

Leukocyte and Platelet Depletion Can Alleviate Reperfusion Injury in Rabbits

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Abstract

To investigate the effect of leukocyte and platelet depletion on reperfusion injury using a leukocyte-platelet removal filter (LRF), several cardiovascular variables were examined in the rabbits during ischemia followed by reperfusion. The rabbits underwent cytoapheresis with LRF (n=5) and were compared with controls without LRF (n=5). LRF was composed of a nonwoven polyester fabric (1.8 μ m, 4.6 gm). Removal of leukocytes and platelets by LRF was 98% for both. A period of 30 min equilibration was allowed before any experimental intervention, at which time the diagonal artery was occluded for 20 min and then reperfused. All arrhythmias were defined and quantified in accordance with the Lambeth Convention. Regional wall thickening was examined by a pulsed Doppler dimension system. No significant differences were observed in hemodynamic variables between the two groups; however, rabbits treated with a LRF demonstrated greater regional wall thickening (LRF group: $17.0 \pm 0.9\%$, control group: $11.0 \pm 0.3\%$, $p < 0.01$), as well as significant improvement in the frequency of ventricular arrhythmias (LRF group: 12.5%, control group: 47.2%, $p < 0.01$). The data suggest that LRF may help prevent arrhythmias and preserve left ventricular contraction during and after intracoronary thrombolysis.

Introduction

Mounting evidence suggests that neutrophil-mediated oxygen free radical release is responsible for the myocardial injury which occurs following intracoronary thrombolysis^{1,2}. It is believed that the majority of injury occurs not during the period of "no flow," but during the initial phase of reperfusion. This functional abnormality is attributed to a burst of oxygen-free radical formation with subsequent lipid peroxidation and membrane damage. Leukocytes are believed to be the source because they have the capacity to produce copious amounts of reactive oxygen species and can also release other mediators that influence cardiac function³.

The role of leukocytes in mediating myocardial necrosis during ischemia, reperfusion, or both is supported by previous studies showing that either leukocyte depletion or pharmacologic inhibition of leukocyte chemotaxis and function during ischemia can reduce infarct size⁴; however, whether leukocyte depletion has been proven effective for

myocardial dysfunction is still controversial. O'Neil and colleagues reported no functional improvement in dogs rendered neutropenic with a specific antiserum⁵. Litt and his coworkers found that neutropenia limited to the perfusion period was associated with significant reductions in the extent of the infarct with no reflow zones after 90 min of ischemia⁶. A careful review of this article, however, reveals that leukocyte depletion obtained by filtering achieved a 30% reduction of platelets.

To clarify the interaction between leukocytes and platelets which might be contributable to reperfusion injury, our study examined the role of leukocytes and platelets in myocardial stunning by removing leukocytes and platelets completely from whole blood using a leukocyte removal filter and assessing whether leukocyte and platelet depletion can augment post-ischemic contractility.

Materials and Methods

Surgical Procedure

All rabbits were housed and experiments performed humanely at the animal facility at Fukui Medical School where all procedures were overseen by an authorized committee corresponding to the American Association for

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the Accreditation of Laboratory Animal Care in the U.S.

Fourteen male New Zealand white rabbits weighing 3.0 to 3.5 kg were anesthetized with intravenous pentobarbital (20 mg/kg) and then were intubated and ventilated with a positive pressure ventilator and oxygen (2 L/min). A left thoracotomy and pericardiotomy were performed and the heart suspended in a pericardial cradle. The large marginal branch corresponding to the left anterior descending coronary artery in humans was compressed with a length of polyethylene tubing held securely in place for 20 min with 5-0 nylon sutures. The polyethylene tubing was removed and reperfusion performed for 50 min. A 2-mm diameter, 10MHz pulsed Doppler probe (DMT 102, Crystal Biotech Inc., Hopkinton, MA) was placed on the epicardial surface at the site to be rendered ischemic. A solid state pressure transducer (SPC-330A, Millar Instruments Inc., Houston, TX) was positioned in the left ventricular (LV) cavity through the left carotid artery to measure LV pressure and its first derivative.

