Original Research

Combined Intravenous Treatment with Ascorbic Acid and Desferrioxamine to Reduce Myocardial Reperfusion Injury in an Experimental Model Resembling the Clinical Setting of Primary PCI

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Introduction: During reperfusion of ischemic myocardium, oxygen-derived free radicals are produced and can cause deleterious effects, known as reperfusion injury. We aimed to determine if a combination of the antioxidant ascorbic acid and an iron-chelating agent desferrioxamine, which reduces the production of the hydroxyl radical via ferrum-catalyzed reactions, can exert a protective action against reperfusion injury.

Methods: Twenty-two young male farm pigs were anesthetized and subjected to 45 mins of ischemia and 3 and a half hours of reperfusion, in the left circumflex coronary artery territory, via the inflation and deflation of an angioplasty balloon. Animals were randomly assigned to receive either an intravenous infusion of 100 mg/kg ascorbic acid and 60 mg/kg desferrioxamine (treatment group, TG) or an equal amount of normal saline (control group, CG). The I/R ratio, the ratio of the infarcted (necrotic) zone (I) to the myocardial area at risk (R) after 3 and a half hours of reperfusion, was calculated using the tetrazolium staining method. Left ventricular end diastolic pressure (LVEDP), number of episodes of ventricular arrhythmias, TIMI flow in the reperfused vessel, and left ventricular ejection fraction (LVEF) were evaluated within the first hour post reperfusion in order to assess further injury severity.

Results: There was no significant difference in the I/R between the TG (27.9 ± 2.2%) and the CG (32.9 ± 2.4%) (p=0.15). In both groups there was a significant reduction in LVEF (-11.6 ± 2.28% for TG and -12.0 ± 2.27% for CG, p<0.01 for both groups) and a significant increase in LVEDP (+3.2 ± 0.9 mmHg for TG and +4.6 ± 0.9 mmHg for CG, p<0.01 for both groups) compared to the baseline values. No significant difference was noted between groups (p=0.61 for LVEF and p=0.60 for LVEDP values, at one hour post reperfusion). In all other parameters measured, no significant difference was observed between the study groups.

Conclusions: Intravenous treatment with a combination of the antioxidant ascorbic acid and the iron-chelating agent desferrioxamine does not provide significant protection against myocardial reperfusion injury.

Early reperfusion is a very important prerequisite for the salvage of ischemic myocardium post myocardial infarction. There is evidence, however, that reperfusion, apart from its major beneficial effect, also results in deleterious effects, known as "reperfusion injury". Reperfusion injury includes myocardial necrosis, arrhythmogenesis, temporary loss of myocardial contractility (myocardial stunning), and endothelial dysfunction (causing the non-reflow phenomenon).[1-7]

Reperfusion injury has been attributed to the excessive accumulation of ox-

ygen-derived free radicals inside the cells during re-oxygenation of ischemic myocardium, through a complex and multi-step pathogenetic cascade. These radicals include superoxide anion, hydroxyl radical, hydrogen peroxide and peroxynitrite.[3,4] The formation of the highly cytotoxic hydroxyl radical is catalyzed by free iron (Fe^{+3})—an abundant ion in myocardial and endothelial cells—via the ferrum-mediated Fenton reactions.[2,8,9] The iron chelator desferrioxamine can reduce the production of this radical by limiting the availability of free iron. Many investigators have tested the effect of desferrioxamine on reperfusion injury and infarct size, with contradictory results.[10-13] Ascorbic acid (vitamin C) also has an antioxidant action, by protecting the cells from the effects of oxygen free radicals.[14-20] However, previous studies have shown only partial protection from the action of the highly toxic hydroxyl radical.[21-24]

A possible explanation for these unsatisfactory results lies in the complexity of the process of reperfusion injury. Reperfusion injury implicates many different mechanisms that apparently cannot be suspended by a single antioxidant.[3] Thus, many investigators have suggested the administration of drug combinations that could have synergistic effects.[4]

In the present study, a combined treatment with ascorbic acid and desferrioxamine was tested in a closed-chest pig model of coronary artery occlusion followed by reperfusion, resembling the clinical scenario of reperfusion with primary percutaneous coronary intervention (PCI). It was hypothesized that desferrioxamine should act synergistically with ascorbic acid, as it reduces the production of the highly toxic hydroxyl radical. Even though these drugs have been studied independently, their combined effect on reperfusion injury protection is not known.

