Limitation of Myocardial Reperfusion Injury by Intravenous Perfluorochemicals
Role of Neutrophil Activation

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Neutrophil activation and infiltration into the ischemic myocardium after reperfusion may limit the amount of salvageable myocardium (reperfusion injury). The effects of intravenous perfluorochemicals (Fluosol-DA) on infarct size, ventricular contractility, and neutrophil function were assessed in an occlusion-reperfusion canine model. Closed-chest dogs were subjected to 90 minutes of left anterior descending artery occlusion followed by 24 hours of reperfusion. Animals were randomized to receive either Fluosol-DA (FDA, n=8) or Ringer’s lactate (CONT, n=10) intravenously over 30 minutes just before left anterior descending artery reperfusion. Neutrophil demargination and infiltration into the myocardium were assessed in vivo with In111. Neutrophil chemotaxis, superoxide radical production, and lysozyme degranulation were evaluated ex vivo at baseline, 1 hour after occlusion, and 1 hour after reperfusion. Perfluorochemicals significantly reduced infarct size expressed as percent of area at risk (FDA, 7±4%; CONT, 24±6%; p<0.01). This was associated with positive wall motion in the jeopardized zone of Fluosol-DA animals compared with dyskinesis in control animals (FDA, +4.4±2.1%; CONT, −1.1±1.5%; p<0.05). Electron microscopy showed reduced neutrophil and erythrocyte plugging of capillaries with relative preservation of endothelial cells in the Fluosol-DA animals. Myocardial blood flow was greater in the ischemic endocardium of Fluosol-DA animals 1 hour after reperfusion (FDA, 1.23±0.21; CONT, 0.62±0.08 ml/g/min; p<0.01). Neutrophil demargination and infiltration into the ischemic myocardium was reduced in the animals treated with Fluosol-DA. (FDA, 2.5±0.7×10⁶; CONT, 14.1±2.7×10⁶ neutrophils/g; p<0.01). Neutrophil chemotaxis and lysozyme release were also markedly suppressed in the Fluosol-DA groups ex vivo. These results show that intravenous Fluosol-DA significantly reduces reperfusion injury with greater salvage of myocardium and improved left ventricular function. The chief mechanism of action of Fluosol-DA appears to be the suppression of neutrophil function. (Circulation 1989;79:645–656)

Early reperfusion of an evolving myocardial infarction has been shown to limit myocardial necrosis and preserve ventricular contractile function, thereby reducing cardiac mortality.¹⁻⁶ However, the introduction of oxygen and cellular elements, particularly neutrophils, into the ischemic myocardium have been postulated to initiate deleterious cascade of events which limit myocardial salvage after reperfusion.⁷⁻⁸ This entity has been termed “reperfusion injury.”⁹ Neutrophils have also been implicated in the progressive reduction of microvascular blood flow in the ischemic region after reperfusion.⁸ Activated neutrophils produce various substances that are both cytotoxic and further amplify the inflammatory response after reperfusion.¹⁰⁻¹³ These include reactive oxygen species, leukotrienes, platelet activating factor, and various potent proteolytic enzymes. The reduction in infarct size in experimental preparations with neutropenia, antiinflammatory agents, and free radical scavengers adds further support to the role of neutrophils in the pathogenesis of reperfusion injury.¹⁴⁻¹⁶

Perfluorochemicals are substances with a small particle size (1–2 μM), low viscosity, and high oxygen-carrying capacity. Neutrophils exposed in

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vitro to the perfluorochemical mixture manifest reduced chemotaxis, adherence, and superoxide anion production.\(^{12,18}\) We have previously shown that intracoronary administration of the perfluorochemical (Fluosol DA-20\%) after reperfusion in dogs subjected to 90 minutes of ischemia results in a 60\% reduction in infarct size and improved regional ventricular function over a 2-week postreperfusion period.\(^{19,20}\) An additional study demonstrated preservation of endothelial cell structure and function in animals treated with perfluorochemicals, and this was associated with qualitative reduction in neutrophil infiltration in the ischemic zone 1 hour after reperfusion.\(^{21}\)

Potentially wide clinical application of intracoronary perfluorochemicals in man would be limited because it results in inherent time delays and requires the availability of an acute cardiac catheterization laboratory. Therefore, we investigated the potential benefit of intravenous administration of perfluorochemicals in a clinically applicable dose given just before reperfusion on infarct size and ventricular function in the closed-chest canine model. The effects of the drug on neutrophil demargination from reserve pools sites to the systemic circulation and chemotaxis were determined in vivo using autogenous In\(^{111}\)-labeled neutrophils. Neutrophil chemotaxis, superoxide radical production, and proteolytic enzyme release were also serially assessed ex vivo. Endothelial cell ultrastructure and the presence of neutrophil plugging in capillaries were determined with electron microscopy.