An extracorporeal circuit was established between the

left carotid artery and the left internal jugular vein. Blood from the cannula leading from the carotid artery was circulated by a roller pump (flow rate: 8-12 ml/min) through a filter (Sepacell R-500[®], Asahi Medical Co., Tokyo, Japan) to deplete both leukocytes and platelets. The filter was composed of nonwoven fabric (1.8 μ m, 4.6 gm). A sham operation was performed with the same procedures with blood being pumped through a bypass circuit to allow whole blood to enter the internal jugular vein.

As a preliminary experiment, specific leukocyte depletion was obtained by the filter (Sepacell-PL[®], Asahi Medical Co., Tokyo, Japan) in an additional three rabbits.

Experimental Protocol

Rabbits were assigned to two groups: 1) leukocyte removal filter treated, and 2) sham operated controls. Leukocyte and platelet depletion was achieved by pumping the blood through two filters 2 hours before coronary occlusion. Equilibration was allowed for 30 min before any experimental intervention. The marginal branch was occluded for 20 min and then reperfused. To quantify arrhythmias, an electrocardiogram was recorded at a paper speed of 5 mm/sec throughout the occlusion phase, at 25 mm/sec during the first 2 min of reperfusion, and at 5 mm/sec for the subsequent 3 min of reperfusion. Simultaneously,

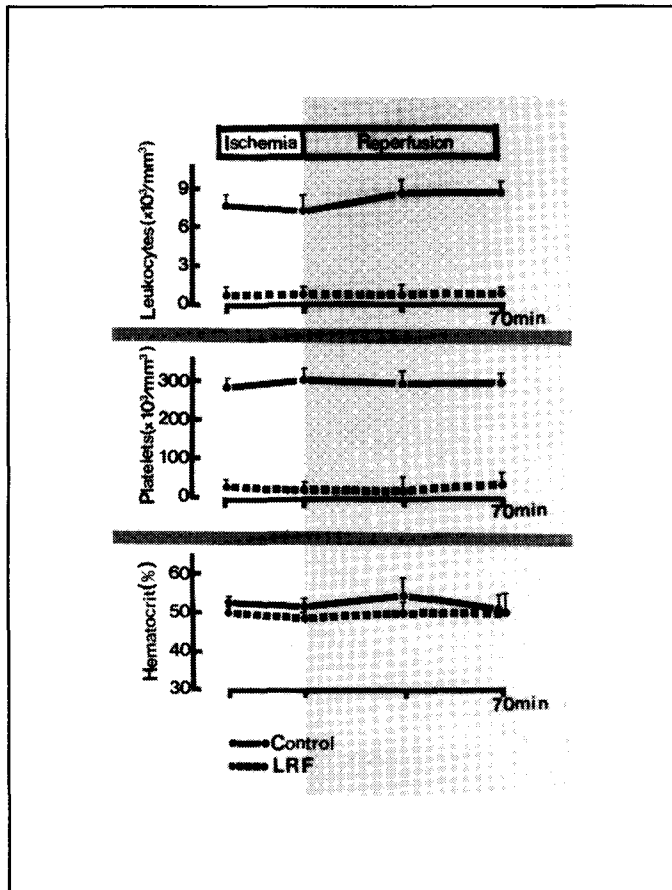


Figure 1
Plot of leukocyte and platelet counts, and hematocrit of the extracorporeal blood in the presence or absence of a filter. Leukocyte removal filter markedly depleted both leukocytes and platelets; it did not change the hematocrit, however. ○ = presence of a filter; ◻ = absence of a filter; LRF: leukocyte-platelet removal filter.

