Failure of nifedipine and reperfusion to reduce infarct size relative to region at risk as measured by NADH fluorophotography

ELYSE FOSTER, M.D., DAPHNE DEJONG, M.D., CAROLYN CONNELLY, PH.D., AND CARL S. APSTEIN, M.D.

ABSTRACT  In this report we describe a new technique for the measurement of region at risk after coronary artery ligation in the rabbit by NADH fluorophotography. We also describe the application of this technique to a study of nifedipine combined with reperfusion in experimental myocardial infarction. In 16 untreated rabbits the epicardial surface area of NADH fluorescence immediately after coronary ligation correlated with infarct size at 24 hr after coronary occlusion, as measured by nitro blue tetrazolium staining (r = .84, p < .001). In 24 rabbits we studied the effect of nifedipine administered immediately after coronary ligation and combined with reperfusion at 1 hr after occlusion. Nifedipine had no significant effect on region at risk or infarct size.


MORTALITY secondary to myocardial infarction is now largely the result of "pump failure," which is directly related to infarct size.1-2 Infarct size can be reduced by early reperfusion (i.e., that less than 3 to 4 hr after occlusion in the dog or man, or at 30 min or before in the rabbit), which primarily salvages the subepicardial region.3-5 The amount of salvage is directly related to the speed with which reperfusion can be achieved. Theoretically, the salvage achieved by reperfusion could be increased if the rate of necrosis was decreased during the time period before the onset of reperfusion. Nifedipine could potentially produce such a protective effect by altering hemodynamics to reduce myocardial oxygen demand, by increasing oxygen supply via increased collateral circulation or relief of coronary vasospasm, or by reducing calcium influx during ischemia and reperfusion.6 We therefore investigated the effect of nifedipine combined with reperfusion on infarct size in the rabbit.

Methods

The overall protocol consisted of (1) definition of region at risk by use of epicardial NADH fluorophotography during a transient coronary occlusion and release, (2) prospective randomization of the animal to a nifedipine or control group, (3) a 60 min coronary occlusion with treatment commencing at 5 min after occlusion, (4) reperfusion for 90 min, and (5) determination of infarct size.

Validation of NADH fluorophotographic technique for determination of region at risk

Surgical preparation. Thirty-three 1 to 2 kg albino New Zealand rabbits were each anesthetized with thiopental (50 mg/kg) and placed on mechanical ventilation. The thorax of each was opened in the fourth or fifth intercostal space; the heart was suspended in a pericardial cradle, and the major marginal branch of the left circumflex artery was identified. In rabbits this artery supplies the anterolateral left ventricular wall with a distribution similar to that of the left anterior descending artery in man.

NADH fluorescent photography. A Polaroid MP3 camera and type 667 Polaroid film were used to take the NADH fluorescent photographs. The high-intensity ultraviolet (UV) light was provided by two Xenon flash tubes, discharged by a 400 W/sec impulse, and fitted with Corning 5840 filters to provide UV light in the range of 330 to 380 nm. Wratten filters No. 4 and 2E were placed over the camera lens to exclude exposure by visible light and to allow the NADH fluorescent emission to pass. This method has been described previously in detail.7-8

Experimental protocol. NADH fluorescent photographs were taken before coronary ligation and, based on these, two animals were rejected because of excessive epicardial fat (which flu-
oesces under UV light). Thirty-one rabbits underwent ligation of the large marginal branch of the left circumflex coronary artery. The site of the ligation along the artery was deliberately varied to produce ischemic regions and infarcts of varying size. The artery was ligated without ligation of the adjacent marginal vein.

Two NADH fluorescent photographs were taken of each rabbit while in both the right and left lateral decubitus positions to allow both the posterior and anterior borders of the fluorescent area, respectively, to be photographed. After the first photograph of the uncovered epicardium, a calibrated acetate grid, premoistened with saline, was gently placed on the surface of the heart over the ischemic region and a second photograph was taken that was used to quantify the area of fluorescence (see below). After the photographs were taken the thoracotomy was closed and the animals were returned to their cages for 24 hr.

**Measurement of NADH fluorescent area.** An area of epicardial NADH fluorescence was detected in 30 of 31 hearts within 2 to 5 min after coronary artery ligation and was measured by counting grid boxes overlying the fluorescent area. The thin sheet of moistened acetate clung to the surface of the heart. The use of this grid compensated for differences in magnification from photograph to photograph and also compensated for the curvature of the heart. Two independent observers made tracings of the fluorescent areas from each heart without knowledge of the resulting infarct size. Agreement between the two observers was good (r = .96, p < .001, n = 16) and their average value was used. Representative NADH fluorophotographs are shown in figure 1.

