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ORIGINAL RESEARCH

Glutathione Infusion Before and 3 Days After Primary Angioplasty Blunts Ongoing NOX2-Mediated Inflammatory Response

Gaetano Tanzilli, MD; Alessio Arrivi, MD; Attilio Placanica, MD; Nicola Viceconte, MD; Vittoria Cammisotto, PhD; Cristina Nocella, PhD; Francesco Barillà, MD; Concetta Torromeo, MD; Giacomo Pucci, MD; Maria Cristina Acconcia, MD; Antonino Granatelli, MD; Stefania Basili, MD; Marcello Dominici, MD; Carlo Gaudio, MD; Roberto Carnevale, PhD*; Enrico Mangieri, MD*

BACKGROUND: Glutathione is a water-soluble tripeptide with a potent oxidant scavenging activity. We hypothesized that glutathione administration immediately before and after primary angioplasty (primary percutaneous coronary intervention) could be effective in modulating immune cell activation, thereby preventing infarct expansion.

METHODS AND RESULTS: One hundred consecutive patients with ST-segment–elevation myocardial infarction, scheduled to undergo primary percutaneous coronary intervention were randomly assigned before the intervention to receive an infusion of glutathione (2500 mg/25 mL over 10 minutes), followed by drug administration at the same doses at 24, 48, and 72 hours elapsing time or placebo. Total leukocytes, NOX2 (nicotinamide adenine dinucleotide phosphate oxidase 2) activation, NO bioavailability, cTpT (serum cardiac troponin T), hsCRP (high-sensitivity C-reactive protein), and TNF-α (tumor necrosis factor α) levels were measured. Left ventricular size and function were assessed within 120 minutes, 5 days, and 6 months from percutaneous coronary intervention. Following reperfusion, a significant reduction of neutrophil to lymphocyte ratio ($P<0.0001$), hsCRP generation ($P<0.0001$), NOX2 activation ($P<0.0001$), TNF-α levels ($P<0.001$), and cTpT release ($P<0.0001$) were found in the glutathione group compared with placebo. In treated patients, blunted inflammatory response was linked to better left ventricular size and function at follow-up ($r=0.78$, $P<0.005$).

CONCLUSIONS: Early and prolonged glutathione infusion seems able to protect vital myocardial components and endothelial cell function against harmful pro-oxidant and inflammatory environments, thus preventing maladaptive cardiac repair and left ventricular adverse remodeling.

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Key Words: immune cells ■ inflammation ■ left ventricular remodeling ■ oxidative stress ■ reperfusion injury ■ STEMI

Following acute myocardial infarction, an appropriate containment and timely resolution of inflammation is mandatory for optimal tissue healing.[1] Early inflammatory activation is a necessary event for the transition to a proper reparative and proliferative program. On the cellular level, the early burst of neutrophil activity is followed by inflammatory monocytes/macrophages recruitment and the ability to remove necrotic tissue. At a later phase, mononuclear cells with less inflammatory phenotypes predominate, and cardiac repair proceeds toward a reparative phase with resolution of inflammation.[2,3] A misbalance of this transition phase may compromise wound healing, thereby favoring maladaptive left ventricular (LV) remodeling.[3,4] Acute reoxygenation

Correspondence to: Roberto Carnevale, PhD, Department of Medical-Surgical Sciences and Biotechnologies, Sapienza University of Rome, 04100 Latina (LT), Italy. E-mail: roberto.carnevale@uniroma1.it

*R. Carnevale and E. Mangieri are co–senior authors.

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CLINICAL PERSPECTIVE

What Is New?

- The effect of glutathione infusion during ST-segment–elevation myocardial infarction reperfusion has not been established.
- Glutathione administration was beneficial for homeostatic control of the immune system pathways and blunted oxidative and inflammatory toxic environment, thereby favoring myocardial cell survival.

What Are the Clinical Implications?

- After ST-segment–elevation myocardial infarction reperfusion, early and prolonged glutathione administration could represent a novel therapeutic target for the prevention of maladaptive left ventricular healing and oxidative damage.
- Strategies aimed to restore glutathione depletion and to recalibrate the leukocyte response toward optimal healing could be of value in counteracting detrimental effects of acute reperfusion on cardiac size and function.

Nonstandard Abbreviations and Acronyms

cTpT	cardiac troponin T
MPG	myocardial perfusion grade
NLR	neutrophil-to-lymphocyte ratio
NOX2	nicotinamide adenine dinucleotide phosphate oxidase 2
p-PCI	primary percutaneous coronary intervention
ROS	reactive oxygen species
TIMI	Thrombolysis in Myocardial Infarction

of ischemic myocardium triggers a heightened and long-lasting oxidant activity resulting in expansion of inflammatory response. A massive leukocyte infiltration of ischemic myocardium occurs, thus promoting further tissue damage and cardiomyocyte loss.[5,6]

Reactive oxygen species (ROS) during myocardial ischemia or reperfusion may originate from the mitochondria, xanthine oxidase, and the phagocytic nicotinamide adenine dinucleotide phosphate oxidase. The NOX2 (nicotinamide adenine dinucleotide phosphate oxidase 2) isoform has a key role for cellular ROS production, and upon cell stimulation, NOX2 releases a small peptide, the soluble NOX2-derived peptide. It has been previously demonstrated that the release of the soluble NOX2-derived peptide in cells, such as platelets and neutrophils, was directly correlated with ROS production and therefore with NOX2 activity.[7]

There is a growing body of evidence supporting the important roles of NOX2-derived ROS in regulating cytokine signaling. Specifically, NOX2 activation can facilitate redox activation of the TNF-α (tumor necrosis factor-α) receptor and then its signaling pathways.[8] TNF-α, a pivotal cytokine in the inflammatory cascade, has been reported to trigger interactions between invading monocytes and vascular endothelial cells, which subsequently induce endothelial apoptosis in the circulation.[9] Moreover, the elevation of TNF-α during acute myocardial infarction may potentially impair NO activity in coronary flow regulation, thus contributing to further deterioration of cardiac function.[10]

Glutathione has emerged as the main defense line for the maintenance of the appropriate cell redox environment to repair oxidative modifications that affect cell function and survival.[11,12] Furthermore, adequate glutathione levels seem essential for the optimal functioning of the immune system.[13,14] During cardiac repair, lymphocyte activation, presumably driven by recognition of cardiac autoantigens, facilitates tissue healing by modulating innate immune cell recruitment to the infarcted myocardium.[15] Deficiency of glutathione system activity prevents lymphocytes from reprogramming their metabolism to meet the rising energy needs to promote the proliferative response and differentiation.[16] We then sought to determine whether early and prolonged glutathione administration after reintroduction of molecular oxygen to the myocardial infarct areas could be effective in modulating immune cell activation, thereby preventing adverse LV remodeling at follow-up. Among total leukocytes, the neutrophil-to-lymphocyte ratio (NLR), by combining the change in neutrophils and lymphocytes recruitment, may be expression of the balance between the sequences of cellular events accompanying cardiac repair.[17,18] Moreover, we evaluated if glutathione administration reduces endothelial damage by reducing NOX2-mediated inflammatory process.

