DOI: 10.1111/eci.12189

ORIGINAL ARTICLE

Dynamics of serum-induced endothelial cell apoptosis in patients with myocardial infarction

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ABSTRACT

Background In patients with ST-segment elevation myocardial infarction (STEMI) reperfused with primary coronary intervention (PCI), the dynamics of endothelial cell (EC) viability, apoptosis and necrosis and its relationship with the structural consequences on the left ventricle have not been addressed so far.

Design In 20 STEMI patients, we incubated human umbilical vein endothelial cells (HUVECs) with serum drawn before reperfusion and subsequently afterwards (24, 96 h, 30 days). Viability, apoptosis and necrosis percentages were evaluated by flow cytometry. Values were compared with 12 age- and sex-matched control subjects with normal coronary arteries. Cardiac magnetic resonance (CMR) was performed during the first week after infarction.

Results Serum from STEMI patients induced a progressive loss of EC viability, with a nadir of 67·7 ± 10·2% at 96 h (baseline: 75 ± 6% and controls: 80·2 ± 3·9%, $P < 0·001$ in both cases). This is due to an increase in apoptosis that peaked at 96 h after reperfusion (15·2 ± 7·1% vs. 11 ± 6 at baseline and 5·8 ± 1·6% in controls, $P < 0·001$ in both cases). However, no significant dynamic changes in EC necrosis were detected. Extensive myocardial oedema (> 30%, median of left ventricular mass) was the only CMR variable significantly associated with a higher percentage of EC apoptosis at 96 h (extensive vs. nonextensive oedema: 18·3 ± 6·8% vs. 12·1 ± 6·3%, $P < 0·05$).

Conclusions Dynamic changes in EC viability occur in the setting of STEMI patients reperfused with PCI, these changes peak late after reperfusion, they are mainly the result of an increase of apoptosis and are associated with the presence of extensive myocardial oedema.

Keywords Acute myocardial infarction, apoptosis, endothelial cells, magnetic resonance.

Eur J Clin Invest 2014; 44 (1): 46–53

Introduction

In acute ST-segment elevation myocardial infarction (STEMI), early and successful myocardial reperfusion by means of primary percutaneous coronary intervention (PCI) is the current state-of-the-art strategy for reducing infarct size and improving patients' outcome. The process of restoring blood flow to the ischaemic myocardium can induce damage, however. This phenomenon, known as myocardial reperfusion injury, can paradoxically reduce the beneficial effects of myocardial reperfusion [1].

In healthy tissues, endothelial cells (ECs) control blood coagulation and regulate vessel wall permeability and circulating leucocytes [2]. Failure of ECs to adequately perform any of these basal functions constitutes 'EC dysfunction'. In the setting of myocardial infarction, loss of EC viability could lead to an increase in microvascular permeability [3] and, as a consequence, reperfusion-derived injury.

In the setting of STEMI, although it has become progressively recognized that apoptosis contributes to myocardial cell death during ischaemia/reperfusion injury [4], the relative contribution of apoptosis and necrosis to the loss of ECs still remains controversial. Moreover, the impact of the dynamics of EC viability on the state of the left ventricle after reperfusion has not been previously addressed. Cardiac magnetic resonance (CMR) represents the ideal noninvasive imaging technique for a comprehensive assessment of the structural repercussion of the infarction on the left ventricle [5].

In this study, we investigate the following: whether the serum of STEMI patients reperfused with PCI modifies EC viability, the relative role of EC apoptosis and necrosis, and the dynamics of this process. The association between EC viability and the CMR-derived structural consequences of the infarction was also evaluated.

Materials and methods

Study population

This investigation conforms to the principles outlined in the Declaration of Helsinki for the use of human subjects. The local ethics committee approved the research protocol, and written informed consent was obtained from all subjects. Reporting of the study conforms to the STROBE statement along with references to STROBE and the broader EQUATOR guidelines [6].