Methods

Animal model and preparation

Twenty-two young male farm pigs (30-35 kg body weight) were subjected initially to occlusion of the left circumflex artery, caused by inflating a coronary angioplasty balloon for 45 min, and subsequently to reperfusion for 60 min, after deflation of the balloon. All animals were anesthetized using pro-anesthetic treatment with azaperone 4 mg/kg and after 15 minutes ketamine 5 mg/kg intramuscularly. Two venous cannulas were placed in the auricle for fluid and drug administration. Anesthesia was induced with intrave-

nous pentothal 7 mg/kg. After endotracheal intubation, mechanical ventilation was started and anesthesia was maintained with sevoflurane 1% and periodic administration of fentanyl and norcuron. ECG and vital signs were monitored during the whole experimental procedure.

Both femoral arteries were surgically exposed and a 6 F sheath was placed in each of them. Then, heparin 80 units/kg was administered intravenously and a 6 F pigtail angiographic catheter was advanced through the sheath of the left femoral artery into the left ventricle under fluoroscopic guidance, in order to provide measurements of the left ventricular end diastolic pressure (LVEDP) and access for the performance of left ventriculography. A 6 F hockey-stick angioplasty guiding catheter was advanced through the sheath of the right femoral artery under fluoroscopic guidance to engage the orifice of the left coronary artery. Through the guiding catheter an angioplasty guide-wire and a 2.5 × 20 mm angioplasty balloon catheter were advanced into the left circumflex coronary artery (LCX).

Experimental protocol

Baseline LVEDP measurements were recorded and ventriculography was performed before the onset of ischemia (baseline values). The end-diastolic and end-systolic volumes of the left ventricle for the calculation of the left ventricular ejection fraction (LVEF) were determined by biplane left ventriculography using the 30° RAO and 60° LAO projections and by tracing the left ventricular silhouette and applying the area-length method.

Then, the angioplasty balloon catheter was inflated in the LCX, just distal to the origin of the first obtuse marginal branch. Vessel occlusion was confirmed by the lack of contrast medium penetration beyond the position of the balloon and by the appearance of ST segment elevation on the ECG. Ischemia duration was 45 minutes. Then, the balloon was deflated (but left in place), in order for reperfusion to take place.

The animals were randomized into two groups: a treatment group (n=11) and a control group (n=11). In the treatment group an intravenous infusion of 100 mg/kg ascorbic acid and 60 mg/kg desferrioxamine, each in 100 ml of normal saline, was administered. The infusion was started 5 minutes before and completed 2 minutes after the onset of reperfusion. The animals on the control group (n=11) received an intravenous infusion of 200 ml normal saline.

Monitoring of ventricular arrhythmias and assessment of blood flow in the reperfused artery, LVEDP and left ventricular systolic function.

During the first 60 minutes of reperfusion the animals were monitored for ventricular arrhythmias. The occurrence of ventricular fibrillation (VF) during the first hour of reperfusion was considered as an endpoint in this study and animals were not resuscitated. Sustained ventricular tachycardia (SVT), defined as VT lasting more than 30 seconds or requiring emergent electrical defibrillation due to hemodynamic compromise, was considered of the same significance as VF and recorded as such. The total number of episodes of non-sustained ventricular tachycardia (NS-VT) and idioventricular rhythm occurring during this time interval was recorded for each animal.

Sixty minutes after the start of reperfusion a measurement of the LVEDP was obtained through the pigtail catheter. Then, angiography was performed via the guiding catheter and flow in the reperfused portion of the LCX was graded according to the TIMI classification (grade 0: zero flow; grade 1: some penetration of the contrast medium beyond the point of the previous obstruction, but without perfusion of the distal coronary bed; grade 2: perfusion of the entire vessel with delayed flow; grade 3: normal flow.)[25,26] Left ventriculography was performed through the pigtail catheter, in order to determine the LVEF, one hour after the start of reperfusion in the same way as at baseline. All data were appropriately stored (continuous ECG recordings, pressure strip-lines for arrhythmias and LVEDP measurements) or record-ed in DICOM format (digital angiographic runs for TIMI flow and LVEF estimation) for later evaluation. Available data were used off-line by an independent operator, who was unaware of the treatment regimen, to perform blinded data measurements.

Assessment of the ischemic area at risk and the proportion of the infarcted (necrotic) myocardium (Figure 1)

Three and a half hours post reperfusion, the angioplasty balloon catheter (which remained in place throughout reperfusion) was re-inflated, in order to re-occlude the vessel at the position of the previous occlusion. Then, 2 ml/kg of 2% Evans blue solution was infused through the pigtail catheter into the beating left ventricle, to delineate the ischemic area at risk (the myocardial territory that was subjected to ischemia and then reperfusion). Three minutes after the administration of Evans blue, the animal was sacrificed with a rapid infusion of a potassium chloride solution into the left ventricle through the pigtail catheter.

