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Effect of perfluorochemical (Fluosol-DA) on infarct morphology in dogs

Frank D. Kolodgie, Albert K. Dawson*, Mervyn B. Forman, and Renu Virmani

Department of Pathology and Cardiology, Vanderbilt University Medical Center; Veterans Administration Medical Center, Nashville, TN 37203; and the Armed Forces Institute of Pathology, Department of Cardiovascular Pathology, Washington DC 20306, USA

Summary. To assess the effect of Fluosol-DA treatment on infarct morphology, detailed histologic examination was performed in 17 dogs with permanent proximal left anterior descending coronary artery occlusion. Two of the three groups of dogs received an equal blood volume exchange (40 ml/kg i.v.) with either Fluosol-DA (F) or heparinized autologous blood (H) 30 min post occlusion while being ventilated with 100% oxygen. A third group received no therapy (C). Animals were sacrificed 3 days post occlusion and sections were obtained for light and electron microscopy. Histologic studies showed that infarct size was statistically smaller in dogs treated with F $54 \pm 7\%$ versus heparin $64 \pm 10\%$ treatment or no therapy $79 \pm 6\%$. Fluosol-DA animals demonstrated decreased inflammatory infiltrate, larger viable subepicardial zones and greater endocardial sparing in the area surrounding the central zone of necrosis. By electron microscopy, perfluorochemical particles were found within endothelial and inflammatory cells in subepicardial zones of infarction. In midmyocardial zones, Fluosol-DA particles were present in capillaries, extracellular spaces and necrotic myocytes. In the normal myocardium Fluosol-DA particles were rarely seen within endothelial cells and never within the interstitium or myocytes. Thus, Fluosol-DA reduces infarct size and alters infarct morphology in the 3 day post permanent coronary occlusion model.

Key words: Perfluorochemical – Myocardial infarction – Permanent coronary occlusion – Infarct reduction – Infarct morphology

* *Present address:* Cardiac Pacemakers, Inc., St. Paul, MN 55112
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Offprint requests to: R. Virmani, Department of Cardiovascular Pathology, Armed Forces Institute of Pathology, Washington DC. 20306, USA

Introduction

Numerous agents have been shown to reduce infarct size in the experimental model, the predominent mechanism of action of most of these compounds is either by decreasing myocardial oxygen demand or by increasing oxygen supply. Perfluorochemical blood substitutes are stable organic compounds with high oxygen and carbon dioxide solubility (Clark and Gollan 1966; Geyer et al. 1968; Mitsuno et al. 1982; Matsumoto et al. 1975; Naito 1980; Templer et al. 1982). Recent animal studies have shown that perfluorochemicals, when administered shortly before or soon after experimental coronary artery occlusion can significantly reduce infarct size (Glogar et al. 1981; Menasche et al. 1984; Nunn et al. 1983). Since perfluorochemicals may have important clinical applications in the treatment of acute myocardial infarction, histologic evaluation of infarct morphology is essential before implementing their clinical use. Light and electron microscopic changes were studied in 3-day permanent coronary occlusion model after the following treatments: Fluosol-DA blood exchange; heparinized autologous blood exchange; or no intervention. The infarct size and the variability in cellular response to necrosis and the sites of entrapment of perfluorochemical particles was evaluated.

Material and methods

Preparation of animal models. Mongrel dogs of either sex, weighing 10–15 kg were anesthetized with an intravenous administration of sodium pentobarbital (Nembutal 25–30 mg/kg), ventilated with a positive pressure respirator, and given 600,000 units of penicillin intramuscularly. Under aseptic conditions, a left thoracotomy was performed at the fifth intercostal space. The pericardium was opened and sutured to the chest cavity to form a temporary cradle. The left anterior descending coronary artery was isolated approximately 5 mm distal to its origin (before the take off of the first diagonal) and a snare, made of surgical monofilament nylon (size 2) enclosed in a polyetheylene sleeve (tubing size PE 320) was placed around the artery and anchored to the epicardium with two small sutures (Dawson et al. 1979). The chest was then closed and the distal end of the snare was buried in a subcutaneous pocket just caudal to the left scapular region. Each animal was allowed at least one week of recovery before inducing myocardial infarction.

Experimental protocol

Prior to commencing the protocol, 22 animals were randomly assigned to Fluosol-DA, heparin or control groups. Fresh suspensions of the perfluorochemical (Fluosol-DA 20%) were obtained commercially (Alpha Therapeutic Corporation, Pasadena, CA) and constituted as recommended by the manufacturer (Naito et al. 1981). Instrumented dogs were anesthetized and ventilated as previously described. EKG leads 1, AVF, and V1 were monitored along with systemic arterial blood pressure. Under aseptic conditions, the distal end of the snare was exposed. Obstruction of the left anterior descending coronary artery was accomplished by gradual occlusion of the snare. Exchange transfusion and oxygen therapy (100% with a positive pressure respirator) was initiated 30 min following the onset of occlusion. Blood volume replacement was accomplished within 30 min by infusing Fluosol-DA or heparinized (low dose 2,500 to 3,500 units) autologous blood 40 ml/kg intravenously while simultaneously withdrawing an equal volume of arterial blood. Blood samples for total leukocyte, platelet count, total CK and arterial oxygen content were obtained at baseline and six hours post myocardial

infarction. Monitoring was ended at six hours post occlusion and oxygen was discontinued. Dogs were then allowed to recover. Of the 22 dogs, 5 died between 24 to 72 h post-myocardial infarction (4 control and 1 heparin).