**Measurement of myocardial infarct size.** Twenty-four hours after coronary artery ligation the animals were killed by decapitation, their hearts were removed, and the isolated left ventricles were “breadloafed” into eight transverse sections approximately 1.5 mm thick. These sections were incubated in nitro blue tetrazolium (NBT) dye for 20 min at 37°C. Myocardial infarction is traditionally defined on the basis of light microscopic findings. Therefore, in preliminary studies, we compared a series of adjacent sections of 24-hr-old infarcts that were stained with either NBT or hematoxylin and eosin and examined under light microscopy. There was excellent agreement between the light microscopic and NBT findings of necrosis. Similar comparisons and validation of the tetrazolium staining technique to distinguish normal from necrotic myocardium have been reported by a number of laboratories. Color slides were then taken of each side of the NBT-stained slices and the average area of infarction per slice and average mass of infarction per left ventricle were calculated. These measurements were made by an independent observer who had no knowledge of the results of the NADH fluorescence studies.

**Nifedipine protocol**

**Surgical preparation.** Thirty-four white male New Zealand rabbits weighing 1 to 2 kg were each anesthetized with pento-

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**FIGURE 1.** Epicardial NADH fluorophotography: measurement of fluorescent surface area. Sequential photographs of the lateral wall of the left ventricle of the rabbit heart in situ in an anesthetized animal. All photographs were taken at the same flash intensity and lens aperture. *A.* Before ligation; *B,* 2 min after ligation approximately one-half of the distance from apex to base on the left marginal coronary artery. The lower half of the ventricle is brighter due to NADH fluorescence. The heart is positioned to view the posterior border of the ischemic region. *C.* The epicardial grid is placed to quantify the amount of fluorescent surface area when the heart is positioned as in *B.* *D.* The animal is positioned so that the anterior border of the ischemic region can be observed. *E.* The epicardial grid is placed for quantification.
barbital (30 mg/kg) and placed on mechanical ventilation. The carotid artery and internal jugular vein were cannulated for blood pressure monitoring and administration of nifedipine or saline control. Lead II of the electrocardiogram was monitored. A lateral thoracotomy was performed as described above and the large marginal branch of the left circumflex artery was identified and encircled with a 4-0 silk suture.

**Determination of region at risk.** We determined the region at risk using NADH fluorescent photography as described above, except that we used temporary preliminary occlusions of 1 min duration, a time previously shown not to result in necrosis.* Five animals were eliminated from the study before randomization into a group because we were unable to define region at risk.

**Experimental protocol.** Ten minutes after definition of the region at risk, 29 rabbits underwent coronary ligation. At 5 min after occlusion the animals were randomly assigned to receive a bolus of 3 μg/kg nifedipine (Pfizer Laboratories; in a vehicle of 15% polyethylene glycol + 15% ethanol) or an equivalent volume of saline followed by an infusion of 0.05 μg/kg/min nifedipine or an equivalent volume of saline. The light-sensitive dye was protected by covering all syringes and tubing with opaque material. At 60 min after occlusion the ligature was released, and 20 min later the infusion was discontinued. Arterial blood pressure and heart rate were monitored throughout the experiment. Tetracycline, 50 mg/kg, was administered over a 10 min period to all animals at 90 min after occlusion (30 min after reperfusion) to determine the necrotic zone and the area of no reflow. The animals were killed 2½ hr after occlusion with an overdose of pentobarbital.

**Measurement of myocardial infarct size.** The heart of each rabbit was excised and the left ventricle was breadloafed into seven to eight horizontal sections. The apical and basal sides of each section were photographed under UV light to delineate the area marked by tetracycline. Tetracycline labels infarcted myocardium by chelating calcium within the necrotic cells. Since the delivery of the antibiotic was dependent on an intact vascular supply to the infarcted region, a region of no reflow was defined by the absence of tetracycline fluorescence and the fluorescence of a portion of the infarct zone demonstrated that reperfusion had occurred when the arterial ligature was released. The sections were then incubated in NBT for 20 min at 37°C and rephotographed as previously described. Each section was weighed.

To summarize, a pictorial representation is shown in figure 2. Normal myocardium did not take up tetracycline but stained violet with NBT and conversely infarcted myocardium was positive for tetracycline but did not stain with NBT; the area of no reflow was always contained within the infarct and was not marked by either tetracycline or NBT.

