A USEFUL CANINE MODEL OF ISCHEMIC MYOCARDIUM WITH CORONARY RETROGRADE FLOW DIVERSION, AND ITS APPLICATION FOR THE STUDY OF ALLOPURINOL ON MYOCARDIAL INFARCT SIZE

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This study was undertaken to evaluate the usefulness of a canine ischemic heart model achieved by coronary retrograde flow (RF) diversion, and to examine the effect of allopurinol on the myocardial infarction. The left anterior descending coronary artery (LAD) was occluded for 90 min followed by 4h reperfusion. Group 1 (n=9) was a simple LAD occlusion group. In group 2 (n=8), retrograde flowing blood from the distal of the occluded LAD was simultaneously diverted during LAD occlusion. In group 3 (n=8), allopurinol was administered 60 min before ischemia with RF diversion followed by its continuous infusion. Infarcted myocardium was determined by triphenyl tetrazolium chloride staining and myocardium at risk by the dye double perfusion technique. RF diversion significantly reduced not only regional myocardial blood flow (RBF) (0.21 ± 0.05 ml/min/g in group 1 vs 0.05 ± 0.01 ml/min/g in group 2; $p < 0.05$) but also its variance ($p < 0.01$). Furthermore, the infarct size (infarct/risk ratio) in the allopurinol treated group was significantly reduced without any significant difference in rate pressure product, risk size or RBF ($89.4 \pm 4.0\%$ in group 2 vs $48.9 \pm 4.5\%$ in group 3; $p < 0.01$). We conclude that the canine RF diversion model is useful for myocardial infarct study because of the minimizing of the difference of collateral flow, and that canine myocardial xanthine oxidase may produce free radicals which take part in myocardial injury after reperfusion.

OXYGEN-derived free radicals have been implicated as agents of cellular damage in ischemia and reperfusion myocardial injury!^{1,2} The xanthine-xanthine oxidase system has been reported to be one of the free radical producing pathways, and some investigations have revealed that allopurinol, a xanthine oxidase inhibitor, could limit myocardial injury after ischemia and

reperfusion.^{3,4} However, these results have been questioned by others.⁵ The reasons for this controversy are thought to be the differences in experimental protocol and the lack of precise analysis of myocardial ischemia, especially the influence of regional myocardial blood flow (RBF) on infarct size.

Infarct size is dependent on the size of area at risk, RBF and cardiac oxygen demand.⁸ The variation in coronary collateral flow (CCF) must be equalized or controlled in a critical infarct size study, particularly when using a canine heart where collateral circulation is well developed!^{10,11} Although

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Fig. 1. Schematic representation of experimental model. The arterial blood was perfused from the left carotid artery to the LAD (left). Simple ischemia for group 1 was achieved by LAD occlusion with clamp A and B (middle). Severe ischemia for groups 2 and 3 was obtained by LAD occlusion with RF diversion with A clamped and B unclamped (right). Retrograde flowing blood was perfused back to the femoral vein.

Fig. 2. Experimental protocol for 90 min ischemia followed by 4h reperfusion. The effect of coronary retrograde flow (RF) diversion on regional myocardial blood flow was compared in groups 1 and 2, and the effect of allopurinol on infarct size was compared in groups 2 and 3.

coronary retrograde flow (RF) has been measured for assessing the CCF, RF collection to divert CCF could also be used to produce a severe ischemic myocardium.¹² Therefore, we examined the validity of the RF diversion model for ischemic myocardium, and investigated the ability of allopurinol to limit infarct size.

SUBJECTS AND METHODS

Twenty-five mongrel dogs of either sex that weighed from 8 to 22 kg were anesthetized with 30 mg/kg of intravenous sodium

pentobarbital. Additional anesthesia was administered during experiment, ad libitum. The dogs were intubated and ventilated at 30 ml/kg/min of room air supplemented with a low flow of oxygen to adjust normal PaO_2 . After a left thoracotomy in the fourth intercostal space, the heart was suspended in a pericardial cradle. The left anterior descending coronary artery (LAD) was cannulated with a low resistance metal cannula (0.05–0.07 mmHg/ml/min) between the first and the second diagonal branch, and perfused with arterial blood from the left carotid artery as a control perfusion state