Histologic studies

Animals were sacrificed 3 days post infarction by ventricular fibrillation, hearts were removed. Samples for electron microscopy were obtained prior to perfusion fixation of the heart from the central zone of necrosis and divided into subendocardial and epicardial regions and from normal myocardium remote from the ischemic zone. Each sample measuring 1 mm^3 was fixed in 3% glutaraldehyde solution with cacodylate buffer, post fixed with osmium tetroxide in sucrose phosphate buffer, dehydrated in a graded series of alcohols and acetone, and embedded in Epon 812. Ultrathin sections were stained with lead citrate and uranyl acetate and examined with a Hitachi 6000 series electron microscope.

To define the anatomic boundary of the left anterior descending coronary artery perfusion bed, the artery was cannulated at the occlusion site and perfused at 100 mm Hg pressure with yellow silicone rubber microvascular dye while simultaneously perfusing the circumflex, and right coronary arteries with a different color dye at similar pressure (Geary et al. 1981). The heart was then allowed to fix in 10% phosphate-buffered formaldehyde for three days. Approximately 8 to 10 serial cross sections of the left ventricle were obtained at 5 mm intervals parallel to its minor axis (posterior atrio-ventricular sulcus), dehydrated and embedded in paraffin. Microscopic sections, 7 μ thick, from each cross section were stained with hematoxylin-eosin and Mallory's trichrome stain. The area of the left anterior descending coronary perfusion bed was visualized on the paraffin block and marked on the corresponding histologic slide by superimposition. The zone of myocardial necrosis was outlined on each slide stained with Mallory's trichrome by microscopic examination at a magnification of 50. An enlarged (X20) tracing was made from each left ventricular cross section with the aid of a Bessler enlarger. The areas of the left ventricle, the perfusion bed, and central zone of infarction were then determined by planimetry of the tracings. These areas were multiplied by the thickness of each cross section. The total volume of the perfusion bed and central zone of infarction were calculated by summation of the volumes of each tissue section and expressed as percent of total left ventricle or of left anterior descending coronary artery bed at risk.

Light microscopic examination was used to identify and quantitate inflammatory infiltrate in the border zones in the 3 basal slices of the left ventricle. An average of 20 high power fields (HPF) per slide were evaluated. An arbitrary semi-quantitative grading scheme as described by Romson et al. (1983) was used to gauge the extent of leukocytic infiltrate associated with myocardial infarction. A score of $++++$ was assigned to the myocardial sample with the most dense and diffuse leukocytic infiltrate. A score of $+/-$ was given to sections in which rare or no extravascular leukocytes were observed. Intermediate scores were assigned based on the varying intensity and extent of leukocyte infiltrate. All sections were coded so that the pathologist was unaware of the treatment given to each dog. The largest diameter of viable myocardium from the epicardium (measurements were only made in areas devoid of penetrating arteries) and endocardium to the areas of necrosis was determined by occular micrometer from 3 basal slices of the left ventricle. Also, the extent of hemorrhage within the area of myocardial infarction was noted.

Grouped data from these experiments are expressed as the mean $\pm$ standard deviation. Data available from two observation points were examined for statistical differences by the unpaired Student's "*t*" test. A two-tailed p value of <0.05 was required for statistical significance.

Results

Laboratory results (Table 1)

Partial pressure of arterial oxygen (PaO_2) concentrations at six hours post-myocardial infarction were similar in both Fluosol-DA and heparin treated

Table 1. Laboratory data in dogs: preinfarction and 6 h postinfarction

Laboratory data		Fluosol-DA	Heparin	Control
TLC	Pre	10.8± 4.3	13.8± 7.4	9.9± 4.3
	6 h	17.1± 7.4	20.0± 7.8	19.3± 7.3
Plt	Pre	218 ± 63	180 ± 67	141 ± 56
	6 h	199 ± 90	152 ± 35	172 ± 66
PaO_2	Pre	91 ± 31	99 ± 28	110 ± 9
	6 h	545 ± 62	526 ± 39	92 ± 7
CK	6 h	674 ±340	1,439 ±545	1,302 ±888

All results expressed as mean ± deviation
Abbreviations: TLC = total leukocyte count; Plt = platelet count; PaO_2 = arterial oxygen tension; CK = creatine phosphokinase (serum)

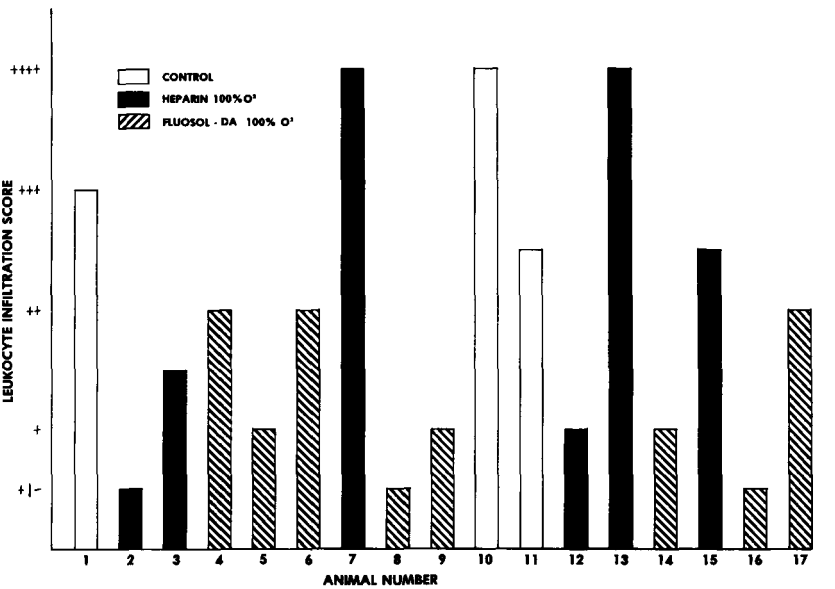


Fig. 1. Results of the histopathologic assessment of leukocyte infiltrate into infarcted myocardium for dogs given Fluosol-DA, or heparin, or no treatment (control). The histologic sections stained with hematoxylin and eosin from the three basal slices of the ventricle were ranked for the extent of leukocytic infiltrate associated with infarcted myocardium on the scale ranging from +/− (rare or no polymorphonuclear leukocytes) to + + + + (assigned to the specimen with the most extensive accumulation of leukocytes). Dogs treated with Fluosol-DA had decreased inflammatory response compared to control and heparin treatment

