0.274	0.028
Neutrophil Count (10 ⁹ cells/L)	3.1 (1.2-8.4)	0.203	0.107
Admission Glucose (mmol/l)	6.29 (5.61-9.44)	0.180	0.148
WBC (10 ⁹ cells/L)	5.9 (3.4-12.2)	0.138	0.275
Homocysteine (mg/l)	13 (10-18)	0.089	0.537
HbA1c (%)	5.7 (5.5-6.1)	0.072	0.600
NT-proBNP (pg/ml)	311 (112-1023)	0.072	0.571
HDL Cholesterol (mg/dl)	0.40 (0.37-0.47)	0.047	0.721
LDL Cholesterol (mg/dl)	1.29 (1.06-1.51)	-0.036	0.789
Total Cholesterol (mg/dl)	2.05 (1.7-2.26)	-0.108	0.411
Creatinine clearance (ml/min)	86 (64-107)	-0.154	0.216
Triglycerides (mg/dl)	1.01 (0.56-1.61)	-0.232	0.367

NT-proBNP : n-terminal pro-b-type natriuretic peptide, CRP : C-Reactive Protein, LDL-C, low density lipoprotein, HDL-C, high density lipoprotein, WBC : white blood cell count.

Table 3: Determinants of the FORT value by multiple linear regression (n=66).

	Total Population	
	Coefficient β	p
LVEF < 40%	+0.421	<0.001
CRP	+0.265	0.023
Diabetes	+0.232	0.039
Peak Creatinine kinase	+0.172	0.177
Age	+0.133	0.301
Neutrophil Count	+0.071	0.589
ST-Elevation MI	-0.033	0.785
β -Blocker on admission	-0.134	0.265
Prior MI	-0.197	0.119
R^2	0.45	*

LVEF: Left Ventricular Ejection Fraction; CRP: C-Reactive protein; STEMI: ST-Elevation Myocardial Infarction.

Figure 1: Direct measurement of H_2O_2 with the FORT system.

Figure 2: Linearity of the Direct measurement of H_2O_2 with the FORT system.

Figure 3: Analysis of the correlation between FORT values and CRP. (n=66).