TABLE 1 SYSTEMIC HEMODYNAMICS, CORONARY HEMODYNAMICS AND MYOCARDIAL INFARCT SIZE IN ALL THREE GROUPS

	Group 1 (n=9)	Group 2 (n=8)	Group 3 (n=8)
<i>Heart rate (bpm)</i>			
Control	156±29	169±19	174±28
Ischemia	156±29	169±17	177±32
Reperfusion	161±31	181±21	196±45
<i>Mean BP (mmHg)</i>			
Control	101±15	109±18	99±21
Ischemia	99±21	107±24	92±28
Reperfusion	96±9	98±24	89±31
<i>RPP (×1000)</i>			
Control	20.1±4.4	22.1±4.7	21.1±6.4
Ischemia	19.0±4.9	21.9±6.3	19.9±7.6
Reperfusion	19.0±4.1	22.3±7.7	21.1±8.5
<i>PCP (mmHg)</i>	25±12	24±8	18±8
<i>CBF (ml/min/g)</i>	0.94±0.57	0.82±0.41	0.71±0.34
<i>RF (% CBF)</i>	23.9±25.6	29.3±20.7	25.1±14.1
<i>CCF (% CBF)</i>	24.8±17.8	36.9±24.8	33.2±18.5
<i>RBF (ml/min/g)</i>	0.21±0.14	0.05±0.04**	0.05±0.01
<i>RBF (% CBF)</i>	24.8±17.8	7.6±5.8*	8.2±5.2
<i>Area At Risk (% LV)</i>	11.7±7.7	17.7±8.4	18.7±10.2
<i>Infarct (% LV)</i>	4.9±4.0	15.3±5.8**	9.6±5.9
<i>Infarct/Risk (%)</i>	46.3±28.9	89.4±11.4**	48.9±12.7++

Values are mean±SD. *: $p<0.05$ vs group 1; **: $p<0.01$ vs group 1; ++: $p<0.01$ vs group 2.

Mean BP=mean aortic blood pressure; RPP=rate pressure product; PCP=peripheral coronary blood pressure; CBF=coronary blood flow; RF=coronary retrograde flow; CCF=coronary collateral flow; RBF=regional myocardial blood flow; LV=left ventricular mass.

(Fig. 1 left). Coronary blood flow (CBF) was measured with an electromagnetic flowmeter which was located in the perfusion line from the carotid artery to the LAD. One catheter was inserted into the femoral artery to measure aortic pressure and another into the femoral vein for administration of saline or drugs. Heart rate, aortic blood pressure, ECG, CBF and coronary blood pressure were recorded throughout the experiment. Heparin (12000 units) was administered intravenously to prevent clotting in the perfusion line, and was supplemented with 3000 units every 30 .

Simple ischemia for group 1 was achieved by LAD occlusion with points A and B clamped on the perfusion line (Fig. 1 middle). Severe and controlled ischemia for groups 2 and 3 was obtained by LAD occlusion with RF diversion, which was achieved by RF collection from the distal segment of the LAD with A clamped and B not

(Fig. 1 right). RF was measured with a graduated cylinder for 60 sec by opening a side branch in the perfusion line to the atmosphere while retrograde flowing blood was perfused back to the femoral vein. The value of RF was measured 1.5 min after ischemia. CCF in the ischemic region during LAD occlusion was estimated by the modified load line method^{12,13} which is reported to be well correlated with that by the 133-Xenon washout method¹⁴ The equation is as follows:

$$CCF = RF(1.61 - PCP/0.8 \times AoP)$$

where PCP is peripheral coronary blood pressure, obtained by clamping both points A and B on the perfusion line, and AoP is mean aortic blood pressure. AoP and PCP were measured simultaneously 1 min after simple LAD occlusion. RBF was estimated as CCF is the simple occlusion, and CCF minus RF in the RF diverted ischemia. RF,

Fig.3. A) Coronary collateral flow (CCF) expressed as a percentage of coronary blood flow (CBF) and regional myocardial blood flow (RBF) expressed as a percentage of CBF. Coronary retrograde flow diversion induced smaller variance and less RBF in group 2 than in group 1.

B) Risk size expressed as a percentage of left ventricular mass (LV), infarct size expressed as a percentage of LV and infarct/risk ratio. The infarct/risk ratio in group 2 was significantly larger and its variance smaller than in group 1.

CCF, and RBF were represented as relative to the weight of the risk area and as a percentage of CBF.

Figure 2 shows the experimental protocol for 90 min myocardial ischemia followed by 4h reperfusion. Group 1 (n=9) was the simple LAD occlusion group. In group 2 (n=8), RF was simultaneously diverted during LAD occlusion. In group 3 (n=8), LAD occlusion with RF diversion was performed the same as in group 2, but allopurinol (50 mg/kg, Sigma Chemical Co. U.S.A.) was injected as a bolus 60 min before ischemia and then followed by continuous infusion of 0.15 mg/kg/min throughout the experiment. Allopurinol was dissolved with sodium hydroxide at a pH of 10.35.¹⁵ In groups 1 and 2, an

Fig.4. A) Effect of regional myocardial blood flow (RBF) expressed as a percentage of coronary blood flow (CBF) on the infarct/risk ratio. In group 1, RBF was variable, and the infarct/risk ratio was inversely correlated with RBF. In group 2, the infarct/risk ratio was almost constant for any given CCF because RBF was well controlled by coronary retrograde flow diversion.