From March 2011 to January 2012, we prospectively included 31 consecutive patients who were admitted to our institution with a first STEMI within the first 12 h since chest pain onset and were treated with primary PCI. We excluded 11 patients because of death (n = 5), reinfarction (n = 4) and clinical instability (n = 2). Therefore, the final study group comprised 20 patients. Medical treatment was left at the discretion of the attending cardiologist. A control group was made of 12 patients who underwent coronary angiography and in whom no heart or coronary abnormalities were detected.

Baseline characteristics and serial blood samples

Clinical characteristics were recorded in all cases upon patient admission.

Percentage ST-segment resolution 90 min after PCI was determined. PCI was performed within 12 h of symptom onset in all patients. Thrombolysis in myocardial infarction (TIMI) before and after PCI was assessed using standard software blinded to CMR results (HM3000; Philips, Best, the Netherlands).

Troponin I (ng/mL, Dimension; Dade Behring, Inc., Newark, NJ, USA) was serially measured before reperfusion and 24, 96 h and 30 days afterwards; the peak value was recorded. At the same time points, blood samples were collected for centrifugation and serum separation, and these were immediately stored at −80 °C until further analyses were performed. A single serum sample was drawn directly after angiography for serum analysis in controls.

Cardiac magnetic resonance

Cardiac magnetic resonance imaging (1·5 T unit, Magnetom Sonata; Siemens, Erlangen, Germany) was performed 7 ± 1 days after STEMI, in accordance with our laboratory protocol and current recommendations [7]. All images were acquired using a phased-array body surface coil during breath holds and were electrocardiographically triggered.

T2-weighted short tau inversion-recovery turbo-spin echo sequences were used to carry out oedema detection. Steady-state free precession sequences were used for cine imaging; these images were used to determine ejection fraction. A segmented inversion-recovery steady-state free precession sequence was used for late gadolinium enhancement (LGE) imaging, which was used to detect infarct size and microvascular obstruction (MVO).

Cardiac magnetic resonance studies were analysed offline in our core CMR imaging laboratory (INCLIVA, Valencia, Spain) by an experienced observer blinded to all patient data, using customized software (QMASS MR 6.1.5; Medis, Leiden, the Netherlands). In T2-weighted sequences, the presence of oedema was quantitatively determined as the percentage of left ventricular (LV) mass with signal intensity 2 standard deviations above the mean signal obtained in the remote noninfarcted myocardium. Myocardial oedema was expressed as percentage of LV mass (extensive if greater than median of LV mass). On a patient basis, the estimation of the area at risk was equivalent to the percentage of LV mass with myocardial oedema. Images were reviewed by the operator and manually corrected if needed. In cine images, ejection fraction (%) was quantified by manual definition of endocardial and epicardial borders of all short-axis slices. Depressed ejection fraction was defined on the basis of accepted reference values according to sex, age and body surface area [8]. In LGE imaging, infarct size was quantitatively determined as the percentage of LV mass with signal intensity 2 standard deviations above the mean signal obtained in the remote noninfarcted myocardium. Infarct size was expressed as the percentage of LV mass showing LGE (extensive if greater than median of LV mass). MVO was defined on a segmental basis as a lack of contrast uptake in the core of a segment surrounded by tissue showing LGE [9]. On a patient basis, the presence of significant MVO was considered if it was detected in more than one segment. LGE images were visually reviewed by the operator and manually corrected if needed.

Human umbilical vein endothelial cells isolation and culture

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cord veins by means of collagenase digestion procedure as previously described [10]. HUVECs were grown in endothelial growth medium (EGM; Lonza, Brusseles, Belgium) supplemented with 10% foetal bovine serum (FBS). The cells were routinely grown in a humidified atmosphere at 37 °C and 5% carbon dioxide (CO_2). HUVECs were characterized by immunostaining for platelet/EC adhesion molecule-1 (Dako, Glostrup, Denmark), incorporation of acetylated low-density lipoprotein (Invitrogen, Molecular Probes, Portland, OR, USA) and lectin binding with *Ulex europaeus* agglutinin-FITC (Sigma, St. Louis, MO, USA).

