

Limitation of infarct size with preconditioning and calcium antagonist (Diltiazem): Difference in ^{99m}Tc -PYP uptake in the myocardium

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Ischemic cell injury and the uptake mechanism of ^{99m}Tc -PYP (Pyrophosphate) were studied with preconditioning and calcium antagonist. Method: The coronary artery of an adult mongrel dog was clamped for 1 hour, followed by reperfusion and ^{99m}Tc -PYP injection. A control group (group C, $n = 8$), a group in which continuous drip infusion of diltiazem (10 mg/kg) (group D, $n = 7$), and a group preconditioned by six 5-minute clampings and perfusions before occlusion (group P, $n = 6$) were compared. Results: Wall motion was fully recovered in group D but not in group P after 2 hours of reperfusion. The ^{99m}Tc -PYP uptake ratio showed a significant ($p < 0.05$) reduction in group D (11.5 ± 3.6 compared with group C), but not in group P (11.5 ± 9.1 , $p = 0.25$). The infarct area was $1.2 \pm 0.6\%$ of the left ventricle in group D, 1.3 ± 0.4 in group P, and 6.4 ± 1.0 in group C ($p < 0.01$ in groups D and P vs. group C). Conclusions: These findings suggest that preconditioning does not alleviate stunning, but it improves cell injury in spite of high uptake of ^{99m}Tc -PYP. Diltiazem protects from both stunning and cell injury, suggesting a different mechanism of myocardial protection from that of preconditioning.

Key words: stunning, calcium-antagonist, ^{99m}Tc -pyrophosphate, preconditioning, myocardial protection

INTRODUCTION

THE DEATH of myocardial cells can be delayed, final infarct size can be reduced, and recovery from stunning after reperfusion can be complete if the myocardium can be protected during ischemia and reperfusion. Stunning itself may be a self-protective response of the ischemic myocardium with reduced ATP turnover,¹ but the final myocardial injury may be reduced by alleviating or shortening the state of stunning. Preconditioning is thought to be an adaptive mechanism reducing ischemic metabolism, and infarct size is thus reduced.

Preconditioning, acidosis on reperfusion,² the administration of certain drugs (e.g., ACE-inhibitors,³ Ca-antagonists,^{4,5} activation of the glycolytic system during ischemia,⁶ and free radical scavengers⁷ are reported to

have protective effects against myocardial cell injury, but in clinical situations we have almost no tools to estimate the contribution of preconditioning to reducing the extent of infarct and injury. We have already reported the usefulness of ^{99m}Tc -PYP uptake in estimating cell injury without infarct,^{8,9} and thought this method to be applicable to the evaluation of them. In this study, we evaluated the myocardial protective effects of preconditioning and calcium antagonist (diltiazem) by comparing cell injury with reference to ^{99m}Tc -Pyrophosphate (PYP), regional blood flow, hemodynamics, wall motion and infarct formation, and discussed the protective mechanism and difference between them.

METHODS

In 21 adult mongrel dogs (body weight, 11.5–27.5 kg), anesthesia was induced by i.m. injection of Ketalar (2.5 mg/kg) and maintained by i.v. injection of Nembutal (25 mg/kg). The animals were intubated, and respiration was controlled with a Harvard respirator. Catheters were inserted into both femoral arteries, one for monitoring blood

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PROTOCOL

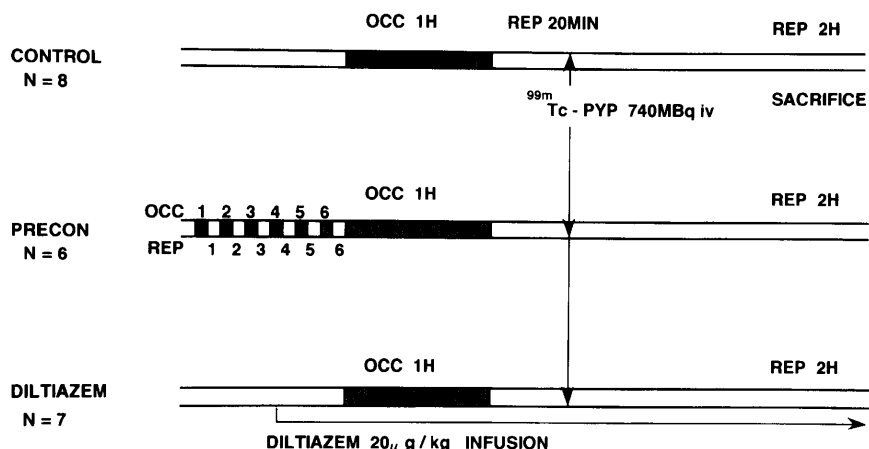


Fig. 1 Figure shows the protocol of the study in these groups. Details are explained in the text.