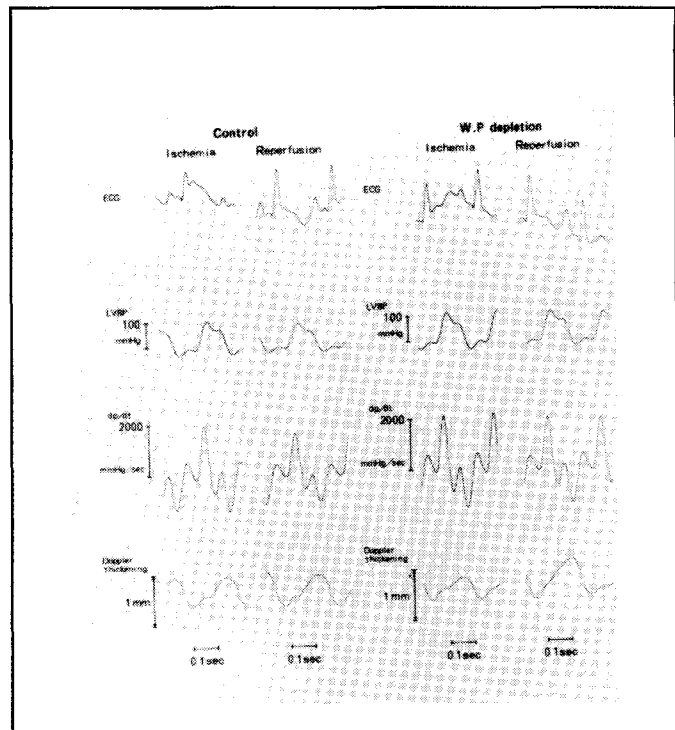


Figure 2
Analog data from the rabbits in two different groups. The amplitude of wall thickening recorded from leukocyte and platelet depleted rabbits shows remarkable recovery at the reperfusion period. W: white blood cell; P: platelet

	LVSP (mmHg)	LV dp/dt max (mmHg/sec)	Heart Rate (beats/min)	% Wall Thickening (%)	Arrhythmias	
					frequency of VPB (%)	Infarct Area (%)
Control (n=5)						
Basal	134 ± 8.0	2010 ± 115	270 ± 10.9		47.2%	
Occlusion 5min	126 ± 7.4	1890 ± 290	280 ± 21.4	11 ± 0.3	(4.6 ± 0.2)	18.2 ± 4.5
Reperfusion 3min	120 ± 9.5	1950 ± 210	273 ± 30.5			
LRF treated (n=5)						
Basal	128 ± 3.5	2200 ± 320	280 ± 14.0		12.5%*	
Occlusion 5min	110 ± 8.1	1990 ± 210	272 ± 27.3	17 ± 0.9*	(1.3 ± 0.4)	19.6 ± 5.1
Reperfusion 3min	110 ± 7.8	1900 ± 315	269 ± 41.0			

*p < 0.01 as compared with control value.

Table 1
Hemodynamic parameters in control and leukocyte removal filter treated group during marginal artery occlusion and reflow.

electrocardiograms were recorded by the data recorder and analyzed for the incidence and duration of ventricular fibrillation. The following cardiovascular parameters were recorded: systemic blood pressure, heart rate, electrocardiogram (ECG) to assess the incidence of premature ventricular contractions, and LV pressure and its first derivative (dp/dt).

³¹P Nuclear Magnetic Resonance (NMR) Spectroscopy

It is technically difficult to obtain coronary flow and maintain satisfactory positioning of the probe throughout the experiment. To assess whether regional ischemia and reperfusion are present, we developed a ³¹P NMR spectroscopy technique that allows myocardial high energy phosphate metabolism to be evaluated.

Phosphorus NMR spectroscopy was performed on a BEM250/80, 2 Tesla (Ohtsuka Denshi, Tokyo, Japan) narrow bore and horizontally positioned spectrometer operating at a phosphorus frequency of 35.3 MHz. Free induction decay (FID) signals were obtained using a 23 μsec radiofrequency pulse and pulse repetition of 2 sec. Six hundred FID signals were accumulated. The observed phosphorus compounds were as follows: inorganic phosphate (Pi), creatine phosphate (CrP), and three peaks of adenosine triphosphate (ATP), where the B-peak was considered to be specific for ATP.

Histopathologic Examination of Myocardial Tissue

The hearts were excised rapidly after 20 min of ischemia and 50 min of reperfusion. Each heart was sectioned into three transverse slices parallel to the atrioventricular groove and then incubated in a 2% solution of

2,3,5-triphenyltetrazolium chloride (TTC) for 20 min at 37°C. Regions that failed to demonstrate brick-red staining with TTC were considered to be positive for infarct myocardium.

Analysis

Myocardial wall thickening, LV systolic pressure (LVSP), LV dp/dt, and ECG (limb lead II) were monitored throughout the experiment on a multichannel recorder (RM 6300, Nihonkoden, Tokyo, Japan). Hemodynamic measurements were determined from a mean of five continuous cardiac cycles. Percent wall thickening was calculated from the formula:

$$\% \text{ wall thickening} = \frac{A(\text{reperfusion}) - A(\text{ischemia})}{A(\text{control})} \times 100$$

Amplitude (A) was measured from 10 to 12 continuous cardiac cycles for each sample period and averaged. The significance of differences between groups for hemodynamic data and differences in percent wall thickening were calculated by repeated measure analysis of variance.