The heart was excised, the atria and the right ventricle were discarded, and the left ventricle was cut from base to apex in parallel slices, each 0.5 cm thick. In each slice the area at risk was recognized as unstained by Evans blue. The border between the area at risk and the rest of the myocardium (which was stained by Evans blue) on the surface of each slice was demarcated with Indian ink, so that it would remain easily discernible after incubation of the slices in TTC (triphenyltetrazolium chloride). The slices

Figure 1. a. Typical appearance of the pig's heart after infusion of Evan's Blue 2% solution before excision. The area which is not stained by the Evan's Blue represents the area-at-risk (R). b. Slice of the heart (0.5 cm thickness). The left ventricular myocardium appears blue, apart from the non-stained area, which represents the area-at-risk (R). c. The previous noted slice (b) after TTC (triphenyltetrazolium chloride) incubation. The demarcated area represents the area-at-risk (R). Within this area (R), two colored-stained areas can be recognized: the red-colored area, which represents the necrotic area (N), and the vivid "brick-red" colored area, which represents the viable myocardium (V).

were then incubated with a 1% TTC solution for 30 minutes at 37° C in a dark room. Then, on the surface of each slice, within the area at risk (Evans blue-unstained), two areas were recognized. The TTC-stained area, with a characteristic brick-red color, represented ischemic but viable myocardium and the TTC-unstained area represented the necrotic (infarcted) tissue.[27,28] The slices were photographed with a digital camera. The areas of the risk zone, the infarcted (necrotic) zone and the total area of each slice were measured using a computer program (UTSHCA Image Tool). By multiplying these areas by the thickness of each slice (0.5 cm), the volumes of the risk zone, the infarcted zone, and the total volume of each slice were calculated, and by summing these volumes for the whole left ventricle the total volume of the risk zone (R), the total volume of the infarcted zone (I) and the total volume of the left ventricular myocardium (LV) were calculated. Then the I/R and R/LV ratios were calculated. The ratio I/R represented the proportion of the myocardium of the risk zone that was necrotic (infarcted). The ratio R/LV represented the proportion of the left ventricular myocardium that was included in the area at risk.

Statistical analysis

All values in the text are presented as mean ± standard error of the mean. Data regarding I/R, EF and the change in LVEDP were analyzed by Student's test (unpaired t-test). The number of episodes of NSVT and idioventricular rhythm during the first hour of reperfusion was analyzed by the Mann-Whitney rank-sum test, due to inequalities of variances among the two groups. The differences between the two groups regarding the number of animals that developed ventricular fibrillation in the first hour of reperfusion, as well as the number of animals having TIMI-3 flow in the LCX 1 hour after reperfusion, were tested by the chi-square test. A value of $p < 0.05$ was accepted as statistically significant for all tests.

Results

Reperfusion ventricular arrhythmias

Of the 11 animals of the control group, 2 developed VF in the first hour of reperfusion. In the treatment group (n=11), 1 animal developed VF (p=0.53). None of the animals developed sustained VT (Table 1).

The remaining variables were assessed only for the animals that did not develop VF (9 animals of the control group and 10 animals of the treatment group). The number of episodes of NSVT and idioventricular rhythm during the first hour of reperfusion was 24.22 ± 7.08 in the animals of the control group (n=9) and 11.40 ± 3.24 in the animals of the treatment group (n=10) (p=0.13) (Table 1).

Left ventricular ejection fraction (LVEF)

One hour after reperfusion, a significant reduction in LVEF was observed in each of the groups compared to the baseline values (from 58.0 ± 3.5 % to 46.2 ± 2.1%, p<0.01 for the control group and from 58.9 ± 8.7% to 47.5 ± 1.4%, p<0.01 for the combination treatment group) (Figure 2). The LVEF was reduced by -11.6 ± 2.3% in the antioxidant combination group and by -12.0 ± 2.3% in the control group. The difference between the two groups was not statistically significant as regards either the absolute values of EF, or the difference from baseline (p=0.61 for the absolute values and p=0.91 for the difference) (Figure 3).