pressure and the other for blood sampling. A catheter was inserted into a femoral vein for fluid infusion and drug administration. Thoracotomy was performed at the 5th intercostal level, and the pericardium was suspended in a cradle. A catheter was advanced from the left atrial auricle and used for infusion of colored microspheres and Evans blue. The origin of the left anterior descending artery or the first diagonal branch, or the left circumflex branch was detached, and the sensor of a Doppler flow meter and an occluder were placed at these sites.

The blood flow was measured before occlusion (control), before reperfusion, 20 minutes after reperfusion, and 120 minutes after reperfusion by means of colored (yellow, red, blue and black) microspheres (E-Z Company) (Fig. 1). The microspheres were well stirred manually and infused from the left atrial auricle at 15 million/time. Blood was drawn from the femoral artery with a continuous aspiration pump at the rate of 10 ml/min and was incubated for 90 seconds. After sacrifice, 12–15 tissue blocks each weighing 1–3 g were cut from the endocardial and epicardial sides of the ischemic and normal regions, and absolute blood flow was calculated by a routine technique.

Left ventricular wall motion was recorded with an echocardiograph (Shimadzu, SVS100) before all treatments (control), before occlusion (30 minutes after diltiazem administration or after preconditioning), before reperfusion and 20 minutes, 1 hour and 2 hours after reperfusion. The probe was applied directly to the epicardium, and short-axis views at the level of the papillary muscles were recorded. The wall motion was analyzed by the centerline method as reported previously.^{8,9} Regions in which the wall motion was reduced to less than 2SD of the mean for normal dogs during the occlusion period were regarded as ischemic regions, and the mean value was calculated in each stage. Changes in wall motion

throughout the observation period were expressed as a percentage of the control value.

To examine myocardial cell injury, 740 MBq of ^{99m}Tc -pyrophosphate (PYP) was infused at 20 minutes after reperfusion from the left atrial auricle, and *in vivo* images were obtained after 2 to 2.5 hours. After sacrifice, 3–4 sections of the heart were obtained along the short axis, and *ex-vivo* images were obtained.

Five tissue samples (1–2 g) were cut from the epicardial side and five from the endocardial side of the infarct region identified by triphenyl tetrazolium chloride (TTC) staining or the area around the infarct region and the normal region. Tissue counting was done in 20 samples from each animal, counts per unit of weight (CPW) were calculated, and the ratio of the count in the ischemic region to that in the normal region was found. Evans blue was infused from the left atrial auricle before sacrifice to determine the risk area after occluding the coronary artery with a snare. After sacrifice, 3–4 slices of the heart were obtained, and the infarct region was confirmed by TTC staining. The ischemic and infarct regions were traced, their areas were calculated, ischemic tissue weight and infarct tissue weight were determined, and their percentages of left ventricular weight were calculated.^{8,9}

The area without TTC staining was strictly defined by 3 observers so as not to include an ambiguous region. In the case of a scattered defect in staining, obvious defect areas and the regions around them including color border zones were also calculated.

The present study was carried out in 3 groups of animals (Fig. 1). In the control group (group C, $n = 8$), 1-hour coronary occlusion and 2-hour reperfusion were performed during continuous infusion of physiologic saline. In the diltiazem group (group D, $n = 7$), diltiazem was infused at 20 $\mu\text{g/kg/min}$ with an infusion pump starting 30 minutes before coronary occlusion, the coro-