**Methods**

**Experimental Preparation**

Thirty-six mongrel dogs of either sex, weighing 15–25 kg and free of common canine diseases, were prepared 5–7 days before the experiment. Under general anesthesia, the heart was exposed by a left thoracotomy and the left anterior descending artery (LAD) was isolated distal to the first diagonal branch. A surgical monofilament ligature was placed around the proximal left anterior descending artery and exteriorized to a subcutaneous pocket within a polyethylene tubing (model 601-325, Dow). The ligature was tightened for 30 seconds to ensure an adequate ischemic zone. If the anterior left ventricular wall did not become pale, the snare was moved proximal to the first diagonal branch. A heparin-filled silastic catheter (model 602-285, Dow) was placed in the left atrial appendage for subsequent radioactive microsphere injection as well as exteriorized to a subcutaneous pocket. The thorax was closed and pneumothorax evacuated. The animals were given two daily doses of prophylactic antibiotics (600,000 units Vetricil-AS) and allowed 5–7 days to recover before instrumentation.

**Experimental Protocol**

Animals were anesthetized with pentobarbital (25 mg/kg i.v.), intubated, and ventilated with room air (Harvard respirator). Fifty milliliters of heparinized venous blood was used to label neutrophils with In\(^{111}\). All procedures were performed under aseptic conditions. The LAD snare and the left atrial catheter were retrieved from the subcutaneous pocket. Both femoral arteries were exposed and cannulated for microsphere flow sampling, measurement of arterial pressure, and left ventricular end-diastolic pressure. A 7F Gensini\(^{\circledR}\) (USCI) catheter was placed in the inferior vena cava for neutrophil sampling. The autogenous In\(^{111}\)-labeled neutrophils were reinfused into the animal and allowed to disperse throughout the circulation for 45 minutes.\(^{22}\) Supplemental anesthesia during the protocol was maintained with morphine sulfate and diazepam. Animals were randomized in subgroups of six animals to receive a 10-ml test dose of either Ringer's lactate or Fluosol-DA (20\%) before commencing the study.

The experimental protocol is shown in Figure 1. After baseline measurements, the snare was tightened in two stages 3 minutes apart and maintained for 90 minutes. Total occlusion of the LAD was confirmed with selective coronary angiography. During the 30 minutes just before reperfusion, the animals received either Fluosol DA-20\% (FDA) or Ringer's lactate (CONT) (0.8 ml/kg/min) intravenously. Animals received lidocaine at occlusion (5 mg/kg bolus followed by 0.12 mg/kg/min maintenance for 30 minutes) and at reperfusion (3 mg/kg bolus followed by 0.12 mg/kg/min maintenance for 15 minutes). Both the Fluosol-DA and Ringer's lactate solution were oxygenated (100\% oxygen) for 20 minutes before infusion. At 90 minutes after occlusion, the snare was gradually released over 3–5 minutes, and 30,000 units streptokinase was given into the left coronary ostium. Animals were ventilated with 100\% O\(_2\) at the start of Fluosol-DA or Ringer's lactate infusion; this was continued for the first 3
hours of reperfusion. Animals were weaned from the ventilator and allowed to recover for 24 hours. The cardiac rhythm (three leads) was continuously monitored during the experiment (model VR-12, Electronics for Medicine). Ventricular fibrillation or hemodynamically significant ventricular tachycardia was treated with electrical DC cardioversion (50–200 J) to revert the rhythm to sinus. All animals underwent left ventriculography in the 30° right anterior oblique projection at baseline, 45 minutes after occlusion, and at 3 and 24 hours after reperfusion. Regional myocardial blood flow was serially evaluated with radioactive microsphere injections into the left atrium. Microspheres were labeled with five isotopes and injected (−2×10⁹/injection) in the following order: ⁴¹Ce, ⁸⁵Sr, ⁴⁶Sc, ¹²⁵I, and ⁵¹Cr (3M Company, St. Paul, Minnesota). Total and differential venous leukocyte counts and lidocaine concentrations were measured serially. Arterial blood gases were monitored (model 158, Corning) and respirator adjusted to ensure normal pH (7.40±0.05) and adequate oxygenation on room air (PO₂>70 mm Hg) and on FiO₂ 100% (PO₂>400 mm Hg).

At 24 hours, the animals were reanesthetized with pentobarbital (15 mg/kg i.v.), the left thorax reopened, and LAD snare ligated under direct vision. Monastral blue dye (Du Pont) was injected through a pigtail catheter positioned in the ascending aorta in a dose of 1 mg/kg 2 minutes after snare ligation. After an additional bolus of pentobarbital and potassium chloride, the hearts were quickly removed and washed to prevent counterstaining.