Determination of viability, apoptosis and necrosis

The dynamics of ECs viability, apoptosis and necrosis were evaluated with a slightly modified *ex vivo* cell culture assay [11,12]. In brief, in 80% confluence cultures, the culture medium was replaced with a modified medium consisting of EGM-2 without FBS. Next, 10% of STEMI serum drawn before and after 24, 96 h and 30 days postreperfusion, respectively, was added to the cultures and incubated during 18 h. A negative control with 10% FBS of survival and a positive control of cell death with FBS deprivation were performed. Following incubation, cells were immediately double-stained for FITC-annexin-V binding and propidium iodide (PI) using Apoptosis Detection Kit (Immunostep, Salamanca, Spain) as previously described [13].

Flow cytometric analysis was used to determine viability, apoptosis and necrosis by dual selectivity with annexin-V and PI. Annexin-V allows for the detection of apoptotic cells, while PI stains dead cells and permits discriminating necrotic cells from viable cells [14]. Percentages of viable cells, apoptotic cells and necrotic cells were determined using a Gallios flow cytometer (standard 2-laser configuration; Beckman Coulter, Brea, CA, USA), and a minimum of 10 000 events was acquired. The analysis of flow cytometry data was conducted using KALUZA software (Beckman Coulter).

Statistical analysis

The Kolmogorov–Smirnov test was applied to test for normal distribution. Continuous variables were expressed as mean ± SD, and comparisons were made using the one-way ANOVA test. Nonparametric data were expressed as the median with the interquartile range and were compared using Mann–Whitney *U*-test. Percentages were compared using the chi-square test and Fisher's exact test when appropriate. Statistical significance was considered for two-tailed *P*-value < 0·05. SPSS 18.0 (SPSS Inc, Chicago, IL, USA) software was used.

On the basis of our previous experience on CMR and post-STEMI structural damage [15], we hypothesized that 30% of patients would display extensive structural damage. On the basis of our preliminary data with EC viability and accepting an alpha error of 0·05 and a beta error of 0·20, the estimated sample size to detect a significant association between EC viability and structural LV damage would be 15 patients. Based on this estimation, we included 20 patients in the final study group.

Results

Baseline characteristics of the study population are displayed in Table 1. No significant differences in baseline characteristics and cardiovascular risk factors between STEMI patients and controls were observed (Table 2).

Table 1 Baseline, electrocardiographic, laboratory and angiographic characteristics of STEMI patients

	STEMI (*n* = 20)
Age (years)	65 ± 13
Male (%)	12 (60)
Diabetes (%)	4 (20)
Hypertension (%)	14 (70)
Hypercholesterolemia (%)	9 (45)
Previous coronary artery disease (%)	0 (0)
Current smoker (%)	9 (45)
Heart rate (beats per minute)	80 ± 17
Systolic blood pressure (mmHg)	126 ± 25
Killip class > 1	3 (15)
ST-segment resolution (%)	87 ± 10
Peak troponin value (ng/mL)	82 (44–100)
Time from chest pain onset to revascularization (min)	212 (140–394)
TIMI 3 flow preangioplasty (%)	3 (15)
TIMI 3 flow postangioplasty (%)	18 (90)
In-hospital medical treatment	
Aspirin	20 (100)
Clopidogrel	20 (100)
IIbIIIa inhibitors (%)	5 (25)
Beta-blockers (%)	11 (55)
ACE inhibitors (%)	10 (50)
Statins (%)	15 (75)
Diuretics (%)	2 (10)

STEMI, ST-segment elevation myocardial infarction; TIMI, thrombolysis in myocardial infarction.

Endothelial cell viability, apoptosis and necrosis

Serum from reperfused STEMI patients induced a significant loss of EC viability compared to that for controls at all time points. A gradual loss of EC viability was detected in STEMI patient sera incubations from prereperfusion to 30 days. The nadir of EC viability occurred 96 h after reperfusion (Fig. 1a).