METHODS

The data, analytical methods, and study materials that support the conclusions of this study will be available to other researchers for the purpose of replicating the procedures and reproducing the results through reasonable request by contacting the corresponding author.

Patients and Study Design

Over a 12-month period (March 2017–March 2018), 100 out of 271 consecutive patients with ST-segment–elevation myocardial infarction (STEMI) aged >18 years, both sexes, referred to the 3 enrolling centers for

primary angioplasty (primary percutaneous coronary intervention [p-PCI]) were screened to enter the glutathione 2014 trial (European Union Drug Regulating Authorities Clinical Trials Database [EudraCT] number 2014-004486-25). Exclusion criteria were: symptom duration >12 hours (n=21), rescue PCI (n=20), cardiogenic shock (n=5), left main disease (n=7), evidence of coronary collateral vessels (Rentrop score of 2 or 3 for the area at risk) (n=7), prior myocardial infarction (n=13), saphenous venous graft occlusion (n=6), estimated glomerular filtration rate <30 mL/min (n=17), acute infection (n=3), treatment with systemic corticosteroids (n=6) or oral anticoagulants (n=13), malignancy (n=4), in-stent thrombosis (n=5), and lack of consent to participate (n=33). Additionally, 11 patients were ineligible because no blood samples were collected before the start of the procedure. Finally, a total of 100 patients were enrolled (Figure 1**)**. The study has been planned according to principles of the Declaration of Helsinki. Agenzia Italiana del Farmaco authorization and single ethic committee approval were obtained from all of the centers participating in the study. The coordinating center designed the protocol. An external core laboratory processed the data. After giving their informed consent, 50 patients were randomly assigned to receive drug therapy, and 50 patients were randomly assigned to receive a placebo. After baseline collection of peripheral blood samples, randomized

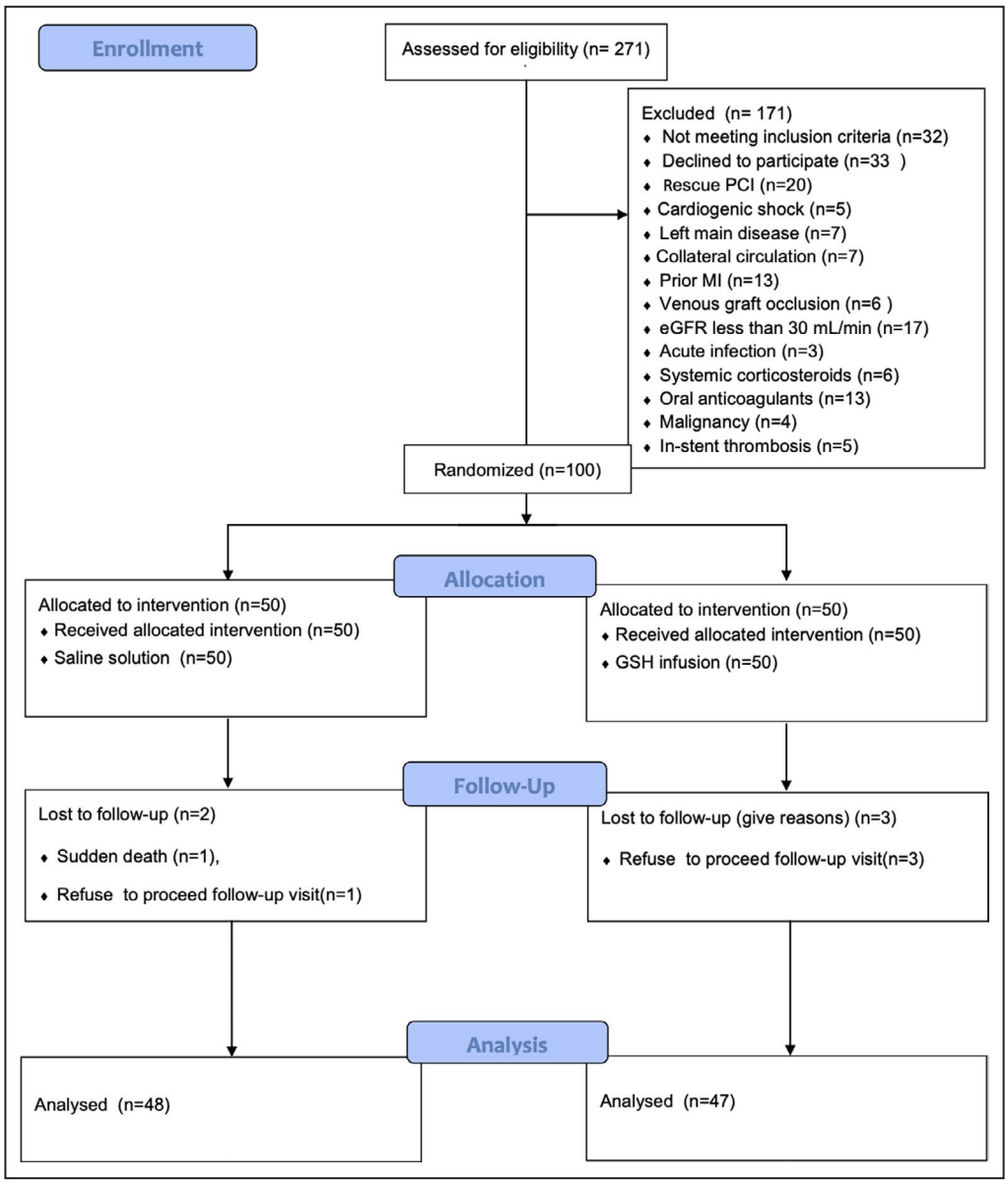

Figure 1. CONSORT flowchart.
CONSORT indicates Consolidated Standards of Reporting Trials; eGFR, estimated glomerular filtration rate; GSH, glutathione; MI, myocardial infarction; and PCI, percutaneous coronary intervention.

patients received an intravenous infusion of glutathione (2500 mg/25 mL of glutathione sodium salt; Biomedica Foscama Group, Rome, Italy) or placebo (saline solution) over 10 minutes before p-PCI. The 2 solutions appeared identical in size and color to ensure blinding. Study participants, investigators, and the laboratory staff remained blinded until the statistical analysis was performed by an independent researcher who was not involved in the study. No side effects were observed during or after glutathione or placebo infusion.

Primary Percutaneous Coronary Interventions

After percutaneous access was obtained, an intravenous bolus of 5000 U of unfractionated heparin was administered, with sufficient supplements (if necessary) to maintain an activated clotting time of ≥250 seconds during interventions. Patients underwent p-PCI according to the standard protocols. The use of thrombus aspiration (glycoprotein IIb/IIIa inhibition) was left to the discretion of the treating physician. Multivessel PCI was performed in a staged fashion 7 to 10 days from the index procedure. All patients had drug-eluting stents implanted in treated vessels. TIMI (Thrombolysis in Myocardial Infarction) flow grading (0–3) and TIMI myocardial perfusion grade (MPG; 0–3) were assessed after p-PCI as previously described.[19] An external core laboratory processed the data. Digital angiograms were analyzed off-line with the use of an automated edge detection system (Cardiovascular Medical System; MEDIS Imaging Systems, Leiden, the Netherlands). Postoperative angiographic no reflow was defined as TIMI flow grade <3 (with any MPG grade) or TIMI flow 3 with MPG 0 to 1. After interventions, glutathione was infused at the same doses at 24, 48, and 72 hours elapsing time. Further blood samples were obtained at the end of the procedure and 5 days from the index procedure. After 60' to 90', a postprocedural 12-lead ECG for ST measurement was performed.