Analysis of Area of Risk and Infarction

The hearts were cut into 1-cm transverse sections parallel to the atrioventricular groove from apex to base and photographed with Ektachrome® (Kodak) to define the area of risk (unstained by Monastral Blue dye). The slices were incubated in 2.0% triphenyltetrazolium chloride (TTC) for 15 minutes at 37°C and rephotographed. The ratio of the infarcted volume to volume at risk was then calculated by a blinded observer using computerized planimetry program as previously described.

Calculations of Regional Myocardial Blood Flow

The second and fourth transverse myocardial slice was sectioned into three zones: central ischemic, lateral ischemic, and nonischemic posterior wall (Figure 2). Each zone was further subdivided into epicardial, midmyocardial, and endocardial sections weighing 0.3–1.0 g. Myocardial sections and reference blood samples were counted for 5 minutes in a multichannel analyzer (Model 5986, Packard Instrument Company, Downers Grove, Illinois) with background correction and the overlapping radioactivity between isotopes corrected using matrix correction method (Compusphere Software®, Packard Instrument, Downers Grove, Illinois). The corresponding sections from the two transverse rings were averaged to give the final flow determination.

Figure 2. The myocardium was sectioned into transverse slices (top) and further sectioned into segments (bottom) to determine regional myocardial blood flow (microsphere), neutrophil infiltration (In¹¹¹ activity), and histologic analysis.

Epicardial collateral flow in the central ischemic zone was calculated by averaging epicardial and midmyocardial flow from both the proximal and distal slice 45 minutes into occlusion.

Analysis of Ventricular Function

Global and regional left ventricular function was measured by digitization of an end-diastolic and end-systolic cineangiogram frame. The left ventricular ejection fraction was calculated by Simpson’s formula and the regional wall motion changes were assessed using a radial shortening method with customized software previously validated in our laboratory.

Neutrophil Labeling

The heparinized blood sample was mixed with 10 ml hetastarch and centrifuged for 20 minutes. The neutrophil rich supernatant plasma was removed and recentrifuged and the pellet sediment was isolated. The neutrophil pellet was washed with normal saline and resuspended in 400 μCi In¹¹¹ for 30 minutes. Most samples retained 50–80% of total In¹¹¹ activity. The viability of In¹¹¹-labeled neutrophils was ensured by Trypan blue exclusion and detection of In¹¹¹ activity in plasma samples drawn throughout the experiment.

In Vivo Neutrophil Chemotaxis

Neutrophil In¹¹¹ activity was determined from epicardial, midmyocardial, and endocardial samples from the central and lateral ischemic regions, the nonischemic region, and reference flow samples. The ratio of neutrophil population in each animal having In¹¹¹ activity was calculated to overcome differences in neutrophil harvesting for label-
ing, In111-labeling efficiencies, and potential injury during in vitro handling. The In111 activity in each segment of myocardium could be corrected to the number of neutrophils per gram of tissue,22 which assumes that the In111-labeled population and the nonlabeled neutrophil populations are sufficiently mixed and have similar ability to demarginate, respond to chemotactic stimuli, and infiltrate into the myocardial tissue.24,25

Ex Vivo Neutrophil Analysis

Heparinized plasma samples were drawn from the animal at baseline, 1 hour after LAD occlusion, and 1 hour after reperfusion. The neutrophils in each sample were isolated by 60-minute gravity sedimentation and Histopaque® (Sigma Chemical, St. Louis, Missouri) density gradient centrifugation. The neutrophils were washed and suspended in 0°C Hank’s balanced buffer solution with calcium (310-4025, Gibco Labs) at a concentration of 2x10⁶/ml. Neutrophil viability was studied by Trypan blue dye exclusion and by LDH release.17,26

Chemotaxis. Neutrophil chemotaxis to 10% zymosan activated plasma was assayed in modified Boyden chambers during a 45-minute incubation period.17 The number of cells migrating across the filter were counted in 20 random high power fields (hpf) (100x oil immersion, Zeiss Microscope) and averaged for the final index of chemotaxis.

Superoxide radical production. Superoxide radical anion production was assessed by the reduction of ferricytochrome C (80 μM, Sigma C-7752) using 2x10⁶ neutrophils in 100 μl. The cells were incubated at 37°C for 30 minutes in the presence of buffer alone, cytochalasin B (Sigma C-6762) and phorbol myristate acetate (3.2x10⁶ M, Sigma 2 P-8139), or cytochalasin B and opsonized zymosan (Sigma 74250) in triplicate. At the end of the incubation period, further cytochrome C reduction was halted by adding superoxide dismutase (20 μg, Sigma 57008). Each sample had an internal control in which superoxide dismutase was added before incubation. Reduced cytochrome C reduction was measured by spectrophotometry at 550-nM wavelength.