Results

Of the 14 rabbits initially selected, four rabbits were excluded because of inadequate leukocyte depletion and death during the experiment.

Cytopheresis

Baseline leukocyte levels were similar in the two groups (8200 ± 1400/μl in control rabbits vs. 7100 ± 2000/μl in filter treated rabbits). Passage of the blood through the extracorporeal circuit did not significantly alter neutrophil, monocyte, eosinophil, or leukocyte counts in the control group; however, there was a mild reduction in the platelet count. In the filter (Sepacell R-500®) treated group, however, there was near total neutropenia (60 ± 5/μl, p < 0.001 vs. control) and severe thrombocytopenia (7200 ± 300/μl, p < 0.001 vs. control); the filter also markedly reduced monocytes and eosinophils. The reduction rate of each blood cell was as follows: polymorphonuclear neutrophil: 98%, lymphocyte: 86%, monocyte: 95%, eosinophil: 62%, and basophil: 81%. Not a single polymorphonuclear neutrophil was detected in the sample taken at the outlet of the filter. Circulating neutrophil levels were assessed by total peripheral leukocyte counts. Severe neutropenia and deficiency of other leukocytes persisted throughout the ischemia and reperfusion periods, as did thrombocytopenia (Figure 1). Peripheral leukocyte levels recovered to baseline within six hours after the experiment. In the preliminary experiment using the Sepacell PL®, the neutrophil level decreased remarkably and reached the same value as that of the Sepacell R-500® with a slight reduction in platelets (neutrophil: 54 ± 11/μl, platelets: 272000 ± 14000/μl).

Criterion	Score
No ventricular arrhythmias	0
Single VPBs only	1
Couplets and/or bigeminy and/or salvos	2
VT	3
VF which was reverted spontaneously to sinus before the end of the experiment	4
VF which was sustained until the end of the experiment	5

Table 2
Scoring system for arrhythmias.

Hemodynamics

Hemodynamic parameters during the occlusion and reperfusion periods are summarized for control and filter treated groups in Table 1. There was no significant change in heart rate, LVSP, and LV dp/dt during the experiment in either group; however, as shown in Figure 2, coronary artery occlusion produced a small, nonsignificant decrease in LVSP and peak dp/dt with no change in heart rate in both groups. The degree of wall thickening post-reperfusion in the leukocyte and platelet depleted groups showed marked recovery; otherwise, there was no change in the control group. As summarized in Table 1, the percentage of wall thickening was low in the control group during the experiment ($11.0 \pm 0.3\%$). In contrast, rabbits in which leukocytes and platelets were depleted showed greater changes ($17.0 \pm 0.9\%$).

In the leukocyte specific depleted group, which was preliminary, the degree of wall thickening increased after reperfusion (right side in Figure 2); however, there was no statistically significant difference.

Arrhythmias

In the control group, severe ventricular arrhythmias began after 4 to 5 min of coronary artery occlusion, peaking after 10 min and shortly after reperfusion (Figure 3). During reperfusion, the mean time to onset of ventricular fibrillation was 305 ± 51 sec, and the mean time to onset of ventricular tachycardia was 354 ± 30 sec. Ventricular premature beat (VPB) was exhibited in 47% of the rabbits, whereas almost 70 % exhibited ventricular tachycardia. In the leukocyte and platelet depleted groups, no ventricular fibrillation was observed in the post-reperfusion period; however, the duration of ventricular tachycardia did not change. The scores for the individual rabbits were averaged as defined by the criteria shown in Table 2, in accordance with the "Lambeth Convention". Both leukocyte and

The frequency of arrhythmias (VPB)

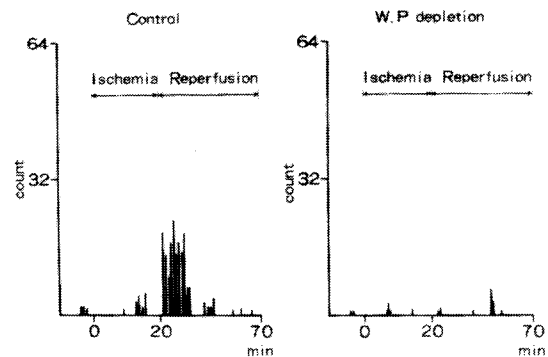


Figure 3
The frequency of ventricular premature beats during ischemia and following the reperfusion period. W: white blood cell; P: platelet.