Randomization and Blinding

An individual not involved in the study assigned codes (using a computer-generated random sequence) to the study treatment with a random allocation of patients to an intravenous infusion of glutathione or placebo (saline solution) before p-PCI. The interventional cardiologists who performed the p-PCI, those who analyzed digital angiograms, and the laboratory technicians were unaware of study-treatment allocation.

Primary End Point

The primary end point consisted of the assessment of the effects of the glutathione infusion on the reduction of the oxidative status and inflammatory cells effector activity after PCI. Time point(s) of evaluation of these end points were before the procedure, and 2 and 5 days from p-PCI.

Secondary End Points

The secondary end points included the assessment of (1) changes in serum cTpT (cardiac troponin T) and hsCRP (high-sensitivity C-Reactive protein) (time point(s) of clinical evaluation of this end point were: at admission, before the procedure, and thereafter once a day up to 5 days) and (2) LV remodeling and its association with the degree of oxidative/inflammatory induced myocardial cell injury. Time point(s) of evaluation of this end point were within 120 minutes, 5 days, and 6 months from p-PCI.

Peripheral Blood Samples

Blood samples were drawn from the antecubital vein before the start of the procedure and after stent deployment in all patients and then collected into tubes without anticoagulant or with 3.8% sodium citrate, lithium heparin, and EDTA, and centrifuged at 300*g* for 10 minutes to obtain the supernatant. All plasma and serum aliquots were stored at −80°C in appropriate cuvettes until assayed. Complete hemochrome was evaluated using standard methods. NLR was calculated by dividing neutrophils by lymphocytes count. Serum cTpT levels were measured using an ELISA kit (Elabscience). Immunochemical luminescent assays (Immulite 2000; Medical Systems SpA, Genoa, Italy) were used to measure hsCRP (detection limit: 0.10–150.0 mg/L).

Echocardiography Data

LV end-diastolic volume (LVEDV; milliliters per square meter), LV end-systolic volume (milliliters per square meter), and LV ejection fraction (percent) were calculated by the biplane Simpson rule. The mean values of the 3 measurements were used for statistical evaluation. Echocardiographic data were stored digitally and analyzed by 2 expert readers blinded to all other clinical data. LV remodeling was calculated according to the following formula: (LVEDV at 6 months−LVEDV at baseline/LVEDV at baseline) × 100.[20] The cutoff value for adverse LV remodeling was set as an increase in LVEDV ≥15%.

Severity of mitral regurgitation was graded from mild to severe using color-flow Doppler, adding supportive signs and quantitative parameters according to the American Society of Echocardiography guidelines.[21]

Soluble Nicotinamide Adenine Dinucleotide Phosphate Oxidase-Derived Peptide Activity Evaluation

NOX2 activity was measured as soluble nicotinamide adenine dinucleotide phosphate oxidase–derived

peptide activity in plasma samples with an ELISA method as previously described.[22] Values are expressed as picograms per milliliter; intra-assay and interassay coefficients of variation are <10%.

Tumor Necrosis Factor Assay

TNF-α concentration in plasma samples was measured using a commercial ELISA kit (Diaclone, Besancon, France). Values are expressed as picograms per milliliter, and the intra-assay and interassay coefficients of variation are 3.2% and 10.9%, respectively.

NO Assay

NO was evaluated in plasma or human umbilical vein endothelial cell supernatant by a colorimetric assay kit (Abcam, Cambridge, UK) used to determine the metabolites of NO (nitrites and nitrates; NOx). Intra-assay and interassay coefficients of variation are 2.9% and 1.7%, respectively.

H_2O_2 Production

H_2O_2 was measured in human umbilical vein endothelial cell supernatant by using a colorimetric assay as described previously.[7]

Cell Culture and Reagents

Human umbilical vein endothelial cells (Lonza; cat. no. N.CC-C2517A) were cultured in complete EGM-2 medium (Lonza; cat. no. CC-3162) and 10% FBS and were plated at a density of 2500 cells/cm^2. The experiments were conducted on 3 different batches of human umbilical vein endothelial cells. An equal number of cells (3×10^3 cells/cm^2) were treated for 2 hours with culture medium in the absence of serum and then treated with culture medium (without serum) with or without different glutathione concentrations (5 mmol/L or 10 mmol/L) for 3 hours, followed by 2 hours of treatment with TNFα (50 ng/mL). Supernatants were removed for NO and H_2O_2 evaluation.

Sample Size

The protocol assumed that the decrease of the NOX2 activation would be 30% for treated patients as compared with controls. Under this assumption, by considering an expected loss to follow-up of 5 patients for each group, a randomized sample size of 100 patients (equal allocation of 50 patients to the glutathione infusion and placebo groups) yielded ≈80% power to declare the treatment provides difference at a 2-sided 0.05 level of significance. Sample size calculation was performed using the software nQuery Advisor version 5.0 (Statistical Solutions, Saugus, MA).

Statistical Analysis

The quantitative variables were expressed as mean+SD; data were synthesized also as median and interquartile range. In the overall population as well as in both groups, all continuous data were checked for normal distribution. In addition, the Levene test was used for testing homogeneity of variance between groups. Because of the lack of normal distribution and homogeneity of variance, both groups were compared by nonparametric methods. Furthermore, at any time, Kruskal-Wallis analyses were used to compare differences between the 2 groups. In individual groups, the differences measured at any time were compared by Wilcoxon test. Bonferroni correction for multiple comparison was applied to control for the increase in the experiment-wise type I error probability. Correlation analysis was performed with Spearman tests. A P value ≤0.05 was considered statistically significant. Statistical analysis was performed using a general-purpose statistical software package.

RESULTS

Ninety-five patients completed the 6 months of follow-up: 1 patient (untreated) died suddenly 5 months after revascularization, and 4 patients (1 untreated and 3 treated) refused to proceed to programmed follow-up visits. Clinical and angiographic characteristics and postoperative medical therapies of enrolled patients are shown in Tables 1, 2, and 3. The baseline characteristics were well balanced between the 2 groups.

Total Leukocytes and Subtypes Count

Total and differential leukocytes counts are summarized in Table 4. On admission, total leucocytes and subtypes count was similar in both groups with increased neutrophils number, decreased lymphocytes, and normal monocytes values. Accordingly, mean NLR value did not differ between treated versus control groups

Table 1. Clinical Characteristics of the Study Population

Variables	Glutathione Group, n=50	Placebo Group, n=50	*P* Value
Age, y, mean±SD	65±10	67.8±10.1	0.11
Men, n (%)	38 (76)	37 (74)	0.82
BMI, mean+SD	26.6±3.1	25.9±4.2	0.15
Killip class ≥3, n (%)	3 (6)	2 (4)	0.64
Diabetes mellitus, n (%)	12 (24)	12 (24)	1.00
Hypertension, n (%)	28 (56)	23 (46)	0.32
Dyslipidemia, n (%)	26 (52)	28 (56)	0.69
Statin use, n (%)	24 (48)	23 (46)	0.84
Smokers, n (%)	34 (68)	30 (60)	0.41

BMI indicates body mass index (weight in kilograms divided by the square of the height in meters).