Lysozyme release. Neutrophil proteolytic enzyme release was assessed by measuring the amount of lysozyme released by 5x10⁸ cells into the media in response to no stimulation, cytochalasin B and phorbol myristate acetate stimulation, or cytochalasin B and opsonized zymosan stimulation. The cells were incubated at 37°C for 30 minutes and then centrifuged at 0°C. Lysozyme activity in the supernatant was measured by its ability to lyse Micrococcus luteus (Sigma M-3770) cell wall as described by Smoleus and Hartsell.27 This assay was validated to ensure lack of interference by Fluosol-DA.

Histology

Light microscopy. The first and third slice from all hearts were fixed in 10% buffered formaldehyde. Sections were taken for light microscopy from central ischemic area, the two border zones (lateral), and from the posterior nonischemic wall extending from the endocardium to epicardium, cut at 6 μm, stained with hematoxylin and eosin and Mallory’s trichrome stains, and examined in a blinded manner. The degree of inflammatory infiltrate (the number of neutrophils within capillaries and in the interstitium), hemorrhage, and the presence of contraction band necrosis in the border zone was assessed. An average of 20 hpf/slide were evaluated in the inner, middle, and outer third of the infarct regions. Neutrophil infiltration was assessed semiquantitatively according to method of Romson et al,14 and the extent of contraction band necrosis was quantitated by the method of Tazelaar et al.28

Electron microscopy. Myocardial biopsies were taken within 60 seconds of sacrifice from the anterior central ischemic zone and the nonischemic zone and divided into endocardial and epicardial halves. The tissue obtained at biopsy was cut into 1-mm² pieces, fixed in 3% buffered glutaraldehyde, and embedded in Epon. Semithin sections were cut, stained with toluidine blue, and examined by light microscopy. Artifact-free areas with the most capillaries were selected for ultrathin section cutting, stained with uranyl acetate lead citrate, and examined with a Zeiss 109 IGF electron microscope.

Statistics

Serial results in the groups (blood pressure, heart rate, myocardial blood flow) were analyzed by repeated measures two-way analysis of variance (BMDP2V, BMDP Software, Los Angeles). If there was statistical difference found between some pair of results, then further pairwise analysis was performed by Duncan’s multiple range test. Comparisons between the two groups (i.e., infarct size) was analyzed by nonpaired Student’s t test with two-tailed discriminant score. Qualitative histologic results were analyzed by χ² tests for ordinal data.

Results

Thirty-six animals underwent randomization (18 Ringer’s lactate and 18 Fluosol-DA). Eight animals (three control and five Fluosol-DA) died at the time of LAD occlusion with intractable ventricular fibrillation before having received either agent. Five animals died at the time of reperfusion (three control and two Fluosol-DA). Four animals were excluded (two control and two Fluosol-DA) due to inadequate myocardial ischemia that was prospectively defined as risk volume less than 20% of the left ventricle, minimal left ventricular wall motion abnormality at occlusion, and epicardial collateral blood flow in the ischemic region of more than 0.5 ml/g/min. One treatment animal was excluded due to a left empyema and technical snare problems. One animal in each group was successfully cardioverted. No animal died between the initial experiment and sacrifice at 24 hours. The results of the remaining
FIGURE 3. Hemodynamic changes during experimental protocol. No significant differences were noted in heart rate (HR), mean blood pressure (mBP), or rate pressure product (RPP) at baseline, 1 hour into occlusion (OCC), 15 minutes after reperfusion (REPER), and at 3 and 24 hours after reperfusion.

eighteen animals (10 control and eight Fluosol-DA) completing the protocol were analyzed.

Hemodynamic and Laboratory Measurements

No significant differences were noted in mean blood pressure, heart rate, or rate-pressure product throughout the experimental protocol (Figure 3). The left ventricular end-diastolic pressure was similarly elevated at occlusion and after Ringer's lactate and Fluosol administration. PaO₂ values were comparable in both Fluosol and Ringer's lactate groups both on room air and on 100% Fio₂. The lidocaine concentrations at 45 minutes after LAD occlusion were 2.7±0.3 and 2.8±0.4 μg/ml (p=NS) in the treatment and control groups, respectively. The lidocaine concentrations at 15 minutes after reperfusion were 2.8±0.6 and 2.4±0.2 μg/ml (p=NS) in the treatment and control groups.

Effect on Infarct Size

The average heart weight, left ventricular volume (data not shown), and area at risk (FDA, 48±4%; CONT, 50±3%; p=NS) were similar in treated and control animals (Figure 4). A significant reduction in infarct size was noted both when expressed as a percentage of the area at risk (FDA, 7±4%; CONT, 24±6%; p<0.01) and as a percentage of the total left ventricle (FDA, 4±2%; CONT, 13±4%; p<0.05) in the treatment group. In both groups, the infarcted area was confined to the subendocardium but was smaller and patchier in the animals treated with Fluosol-DA.