In a preliminary study comparing infarct size as measured by NBT and tetracycline in individual sections studied at 90 min and 24 hr after occlusion, we demonstrated that they were nearly identical, with r values of .94 and .93, respectively (figure 3). To assess the effect of nifedipine, we used the tetracycline photographs because the boundaries were more clearly defined, but the NBT photographs were used as a reference.

**Statistical analysis.** The relationship between the NADH fluorescent area and subsequent infarct size was examined by regression analysis. The significance of differences between the treated and control groups for all except the hemodynamic measurements were examined by Student’s t test for unpaired samples. The hemodynamic measurements were examined by analysis of variance for independent groups with repeated measures. All values are given as mean ± SEM.

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*Foster E, et al.: Unpublished observations.
Results

Protocol I (NADH as a measure of region at risk). Two of the initial 33 rabbits could not be used because the control preligation UV photograph had fluorescence due to epicardial fat. This problem was subsequently minimized by the use of relatively young animals. However, four animals were excluded because of our inability to trace the fluorescent area accurately. A fluorescent epicardial region was observed within 2 to 5 min after coronary artery ligation in 30 of 31 animals. Eleven rabbits died in their cages before the scheduled time of sacrifice. These animals were eliminated from the study because infarct size could not be accurately determined; NBT staining must be done in the immediate postmortem period.

In the 16 animals that survived for 24 hr after ligation, infarct size correlated with the area of postligation epicardial NADH fluorescence when infarct size was expressed as a percent of left ventricular weight (r = .74, p < .001; figure 4). These results showed that the epicardial NADH fluorescent area measured immediately after coronary artery ligation can serve as a reliable predictor of the amount of myocardial necrosis that will occur over the subsequent 24 hr.

In all animals several photographs were taken during a 45 min postligation period, before the thoracotomy was closed. The position of the animals was changed so that all regions of epicardial fluorescence could be imaged and quantified. Despite the different animal positions in these sequential photographs, qualitative inspection of the total distribution of epicardial fluorescence was possible and revealed a stable pattern in 15 of 16 hearts. In the one exception, a region of fluorescence was present on the posterolateral wall from 2 to 8 min after ligation, but this region was no longer fluorescent at 28 min after ligation. In five hearts we were able to quantify the epicardial fluorescent area on early (14 ± 1.6 min) and late (28 ± 2.4 min) photographs of the same region of the heart in the same projection. The pattern and extent of fluorescence was highly stable over this time range. The fluorescent area measured on the early photographs correlated significantly with the area on the late photographs (average difference = 0.001 cm²; r = .999, p < .001, n = 5).

Protocol II (nifedipine vs saline control). The two groups were equivalent with respect to all baseline parameters, including weight, pentobarbital dose, and amount of saline administered for flushing catheters (table 1).

Hemodynamics (figure 5). Heart rate significantly decreased throughout the experiment, but there were no significant differences between the two groups at any time. Blood pressure also declined during the experiment. The only significant difference between the two groups was a lower mean arterial pressure in the rabbits in the control group immediately before they were killed (p < .05). The normal systolic blood pressure for the New Zealand white rabbit in an unanesthetized state is 85.6 ± 7.1 mm Hg (mean ± SD; n = 89).* Thus, the blood pressures were, for the most part, in the low normal range.

Region at risk, infarct size, and area of no reflow (figures 6 and 7). Region at risk and infarct size were statistically equivalent for the two groups (figure 6). Infarct size (% total left ventricular weight) was 20.7 ± 3.3 for the nifedipine group and 16.0 ± 2.6 for the control group (p = NS). The area of no reflow was not significantly different when expressed as a percent of infarct size (nifedipine 19 ± 4; control 16 ± 3) or as a percent of the total left ventricular weight (nifedipine 4.0 ± 1.3; control 2.8 ± 1.0; figure 7).

*Chobanian A, Shippman J: Personal communication.
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FIGURE 5. Heart rate (HR) and blood pressure (MAP, mean arterial pressure) response during nifedipine protocol (see text for details).

Discussion

Our results demonstrate that the size of the epicardial NADH fluorescent area, measured in the immediate postcoronary ligation period, accurately predicts the extent of myocardial infarction that subsequently occurs. This relationship allows the NADH fluorophotograph to serve as a prospective measure of region at risk. This method for assessing region at risk was adopted from the work of Barlow and Chance and their colleagues7, 8, 13, 14 and is based on the increase in the NADH:NAD ratio that occurs during tissue hypoxia. In buffer-perfused hearts subjected to coronary ligation the presence of an increase in NADH fluorescence correlated with morphologic ischemic damage.15-17

Our results extend this relationship to the blood-perfused heart in vivo.