Left ventricular end-diastolic pressure (LVEDP)

When LVEDP was compared to the baseline values, there was a significant increase at the end of the 1st hour post reperfusion in both groups (p<0.01) (Figure 4). The change in LVEDP however, did not differ significantly between the two groups (+4.6 ± 0.9 mmHg in the control group and +3.2 ± 0.9 mmHg in the treatment group, p=0.30). The change in LVEDP was also expressed as a percentage of its initial value (%LVEDP change) (Figure 5). The %LVEDP change was +71.3 ± 13.6% in the control group and +52.9 ± 13.0% in the treatment group (p=0.34).

TIMI flow in the reperfused coronary artery

Among the 9 animals of the control group that survived one hour after the onset of reperfusion, 4 animals (44.4%) had TIMI grade 3 flow. In the treatment group (n=10) 6 animals (60%) had TIMI grade 3 flow one hour after the onset of reperfusion. The difference between the two groups was not significant (p=0.50).

Infarct size (I/R ratio)

The infarct size (the area of necrotic myocardium) as a percentage of the area at risk (I/R) three and a half hours after the onset of reperfusion was 32.9 ±

Table 1. Detailed representation of the parameters studied.

| #	Group	VF	I/R %	NSVT /IVT	EF %	Baseline LVEDP	LVEDP	LVEDP change	%LVEDP change	TIMI Flow	R/LV %
1	CG	No	31.2	65	38	7	12	5	71.4	3	22.4
2	CG	No	34.3	37	55	5	8	3	60	3	15.6
3	CG	Yes				7					
4	CG	No	40.5	19	39	7	14	7	100	3	19.7
5	CG	No	36.2	0	40	7	15	8	114.3	0	20.4
6	CG	Yes				6					
7	CG	No	19.4	13	46	7	10	3	42.9	2	21.2
8	CG	No	41.4	32	53	6	8	2	33.3	2	17.7
9	CG	No	38	5	48	5	11	6	120	2	17.5
10	CG	No	26.9	15	52	7	7	0	0	3	19.5
11	CG	No	28	13	45	7	14	7	100	2	21
12	TG	No	37.2	32	44	4	8	4	100	3	17.4
13	TG	No	27.9	5	46	8	12	4	50	2	18.9
14	TG	No	25.1	15	52	8	17	9	112.5	1	20.3
15	TG	No	23.4	13	48	6	9	3	50	3	22
16	TG	No	18.3	3	47	6	6	0	0	2	19.4
17	TG	No	22.8	11	50	5	7	2	40	3	19.1
18	TG	No	19.8	0	56	7	7	0	0	3	15.8
19	TG	No	34	0	48	5	8	3	60	3	20.1
20	TG	No	36.1	23	41	6	7	1	16.6	3	17.6
21	TG	No	34.7	12	43	6	12	6	100	2	19.6
22	TG	Yes				5					

CG – control group; TG – treatment group; NSVT – non-sustained ventricular tachycardia; IVT – idioventricular tachycardia; EF – ejection fraction; LVEDP – left ventricular end-diastolic pressure.
R/LV represents the ratio of the area-at-risk (R) to the total left ventricular area (LV). NSVT and IVT are measured in runs, LVEDP in mmHg. LVEDP values were measured one hour post reperfusion.
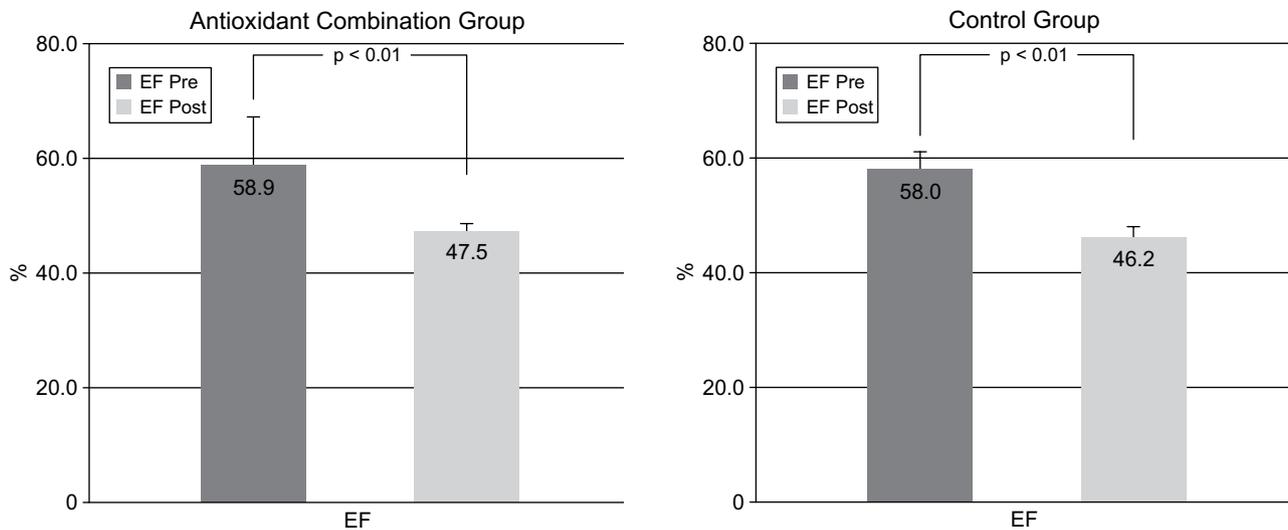
Figure 2. There was a significant decrease in left ventricular ejection fraction (LVEF) in each of the groups ($p<0.01$ for both groups). EF pre: ejection fraction as estimated at baseline, EF post: ejection fraction as estimated at one hour post reperfusion.