Table 2. Angiographic Parameters

Variables	Glutathione Group, n=50	Placebo Group, n=50	*P* Value
Ischemia time, min, mean±SD	247±48	262±67	0.77
Thrombus burden ≥3, n (%)	21 (42)	23 (46)	0.69
Thrombus aspiration, n (%)	20 (40)	20 (40)	1.00
GP IIb/IIIa inhibitors, n (%)	7 (14)	6 (12)	0.21
MVD			
2 vessels, n (%)	14 (28)	14 (28)	1.00
3 vessels, n (%)	9 (18)	5 (10)	0.25
Staged PCI, n (%)	22 (46)	18 (38)	0.76
IRA			
LAD, n (%)	20 (44)	22 (47)	0.86
LCx, n (%)	12 (25)	15 (32)	0.73
RCA, n (%)	15 (31)	11 (21)	0.58
Postprocedural TIMI flow grade, mean±SD	2.93±0.25	2.91±0.27	0.72
No reflow, n (%)	4 (8)	9 (18)	0.23
MPG, mean±SD	22.55±0.58	2.33±0.64	0.1
MPG=3	28 (56)	17 (34)	0.044

Glutathione means reduced glutathione. Ischemia time was defined as the timing between symptom onset and balloon inflation. GP indicates glycoprotein; IRA, infarct-related coronary artery; LAD, left anterior descending coronary artery; LCx, left circumflex coronary artery; MBG, myocardial perfusion grade; MVD, multivessel coronary artery disease; PCI, percutaneous coronary intervention; RCA, right coronary artery; and TIMI, Thrombolysis in Myocardial Infarction.

(5.33±1.9 versus 5.1±1.6, respectively; *P*=0.24). Five days after reperfusion, in the treated group, NLR significantly decreased compared with baseline (2.68±1.15 versus 5.33±1.9, respectively; *P*=0.00001), with an opposite behavior of cell subtypes characterized by the neutrophils decrease (5.8±1.45 versus 7.44±2.27, respectively; *P*<0.00001), lymphocytes increase (2.3±0.57 versus 1.63±0.6, respectively; *P*<0.00001), and slight reduction of monocytes (0.59±0.47 versus 0.71±0.45, respectively; *P*=0.21). Conversely, total and differential cell count remained unchanged in controls. Accordingly, in untreated patients, mean NLR values remained significantly higher than those observed in treated patients (4.9±1.3 versus 2.68±1.15, respectively; *P*<0.00001; Figure 2).

Indexes of Oxidative Stress, Inflammation, and Endothelial Dysfunction

Baseline soluble NOX2-derived peptide levels did not differ between treated patients and controls. After PCI, in the glutathione group, a progressive significant reduction of soluble NOX2-derived peptide generation was observed as compared with the controls, in which sustained serum levels persisted at the prespecified time points (23.2±6.8 pg/mL versus 33.2±8.7 pg/mL, respectively; *P*<0.001) (Table 5 and Figure 3A). Similarly, a significant reduction of TNF-α after PCI in glutathione group was observed as compared with the control group (45.6±12.4 pg/mL versus 64.4±11.6 pg/mL, respectively; *P*<0.0001) (Table 5 and Figure 3B). In both

Table 3. Medication at Discharge

Discharge Medication	Glutathione Group, n=50	Placebo Group, n=50	*P* Value
Aspirin, n (%)	50 (100)	50 (100)	1.00
P2Y12 inhibitors, n (%)	50 (100)	50 (100)	1.00
Statins, n (%)	49 (98)	48 (96)	0.56
β-Blockers, n (%)	45 (90)	47 (94)	0.46
ACE inhibitors, n (%)	43 (86)	44 (98)	0.77
ARBs, n (%)	6 (12)	6 (12)	1.00
Calcium channel blockers, n (%)	7 (14)	7 (14)	1.00
Organic nitrates, n (%)	6 (12)	4 (8)	0.50
Diuretics, n (%)	6 (12)	5 (10)	0.75
Spironolactone, n (%)	4 (8)	4 (8)	1.00

Glutathione means reduced glutathione. ACE indicates angiotensin-converting enzyme; and ARB, angiotensin II receptor blocker.

Table 4. Total and Differential Leukocytes Count

		P Value		*P* Value	*P* Value
Variables	Treated, n=50	Within Group	Controls, n=50	Within Group	Between Group
Leukocytes, ×10³/μL					
t0	10.27±2.69		10.84±3.8		0.69
		0.0016		0.10	
t5	8.71±1.87		9.72±2.9		0.004
Neutrophils, ×10³/μL					
t0	7.94±2,27		8.18±2.6		0.60
		0.00001		0.06	
t5	5.8±1,45		7.43±2.1		0.00001
Lymphocytes, ×10³/μL					
t0	1.63±0.6		1.76±1.2		0.35
		0.00001		0.31	
t5	2.3±0.57		1.58±0.73		0.00001
Monocytes, ×10³/μL					
t0	0.71±0.45		0.88±0.60		0.11
		0.21		0.64	
t5	0.59±0.47		0.93±0.53		0.002
NLR	5.33±1.9		5.1±1.6		0.24
t0					
		0.00001		0.40	
t5	2.68±1.15		4.9±1.3		0.00001

Results are presented as mean±SD. $P<0.05$ is statistically significant. t0 indicates baseline; t5, 5 days after reperfusion; and NLR, neutrophil to lymphocyte ratio.

groups, the concentration of hsCRP, the classic acute-phase plasma protein, had a progressive increase and reached peak value 5 days after reperfusion (4.3±3.4 mg/L versus 80.1±63.2 mg/L for control group; $P<0.001$ and 5.6±4.7 mg/L versus 31.6±54.4 mg/L for treated group; $P<0.001$) (Table 5 and Figure 3C). Despite that, in treated patients, the protein generation was significantly blunted as compared with controls (31.6±54.4 mg/L versus 80.1±63.2 mg/L, respectively; $P<0.005$) (Table 5 and Figure 3C).

A significant correlation between TNF-α and NOX2 ($r=0.05$, $P<0.001$) was found supporting the role of NOX2-mediated cytokine production. Moreover, a significant correlation between increased CRP plasma levels and higher NLR was found, confirming its potential role as a marker of a persistent toxic inflammatory environment ($r=0.62$, $P<0.01$).

Finally, a significant increase of NO was observed in the glutathione group after PCI compared with the control group (33.5±6.8 μmol/L versus 23.1±11.6 μmol/L, respectively; $P<0.0001$) (Table 5 and Figure 3D). A significant inverse correlation between increased TNF-α levels and reduced NO ($r=-0.31$, $P<0.001$) and between increased NOX2 levels and reduced NO was found ($r=-0.31$; $P<0.001$).