animals. No statistically significant differences were noted in total leukocyte and platelet counts between Fluosol-DA, heparin and control groups. Fluosol-DA treated animals had statistically significant lower CK values (674 ± 340 I.U./L), as compared to heparin (1,439 ± 545 I.U./L) and control groups (1,302 ± I.U./L). (F vs H $p<0.05$; F vs C $p<0.05$; H vs C $p=$ not significant).

Table 2. Effect of Fluosol-DA on the size of myocardial infarction

Group	% of LV infarcted	% of total LV at risk (LAD bed)	% LAD bed infarcted	Largest viable epicardial diameter (mm)	Largest viable endocardial diameter (mm)
1. Fluosol-DA (F) ($N=8$)	32.4± 5.9	59.7±8.4	54± 7	4.4±1.9	1.6±0.8
2. Heparin (H) ($N=6$)	39.6± 5.9	62.1±6.0	64±10	2.5±0.7	0.7±0.7
3. Control (C) ($N=3$)	54.3±11.2	68.2±9.2	79± 6	2.3±0.5	0.2±0.2
4. p value	F vs H $p<0.05$ F vs C $p<0.01$ H vs C $p<0.05$	Not Significant (NS)	F vs H $p<0.05$ F vs C $p<0.01$ H vs C $p<0.05$	F vs H $p<0.05$ F vs C NS H vs C NS	F vs H $p<0.05$ F vs C $p<0.02$ H vs C NS

All results are expressed as a mean±standard deviation
Abbreviations: LAD = left anterior descending coronary atery; LV = left ventricle

Delineation of infarct size (Table 2)

Infarct size in all 3 groups was normalized as a percentage of the left anterior descending coronary artery bed. The mean area at risk expressed as a percentage of the total left ventricle showed no statistical difference in Fluosol-DA (60±8%), heparin (62±6%) and control groups (68±9%). However, the area of necrosis expressed as the percentage of myocardium at risk that became infarcted was statistically smaller in Fluosol-DA treated dogs (54±7%), as compared to heparin (64±10%) and control (79±6%). (F vs H $p<0.05$; F vs C $p<0.01$; H vs C $p<0.05$).

Light microscopy

Infarction produced by permanent coronary artery occlusion in all three groups was characterized by three zones which could be distinguished both grossly and microscopically. Microscopically, a central area of coagulation necrosis devoid of inflammation and hemorrhage was present. Surrounding this central zone of necrosis was a zone of inflammation with focal interstitial hemorrhage. Hemorrhagic zones were most notable in Fluosol-DA treated animals as compared to heparin or no treatment. However, the Fluosol-DA treated group demonstrated a decrease in the extent of inflammatory infiltrate in the border zone (Figs. 1, 2). In the peripheral zones of the infarct, the vasculature appeared intact in control animals while 2 of 6 heparin and 4 of 8 dogs with Fluosol-DA treatment showed presence of hemorrhage in this zone. The subepicardial and lateral border zones in the Fluosol-DA as compared to the heparin and control groups revealed larger viable myocardium and prominent contraction band necrosis with peninsulas of normal myocardium interspersed with necrotic myocardium