The region at risk after coronary occlusion has been assessed by a variety of techniques, such as mapping of the ST segment,18, 19 measurement of regional coronary blood flow with radioactive microspheres,20, 21 postmortem infusion of dyes into the ligated or unoccluded coronary vascular bed,22 and stereoscopic anal-

FIGURE 6. Effect of nifedipine on infarct size. There was no significant difference in region at risk (measured by NADH fluorescence) or infarct size between nifedipine and control groups.

FIGURE 7. Effect of nifedipine on the area of no reflow. There was no significant difference between the two groups.
ysis of the anatomic distribution of the occluded vascular bed after postmortem injection of contrast material.\textsuperscript{23} The NADH fluorophotographic technique has the advantages of being relatively simple and inexpensive and of not requiring radioactive materials or injection of any dyes.

However, use of the NADH fluorophotographic method for determination of region at risk also has some significant disadvantages. The heart must be exposed and the animal must be photographed in both decubitus positions to include all borders of the fluorescent region. Also, only superficial epicardial ischemia of the ventricular free wall is detected; neither the deeper myocardial layers nor the interventricular septum can be assessed by this technique. Nonetheless, as our results show, when the ischemic region is limited to the anterolateral wall, the epicardial NADH image is a reliable predictor of infarct size.

The NADH fluorophotography technique appears to define a “functional” rather than “anatomic” risk region, since the regression line in figure 4 passes close to the x and y origins. In contrast, when postmortem angiography was used to define an anatomic region at risk in the dog, it was found that a region of 0 to 20% of left ventricular mass did not result in an infarct; infarcts only occurred when the region at risk exceeded 20% of left ventricular mass.\textsuperscript{23} On the other hand, when functional region at risk was defined by myocardial perfusion measurement, no minimal region at risk was demonstrated.\textsuperscript{24} In contrast to the dog, however, in the rabbit there is probably little difference between anatomic and functional risk regions.\textsuperscript{25}

Nifedipine had no beneficial effects in our experiments. Infarct size was not affected by the drug, nor was the area of no refux within the infarct region. The area of no refux was defined by lack of tetracycline fluorescence and corresponded to the area of hemorrhage observed on gross inspection of the ventricular slices. Thus, nifedipine did not reduce damage to either myocytes or vasculature.

It seems unlikely that a different dosage of nifedipine would have resulted in a different result. We selected our dosage by titrating the hemodynamic effects of nifedipine in a preliminary series of experiments and we used the largest dose that allowed arterial hypotension to be avoided. Thus, we could not increase dosage without compromising coronary perfusion pressure. However, we cannot absolutely rule out the possibility that nifedipine could be beneficial if given at a different dosage.

The potential of nifedipine to reduce infarct size may depend on the amount of collateral circulation to the ischemic zone. Harken et al.\textsuperscript{25} compared the borders of the perfusion defect after coronary occlusion in rabbits with the borders of the anoxic region defined by NADH fluorophotography and these two zones were virtually identical, suggesting minimal collateral flow in the rabbit. Nifedipine also failed to reduce infarct size in the baboon, despite the use of a dosage greater than that in our studies,\textsuperscript{26} and the baboons studied also have poorly developed collateral circulation.\textsuperscript{19} In contrast to these results in the rabbit and the baboon, most studies in the dog, an animal with a well-developed collateral circulation, have shown that nifedipine decreases ischemic injury or infarct size.\textsuperscript{22, 27, 28} In a recent study by Melin et al.\textsuperscript{29} nifedipine reduced infarct size in the conscious dog and induced a mean increase in collateral flow, but in the four dogs in which the drug caused the greatest improvement in collateral flow there was the greatest protective effect against necrosis.

In summary, our results and the results of other investigators seem to suggest that nifedipine is most beneficial when extensive collateral circulation is present, as in the dog. In the rabbit and baboon, both of which have less collateral flow than the dog, nifedipine had no protective effect. Our results do not preclude a role for nifedipine in certain patients with acute myocardial infarction in whom extensive collateral vessels or coronary spasm may be present. However, in a recent randomized clinical study, nifedipine did not alter infarct size in man.\textsuperscript{30} This conclusion is consistent with results of our rabbit study.

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