DOUBLE PRODUCTS

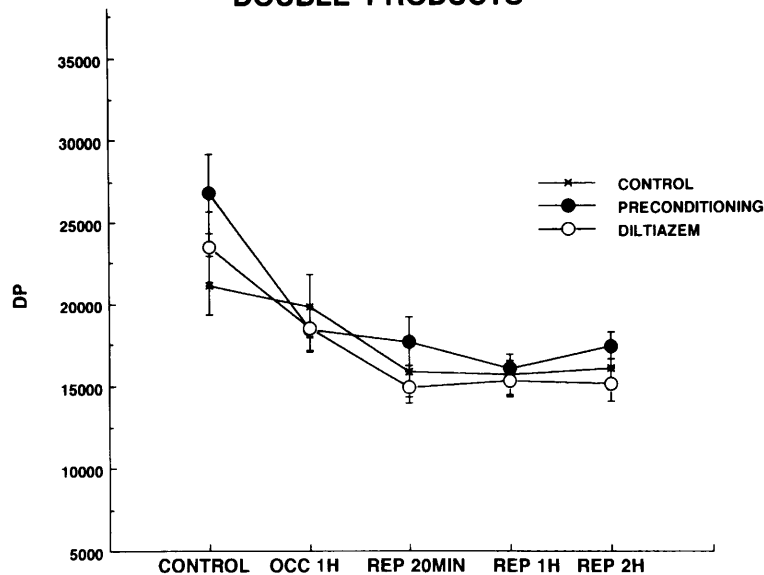


Fig. 2 Double products through the study are shown in this figure. Double products in the group with diltiazem showed lower value after reperfusion than other 2 groups, but without significant difference. REP = reperfusion, DP = double products

Table 1 Regional blood flow (%)

		Control	Diltiazem	Preconditioning
Transmural	OCC	20 ± 3	23 ± 4	24 ± 4
	R 20 M	119 ± 13	136 ± 28	133 ± 72
	R 2 H	84 ± 44	106 ± 17	71 ± 21
EPI	OCC	31 ± 7	24 ± 6	22 ± 5
	R 20 M	112 ± 22	104 ± 23	82 ± 27
	R 2 H	103 ± 57	139 ± 0	66 ± 20
END	OCC	6 ± 2	16 ± 4	18 ± 4
	R 20 M	129 ± 3	190 ± 53	231 ± 149
	R 2 H	65 ± 31	108 ± 0	95 ± 33

(Control: 100%)

EPI: epicardium, END: endocardium, OCC: occlusion, R 20 M: 20 minutes after reperfusion, R 2 H: 2 hours after reperfusion

nary artery was occluded for 1 hour, then reperfusion for 2 hours. Diltiazem infusion was maintained throughout the protocol. In the preconditioning group (group P, n = 6), 5-minute occlusion and 5-minute reperfusion were repeated 6 times, followed by 1-hour coronary occlusion and 2-hour reperfusion (Fig. 1).

Statistical analysis: The mean and standard deviation values were calculated from the data obtained, their differences were examined by Student's t-test, and their changes with time were examined by ANOVA at the $p < 0.05$ level of significance. Data in the figures are expressed as the mean \pm SE.

RESULTS

Hemodynamics (Fig. 2): In group C, the double products (DP) decreased after reperfusion due to decreases in

the heart rate (HR) and blood pressure (BP). In group D also, it decreased significantly from 20 minutes after reperfusion because of a significant decrease ($p < 0.01$) in HR as well as BP. In group P, DP decreased significantly ($p < 0.01$) during occlusion, and BP increased slightly after reperfusion because of a significant decrease ($p < 0.01$) in HR, which began during occlusion. Double products decreased much in the group D compared to other 2 groups during the study because of decreased HR & BP caused by the drug, but there was no statistical difference because of the large SD of the groups.

Regional blood flow: During coronary occlusion, the blood flow in the ischemic region was significantly smaller ($p < 0.01$) than that in the normal region in all groups compared with the control ($20 \pm 3\%$ in group C, $23 \pm 4\%$ in group D, and $24 \pm 4\%$ in group P), but it recovered after 2 hours of reperfusion ($84 \pm 44\%$ in group C, $106 \pm 17\%$