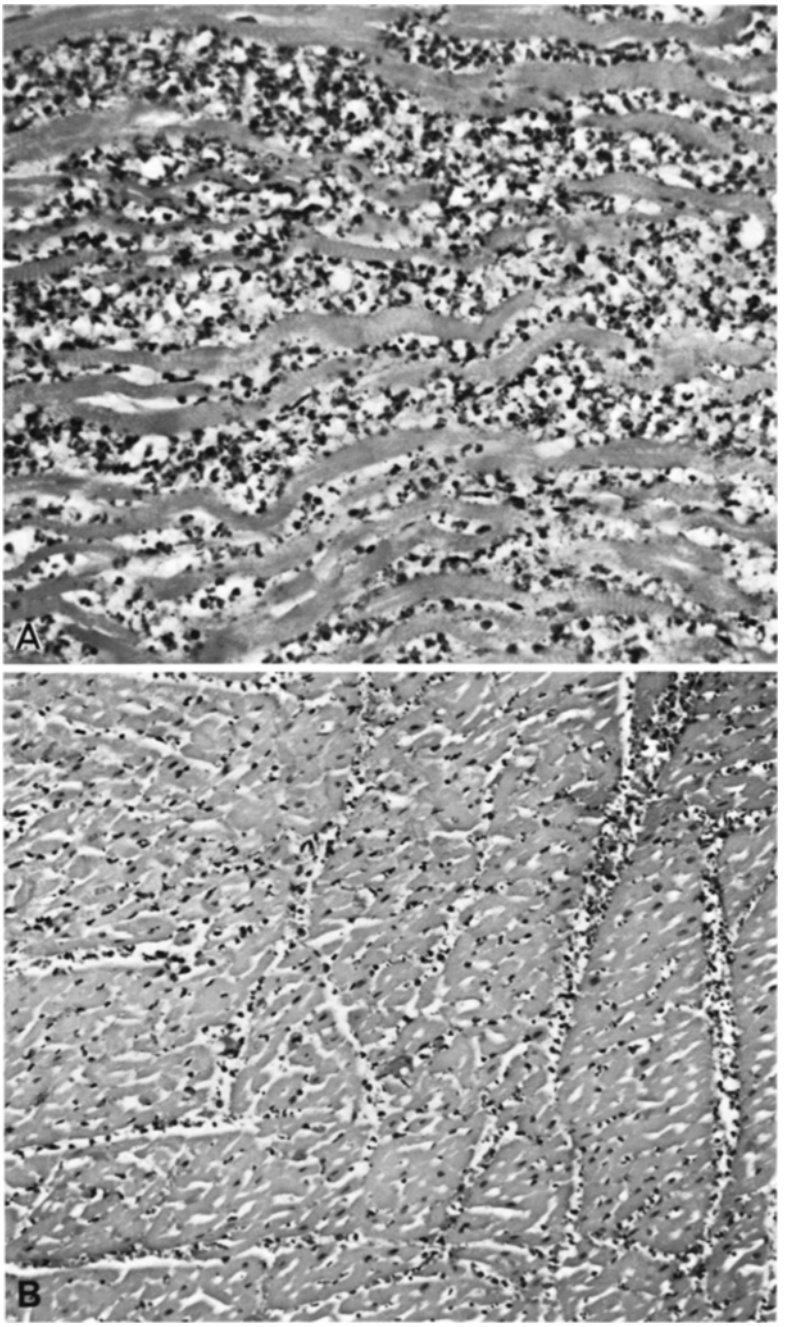


Fig. 2A–C. Region of inflammation in a control **A** and a Fluosol-DA treated dog **B** 3 days post-permanent coronary occlusion. Note massive inflammation (+ + + +) in an control dog **A** compared to ± and +inflammation seen in a dog treated with Fluosol-DA (**B, C**). (H & E: **A, B, C** × 250)

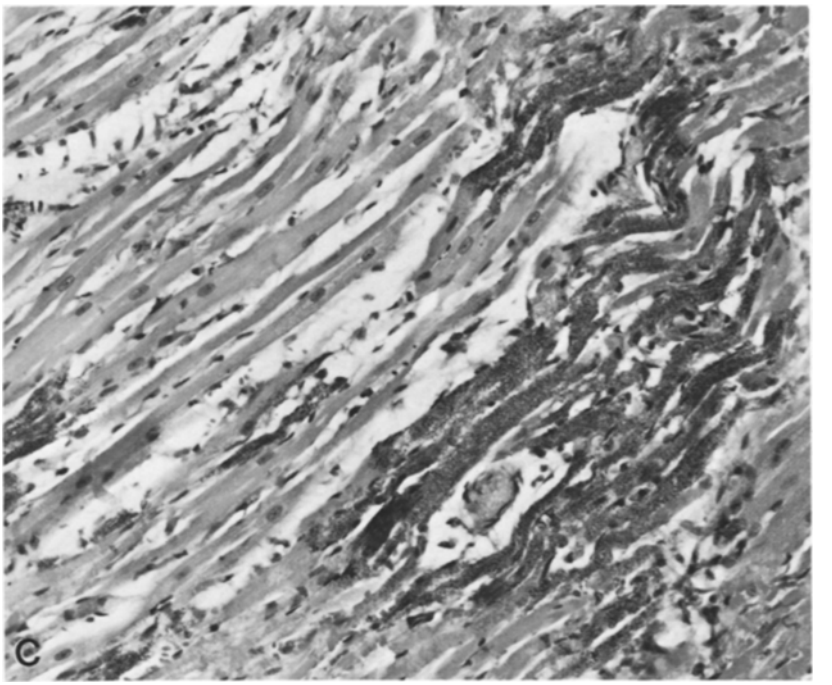


Fig. 2C

(Table 2). Again, the endocardial zones in the area of infarction in Fluosol-DA treated dogs demonstrated either total absence or the presence of mild inflammation and larger viable subendocardial regions with the formation of islands of normal myocardium focally. Heparin and control animals showed greater inflammation and sparing of only 2 to 3 layers of subendocardial myocytes (Fig. 1 and Table 2).

Electron microscopy

Ultrastructural features of myocardial necrosis that were common to all three study groups in the central zone of infarction consisted of marked disruption of cellular architecture. This was characterized by extensive interstitial edema with extravasation of red cells (more prominent in Fluosol-DA treated dogs), cell swelling, and disruption of sarcolemmal membranes with the formation of subsarcolemmal blebs of varying sizes. Also present were changes of myofibrillar fragmentation and loss, sarcoplasmic reticulum and transverse tubular disruption, lipid droplets, densities in the matrix spaces of the mitochondria, loss of cristae, abnormally clear matrices, and formation of myelin figures (Fig. 3). Myocardial nuclei showed extensive margination of chromatin. The changes in the surrounding subepicardial border zone of inflammation and hemorrhage varied from early changes of ischemia with depletion of glycogen, swelling of mitochondria, and focal dilatation