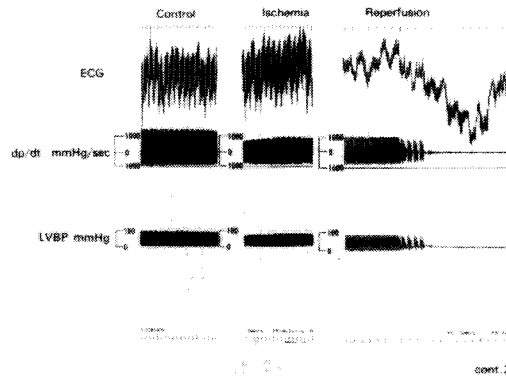


Figure 4
Continuous recordings of ECG, left ventricular dp/dt and left ventricular blood pressure (LVBP) from the control rabbit. Ventricular fibrillation occurred four minutes after reperfusion.

platelet depletions had no significant effect on the duration of ventricular tachycardia, but the incidence of ventricular fibrillation and VPB was markedly reduced (Figures 4,5). In the specific leukocyte depletion group, the frequency of VPB was reduced compared to the control group; however, the reduction was not as great as the same values of the leukocyte and platelet depletion group (Figure 3).

Histopathologic Findings

The proportion of the left ventricle involved in myocardial infarction was the same in the control ($19.6 \pm 5.1\%$) and leukocyte and platelet depleted ($18.2 \pm 4.5\%$) groups (Figure 6). Comparison of the infarct sizes indicated that the

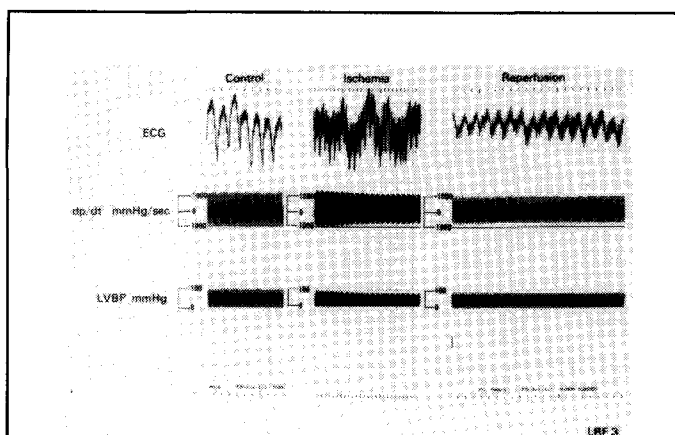


Figure 5
Continuous recordings of ECG, left ventricular dp/dt and left ventricular blood pressure (LVBP) from the leukocyte and platelet depleted rabbit. There are no significant arrhythmias before or after the reperfusion period.