REGIONAL WALL MOTION

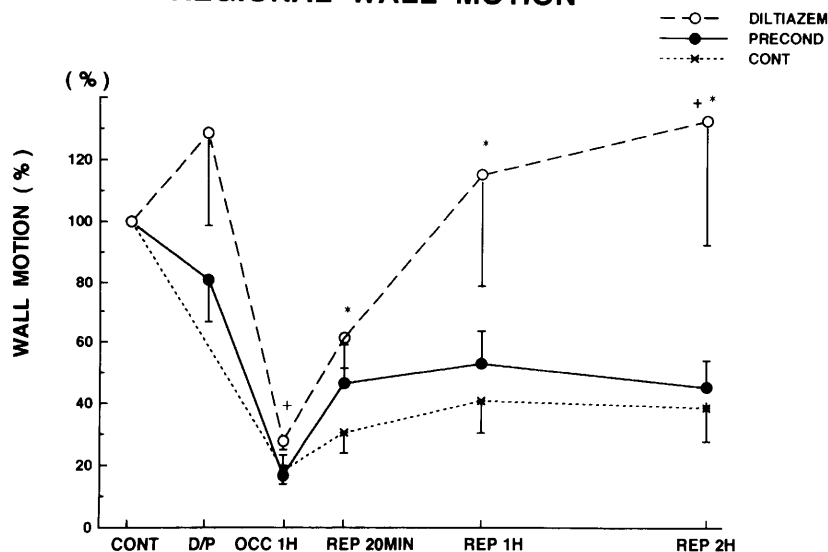


Fig. 3 Regional wall motion during the study is shown. The group with diltiazem treatment showed marked and significant improvement after occlusion-reperfusion. OCC: occlusion, REP: reperfusion, CONT: control, D/P: wall motion calculated in a group with diltiazem and preconditioning just before occlusion. *: $p < 0.05$ vs. control group, +: $p < 0.05$ vs. preconditioning group

in group D, and $71 \pm 21\%$ in group P). Regional blood flow including epi- and endocardial side is shown in Table 1. In group C, the blood flow in the normal region was slightly greater than that in the control during occlusion ($119 \pm 25\%$). In group D, it was relatively unchanged at all measuring points ($97 \pm 0\%$ at the control measurement, $95 \pm 12\%$ 1 hour after occlusion, $97 \pm 17\%$ 20 minutes after reperfusion, and $101 \pm 10\%$ 2 hours after reperfusion). In group P, the blood flow in the normal region was smaller during occlusion than at the control measurement in all but one dog, and the mean value for all animals was $92 \pm 49\%$. This slight reduction in the blood flow was also observed 20 minutes after reperfusion ($59 \pm 9\%$) and 2 hours after reperfusion ($81 \pm 9\%$).

Regional wall motion (Fig. 3): In group P, the regional wall motion decreased to $81 \pm 15\%$ of the control after preconditioning. After reperfusion also it remained $47 \pm 12\%$ of the control at 20 minutes, $53 \pm 23\%$ at 60 minutes, and $45 \pm 20\%$ at 120 minutes, indicating stunning, although the values were higher than in group C. In group D, on the other hand, the wall motion increased to $129 \pm 43\%$ of the control after diltiazem administration alone. This improvement of the wall motion was also observed visually during monitoring of echocardiograms in nearly all animals in group D. The decrease in the wall motion during occlusion was smaller in group D than in group C or group P ($28 \pm 3\%$ in group D, $18 \pm 4\%$ in group C, $16 \pm 3\%$ in group P), and the difference compared with group P was significant ($p < 0.05$). The improvement in the wall motion after reperfusion was noticeable; it became better than the value prior to the drug administration 60 minutes after reperfusion, and was significantly better

($p < 0.05$) than in group C at all measurements.

Injured area: Figure 4 shows the ^{99m}Tc -PYP uptake ratio, which is an index of cell injury with or without infarct, in and around the area where myocardial infarction could be confirmed by TTC staining. The value on the endocardial side (End) increased similarly in groups P and C (14.6 ± 3.7 in group C and 14.0 ± 3.4 in group P). The value on the epicardial side (Epi) remained low (7.8 ± 2.0 in group C and 4.0 ± 1.1 in group P) although the difference was not significant. In group D, the uptake was even lower than in group P in both Epi and End (5.2 ± 0.8 and 1.8 ± 0.2 , respectively). Representative cases of ^{99m}Tc -PYP *ex-vivo* imaging, TTC and Evans Blue staining are shown in Figures 5 to 7. The correlations between myocardial blood flow at 20 minutes after reperfusion and the uptake ratio of ^{99m}Tc -PYP injected at the same time were -0.39 , -0.20 and -0.20 in group with control, diltiazem and preconditioning, respectively.

Figure 8 shows the ischemic region (weight) and infarct region (weight) percentages of the entire left ventricle and the infarct region percentage of the ischemic region. The ischemic region percentage of the left ventricle was $20.2 \pm 1.9\%$ in group C, $23.8 \pm 3.3\%$ in group D, and $20.4 \pm 2.0\%$ in group P, showing no significant difference among them. The infarct region percentage was similarly and significantly smaller ($p < 0.01$) in group P and group D than in group C ($6.4 \pm 1.0\%$ in group C, $1.3 \pm 0.4\%$ in group P, and $1.2 \pm 1.5\%$ in group D). The percent region percentage of the ischemic region was noticeably smaller in groups P and D than in group C, but the visual findings obtained by TTC staining were different in groups D and P. Infarct regions were observed as focal areas in group D