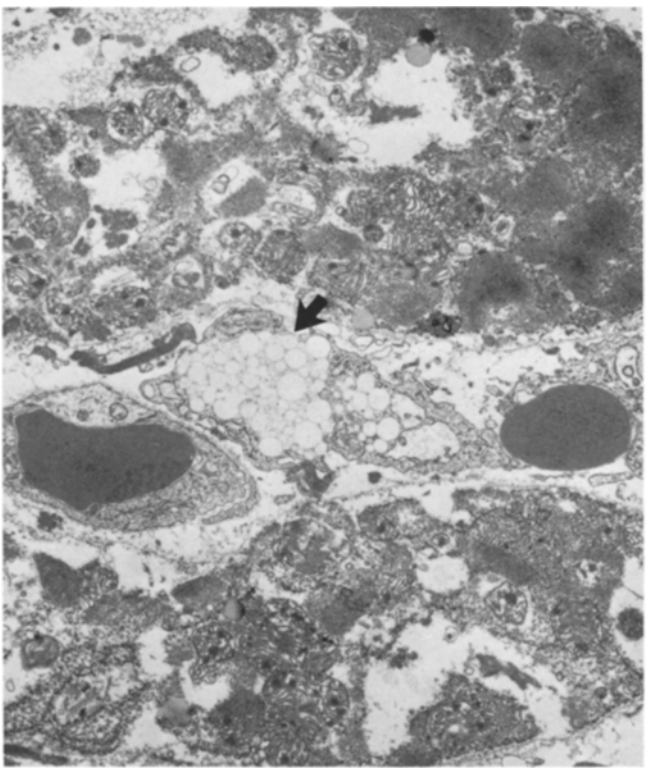
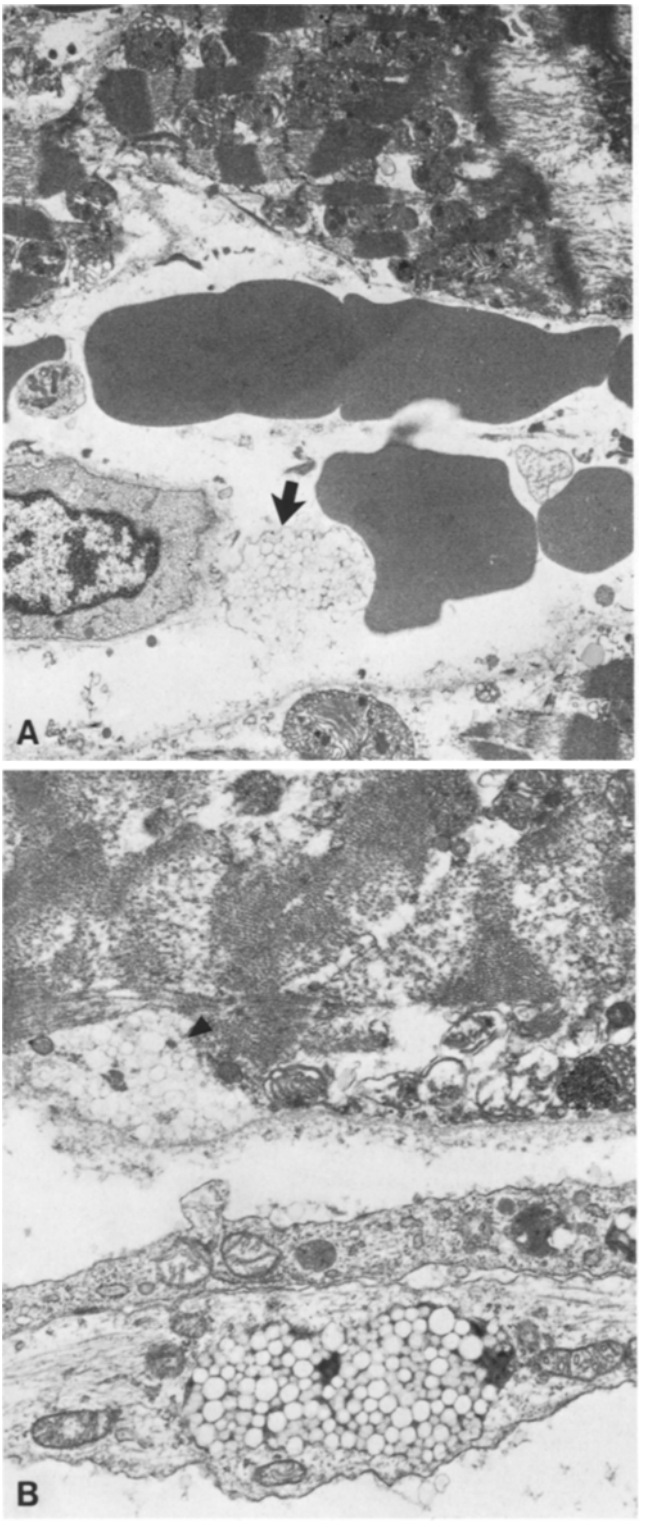


Fig. 3. Electron micrograph of the infarcted region of the myocardium in a dog treated with Fluosol-DA. Note the marked disruption of cellular architecture with myofibrillar fragmentation and loss, edema, presence of occasional lipid droplets and Fluosol-DA particles. The sarcolemmal membrane appears focally disrupted. The mitochondrial cristae are separated, there is clearing of matrix, focal deposition of flocculent densities and formation of myelin figures. The interstitium is edematous and shows presence of occasional red blood cells, and a portion of a macrophage containing Fluosol-DA particles. (× 8,000)

of the sarcoplasmic reticulum and transverse tubules to changes of irreversible damage. Myofibrils were often strongly contracted and this change was most marked in dogs treated with Fluosol-DA. However, no changes of ischemia were noted in the normal myocardium away from the area of necrosis. Fluosol-DA treated dogs demonstrated perfluorochemical particles of varying sizes (0.1 μm and greater) within capillaries, endothelial cells, interstitium and necrotic myocytes in the zone of necrosis (Figs. 4, 5, 6). Neutrophils (Fig. 7) and macrophages (Fig. 5) in the zone of inflammation showed presence of membrane bound perfluorochemical particles in the

Fig. 4A, B. Necrotic myocytes surrounding interstitial space showing presence of Fluosol-DA particles (*arrow*) and red cells **A**. Also, Fluosol-DA particles are seen within myocyte (*arrowhead*) **B**. (**A** × 8,000, **B** × 19,200)