Serological Signs of Myocardial Injury

Baseline cTpT mean values were similar between the glutathione and placebo groups (177.0±60.8 pg/mL versus 180.9±51.9 pg/mL, respectively) At 24 hours, they increased in both groups. Starting from 5 days after reperfusion, glutathione-treated patients showed a progressive significant decrease of cTpT levels compared with baseline (136.3±64.9 pg/mL versus 177.0±60.8 pg/mL; $P<0.005$) (Table 5). Differently, a persistence of high cTpT values was observed in the placebo group 5 days after reperfusion compared with baseline (182.7±45.7 pg/mL versus 180.9±51.9 pg/mL) (Table 5). A modest correlation between delta changes of NOX2 and cTpT from baseline to 5 days ($r=0.13$, $P<0.05$) and a significant correlation between delta changes of TNF-α and cTpT from baseline to 5 days ($r=0.11$, $P<0.001$) were found.

Angiographic Indexes of Myocardial Reperfusion

A high grade of flow restoration of the infarct-related coronary artery was obtained in both groups. Overall, 13 patients, 4 treated and 9 untreated, showed the no-reflow phenomenon ($P=0.23$). Despite that, a significant impairment of myocardial microcirculation was

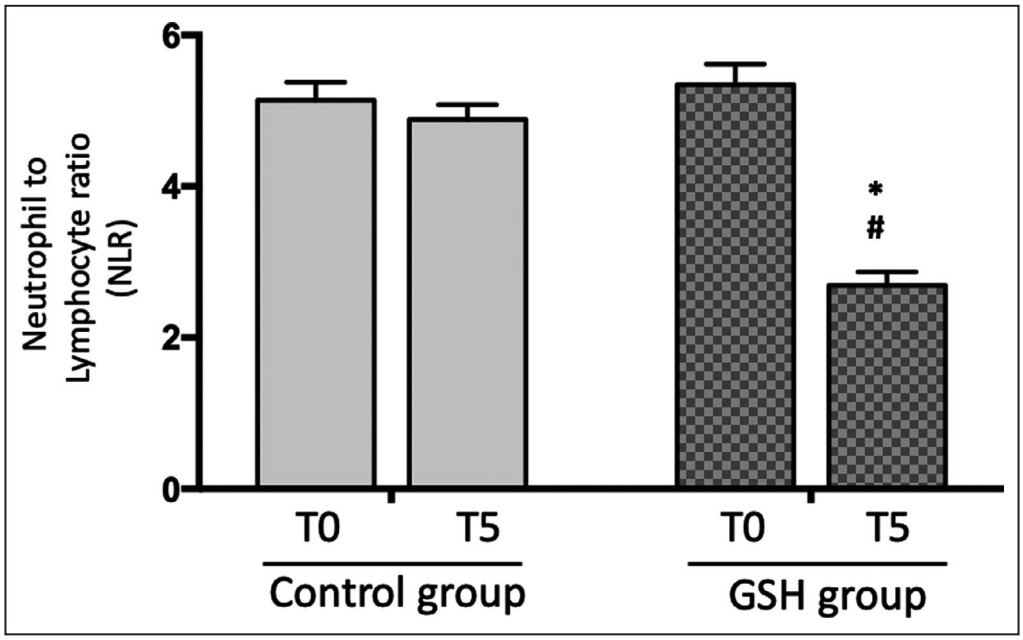

Figure 2. Neutrophil-to-lymphocyte ratio (NLR) at admission (t0) and after 5 days from reperfusion (t5) in patients who received glutathione (GSH) or placebo.
*P<0.0005 vs controls. #P<0.0005 vs t0. Interaction group time P<0.0001.

observed in controls as compared with glutathione-treated patients (2.27±0.71 versus 2.55±0.58; P=0.036). Of note, a larger number of treated patients than controls reached normal tissue perfusion, as assessed by MPG=3 (28 patients [56%] versus 17 patients [34%], P=0.044). Postreperfusion MPG values showed a significant correlation with changes of NO levels from baseline (r=0.63, P=0.021).

Echocardiographic Data

Table 6 depicts echocardiographic data. LV parameters were similar between groups at baseline. In the early days after p-PCI, LV size and function were not different between groups, although in glutathione patients, a slight decrease of LVEDV and improvement of LV ejection fraction were observed (Table 6). At 6 months in treated patients, LVEDV decreased compared with controls group (110.7±16.4 mL/m^2 versus 133.1±18.1 mL/m^2, respectively; P<0.00001) and LV ejection fraction improved (52.3±5.5% versus 48.1±5.1%, respectively; P=0.00013). Of note, adverse LV remodeling (>15% increase in LVEDV) was observed in 21 of 48 controls (43.7%) and 3 of 47 treated patients (6.4%) (P<0.00003). In the glutathione-treated group, the improvement in LV size and function parallels the reduction of serum inflammatory markers and troponin release induced by glutathione administration (r=0.78, P<0.005 and r=0.62, P<0.05; respectively). Post–p-PCI mitral regurgitation was absent in 62 patients (32 treated/30 untreated), mild in 32 patients (14 treated/18 untreated), and moderate in 6 (4 treated/2 untreated) (P=0.83). At 6-month follow-up, mitral regurgitation was documented in 28 patients out of 95 patients (29%), with mild-to-moderate mitral regurgitation in 12 treated and 16 untreated patients (P=0.4).

In Vitro Study

To evaluate the effect of glutathione on TNF-α–mediated endothelial function, we treated human umbilical vein endothelial cells with 2 concentrations of glutathione in presence of TNF-α as an inflammatory stimulus. Results showed that compared with TNF-α–stimulated cells, pretreatment with glutathione significantly increases NO levels in a dose-dependent manner (34.2±5.1 µmol/L versus 20.8±4.7 µmol/L for 5 mmol/L of glutathione; P<0.01 and 41.0±4.9 µmol/L versus 20.8±4.7 mmol/L for 10 mmol/L of glutathione; P<0.0001) (Figure 4A). Conversely, glutathione treatment reduces in a dose-dependent manner oxidative stress as indicated by the reduction in H_2O_2 levels (23.6±6.1 µmol/L versus 39.8±7.3 µmol/L for 5 mM of glutathione; P<0.01 and 17.2±5.8 µmol/L versus 39.8±7.3 mmol/L for 10 mmol/L of glutathione; P<0.0001) (Figure 4B).

DISCUSSION

Data from our study outline the role of glutathione with respect to the defense of myocardial and endothelial cells against toxic oxidative and inflammatory environment following timely STEMI reperfusion.