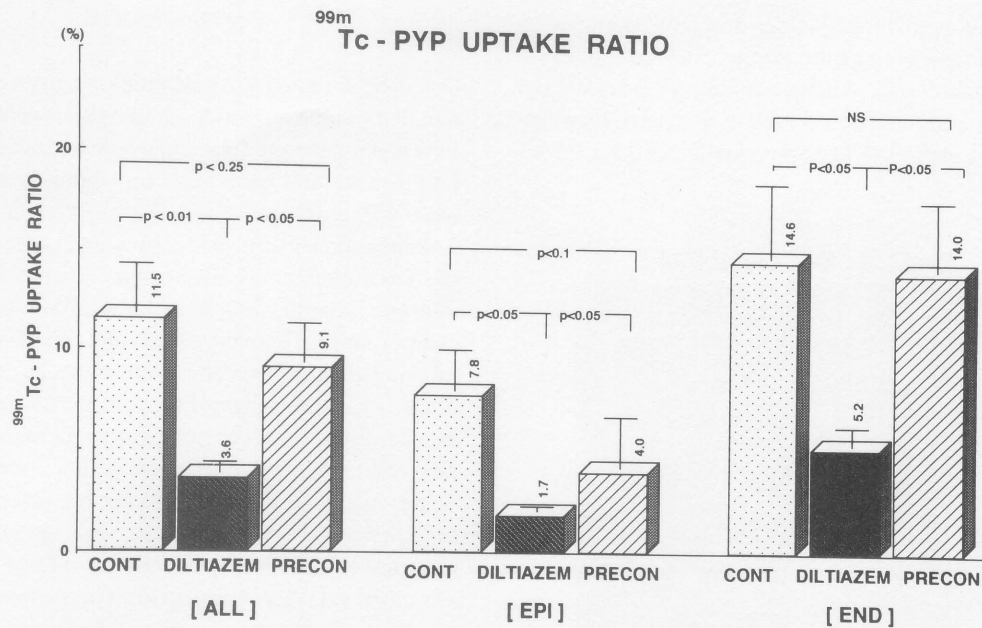


Fig. 4 ^{99m}Tc-PYP uptake ratio is shown. ALL: transmural uptake, EPI: epicardial uptake, END: endocardial uptake. Uptake ratio is shown as an uptake compared to non-ischemic control area.

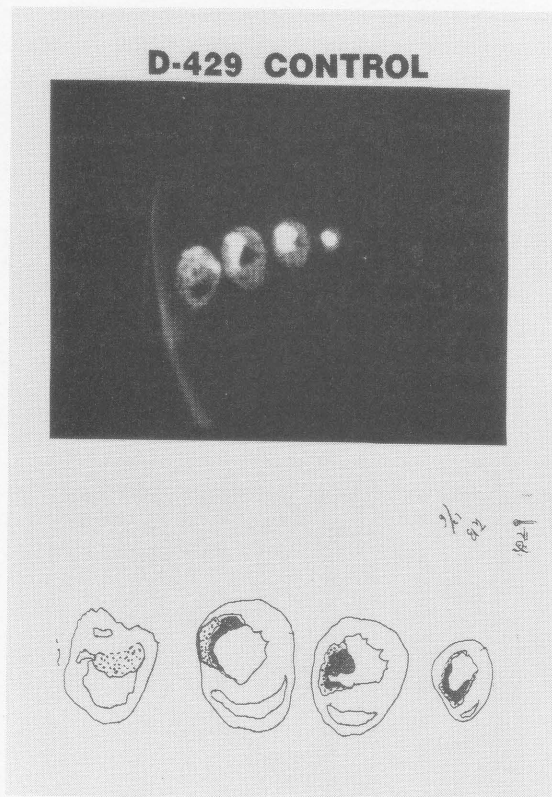


Fig. 5 *Ex vivo* imaging of ^{99m}Tc-PYP, Evans Blue and TTC staining with the control dog. Marked ^{99m}Tc-uptake was visualized at the risk region (dotted area), and coincide with TTC negative stained area (dark region).