A
B

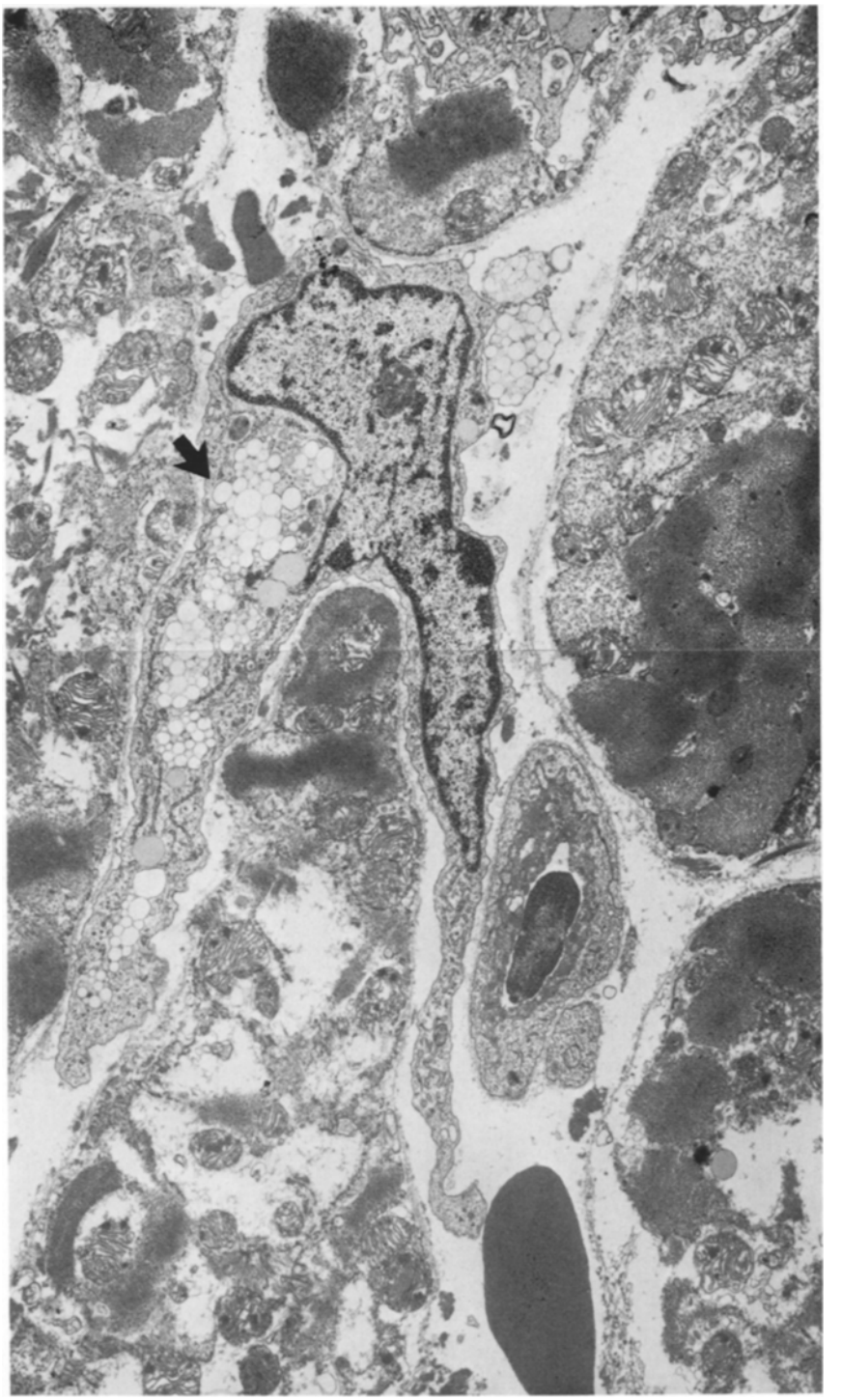


Fig. 5. Interstitial space showing presence of macrophage containing perfluorocarbon particles (*arrow*). (× 8,000)

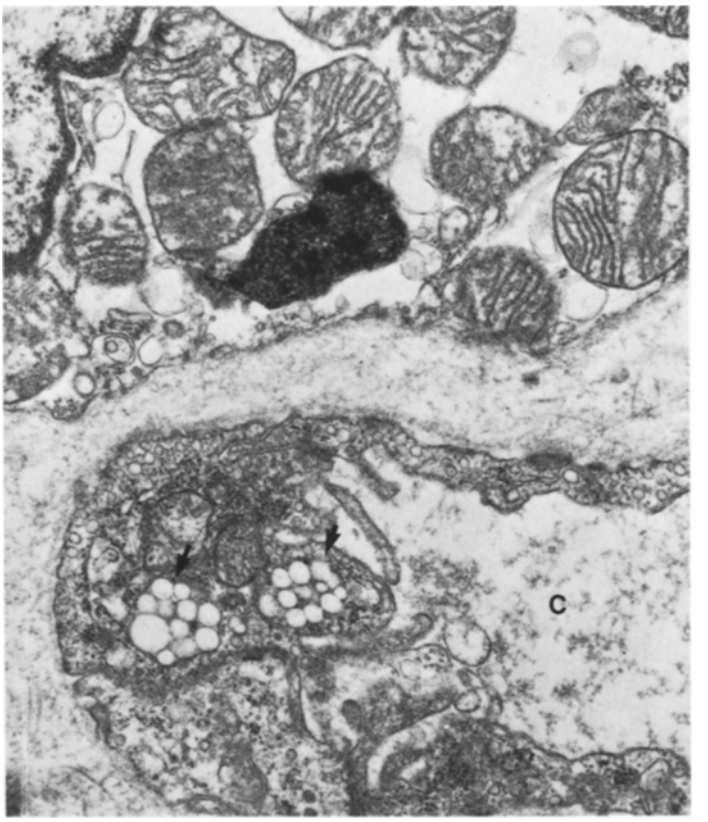


Fig. 6. Electron micrograph of a capillary (c) surrounded by reversibly damaged myocardium showing Fluosol-DA particles (*arrow*) within endothelial cell (× 27,600)

cytoplasm. In the normal myocardium, (away from the site of infarction), particles of Fluosol-DA were rarely seen within endothelial cells and never within myocytes or in the interstitium.