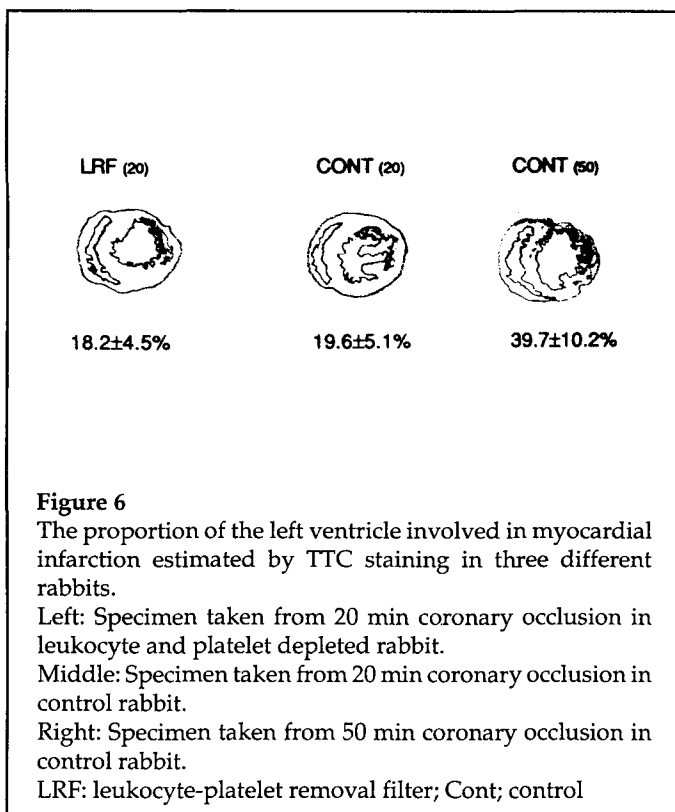


Figure 6
The proportion of the left ventricle involved in myocardial infarction estimated by TTC staining in three different rabbits.
Left: Specimen taken from 20 min coronary occlusion in leukocyte and platelet depleted rabbit.
Middle: Specimen taken from 20 min coronary occlusion in control rabbit.
Right: Specimen taken from 50 min coronary occlusion in control rabbit.
LRF: leukocyte-platelet removal filter; Cont; control

percentage of infarct area which was caused by 20 min ischemia was less than that caused by 50 min ischemia (39.7 ± 10.2%). TTC positive areas demonstrated no more transmural infarction between control and leukocyte and platelet-depleted groups after 20 min of coronary occlusion.

Discussion

Many studies have examined the role of neutrophils in the pathogenesis of myocardial infarction. There is considerable accumulation of neutrophils in the infarct region during the first 24 to 48 hr after reperfusion⁸. Treatment with antiinflammatory agents, prostacyclin, or its analogue, iloprost, before ischemia reduces this neutrophil accumulation in infarcted myocardium and is associated with up to a 40% reduction of infarct size^{9,10}. This beneficial effect appears to be related to inhibition of the lipoxygenase pathway of arachidonic acid metabolism, resulting in reduced neutrophil activation. Other studies using rabbit antineutrophil serum to produce neutropenia in dogs and leukocyte depleted dogs obtained by filtering demonstrated a mean reduction in infarct size¹¹. These studies also revealed that neutropenia during the reperfusion period reduced infarct size. Neutrophils, thus, appear to be a cause of jeopardized myocytes after coronary occlusion and subsequent reperfusion.

The important difference between these previous reports and the present study is that we elucidated the change of regional wall motion which might be influenced by myocardial ischemia in the pre- and post-reperfusion stages. We also investigated the effect of platelets on reperfusion injury using two different types of filters: Sepacell-R[®] which can remove both leukocytes and platelets¹², and Sepacell-PL[®] which can remove leukocytes specifically¹³.

In previous studies, neutropenia during reperfusion had no significant effect on cardiac performance, although myocardial necrosis was diminished. A careful review of these articles revealed that coronary occlusion times were longer than 90 min. After longer coronary occlusion, neutrophil depletion did not noticeably affect cardiac function, most likely due to the severity of myocardial injury related to prolonged ischemia. From these standpoints, we performed coronary ischemia by 20 min occlusion. In fact, 50 min coronary occlusion of the rabbit without collateral circulation showed marked transmural myocardial infarction; however, 20 min coronary occlusion demonstrated localized endocardial infarct.

LV wall thickening is a widely used index of regional myocardial function in experimental animals and humans. In animals, wall thickening is conventionally measured by the transit time technique, which uses two ultrasonic crystals, one sutured to the epicardium and the other inserted into the subendocardium. An important limitation is that a crystal in the myocardium causes considerable trauma and may result in inaccurate measurement. In addition, it is technically difficult to obtain and maintain alignment of the two crystals throughout the experiment. Furthermore, coronary flow cannot be measured in rabbits using the Doppler probe due to heart size. To overcome these limitations we used a single epicardial transducer which provided a simple, atraumatic and accurate means