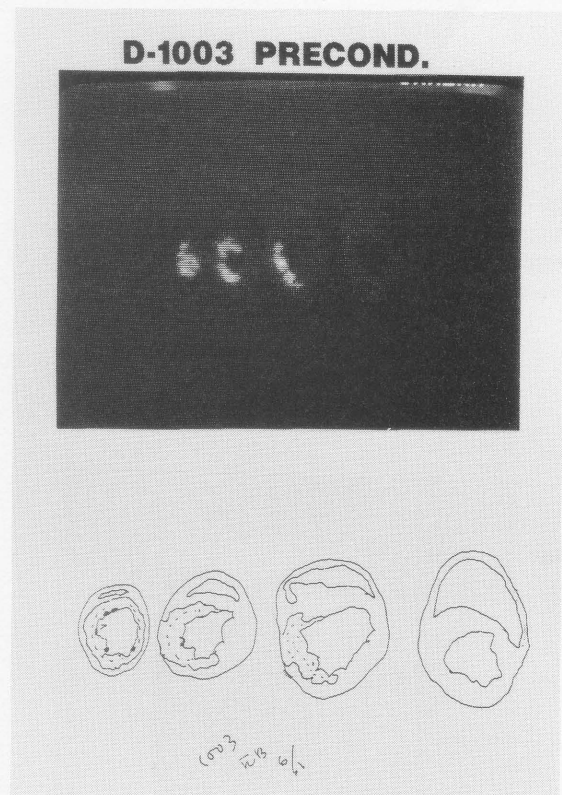


Fig. 6 *Ex vivo* imaging of ^{99m}Tc-PYP, Evans Blue and TTC staining with the dog of preconditioning. Moderate uptake of ^{99m}Tc-PYP was seen especially at the endocardial side of risk region (dotted area), but small infarct was detected (small dark region).

but invariably as diffuse "pepper-and-salt" patterns in group P, although both of the groups showed a marked reduction of infarct size. When the infarct region is strictly defined as the complete defect area in this patchy region, the infarct area in group P becomes smaller, or $0.8 \pm 0.2\%$.

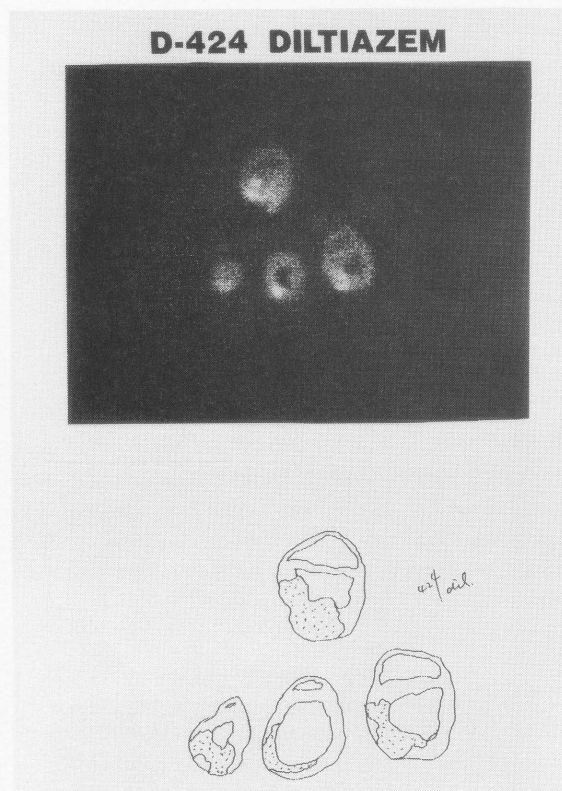


Fig. 7 *Ex vivo* imaging of ^{99m}Tc -PYP, Evans Blue and TTC staining with the dog using Diltiazem. Slight ^{99m}Tc -uptake was visualized at the risk area (Evans Blue). No negative TTC staining was seen (no infarct).

DISCUSSION

Our study revealed that preconditioning can reduce infarct size to the same level as a calcium antagonist even though flow and double products did not differ greatly, but ^{99m}Tc -PYP uptake and recovery from stunning in them were quite different.

Contraction insufficiency (stunning) caused by ischemia of short duration may often occur clinically in the form of multiple ischemic attacks including silent ischemia (preconditioning). There have also been reports on the effects of a single^{10,11} or repeated ischemic attacks on the myocardium. Preconditioning has a protective effect on the myocardium: it delays myocardial necrosis and eventually reduces the size of myocardial infarction, but is widely believed to have no favorable effect on the contractility of the myocardium.¹²⁻¹⁶ Preconditioning is also reported to reduce the utilization rate of high energy phosphates (HEP)¹⁷⁻¹⁹ and to limit mitochondria swelling, which is caused even by short term ischemia,²⁰ and to result in non-irreversible injury.²¹ Moreover, preconditioning is reported to delay cell necrosis by reducing anaerobic glycolysis and catabolite burdens (lactate, K^+ , H^+), thus avoiding the increases in the products of glycolysis, extracellular Ca^{2+} ,²² exacerbation of acidosis, and reduction in pH ²³ during ischemia. Concerning the segmental shortening and wall thickening, there is a report stating that the recovery of wall motion after reperfusion was significantly better in a preconditioned group than in an unpreconditioned control group,²⁴ but preconditioning is generally not considered to have a protective effect on the wall motion of larger animals, as our results also indicate.