Discussion

Perfluorochemicals

Recent studies have shown that inert organic particles of perfluorochemicals can adequately substitute for hemoglobin as oxygen and carbon dioxide transport media in animals (Geyer et al. 1968; Matsumoto et al. 1975) and man (Mitsuno et al. 1982; Trempler et al. 1980; Trempler et al. 1982). Because of their unique ability to transport gases, low viscosity, and small particle size (90% <0.2 μm) their potential beneficial effects on ischemia in animal models have been explored. Infarct size reduction has recently been shown with perfluorochemical treatment prior to or shortly after coronary

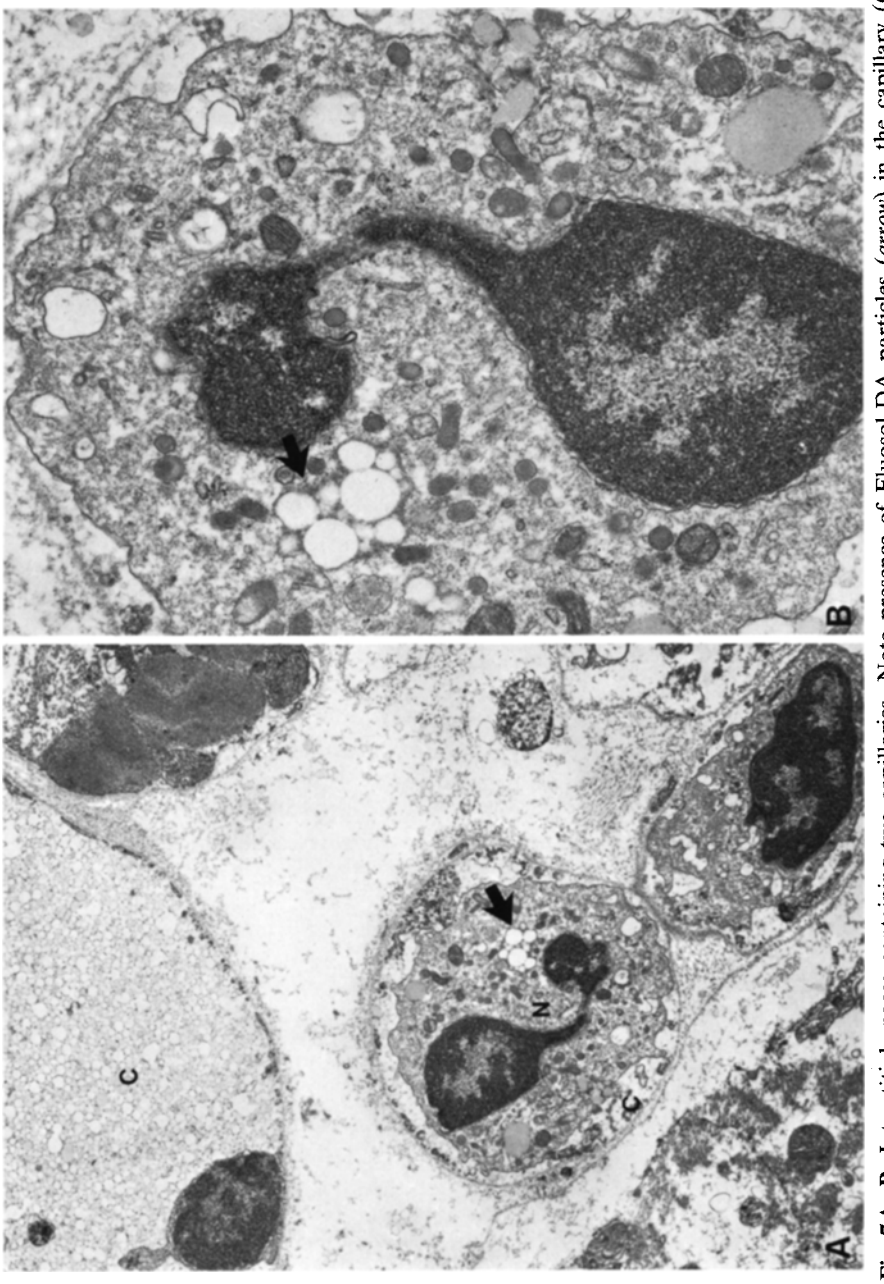


Fig. 7A, B. Interstitial spaces containing two capillaries. Note presence of Fluosol-DA particles (*arrow*) in the capillary (*C*) and within the neutrophil (*N*) seen in the capillary lumen. Fluosol-DA particles are seen within membrane bound lysosomes **B**. (**A** × 8,230, **B** × 25,700)

occlusions (Glogar et al. 1981; Menasche et al. 1984; Nunn et al. 1983). All studies were performed up to six hours post infarction and lacked microscopic evaluation.