Dynamic changes in EC viability were mainly due to a significant increase in apoptosis in STEMI patient sera that gradually increased and peaked 96 h after reperfusion (Fig. 1b).

Table 2 Baseline characteristics of the study and control groups

	STEMI (n = 20)	Controls (n = 12)	P
Age (years)	65 ± 13	71 ± 12	n.s.
Male (%)	12 (60)	9 (75)	n.s.
Diabetes mellitus (%)	4 (20)	4 (33)	n.s.
Hypertension (%)	14 (70)	6 (50)	n.s.
Hypercholesterolemia (%)	9 (45)	8 (66)	n.s.
Current smoker (%)	9 (45)	4 (33)	n.s.
Previous coronary artery disease (%)	0 (0)	0 (0)	n.s.

STEMI, ST-segment elevation myocardial infarction; n.s., not significant.

Endothelial cell necrosis, on the contrary, showed no significant differences in the sera of STEMI patients when compared with either controls or the dynamics of this parameter upon analysis after reperfusion (Fig. 1c).

A dynamic and progressive loss of EC viability occurred in reperfused STEMI patient sera. This tendency peaked at 96 h postreperfusion and returned to normal at 30 days. Conversely, EC necrosis remained unaltered throughout the entire follow-up.

There were no significant differences between negative controls, corresponding to foetal calf serum incubation and to incubation that was done with the serum of control patients (data not shown).

Endothelial cell dynamics and cardiac magnetic resonance data

Cardiac magnetic resonance characteristics of the patients included in the study are shown in Table 3.

Extensive oedema (greater than median, 30% of LV mass) was detected in 10 patients (50%), depressed ejection fraction in 11 patients (55%), extensive infarction (greater than median, 12% of LV mass) in seven patients (35%) and MVO (in > 1 segment) in five patients (20%).

The presence of altered CMR data did not significantly associate with either the amount of EC viability or EC necrosis.

Similarly, the presence of depressed ejection fraction or MVO did not relate to the degree of EC apoptosis. A nonsignificant trend towards more EC apoptosis occurred in patients with an extensive infarct size (Fig. 2).

The relationship between EC apoptosis and myocardial oedema represented the only significant association between the CMR-derived structural consequences of STEMI and EC dynamics. A significant increase in EC apoptosis was detected both at 24 h (17 ± 8 vs. 10 ± 6, $P < 0{\cdot}05$) and at 96 h (18 ± 7 vs. 12 ± 6, $P < 0{\cdot}05$) in patients when compared with those without extensive oedema (Fig. 2).

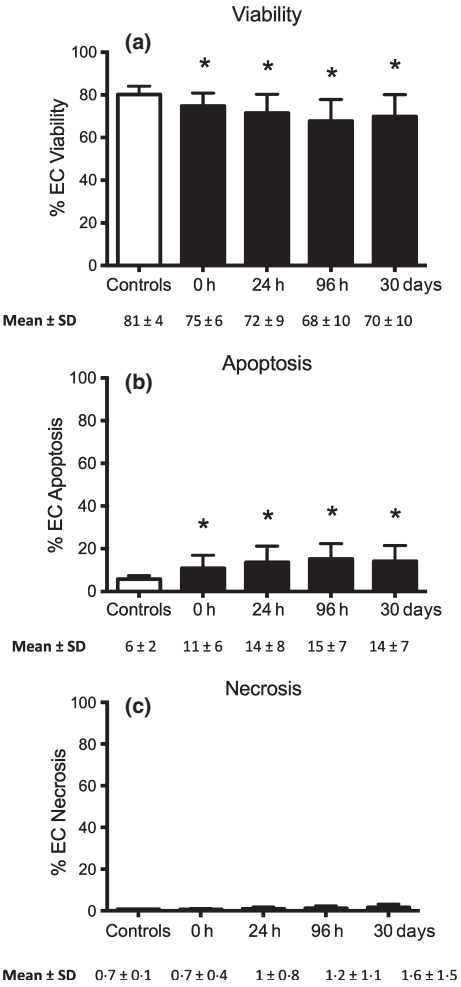

Figure 1 Percentages of EC viability (a), apoptosis (b) and necrosis (c) determined by flow cytometry, when cells were incubated with control serum or STEMI patient serum before reperfusion and 24, 96 h and 30 days afterwards. Data show mean ± SD. $P < 0{\cdot}05$. EC, endothelial cells; SD, standard deviation; STEMI, ST-segment elevation myocardial infarction.