2.4% in the control group (n=9) and 27.9 ± 2.2% in the treatment group (n=10) (p=0.15) (Figure 6). The area at risk as a proportion of the whole left ventricular myocardium (R/LV) was 19.4 ± 0.7% in the control group and 19.0 ± 0.6% in the treatment group (p=0.64) (Figure 7).
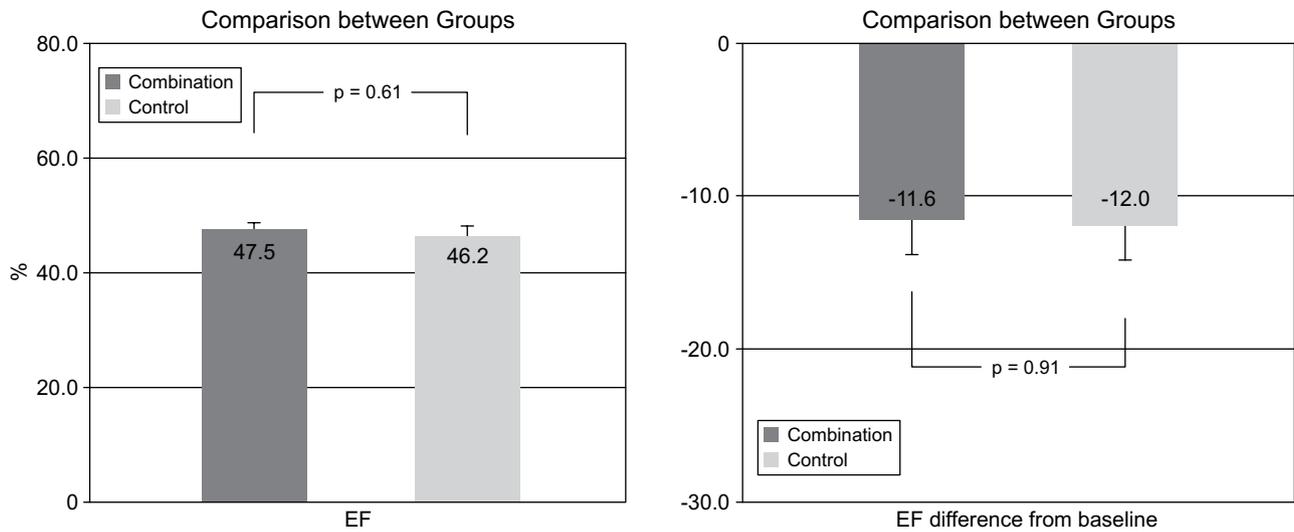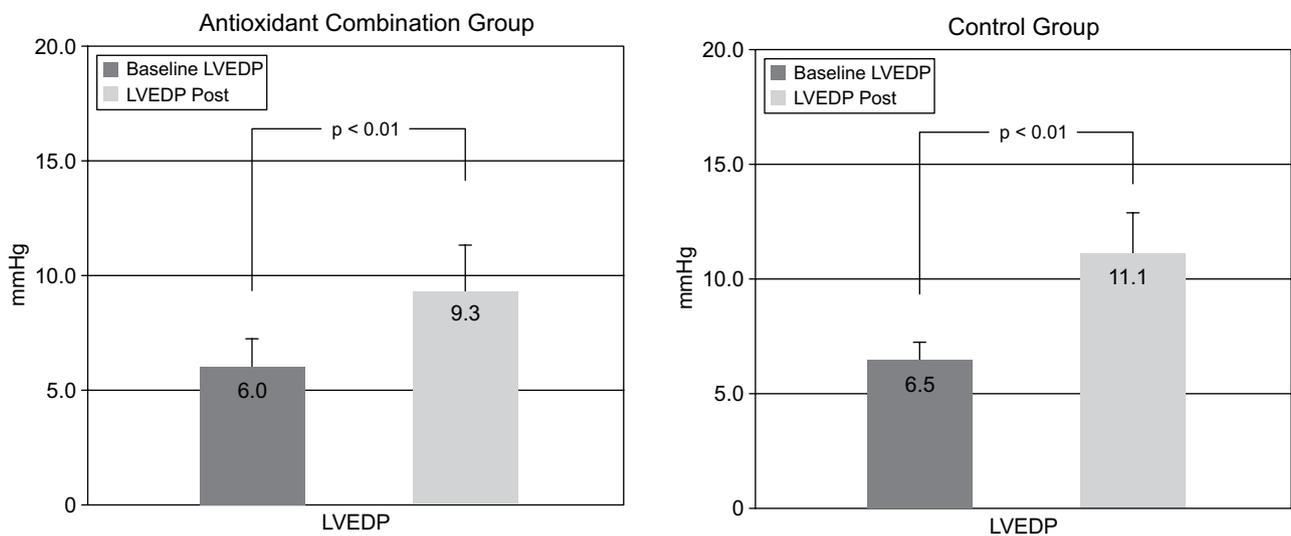
Figure 3. There was no significant difference between the left ventricular ejection fraction (LVEF) of the two groups one hour post reperfusion (p=0.61). Similarly, no difference was noted in the reduction of the LVEF in both groups (p=0.91).