Table 5. Serological Markers of Oxidant Status, Inflammation, and Myocardial Injury

Variables	Overall, n=100, mean±SD	Controls, n=50, mean±SD	Glutathione, n=50, mean±SD	Multiple Comparison		
				Between Group	Within Group vs t0	
					Controls	Glutathione
sNOX2dp, pg/mL						
Admission	36.5±7.8	35.6±8.9	37.3±6.7	NS		
After p-PCI 2 h	31.9±9.0	35.5±9.0	28.4±7.5	<0.001	NS	<0.0001
After p-PCI 5 d	28.2±9.3	33.2±8.7	23.2±6.8	<0.001	NS	<0.0001
δ_NOX2 _5 d	−8.3±12.3	−2.5±13.4	−14.0±6.9			
TNF-α, pg/mL						
Admission	70.07±12.5	68.9±12.3	71.1±12.8	NS		
After p-PCI: 2 h	63.93±11.5	67.1±11.9	60.8±10.3	<0.05	NS	<0.0001
After p-PCI 5 d	55.06±15.2	64.4±11.6	45.6±12.4	<0.0001	NS	<0.0001
δ_TNF-α _5 d	−15.0±15.6	−4.5±10.56	−25.5±12.5			
NO, μmol/L						
Admission	16.6±4.4	16.7±4.3	16.61±4.6	NS		
After p-PCI: 2 h	24.1±7.2	22.7±7.9	26.0±5.9	<0.0001	NS	<0.001
After p-PCI 5 d	28.3±10.1	23.1±11.6	33.5±6.8	<0.0001	NS	<0.001
δ_NO_5 d	11.6±11.3	6.4±11.8	16.8±7.9			
hsCRP, mg/L						
Admission	4.9±4.1	4.3±3.4	5.6±4.7	NS		
After p-PCI 2 h	26.3±34.5	22.5±16.9	30.1±45.7	NS	<0.001	<0.001
After p-PCI 5 d	55.8±63.5	80.1±63.2	31.6±54.4	<0.005	<0.001	<0.001
δ_hsCRP_5 d	50.8+63.5	75.7±62.9	26.0±54.0			
cTpT, pg/mL						
Admission	178.9±40.7	180.9±51.9	177.0±60.8	NS		
After p-PCI 2 h	205.1±55.3	204.7±57.5	205.5±53.7	NS	<0.0005	<0.0005
After p-PCI 5 d	159.5±60.6	182.7±45.7	136.3±64.9	<0.005	NS	<0.005
δ_cTpT_5 d	−19.4±55.4	1.7±28.7	−40.7±66.8			

cTpT indicates cardiac troponin T; hsCRP, high-sensitivity C-reactive protein; NS, not significant; p-PCI, primary percutaneous coronary intervention; sNOX2dp, soluble nicotinamide adenine dinucleotide phosphate oxidase 2–derived peptide; t0, baseline; and TNF-α, tumor necrosis factor α.

In particular, prophylactic and prolonged glutathione infusion is able to reduce NOX2 activation and modulate inflammatory effector cell response as assessed by changes of differential leukocytes count and TNF-α production over the first 5 days after STEMI. In treated patients, the balance of innate and adaptive immune response resulted in improvement of LV function at follow-up. Lower NLR values in the early phases of cardiac repair were tightly linked to better LV remodeling and contractility at late follow-up.

Following STEMI, the initial inflammatory response is required for tissue healing and scar formation. However, after acute reperfusion of injured myocardial areas, both sustained and unbalanced activation of inflammatory effector cells may promote infarct expansion.[1–4] A recent patient-level meta-analysis has confirmed the pivotal importance of LV remodeling within 1 month after p-PCI as a determinant of all-cause mortality and hospitalization for heart failure at 1 year.[23] On the cellular level, the early phase of cardiac repair is characterized by massive neutrophil infiltration that induces tissue injury by releasing oxidants and proteases in reoxygenated myocardial areas.[24] The extent of damaged myocardium has been widely correlated with recruitment of neutrophils in the infarct zone, and the increase in neutrophil accumulation parallels the increase in infarct size from 6 to 24 hours.[25,26] Of note, neutrophils are a primary source of ROS, which in turn provoke an additional damaging effect on myocardial cells.[27] In our study, the early and prolonged infusion of glutathione induced a prompt and sustained reduction of NOX2 activation production, a reliable marker of oxidant activity, that showed a slight correlation with delta changes of cTpT values from baseline to 5 days. Aside from neutrophils, monocytes are among the earliest responders.[28] Recruitment of monocytes gives rise to inflammatory followed by less-inflammatory macrophages that promote adequate tissue regeneration

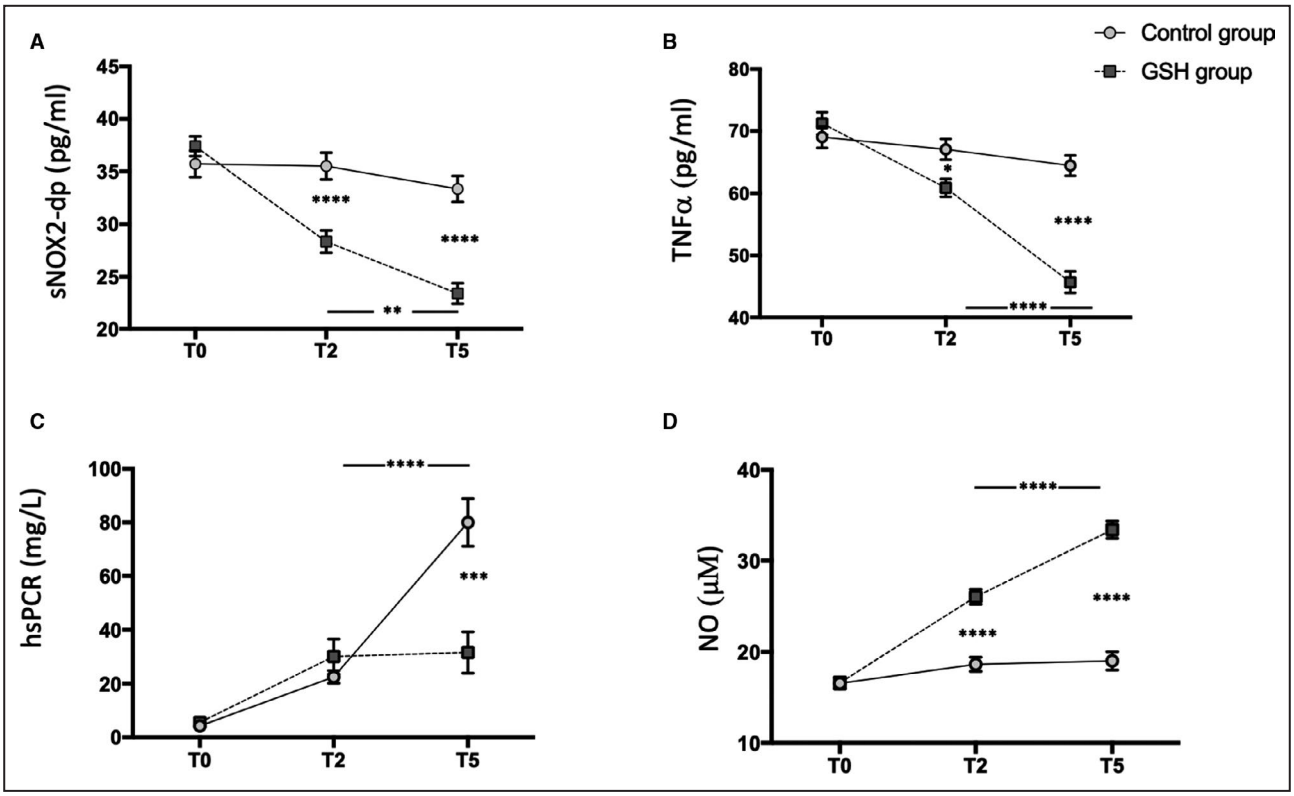

Figure 3. sNOX2-dp) levels (A), TNF-α (tumor necrosis factor α) levels (B), hsCRP (high-sensitivity C-reactive protein) (C), and NO (D) at admission (t0), after 2 hours (t2), and after 5 days (t5) from reperfusion in patients who received glutathione (GSH) (n=47, dashed line) or placebo (n=48, continuous line).
***P*<0.01. ****P*<0.001. *****P*<0.0001.