Discussion

The main finding of the present study is that serum from STEMI patients reperfused with primary PCI induces a progressive loss of EC viability due to a dynamic increase in EC apoptosis. The latter relates to the presence of extensive myocardial oedema.

Table 3 Cardiovascular magnetic resonance imaging data of STEMI patients

	STEMI (n = 20)
Presence of myocardial oedema (%)	20 (100)
Median myocardial oedema (% of LV mass)	27·9 (1·4–49·7)
Ejection fraction (%)	55 ± 12
Depressed EF (%)	11 (55)
End-systolic volume (mL/m^2)	36 ± 17
End-diastolic volume (mL/m^2)	79 ± 19
LV mass (g/m^2)	73 ± 18
Anterior infarction (%)	6 (30)
Nonanterior infarction (%)	14 (70)
Infarction size (% of LV mass)	14 ± 10
Extensive infarction (%)	7 (35)
Median MVO (segments)	1·2 (0–8·3)
Presence of MVO (%)	5 (20)

EF, ejection fraction; STEMI, ST-segment elevation myocardial infarction; LV, left ventricular; MVO, microvascular obstruction.

Progression of endothelial cell apoptosis

Endothelial cell ordinarily perform several key homeostatic functions such as controlling blood coagulation, regulating vessel wall permeability and circulating leucocytes. Irreversible damage to the integrity of the cell may result in loss of endothelial viability and consequently EC death [3].

Our results indicate that a reduction in EC viability due to apoptosis, but not to necrosis, occurs when cells were incubated with sera from STEMI patients treated with PCI. In a different setting, Agnoletti *et al.* [11] observed that HUVEC incubated with serum derived from heart failure patients suffered a loss of viability due to apoptosis but not to necrosis. Aebert *et al.* [16] performed an *ex vivo* assay with HUVEC to confirm the pro-apoptotic effect of serum derived from patients who underwent cardiac surgery. The potential for serum to induce apoptosis in scenarios different from STEMI has already been addressed [17].

Data from acute coronary syndromes are limited, however. To our knowledge, this is the first study in humans that assesses the dynamics of EC viability/apoptosis/necrosis in STEMI patients reperfused with PCI and its impact on the structural consequences of the infarction as derived from the