Effect on Regional Myocardial Blood Flow

Baseline epicardial and endocardial blood flow was similar in the two treatment groups (Table 1). Endocardial blood flow in the central ischemic zone was markedly reduced to less than 10% of baseline flow in both Fluosol-DA and Ringer's lactate animals 45 minutes after occlusion, suggesting comparable levels of severe ischemia (FDA, 0.07±0.01; CONT, 0.06±0.01 ml/g/min; p=NS). No significant differences in flow in the central and lateral ischemic zones were noted during the occlusion period before or after Fluosol-DA administration suggesting that treatment did not alter collateral blood flow. Both groups exhibited reactive hyperemia during reperfusion. Control animals manifested a significant reduction in both endocardial and epi-

Table 1. Myocardial Blood Flow (ml/g/min)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>OCC (45 min)</th>
<th>OCC (90 min)</th>
<th>REP (15 min)</th>
<th>REP (1 hr)</th>
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</thead>
<tbody>
<tr>
<td>Nonischemic zone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epicardium</td>
<td>Control</td>
<td>0.90±0.07</td>
<td>0.79±0.04</td>
<td>1.16±0.15</td>
<td>1.04±0.05</td>
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<tr>
<td></td>
<td>Fluosol</td>
<td>0.85±0.06</td>
<td>0.71±0.06</td>
<td>0.80±0.08</td>
<td>0.75±0.10</td>
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<tr>
<td>Endocardium</td>
<td>Control</td>
<td>0.71±0.06</td>
<td>0.62±0.04</td>
<td>0.91±0.11</td>
<td>0.88±0.05</td>
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<tr>
<td></td>
<td>Fluosol</td>
<td>0.62±0.03</td>
<td>0.11±0.03</td>
<td>0.10±0.03</td>
<td>1.43±0.13</td>
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<tr>
<td>Ischemic zone</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epicardium</td>
<td>Control</td>
<td>0.71±0.06</td>
<td>0.13±0.03</td>
<td>0.11±0.03</td>
<td>1.30±0.19</td>
</tr>
<tr>
<td></td>
<td>Fluosol</td>
<td>0.66±0.06</td>
<td>0.06±0.02</td>
<td>0.03±0.01</td>
<td>2.52±0.58</td>
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<tr>
<td>Endocardium</td>
<td>Control</td>
<td>0.72±0.05</td>
<td>0.07±0.01</td>
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<td>2.07±0.24</td>
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<tr>
<td></td>
<td>Fluosol</td>
<td>0.62±0.03</td>
<td>0.11±0.03</td>
<td>0.10±0.03</td>
<td>1.43±0.13</td>
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</table>

OCC, occlusion; REP, reperfusion; Control, Ringer's lactate.
*p<0.05; t p<0.01.
cardiac blood flow in the central ischemic region 1 hour after reperfusion.

**Relation of Infarct Size to its Baseline Predictors**

The relation between infarct size as a percentage of the area at risk and mean collateral blood flow (epicardial and midmyocardial in the central ischemic zone) in treated and control animals is shown in Figure 5. Only a rough linear relation was noted between these variables ($r=0.46$, $p=NS$). Although, the regression line for the Fluosol-DA group shows a lower y-axis intercept and a flatter slope, this was not statistically different from the control animals. No difference was noted between the relation of infarct size and area of risk or rate-pressure product.

**Effect on Ventricular Function**

The results of regional and global ventricular function are shown in Figure 6. No significant differences were noted in global ejection fraction in both groups throughout the experimental protocol. Although Ringer's lactate animals demonstrated greater regional radial shortening at baseline, both groups developed anterior wall dyskinesis during occlusion (FDA, $-2.6\pm1.1\%$; CONT, $-2.0\pm2.0\%$; $p=NS$). A significant improvement in regional function was noted in Fluosol-DA animals at 24 hours after reperfusion (FDA, $+4.4\pm2.1\%$; CONT, $-1.1\pm1.5\%$; $p<0.05$).

**Leukocyte Demargination**

Serial venous plasma neutrophil counts are shown in Figure 7. The number of neutrophils increased minimally during occlusion in both groups. Neutrophil counts tended to be lower at the end of the Fluosol infusion compared with control animals. Control animals manifested an abrupt increase in the number of neutrophils immediately after reperfusion, whereas the treated animals showed a delayed increase that was significantly lower during the 1st hour of reperfusion. Both groups demonstrated a progressive rise over the ensuing 3 hours of reperfusion, with the number of neutrophils remaining lower in Fluosol-DA animals.