and resolution of inflammation.[29] Experimental studies revealed that a misbalance of these sequential phases, as a consequence of expanded inflammatory cell supply, affects infarct healing and leads to adverse remodeling.[3] Optimal healing requires a progressive and coordinated cell activation that removes dead tissue and strengthens the wound, thus avoiding the expansion of the initial ischemic myocardium.

In this context, lymphocytes play a pivotal role by modulating innate immune cell recruitment to the infarcted myocardium. In a mouse experimental model of myocardial infarction, CD4+ T-lymphocyte cell deficiency delayed the monocytes' transition and impaired the healing of the heart.[15] Of note, heightened metabolic activity accompanying T-cell activation drives increased production of ROS that requires the antioxidative glutathione upregulation to prevent cellular damage.[16] Upon early T-cell activation, glutathione tissue content is enough for adequate scavenging activity. The heightened oxidative status following sustained lymphocyte activation overwhelms their glutathione antioxidant potential, thus affecting cell growth and differentiation.[30,31] In an experimental model, deficiency of glutathione system activity prevented T cells from reprograming the metabolism to meet the rising energy needs, thus hampering their proliferative response.[15] Interestingly, in our treated patients 5 days after reperfusion, an increased serum value of lymphocytes along with lower neutrophil count was observed, resulting in the significant reduction of NLR. In previous studies, higher NLR has been associated with adverse remodeling and lower ejection fraction after STEMI.[32–34] Thus, its determination seems a more reliable and simpler marker of maladaptive immune response than any other leukocyte subtypes. As observed in our study, it is conceivable that prophylactic and prolonged glutathione administration has played an unexpected role in balancing innate and adaptive immune response, thereby reducing the deleterious effect of persistently heightened systemic inflammation on myocardial cell function and survival.

As widely reported, CRP production closely reflects the magnitude of inflammatory activity, and its response provides a useful objective marker in monitoring the time course of inflammation.[35] Overall, in our population, CRP generation progressively increased by reaching peak values 4 days after reperfusion. Despite that, in treated patients, we observed a

Table 6. Echocardiographic Parameters on Admission and at Follow-Up

Variable	Placebo	Glutathione	*P* Value
Admission	n=50	n=50	
LVEDV, mL/m^2	117.8±14.5	117.9±22.2	0.98
LVESV, mL/m^2	65.6±17.8	67.9±19.9	0.55
LVEF, %	45.3±6.1	43.4±7.3	0.16
MR	20 (40)	18 (36)	0.68
Mild, n (%)	18 (36)	14 (28)	0.38
Moderate, n (%)	2 (4)	4 (8)	0.39
FU 5 d	**n=50**	**n=50**	
LVEDV, mL/m^2	119.8±15.9	114.0±18.1	0.09
LVESV mL/m^2	64.9±12.3	62.4±12.4	0.33
LVEF, %	45.9±5.4	45.2±5.5	0.51
FU 6 mo	**n=48**	**n=47**	
LVEDV mL/m^2	133.1±18.1	110.7±16.4	0.00001
LVESV mL/m^2	69.3±12.7	52.2±8.1	0.019
LVEF, %	48.1±5.1	52.3±5.5	0.00013
LVR, %, mean±SD	12.8±7.2	−5.6±7.4	0.00001
Adverse LVR, n (%)	21 (43.7)	3 (6.4)	0.00003
MR	16	12	0.40
Mild	15	12	0.53
Moderate	1	0	0.96

Glutathione means reduced glutathione. FU indicates follow-up; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; LVESV, left ventricular end-systolic volume; LVR, left ventricular remodeling; and MR, mitral regurgitation.

blunted acute-phase protein production, thus confirming the positive effects of glutathione administration on amplitude and duration of inflammation and its timely attenuation. To support the role of immune-mediated inflammation, we also evaluated TNF-α levels, the master regulator of the immune response, as the major contributor during the development and progression of heart failure. In treated patients, we found a significant reduction in TNF-α production confirming the positive effect of glutathione administration on inflammation. TNF-α production during acute myocardial infarction may potentially contribute to further deterioration of cardiac function by regulating the role of NO in coronary flow regulation. We found a significant inverse correlation between systemic TNF-α and NO levels. This relationship was also confirmed by an in vitro

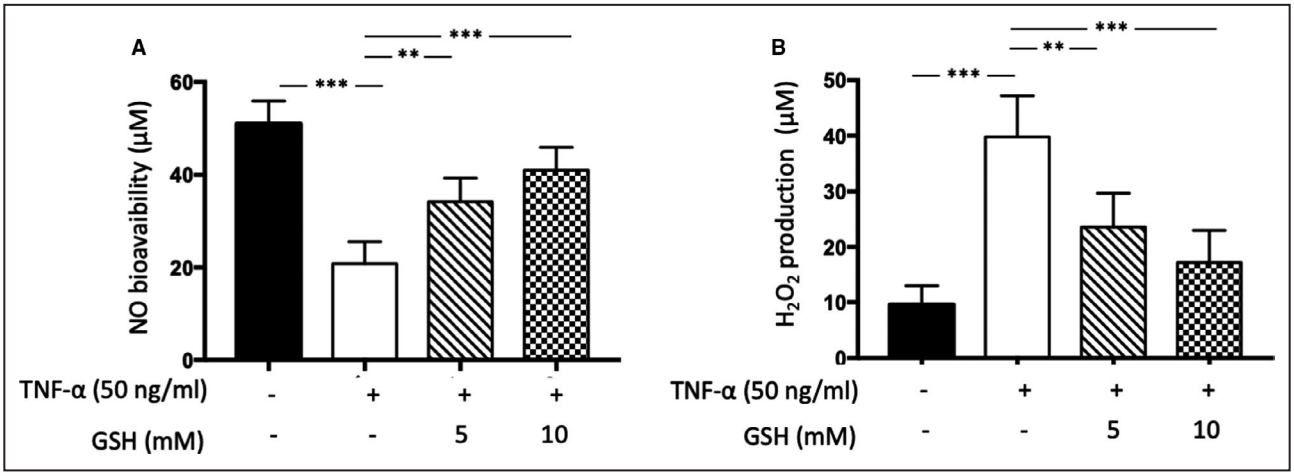

Figure 4. Effect of glutathione (GSH) pretreatment (5 mmol/L or 10 mmol/L) on NO bioavailability (A) and H_2O_2 levels (B) in human umbilical vein endothelial cells stimulated with TNF-α (tumor necrosis factor α) (50 ng/mL).
***P*<0.01. ****P*<0.001.

study in TNF-α–stimulated human umbilical vein endothelial cells; in these cells, glutathione pretreatment reduced H_2O_2 and increased NO production. In this context, well-preserved myocardial perfusion at the microcirculatory level, by strategies protecting endothelial dilatory function, has the potential to become a new treatment for myocardial ischemia.