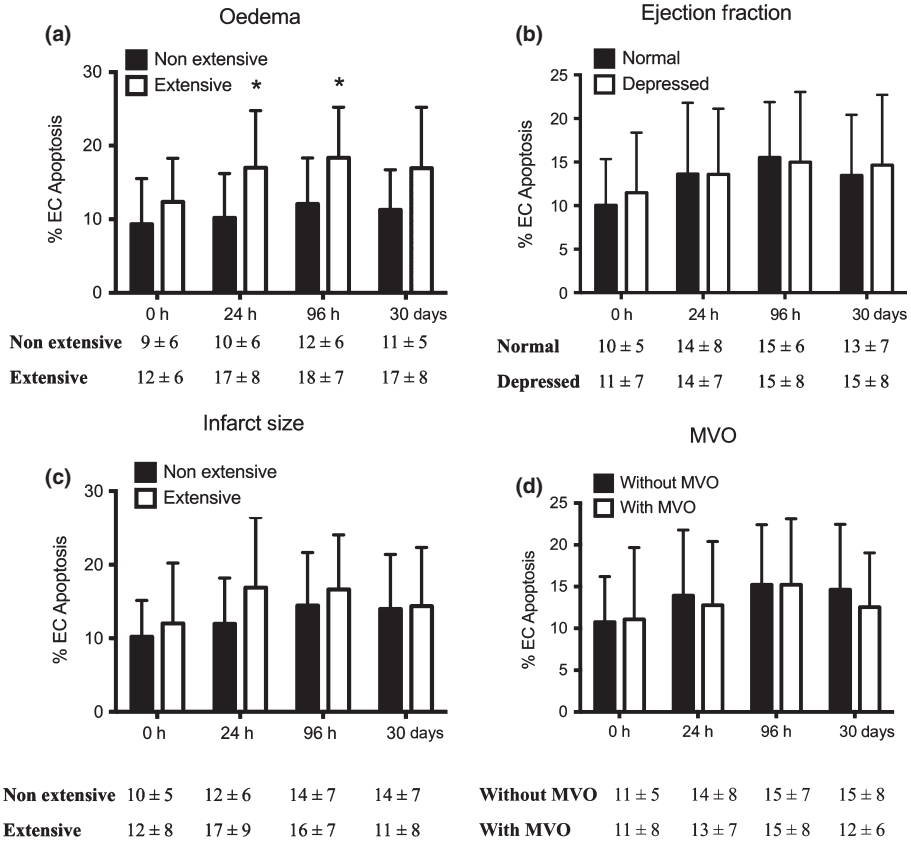

Figure 2 Changes in EC apoptosis percentages determined by flow cytometry when incubated with STEMI patient sera before reperfusion and 24, 96 h and 30 days afterwards with respect to the extension of myocardial oedema (a), severity of ejection fraction (b), extension of infarct (c), existence (or not) of microvascular obstruction (d). Data show mean ± SD. $P < 0{\cdot}05$. EC, endothelial cells; SD, standard deviation; STEMI, ST-segment elevation myocardial infarction.

current gold-standard noninvasive imaging technique, namely CMR.

According to our results, EC apoptosis was already present in incubations with sera before angioplasty and it augmented with reperfused sera. This result suggests that ischaemia could induce EC apoptosis, and reperfusion augmented this effect. In experiments of ischaemia/reperfusion with mongrel dogs, Freude *et al*. [4] suggested that apoptosis was already present in infarcted myocardium within first hours of ischaemia, and reperfusion was necessary to complete the apoptotic cascade.

Cardiac magnetic resonance-derived structural consequences in ST-segment elevation myocardial infarction patients and endothelial cell apoptosis

Cardiac magnetic resonance is being increasingly used as a standard tool in the evaluation of myocardial structure and function and it has become the state-of-the-art imaging technique for evaluating the consequences of infarction.

Cardiac magnetic resonance imaging permits both the evaluation of myocardial oedema associated with acute coronary occlusion and reperfusion, and the analysis of its spatial distribution. Similarly, CMR has emerged as the gold standard for assessing systolic function, global ventricular volumes and ejection fraction [18]. It also allows for a comprehensive assessment after myocardial infarction with accurate detection of infarct size and MVO [19] in LGE sequences. CMR is the reference noninvasive imaging technique for a comprehensive evaluation of patients after STEMI [20], and its prognostic usefulness has already been validated [15]. Accordingly, in the present study, we used CMR to analyse the association of EC dynamics with the structural consequences of STEMI.

Overall, little is known about the potential damaging effects of reperfusion serum from STEMI patients on ECs. This is a relevant issue because it could partly explain some of the deleterious consequences of the ischaemia/reperfusion injury, thus opening new pathophysiological pathways into the understanding of this process.

Using an isolated perfused rat heart model, Scarabelli *et al.* [21] demonstrated that microvascular EC apoptosis preceded myocardial apoptosis, with radial spread from microvessels to myocyte apoptosis. These data suggest that apoptotic ECs release pro-apoptotic substances into the surrounding myocardium and cause myocyte apoptosis in a paracrine fashion. We did not find any significant correlation between infarction size, ejection fraction or MVO and EC viability, apoptosis or necrosis. The fact that complete reperfusion was accomplished in all patients (all of them recovered TIMI 3 flow in the epicardial artery) might have attenuated the deleterious effect of EC disruption.