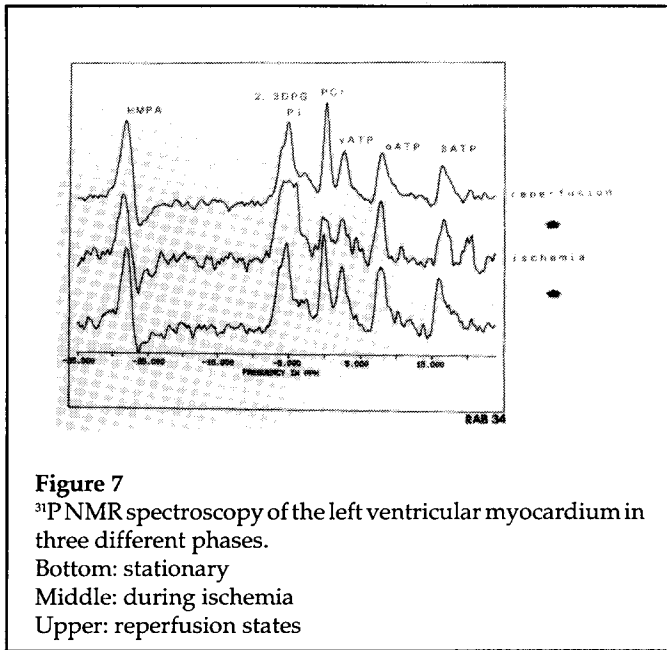


Figure 7
 ^{31}P NMR spectroscopy of the left ventricular myocardium in three different phases.
 Bottom: stationary
 Middle: during ischemia
 Upper: reperfusion states

for measuring myocardial function, both transmurally and in selected layers of the LV wall¹⁴.

To assess whether myocardial ischemia could be produced by coronary occlusion, we used ^{31}P -MNR spectroscopy as described in the Methods section. The values for creatine phosphate and ATP decreased during ischemia and reached control values during reperfusion (Figure 7). Previous studies have used dogs as experimental subjects, but some developed collateral circulation, making it difficult to evaluate and confirm the area at risk. To obviate the problems associated with collateral circulation, we used rabbits in which a rapid depletion of leukocytes was possible.

In seeking to define the mechanisms underlying the genesis of serious ventricular arrhythmias during both ischemia and reperfusion, it is probably reasonable to state that the establishment of a reentry circuit and possibly enhanced automaticity are the ultimate electrophysiologic aberrations responsible for manifestation of the arrhythmias¹⁵, and regional heterogeneity of tissue injury or recovery is probably a progenitor for reentry circuits. In the present study, we have shown that leukocyte and platelet depletion in an in vivo rabbit model significantly reduces the incidence and severity of ischemia-induced reperfusion arrhythmias. We have not directly measured free radicals; however, many studies report radical scavengers as having arrhythmogenic effects with a reduction in free radical formation¹⁶. If free radical formation initiates a sequence of events leading to an arrhythmia, it is necessary to ask whether there is a direct electrical effect of these charged intermediates, or if they act via some intermediate process. At the present time, this question cannot be definitively answered.

This study suggests that using a filter to deplete leuko-

cytes and platelets from blood abrogates myocardial stunning. There are a number of potential mechanisms whereby removal of leukocytes and platelets could improve segment function during reperfusion. Polymorphonuclear leukocytes are powerhouses of free radical production and can also release other mediators capable of influencing myocardial function, integrity, or both, including platelet activating factor (PAF), metabolites of arachidonic acid (AA), and proteolytic enzymes. PAF and leukotriene (LT) C4 reduce contractility of isolated papillary muscles; moreover, PAF, AA, and LTB₄ enhance superoxide anion formation by neutrophils¹⁷. An alternative mechanism by which leukocytes may compromise post-ischemic contractility is the plugging of small coronary arterioles and capillaries resulting in underperfusion of discrete regions of the myocardium¹⁸. Our preliminary study using the Sepacell-PL[®], which removed leukocytes specifically, indicated that platelets played an important role in reperfusion injury. During the "super-acute" phase of the reperfusion period, microvascular contraction might have occurred due to interaction between platelets and the coronary endothelium. An additional means by which the filter could improve post-ischemic contractility is a reduction in blood viscosity secondary to leukocyte and platelet depletion, resulting in enhanced tissue perfusion. Neutrophils are large cells that must deform to pass through the microcirculation, and the resistance imposed by one neutrophil is approximately 2000 times that of a red blood cell. Consequently, neutrophils can have a large impact on vascular resistance¹⁹.

The present study indicates that a filter for clinical use may represent an important adjunctive treatment for patients with acute myocardial infarction undergoing reperfusion therapy.

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