**Ex Vivo Neutrophil Function**

Neutrophils from the animals receiving Ringer's lactate and Fluosol-DA at baseline and 1 hour after LAD occlusion showed similar ability to migrate in response to a potent chemotactic stimulus (Figure 8). At 1 hour after reperfusion, the neutrophils from the control animal manifested a significantly greater ability to migrate, whereas neutrophils from the Fluosol-DA animals showed marked impairment of chemotactic ability. No significant differences in superoxide anion production was noted in either the unstimulated or stimulated state (Table 2). Neutrophils from both groups showed similar ability to release lysozyme at baseline and occlusion in the unstimulated and stimulated state (Figure 9). However, 1 hour after reperfusion, lysozyme release
was significantly reduced in neutrophils exposed to Fluosol-DA in vivo.

**Myocardial Neutrophil Infiltration**

In the ischemic myocardium of control animals, there was a significant gradient of neutrophil infiltration increasing from the epicardium to the endocardium, which was absent in Fluosol animals (Table 3). In the control animals, there was nearly a sixfold increase in the number of neutrophils between the ischemic and nonischemic endocardium, whereas neutrophil numbers were equally distributed in all segments of the ischemic zone in Fluosol animals. In animals treated with Fluosol-DA, the number of neutrophils in the myocardium was significantly less in comparison with control animals, especially in the ischemic endocardium segments.

**Histology**

**Light microscopy.** Hearts from nine control animals and eight Fluosol-DA animals were examined by light microscopy. The inflammatory infiltrate (interstitial and intravascular) was far more extensive in the control than Fluosol-DA animals (Figure 10). The infiltrate was greatest in the inner third of all animals and was rarely seen in the subepicardial region. Neutrophil infiltrate was more extensive in the interstitial areas of control animals, whereas neutrophils were confined mostly to intravascular space in the treated animals. Endocardial hemorrhagic infarction was observed in seven control animals and three treated with Fluosol-DA (Figure 11). Moderate-to-severe contraction bands were present in all nine control animals but in only four of the eight treated animals in the border zones of the infarct.

**Electron microscopy.** A total of 56 specimens were examined by electron microscopy from seven control animals and seven Fluosol animals. Animals from the control and treated groups showed prominent changes of irreversible and reversible injury in the subendocardium in the ischemic zone with myocyte blebs, disruption of the plasmalemma, and mitochondrial swelling with and without disruption of the cristae and amorphous matrix densities (Figure 12). The subepicardial zones rarely showed changes of severe ischemia. The most prominent change in the control animals consisted of polymorphonuclear leukocyte infiltration that was seen both in the interstitium and intravascular areas. The capillaries were often disrupted with erythrocytes seen in the interstitium as well as fibrin deposition and membrane bound vesicles representing disrupted endothelial cells. The Fluosol-DA–treated animals more often showed intact capillaries with swollen endothelial cells and decreased pinocytotic vesicles. Occasional endothelial cells showed Fluosol-DA particles within the cytoplasm. Neutrophils, which were rarely seen in the intravascular or interstitium areas, also contained phagocytosed perfluorochemical particles.

**Discussion**

**Present Study**

This study demonstrates that administration of intravenous Fluosol-DA (20%) over 30 minutes just before reperfusion significantly reduces myocardial reperfusion injury in the closed-chest canine model. This was manifested by a marked reduction in infarct size expressed as a percentage of area at risk and was associated with relative preservation of regional left ventricular contractile function. This study also gives insights into the mechanisms of reperfusion injury and the ability of perfluorochemicals to salvage myocardial tissue. The treated animals had delayed increase in venous neutrophil counts with marked suppression of neutrophil chemotaxis, proteolytic enzyme degranulation, and myocardial infiltration. Endocardial blood flow in the ischemic zone decreased significantly in control animals at 1 hour after reperfusion but was main-

<table>
<thead>
<tr>
<th>Table 2. Neutrophil Superoxide Radical Production</th>
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<tr>
<td><strong>Baseline</strong></td>
</tr>
<tr>
<td>Not stimulated</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Phorbol myristate</td>
</tr>
<tr>
<td>stimulated</td>
</tr>
<tr>
<td>Opsonized</td>
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<tr>
<td>zymosan–stimulated</td>
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</table>

Results are expressed as nanomoles of ferricytochrome C reduction/1×10⁶ neutrophils.
Neutrophils and the No-Reflow Phenomenon

While the concept of reperfusion injury is not universally accepted, studies such as this one that temporally link an intervention in the perireperfusion period to myocardial salvage lend strong support to such a phenomenon. Neutrophils have been previously implicated in causing reperfusion injury in the myocardium and other organs.11,30 Reperfusion results in rapid influx of neutrophils into the ischemic region compared with permanent occlusion models.22,31,32 The production of neutropenia in reperfusion models with antisera, chemotherapy (hydroxyurea), and lipoxygenase inhibitors (BW 755C) attenuates neutrophil infiltration into the ischemic myocardium and reduces infarct size.14,15