In this study, we employed ^{99m}Tc -PYP to evaluate the protective effect of preconditioning on myocardial viability.

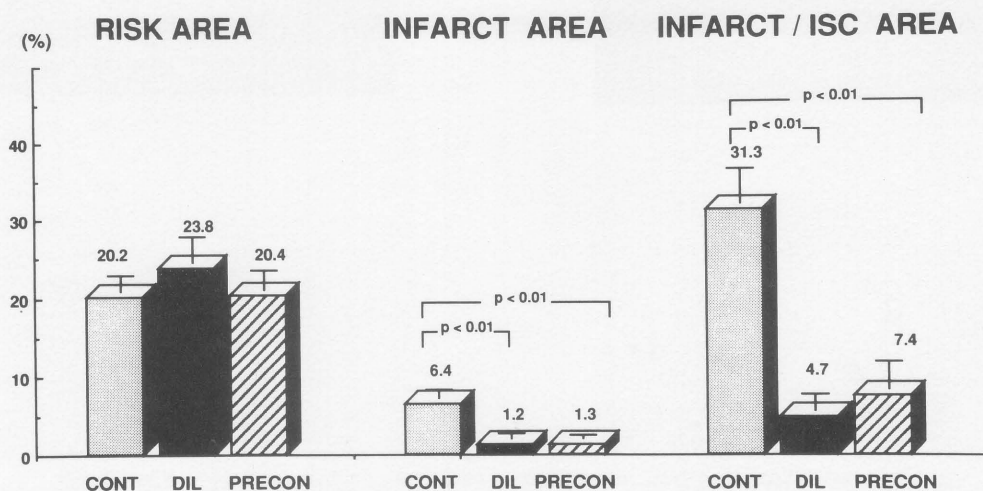


Fig. 8 Size of risk area, infarct area and % of infarct/ischemic area are shown. Although risk area were comparable among the three groups, infarct size showed marked differences between control and diltiazem or preconditioned groups. CONT: control group, DIL: diltiazem, PRECON: preconditioned group

ity, the state of cell injury, and the mechanical difference in protection from infarction with a calcium antagonist. When ^{99m}Tc -PYP was clinically used beforehand, ^{99m}Tc -PYP was a marker for determination of myocardial infarction and for confirmation of the size of the infarction,²⁵⁻²⁹ but we demonstrated that ^{99m}Tc -PYP uptake occurs not only due to necrotic myocardium but also injured myocardium without infarct.^{8,9} In *in-vivo* studies, ^{99m}Tc -PYP was taken up by pre-infarcted injured myocardium after a 10-minute or 30-minute occlusion of LAD or Cx.^{8,9} Clinically, also, we confirmed ^{99m}Tc -PYP uptake in areas where ^{201}Tl perfusion was observed in cases successfully reperfused in an early stage of acute myocardial infarction,³⁰ and suggested the application of ^{99m}Tc -PYP to the evaluation of the myocardial viability and recognition of the risk area in combination with a perfusion tracer. In the present study, the ^{99m}Tc -PYP uptake ratio was noticeably low in the diltiazem group, where the infarct was quite small. In the preconditioned group, the uptake (cell injury) on the endocardial side was similar to the control group, but that on the epicardial side was reduced. This is considered to have played a part in the reduction in the infarct size to a level comparable to that in the diltiazem group.

^{99m}Tc -PYP is considered to be taken up by injured myocardium without infarct, to bind with hydroxy apatite, in which Ca^{2+} content is changed by ischemia, and to reflect its behavior.⁹ In evaluating its uptake, we divided the animals into 3 groups (control group, preconditioned group, and calcium-antagonist treated group). There are reports that contraction of the stunned myocardium was improved by the administration of an ACE-inhibitor (captopril), and a calcium antagonist (verapamil),³¹ and our diltiazem.⁸ It completely suppressed stunning and improved wall motion after reperfusion to a greater extent than the pre-administration control state. This effect of diltiazem may be due to maintaining the balance between the intracellular and extracellular Ca^{2+} concentrations and the increasing extracellular Ca^{2+} during ischemia³² rather than the increase in coronary blood flow. The protective effect of preconditioning against myocardial necrosis and the suppressive effect of calcium antagonists against stunning are considered to disappear as ischemia is sustained for more than a few hours.^{1,17} Since more than a few hours is needed in many clinical cases until reperfusion after the onset of sustained ischemia following preconditioning by repeated ischemic episodes of varying degrees including silent ischemia, further studies are needed on the effects of preconditioning over a longer period of ischemia. Both the segmental shortening and the infarct size were reduced by preconditioning and cyclic flow variation (CFV), which is similar to clinical attacks of angina, after 60-minute ischemia followed by 4-hour reperfusion, but the infarct size was significantly reduced by CFV after 90-minute ischemia, indicating a greater protective effect.¹⁴ Unstable angina may therefore pre-