Discussion

There was no significant difference between the two groups of animals regarding the infarct size (expressed as percentage of the area at risk). Thus, the combined intravenous treatment with desferrioxamine and ascorbic acid, administered before the onset and during the first minutes of reperfusion, did not significantly reduce the size of the infarcted (necrotic) zone. Moreover, no significant difference in the number of animals with TIMI grade 3 flow in the reperfused vessel was observed between the two

groups. Thus, the combination treatment failed to provide reduction of the infarct size, or improvement of blood flow in the reperfused artery. Furthermore, no benefit was noted regarding LVEF and there was no significant improvement in left ventricular hemodynamics (assessed by the LVEDP change) in the treatment group. The treatment combination also did not provide a significant reduction in the number of ventricular arrhythmias.

The notion of free radical-generated reperfusion injury, when oxygen is reintroduced to ischemic tissue, is supported by a large body of experimental evi-

Figure 4. At one hour post reperfusion there was a significant increase in left ventricular end-diastolic pressure (LVEDP) in each of the groups.
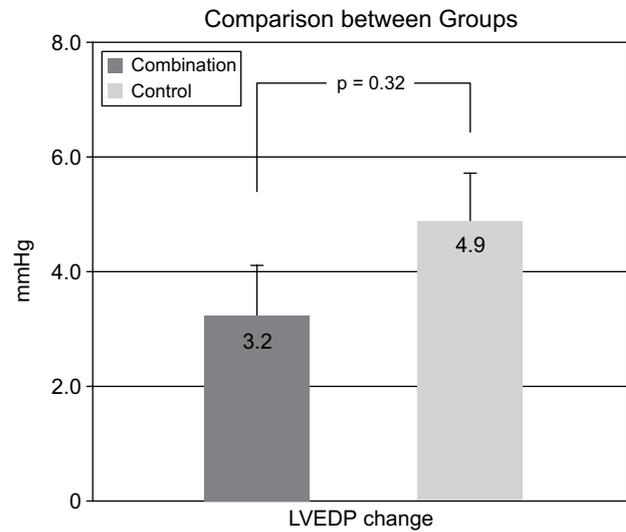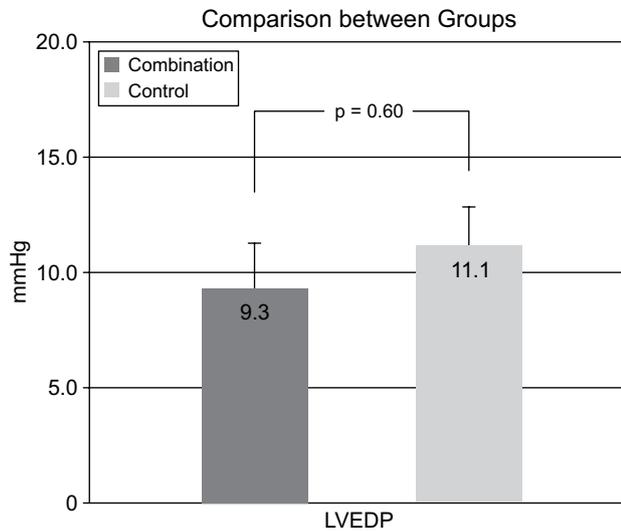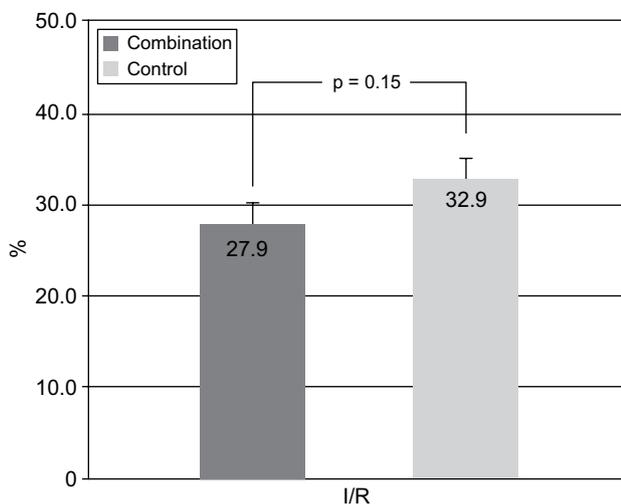
Figure 5. There was no significant difference between the groups in either the absolute values or the change in the left ventricular end-diastolic pressure (LVEDP) as measured at one hour post reperfusion.