Infarct morphology

The early ischemic areas, less than 40 minutes postocclusion, involve 2 zones of myocardial damage; a central zone with very low blood flow and a surrounding marginal zone (Cox et al. 1968; Factor et al. 1978; Hearse et al. 1977; Hillis and Braunwald 1977; Jennings et al. 1965; Reimer et al. 1977). However, by 24 h, necrosis becomes nearly transmural. The marginal zone may be up to 8 mm wide in the epicardial region depending upon collateral flow and it is this area which is potentially salvagable by various interventions. Permanent left anterior descending coronary artery ligation in our study showed a 32% reduction in infarct size with Fluosol-DA treatment. Not only were subepicardial and lateral border zones wider with Fluosol-DA treatment but larger regions of the subendocardium were spared (mean 1.6 mm) with the formation of islands of viable myocardium. However, significant differences were not noted between Fluosol-DA and control animals for subepicardial regions, this probably resulted from fewer survivors in the control group. Inflammatory response was much less in dogs treated with Fluosol-DA compared to heparin or no intervention. Also, contraction band formation was greatest in Fluosol-DA treated dogs. Electron microscopy demonstrated perfluorochemical particles within infarcted necrotic myocytes, extracellular spaces, and capillaries in the zone of infarction. The presence of perfluorochemical within areas of irreversible damage would indicate that perfluorochemicals reach areas of necrosis via collaterals and that the ischemic region closest to viable myocardium is salvaged and not the central zone of infarction. The leakage into interstitial spaces and necrotic myocytes may occur from damaged capillaries and cell membranes. The endothelial and inflammatory cell in the epicardial and mid-myocardial zones showed presence of perfluorochemical particles. Some Fluosol-DA particles in the myocardium were larger than the infused particles (0.1 to 0.6 μ) due to their coalescence over time.

Transmural progression of myocardial injury "The Wavefront Phenomenon" as described by Reimer et al. (1977) was noted in our histologic findings with the exception of 1.6 mm region of endocardial sparing in the Fluosol-DA treated group. This effect may involve enhanced oxygen diffusion capacity from perfluorochemicals within the ventricular cavity. The ability of Fluosol-DA to salvage subepicardial "border zones" may result from low viscosity and small particle size, enabling perfluorochemicals to travel through capillaries where red cell sludging may occur due to anoxia in the absence of Fluosol-DA.

Mechanisms of myocardial salvage

The exact mechanism by which Fluosol-DA reduces infarct size remains conjectural. Rude et al. (1982; 1984) compared changes in intramyocardial

$PmCO_2$ and PmO_2 produced by temporary coronary occlusion, or no occlusion, in dogs infused with either perfluorochemical, saline or dextran. Perfluorochemical-oxygen treatment reduced myocardial ischemia by augmented myocardial oxygen availability in the central ischemic zone. They also concluded that the beneficial effects of Fluosol-DA on myocardial ischemia were most likely due to enhanced oxygen delivery rather than reduced oxygen demand. In a recent study, we measured regional myocardial blood flow before and after acute myocardial infarction in dogs treated with intracoronary perfluorochemical or saline. Blood flow was similarly reduced at occlusion in both groups in the epicardium and endocardium of the central ischemic zone. However, relative endocardial blood flow was significantly greater immediately after reperfusion with Fluosol-DA compared to saline but this difference was not maintained 1 hour after reperfusion (Forman et al. 1985). Therefore, the mechanism of reduced ischemic injury is not secondary to differences in blood flow.

In the present study, we noted decreased inflammation in the area surrounding the central zone of necrosis in dogs treated with Fluosol-DA. Romson et al. (1983) have shown that the neutrophil accumulation in the acute inflammatory phase in response to temporary coronary artery occlusion may exacerbate tissue injury through the release of cytotoxic activated oxygen free radicles. Although the mechanism by which Fluosol-DA exerts cardioprotective effect is not known, their influence on neutrophil function may be an important mechanism of salvaging ischemic myocardium.

Decreased phagocytosis by monocytes and neutrophils exposed in vivo and in vitro to perfluorotributylamine has been shown by us (Virmani et al. 1983) and this correlated with the presence of perfluorochemical particles within monocytes and neutrophils by electronmicroscopy. We have also observed (Virmani et al. 1984) decreased superoxide generation and adherence to glass surfaces, inhibition of chemotaxis (Lane and Lamkin 1984), and suppression of phagocytosis of neutrophils after exposure to Fluosol-DA and perfluorotributylamine as compared to Hank's balanced salt solution. Vercellotti et al. (1982) reported that Fluosol-DA activates complement which results in neutrophil aggregation with resultant pulmonary leukostasis. Also, products of activated complement markedly alter neutrophil metabolic responses by several nonchemotactic stimuli (Stossel 1974). Similar interactions in vivo could disrupt neutrophil inflammatory responses to injured myocardium. Polymorphonuclear leukocytes play an important role in the demolition and repair of infarcted myocardium (Lautsch 1979; Mallory et al. 1939), and are an important determinant of the ultimate extent of tissue necrosis by either local production of oxygen metabolites (such as superoxide anion, hydroxyl radical, hydrogen peroxide and singlet oxygen) or by release of lysosomal enzymes capable of proteolytic disruption (Romson et al. 1983). Romson et al. (1983) have shown that myocardial cell damage in response to ischemia may, in part, be mediated by the extent of neutrophil accumulation in the ischemic myocardium. Therefore, if neutrophil functions are depressed in the presence of Fluosol-DA it is conceivable that the ultimate extent of myocardial injury induced by ischemia is reduced.

In summary, Fluosol-DA reduces experimental myocardial infarct size in the permanent coronary occlusion dog model and alters infarct morphology by increasing viable epicardial zones, sparing endocardium, reducing inflammation, and producing a hemorrhagic infarction. We suggest further evaluation of Fluosol-DA during the early and late phase of healing to ensure normal tensile strength of the infarcted myocardium.

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