On the contrary, we observed that EC apoptosis was significantly associated with an extensive CMR-derived myocardial oedema, one of the main markers of reperfusion injury. Acute changes in the myocardium after coronary occlusion can be assessed using T2 CMR sequences that reveal increased water content in tissue [22]. The appearance of oedema after STEMI is a common event that has been demonstrated in animal [21] and in patient [23] studies. The mechanisms leading to postreperfusion myocardial oedema are not fully understood, but an increase in local vascular pressure, cells plugging or inflammation seems to play a role. Moreover, according to our results, EC apoptosis (and subsequently, loss of endothelial integrity) could also lead to an increase in tissue water content.

Tissue oedema is not benign: the accumulation of parenchymal and interstitial fluid could impair heart function by increasing the distance required for the diffusion of oxygen and by compromising microvascular perfusion because of augmented interstitial pressure. On the other hand, EC apoptosis could also be an epiphenomenon associated with a massive release of pro-apoptotic substances.

In the present study, the primary end point was to assess the alterations of EC viability induced by STEMI serum and their association with the CMR-derived structural LV damage in the context of the ischaemia/reperfusion injury; thus, the potential mechanistic role of different apoptosis biomarkers was not evaluated.

An important finding of this study is that EC viability nadir and EC apoptosis peak occurred relatively late after reperfusion (96 h). This, along with the association of EC apoptosis with the extension of myocardial oedema, might indicate that novel therapies addressing the preservation of EC could represent a potential adjunctive therapy to mechanical reperfusion in STEMI patients. As opposed to the latter's tight time window for saving myocardial cells (a few hours), new treatments focusing on EC preservation could be applied over a longer period of time.

Limitations

Although apoptosis is recognized as a new contributor to cell death in ischaemia/reperfusion injury, there are no models to show how EC apoptosis is operative *in vivo*. One limitation in our study is the reduced sample size. Further studies with larger series of patients will be needed to clarify (i) the prognostic significance of the loss of EC viability and necrosis on EC provoked by the serum of reperfused STEMI patients and (ii) the potential influence of EC apoptosis on infarct size.

Conclusions

Sera of STEMI patients reperfused with primary PCI exert dynamic changes in EC viability. These changes peak late after reperfusion, are the consequence of an increase in EC apoptosis and associate with the presence of extensive myocardial oedema. These findings are hypothesis-generating and could be useful in advancing the understanding of the pathophysiology

of myocardial ischaemia/reperfusion injury and, in future, in exploring new therapeutic options to minimize this process.

Acknowledgements

The research was supported by grants from PROMETEO 2013 007, Plan Nacional de I+D, Instituto de Salud Carlos III (FIS PI11/02323, FIS PI10/00518 and Red Cardiovascular RD12/0042/0052).

Conflict of interest

None.

Authors contribution

Maria J Forteza involved in drafting of the manuscript, experimental work, acquisition of data, statistical analysis and interpretation of data. Susana Novella involved in statistical analysis and interpretation of data. Carlos Hermenegildo involved in statistical analysis and critical revision of the manuscript for important intellectual content. Amparo Ruiz-Sauri involved in critical revision of the manuscript for important intellectual content. Fabian Chaustre involved in acquisition and interpretation of CMR data. Isabel Trapero involved in acquisition of clinical data. Clara Bonanad involved in acquisition of clinical data. Ricardo Oltra involved in acquisition of clinical data and recruitment of patients. Lorena Palacios involved in recruitment of patients. J. Enrique O'Connor involved in critical revision of the manuscript for important intellectual content. F. Javier Chorro involved in acquisition of clinical data and critical revision of the manuscript for important intellectual content. Vicente Bodi involved in study concept and design, and critical revision and drafting of the manuscript for important intellectual content.

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Received 16 July 2013; accepted 7 October 2013

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