condition the myocardium and protects against sustained myocardial ischemia. The results of our study suggest that injury to the preconditioned myocardium represented by ^{99m}Tc -PYP is reversible, especially on the epicardial side, and that preconditioning protects the myocardium against ischemia by delaying myocardial necrosis and reducing the infarct size despite the occurrence of stunning.

^{99m}Tc -PYP uptake in the preconditioned group did not differ greatly from that in the groups treated with calcium antagonist, in spite of the same reduction in infarct size.

^{99m}Tc -PYP uptake especially on the endocardial side was almost the same in the control and the preconditioned group, although infarct size was greatly reduced in the preconditioned group.

These discrepancies may be explained in the following way:

1) Stunning and infarct size were well controlled by calcium antagonist, but the mechanism of preconditioning to reduce infarct size is completely different from that of calcium antagonist in terms of Ca^{2+} metabolism. ^{99m}Tc -PYP uptake was closely related to the suppression of stunning,⁸ suggesting that stunning is controlled by Ca^{2+} metabolism. The mechanism of preconditioning to suppress myocardial infarction is mediated by A1 adenosine receptor in rabbit heart,³² or ATP-sensitive potassium channels in dogs,³³ which is not directly related to calcium metabolism. Several other factors including decreased demand of the ischemic heart, inhibitions of metabolite, and heat shock protein with preconditioning appear to reduce infarct size, but not all of them may reduce ^{99m}Tc -PYP uptake. The mechanism involved in reducing infarct size is different from the effect of calcium antagonist as is shown in our paper.

2) In the preconditioned group, ^{99m}Tc -PYP uptake was at a higher level, but infarct size was greatly reduced. It may be due to a scattered form of the ^{99m}Tc concentration as the infarct was also in a scattered form, and homogenized myocardial tissue ^{99m}Tc -PYP counts showed a higher level in spite of the small size of the total infarct with injury reversible myocardium and moderately increased uptake of ^{99m}Tc -PYP around the scattered infarct.

3) It is believed that preconditioning, by unifying A1 adenosine receptor and ATP-regulated K^{+} channels to reduce ischemic metabolism, slows infarct formation. Thus, even if ^{99m}Tc -PYP uptake, which is related to phospholipid depletion during ischemia and a subsequent Ca^{2+} permeability defect resulting in increased intracellular Ca^{2+} , might cause a disorder of reversal metabolic change with Ca^{2+} permeability may follow, and lethal cell death will be delayed.

The implications of our findings are:

1) preconditioning resulted in a greater uptake of ^{99m}Tc -PYP in spite of reduced infarct size, suggesting clinical limitations in infarct estimation with ^{99m}Tc -PYP in a situation analogous to preconditioning, such as unstable angina pectoris.^{26,27}

2) The risk area may be easily recognized even if no infarct is clinically revealed, suggesting higher uptake of ^{99m}Tc -PYP in frequent ischemia such as silent ischemia and unstable angina pectoris.

3) ^{99m}Tc -PYP uptake may be a marker in estimating a factor limiting infarct size, whether it is due to Ca^{2+} metabolism or to another protective mechanism.

4) Preconditioning is effective in reducing infarct size, but it is not at all effective in stunning, which is generally accepted, as previous reports show.^{34,35} The results of our study also strongly suggest this and offer a clue to finding the mechanical difference between stunning and preconditioning.

Experimental limitations

The myocardial infarct was rather small in our experiment, and may show the limitation of TTC staining especially in patchy distribution of the infarct. The results would be clearer if we used a more precise method because even a gross visual method provides clear findings. We calculated the non-stained TTC area very carefully, and in the case of patchy regions we tried to calculate the defect region and the vague region around them separately, and then checked our results, although we have not used microscopy to confirm our findings. Further evaluation with more sensitive methods is awaited.

CONCLUSION

Our results indicated that the mechanism of protection from myocardial injury with calcium antagonist is different from that with preconditioning, and also suggested the limited value of clinical ^{99m}Tc -PYP imaging in evaluating myocardial necrosis.

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