dence.[4,5,29] During ischemia antioxidant defenses are eroded. Thus, the ability of the tissues to tolerate increased production of free oxygen radicals during reperfusion is diminished by the preceding ischemic injury.[2] The reintroduction of oxygen during reperfusion evokes a burst of free radicals. This has been demonstrated in experimental studies, as well as in patients with acute myocardial infarction treated with either thrombolysis or PCI.[4,30] Direct identification of free radicals has been possible in experimental studies of ischemia and reperfusion with the technique of electron spin resonance spectroscopy and spin trap-

ping.[3,31] *In vitro* studies have shown that oxygen-derived free radicals can cause damage to myocardial cells.[3,16] Free radicals interact with phospholipids and proteins of the cell membrane causing lipid peroxidation and oxidation of thiol groups. This has deleterious effects on membrane ultrastructure and function.[4] Oxygen-derived free radicals also stimulate the endothelial release of platelet activating factor, which attracts neutrophils to the reperfused area. Neutrophils contribute to the pathogenesis of reperfusion injury and to further production of oxidant radicals.[7]

Antioxidants, such as superoxide dismutase, bu-

Figure 6. No significant difference as regards the infarcted area was observed between the two groups (p=0.15). I/R – infarcted area (I) / area-at-risk (R).
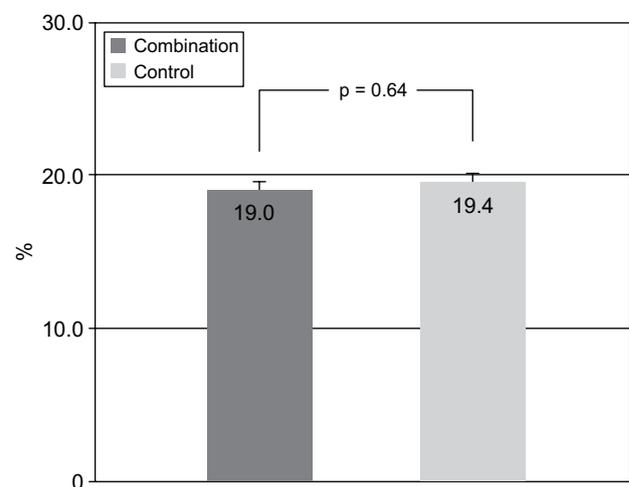
Figure 7. The proportion of area-at-risk (R) within the area of the left ventricle (LV) was equal in both groups (p=0.64).

cillamine, N-acetylcysteine, allopurinol, vitamin E, and the combination of ascorbic acid with glutathione, have been shown to reduce reperfusion injury in experimental studies.[1,7] Nevertheless, there are also studies showing the failure of several antioxidants to protect from reperfusion injury in experimental or clinical settings.[13,18,19] These results have led to the hypothesis that in many cases a single antioxidant may not be sufficient to exert a potent therapeutic effect. Thus, some investigators have used drug combinations in order to block simultaneously different steps of the pathogenetic process.[4,6,17,20] Antioxidant combinations have shown promising results in some experimental models of myocardial reperfusion injury.[15,17,20,32,33]