The first step in neutrophil accumulation in the heart involves interaction between neutrophils and the endothelium (adherence).33,34 Because neutrophils are large poorly compliant cells, plugging of normal capillaries is prevented by shunting through high flow channels, surface charges on the cells causing electrostatic repulsion, and endothelial release of antiinflammatory mediators (adenosine).35–39 Several types of endothelial injury, including anoxia, may increase neutrophil adherence.33 Damaged cells express membrane receptors for immunoglobulin and complement fragments that promote adherence; it has been shown that the complement fraction C1d localizes to ischemic myocardium in vivo.40,41 Depletion of endothelial stores of antiinflammatory mediators (adenosine, cyclic AMP, and prostacyclin) during ischemia may be exacerbated by washout at reperfusion, thereby contributing to increased neutrophil accumulation.38,42,43

Reperfusion after more than 40 minutes of ischemia results in a progressive decrease in blood flow to previously ischemic myocardium, no-reflow phenomenon.44 Histologic studies demonstrate mechanical plugging of capillaries with neutrophils, which is prevented by leukopak filters.7,8,21 The release of vasoactive substances such as leukotrienes and platelet activating factor by activated neutrophils in association with inability of damaged endothelial cells to release relaxing factors may contribute to continual neutrophil plugging and vascular injury after reperfusion.21,45–47 This study demonstrates that intravenous Fluosol reduced neutrophil plugging of capillaries in the ischemic zone; this was associated with relative preservation of endothelial structure. In contrast to a previous study with intracoronary perfluorochemical, improved regional myocardial blood flow was noted 1 hour after reperfusion in the treated group.19 This discrepancy may be due to differences in the time, dose, and route of administration of the drug in this study.

### Table 3. Neutrophil Infiltration Into Ischemic and Nonischemic Myocardium

<table>
<thead>
<tr>
<th></th>
<th>Epicardium</th>
<th>Midmyocardium</th>
<th>Endocardium</th>
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<tbody>
<tr>
<td><strong>Nonischemic region</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.9 ± 0.3</td>
<td>2.3 ± 0.5</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>Fluosol</td>
<td>2.1 ± 0.7</td>
<td>2.0 ± 0.6</td>
<td>2.5 ± 0.6</td>
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<tr>
<td><strong>Lateral ischemic region</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.4 ± 1.0</td>
<td>6.7 ± 2.9</td>
<td>11.0 ± 4.4*</td>
</tr>
<tr>
<td>Fluosol</td>
<td>1.8 ± 0.5</td>
<td>1.8 ± 0.4</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td><strong>Central ischemic region</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.8 ± 1.9</td>
<td>9.6 ± 3.8†</td>
<td>14.1 ± 4.2*</td>
</tr>
<tr>
<td>Fluosol</td>
<td>2.2 ± 0.8</td>
<td>1.9 ± 0.5</td>
<td>2.5 ± 0.7</td>
</tr>
</tbody>
</table>

Neutrophils, (×10⁹/Gm myocardium).

* p < 0.01; † p < 0.05.
Effect of Perfluorochemicals on Neutrophils

Activated neutrophils cause tissue injury by release of reactive oxygen species, such as hypochlorous acid from myeloperoxidase and numerous potent proteolytic enzymes.11-13 Leukotrienes released by neutrophils can enhance chemotaxis and adhesion of other neutrophils, thereby increasing the inflammatory response in an exponential fashion.10,45 Normal cells are protected from oxidant injury by numerous endogenous enzymes, which are depleted by ischemia.48 Both lysosomal enzymes such as elastase and reactive oxygen species have been shown to disrupt endothelial cell basement membranes in vitro.49,50 Neutrophil-derived oxidants may also inactivate antiproteases present in the serum.51 Neutrophil degranulation and free radical release may, therefore, permit an unchecked activity of proteolytic enzymes on cellular membranes.

The lack of neutrophil infiltration into the ischemic myocardium of Fluosol-DA animals is due to marked suppression of neutrophil demargination and chemotaxis during the 1st hour of reperfusion. This and previous in vitro studies found marked suppression of neutrophil chemotaxis.17,18 Lane and Lamkin18 were able to reverse this neutrophil chemotaxis suppression by washing neutrophils three times 1 hour after exposure to Fluosol-DA but not at 18 hours. Although we washed the neutrophils in this study, the first washing was not until 3 hours after neutrophils were first exposed to Fluosol-DA. At 24 hours, the Fluosol-DA particles were seen to be ingested by neutrophils by electron microscopy, but the exact time course of perfluorochemical internalization by neutrophils is not known.