Given that early and prolonged attenuation of the heightened toxic environment following acute myocardial reoxygenation is necessary for better cardiac repair, the rapid and sustained increase of antioxidant activity seems to be mandatory for an effective and timely approach to limiting both oxidative myocardial and endothelial cell injury. Among others, vitamin E and its derivatives are known for significant antioxidative and anti-inflammatory properties. In particular, preclinical studies of ischemia/reperfusion injury have shown the potential of vitamin E supplementation in hampering the detrimental effects on myocardium of unbalanced oxidative and inflammatory status.[36,37] Despite that, the translation of benefits in the clinical setting is controversial. Critical for extensive clinical use and success of vitamin E therapy, route of administration, dosage, duration of supplementation, and mechanisms against ROS-induced cell damage may be considered. Notably, along with reduction of non–antioxidant-induced apoptosis,[38] α-tocopherol cardioprotection relies on increased antioxidative enzyme systems such as catalase and glutathione peroxidase.[39] Following ischemia/reperfusion, the higher vitamin E consumption and the concomitant decrease of glutathione peroxidase activity result in marked attenuation of protection against ROS. In addition, donation of α-tocopherol's hydrogen atom results in its oxidation and loss of antioxidant activity. This α-tocopheroxyl radical gets reduced back to α-tocopherol by ascorbic acid. Thus, ascorbate acts as a cofactor, and its coadministration seems to be necessary in regeneration of tocopherol.[40] Of note, ascorbate consumes glutathione to exert its antioxidant activity.[41,42] Thus, it is conceivable that early supplementation of thiol compounds may play a pivotal role in activating enzymatic part of antioxidant defense system. Relying on this concept, the role of N-acetyl-L-cysteine in counteracting oxidative-induced thiol loss may be of value. N-acetyl-L-cysteine acts as a precursor for synthesis of glutathione, thus replenishing glutathione that has become depleted through the use of this peptide in detoxification routes.[43] In STEMI patients undergoing p-PCI, high-dose intravenous N-acetyl-L-cysteine was able to reduce oxidative stress but did not provide an additional clinical benefit to placebo with respect to myocardial reperfusion injury as measured by the magnetic resonance myocardial salvage index.[44] On the contrary, in the NACIAM (N-Acetyl Cysteine in Acute Myocardial Infarction) trial, myocardial infarct size was significantly reduced, and myocardial salvage increased with the early use of N-acetylcysteine combined with nitroglycerin in patients with acute STEMI undergoing p-PCI. It is noteworthy that no beneficial effects of post–myocardial infarction LV remodeling and no change in either LV dimensions or ejection fraction at 3-month follow-up were observed.[45] The reason for the discrepancy may be related to the more effective tissue reperfusion provided by combined drug administration than preservation of myocardial cell integrity and function. Accordingly, relying on our results, it can be hypothesized that direct glutathione infusion, through both activation of endogenous antioxidant defense mechanisms and attenuation of inflammatory cell recruitment, is able to protect vital myocardial components and endothelial cell function against a harmful pro-oxidant and inflammatory environment, thus preventing maladaptive cardiac repair and LV adverse remodeling.

Nevertheless, this study has several limitations. Immune-cell response modulation is mandatory to prevent progressive LV dysfunction. However, in myocardial tissue, a remarkable heterogeneity and plasticity in monocyte/macrophage development, phenotype, and function has been demonstrated. Our clinical data are only representative of the systemic inflammatory effector cell response and have not addressed the recognition of a subtype cell population inside myocardium following reperfusion.

In addition, we used troponin release as a marker of cardiomyocytes loss and infarct size. The amount of necrotic as well as viable ischemic myocardium is better defined by morphological imaging. However, in untreated patients, higher cTpT values paralleled with more remodeled LV at follow-up. It suggests that early dynamic changes of enzymes better fit with functional and morphological late LV changes. Moreover, it appears from the means of ejection fraction that most patients remained with mildly reduced LV function. Thus, the study likely includes patients with relatively small infarcts, which is not reflective of the general STEMI population. Finally, we cannot exclude the role of other enzyme systems than NOX2 in the increase of oxidative stress such as superoxide dismutase.

CONCLUSIONS

Taken together, glutathione administration was beneficial for homeostatic control of the immune system pathways and blunted oxidative and inflammatory toxic environment, thereby favoring myocardial cell survival.

After STEMI reperfusion, early and prolonged glutathione administration, by reducing inflammation and oxidative stress and by balancing innate and adaptive immune system activity, could represent a novel

therapeutic target for the prevention of maladaptive LV healing and oxidative damage. In particular, strategies aimed to restore glutathione depletion and to recalibrate the leukocyte response toward optimal healing could be of value in counteracting detrimental effects of acute reperfusion on cardiac size and function.

ARTICLE INFORMATION

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Affiliations

Department of Clinical, Internal Medicine, Anesthesiology, and Cardiovascular Sciences, Sapienza University of Rome, Rome, Italy (G.T., N.V., C.N., F.B., C.T., M.C.A., C.G., E.M.); Department of Cardiology, Interventional Cardiology Unit, "Santa Maria" Hospital, Terni, Italy (A.A., M.D.); Department of Cardiology, "San Giovanni Evangelista" Hospital, Tivoli, Italy (A.P., A.G.); Department of General Surgery and Surgical Specialty Paride Stefanini, Sapienza University of Rome, Rome, Italy (V.C.); Internal Medicine Unit, "Santa Maria" Hospital, Terni, Italy (G.P.); Department of Translational and Precision Medicine, Sapienza, University of Rome, Rome, Italy (S.B.); Department of Medical-Surgical Sciences and Biotechnologies, Sapienza University, Latina, Italy (R.C.); and Mediterranea Cardiocentro, Napoli, Italy (R.C.).

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Author contributions: Conceptualization: Drs Tanzilli and Mangieri; Data curation: Drs Arrivi, Dominici, Placanica, Viceconte, Cammisotto, Nocella, and Granatelli; Formal analysis: Drs Carnevale and Acconcia; Investigation: Drs Arrivi, Placanica, Viceconte, Granatelli, and Dominici; Methodology: Drs Torromeo, Barillà, Carnevale, and Pucci; Project administration: Drs Tanzilli, Mangieri, and Gaudio; Resources: Dr Gaudio; Supervision: Drs Tanzilli, Mangieri, Arrivi, Dominici, and Basili; Validation: Drs Basili and Viceconte; Writing—original draft: Dr Tanzilli; Writing—review and final editing: Drs Tanzilli, Arrivi, Carnevale, and Mangieri.

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Disclosures

None.

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