The combination of ascorbic acid and desferrioxamine would be expected to have a synergistic action, because both drugs attenuate the effect of oxygen free radicals, but with a different mode of action. Ascorbic acid (vitamin C) is a potent, water-soluble antioxidant, because it scavenges reactive oxygen radicals, protecting many substrates from oxidative damage,[14-20] while desferrioxamine may ameliorate oxidative stress by reducing the availability of Fe+ and thus the production of hydroxyl radicals during reperfusion via Fenton reactions.[2,8-11]

In the present study, however, the above drug combination did not show a significant effect on infarct size, arrhythmias, or blood flow. There are several reasons that may explain this negative result. A possible explanation lies in the route and time of administration. The intravenous route was selected because it is convenient and can be performed safely in everyday clinical practice. Additionally, the present experimental protocol was selected in order to resemble the clinical scenario of myocardial infarction and reperfusion by PCI. Therefore, in order for the results to be clinically meaningful, the tested drugs had to be administered just before reperfusion and during the first minutes of reperfusion. Moreover, previous studies have shown that in order to achieve a significant reduction in reperfusion injury, high concentrations of a suitable drug must be present in the reperfused tissue very early, at the onset of reperfusion.[4,5] In the present study the dosage of the two drugs was in excess compared to their usual therapeutic dosage and the infusion was started well before the onset of reperfusion. Thus, the drugs were already present in the blood in order to enter the myocardium immediately at the onset of reperfusion. However, it is possible that intravenous administration might not achieve the high drug concentrations needed in the reperfused tissue, at the onset of reperfusion, in order to produce a significant effect.[34]

Another explanation for the findings of the present study could lie in the effects of the administered drugs. The already published evidence regarding the effects of ascorbic acid, either independently, or in combination with other substances, is contradictory. In some studies it has been shown to reduce reperfusion injury, while in other studies it failed to provide any beneficial effect.[14,17,19,35-37] Similarly, contradictory findings exist regarding the effects of desferrioxamine on myocardial reperfusion injury.[12,13] This leads to the conclusion that the effect of antioxidants on reperfusion injury is greatly influenced by the specific experimental conditions and the drug combination used. Factors such as dosage, route and timing of administration, as well as the animal model and the duration of the preceding ischemia, may play an important role in determining the results of the study. Moreover, selection of the appropriate antioxidant composite in such a way that there is no negative interaction between the antioxidants is crucial. This has been shown in a previous experimental study, in which the combination of ascorbic acid, desferrioxamine and N-acetyl-cysteine (NAC) did not offer any protection from reperfusion injury.[38]

Finally, oxygen free radicals can cause damage, but they also trigger some protective cellular functions. An example of such a protective function is ischemic preconditioning.[39] Recent studies have shown that low concentrations of tumor necrosis factor alpha (TNFa) can offer cardioprotection, and that free radical signaling is involved in TNFa-induced cardioprotection.[40] The generation of free radicals in tissues is now recognized as a signaling mechanism for a vast range of metabolic pathways.[41,42] Cells maintain a delicate balance between protective oxidant signaling and the detrimental effects of oxygen free radicals.[2] It is possible that some antioxidant combinations interfere with this balance and abolish not only the detrimental effects, but also some protective effects of oxidant signaling.

Limitations

A limitation of the present study is that administration of the studied drug combination through another route was not tested (e.g. intracoronary or retrograde cardiac venous route). However, this would probably reduce the clinical meaning of the results, since the administration or infusion of therapeutic agents ei-

ther intracoronary or through the cardiac veins is rare and thus clinical extrapolation of the results of such a study would be more difficult.

Another limitation of the study is the fact that an assessment of apoptosis was not included. A number of cells that were not necrotic at the time we estimated the infarct size could have been damaged and programmed to die in the near future. Experimental studies have shown that reperfusion injury can initiate apoptosis, probably as a consequence of oxidative stress.[43-45] Thus, an assessment of apoptosis could provide additional evidence.

Conclusions

The combined intravenous treatment with ascorbic acid and desferrioxamine does not provide efficient protection against reperfusion injury in this experimental model of myocardial ischemia and reperfusion. More evidence from experimental and clinical studies is needed to expand our knowledge of the pathophysiology of reperfusion injury and to achieve the goal of finding an effective treatment.

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