We have previously shown Fluosol-DA to markedly inhibit oxygen radical production in vitro, but we did not see such inhibition in this study.17 Lane and Lamkin18 did not observe suppression of oxygen radical production by Fluosol-DA. The etiology of these discrepancies is unclear, but multiple mechanisms for the induction of superoxide production are known to exist and are complex and stimulus dependent. Therefore, it is conceivable that technical variations among studies, such as washing techniques and whether Fluosol exposure occurs in vivo or in vitro, may account for the variable results. Also, the time course of reversal of neutrophil suppression by washings may be different for chemotaxis, oxygen radical production, and proteolytic enzyme degranulation.

Neutrophils have been shown to extrude potent proteolytic enzymes from azurophilic and specific granules with reverse endocytosis.52,53 Proteolytic enzymes may be even more important than oxygen radical species in mediating endothelial injury.49 We found marked suppression of lysozyme release ex vivo. Lane and Lamkin did not see any significant suppression of β-glucuronidase release by Fluosol-DA in vitro.18 The differences may be due to the use of cytochalasin B to cause neutrophils to release a greater amount of proteolytic enzyme, thereby mimicking in vivo maximal activation. Although both β-glucuronidase and lysozyme are present in azurophilic granules, lysozyme is also present in specific granules that comprise a larger percentage of total granules. Specific granules fuse with cell membrane with extracellular extrusion better than azurophilic granules.54 The mechanism of ex vivo suppression of lysozyme release is unlikely to be artifactual due to previous degranulation of neutrophils in vivo because we also found similar suppression of lysozyme release by Fluosol-DA in a separate in vitro study.55 In addition, baseline enzyme release was similar in control and treated animals.

**Figure 10.** Qualitative histologic assessment of neutrophil infiltration in the interstitium and intravascular areas is shown for the endocardium, midmyocardium, and epicardium in both groups. Vertical axis represents the number of animals showing the neutrophil infiltration. Horizontal axis represents the degree of neutrophil infiltration with 0 when rare or no neutrophils were present and 4+ with severe infiltration.

**Figure 11.** The severity of contraction band necrosis and endocardial hemorrhagic infarction is shown for both groups.
Figure 12. Electron microscopy photomicrographs from a control animal (Panels A and B) and a Fluosol-DA–treated animal (Panels C and D) are shown. In the control animal, Panel A shows two leukocytes (arrowheads) adjacent to irreversibly injured myocytes, and Panel B shows capillary disruption with extravasated erythrocytes (R), fibrin deposition (arrowheads), and membrane bound vesicles (arrows). In contrast, Fluosol-DA–treated animal shows a neutrophil with ingested perfluorocarbon microparticles (Panel C, arrowheads) adjacent to a viable myocyte. Panel D shows a swollen, but intact capillary membrane with decreased pinocytic vesicles in the Fluosol-DA–treated animal.
Methodology

Several considerations need to be addressed before these data can be extrapolated to humans. The canine model is a highly variable one with much variability in collaterals between LAD and circumflex territories as well as between animals.\(^{56-57}\) The anatomic and physiologic difference between acute occlusion of normal canine coronary arteries and chronically diseased human coronary vessels allows for only crude comparisons. We evaluated this with serial coronary blood flow in both groups of animals and found no significant differences.

The use of lidocaine during the acute occlusion and reperfusion may alter neutrophil function and has been shown to reduce infarct size.\(^{58-61}\) We used lidocaine in an attempt to decrease animal mortality and to simulate the clinical condition. Because lidocaine is generally used prophylactically in acute myocardial infarction, we wanted to assess the ability of Fluosol-DA to preserve myocardium and affect neutrophils above and beyond that of lidocaine alone. The two groups were given lidocaine by identical protocol with lidocaine concentrations being similar. Neutrophils from the Fluosol group showed suppression of neutrophil function, whereas the control group demonstrated activation 1 hour after reperfusion despite receiving lidocaine.

Implications

Previous reperfusion studies and this study suggest an important role of reperfusion injury in the coronary occlusion-reperfusion model. Total myocardial necrosis in the experimental canine model (wavefront phenomenon\(^{62}\) is a summation of two separate processes, anoxic myocardial injury and reperfusion-induced cardiac injury, which likely have their own time-dependent courses. The time course of reperfusion injury and its contribution to final infarct size after various occlusion periods requires clarification. This study corroborates previous work from our laboratory that reperfusion injury contributes significantly to infarct size in the dog model with 90 minutes of LAD occlusion. Suppression of neutrophil activation, especially chemotaxis, is an ideal step to reduce this component from the inflammatory response in the ischemic myocardium after reperfusion. Although experimental data cannot be fully extrapolated to humans, prevention of reperfusion injury by aggressive clinical intervention could significantly enhance myocardial salvage.

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