B) The correlation between infarct size expressed as a percentage of left ventricular mass (LV) and risk size expressed as a percentage of LV. Infarct size was linearly correlated with risk size in group 2, in which regional myocardial blood flow was well controlled.

equal volume of saline was infused. The dogs were sacrificed with an overdose of sodium pentobarbital after reperfusion.

The perfused area at risk was determined by the dye double perfusion technique, in situ. A cannula was inserted into the ascending aorta, and the aortic branches and the descending aorta were ligated. Both the LAD and aorta were perfused with saline under the same perfusion pressure, from 100 to 120 mmHg. The LAD bed was perfused with saline alone, and the remainder of the heart was perfused with saline plus fluores-

Fig.5. A) Coronary collateral flow (CCF) expressed as a percentage of coronary blood flow (CBF) and regional myocardial blood flow (RBF) expressed as a percentage of CBF. RBF and its variance were the same in both groups as were CCF and its variance.

B) Risk size expressed as a percentage of left ventricular mass (LV), infarct size expressed as a percentage of LV and infarct/risk ratio. No significant difference in risk size was found between the 2 groups. The infarct/risk ratio in group 3 was significantly smaller than in group 2.

Fig.6. A) The correlation between infarct/risk ratio and regional myocardial blood flow (RBF) expressed as a percentage of coronary blood flow (CBF). RBF in both groups was well controlled and almost constant. However, the infarct/risk ratio in group 3 was smaller than in group 2.

B) The correlation between infarct size expressed as a percentage of left ventricular mass (LV) and risk size expressed as a percentage of LV. A significant correlation was found in both groups. However, the regression line in group 3 was shifted downward significantly from that in group 2.

cent particles (10–20 micrometers in diameter, Duke Scientific Co. U.S.A.). After the heart was excised and frozen, ventricles were sliced into 4 mm transverse serial sections. In each section, the area at risk was visualized as the lack of fluorescent particles under ultraviolet light, and traced on a thin acetate sheet. The infarct region was determined by triphenyl tetrazolium chloride (TTC) staining, which leaves the necrotic tissue unstained, whereas viable tissue is stained dark red. The mass of the infarct of the risk region was calculated by planimetry the area of infarct or risk in each slice, multiplying it by the thickness and by the specific gravity of myocardium (1.05), and

summing the values for the entire heart. The risk size was expressed as a percentage of left ventricular mass (% LV), and the infarct size was also expressed as a percentage of left ventricular mass (% LV) and risk size (infarct/risk ratio).

Statistical analysis

All data are expressed as mean $\pm$ SD. Significant difference was considered as $p < 0.05$. The analysis of the variance between any 2 groups was performed with an F test, the average with a Student's t test or Welch's t test, and the significance of the correlation coefficient with a t test. The regression line was determined by the least

square method, and the analysis of covariance was used for the difference between 2 regression lines.

RESULTS

A total of 29 mongrel dogs were used for this study. Two dogs died post-operatively due to hemorrhage or ventricular fibrillation. Two dogs were excluded from the study because of unsatisfactory TTC staining. At the end of the experiment, statistical analysis was performed on 25 dogs with 9 dogs in group 1, 8 in group 2, and 8 in group 3. All data are shown in Table I.

1. Coronary retrograde flow diversion model

At first, we examined how RF diversion influenced the RBF of ischemic myocardium (group 1 vs group 2).

There was no significant difference in heart rate, mean aortic blood pressure, nor rate pressure product between groups 1 and 2 (Table I).

Figure 3-A shows the coronary hemodynamics. CCF was not significantly different between group 1 and group 2 ($24.8 \pm 17.8\%$ CBF and $36.9 \pm 24.8\%$ CBF, respectively). In group 2, RBF was significantly smaller than in group 1 ($7.6 \pm 5.8\%$ CBF in group 2 vs $24.8 \pm 17.8\%$ CBF in group 1; $p < 0.05$), and also its variance was statistically smaller than in group 1 ($p < 0.01$). RF diversion produced severe myocardial ischemia with extremely small variation. The infarct and risk size are shown in Fig. 3-B. Compared with group 1, the infarct/risk ratio in group 2 was larger ($46.3 \pm 28.9\%$ vs $89.4 \pm 11.4\%$; $p < 0.01$) and the variance was smaller ($p < 0.05$). These results may depend on only the reduction of RBF via collecting the RF from the occluded LAD because the hemodynamics and risk size were the same between the 2 groups.

The infarct/risk ratio was plotted against RBF in both groups (Fig. 4-A). In group 1, the value of RBF varied from low to near normal, and the infarct/risk ratio was found to be inversely correlated with RBF ($Y = 86.5 \times e^{-0.03X}$; $r = -0.84$, $p < 0.01$). On the other hand, in group 2, RBF was found to be consistently low with small variation. Since RBF was very low in the dog heart

with RF diversion, the infarct/risk ratio was very high. Consequently, infarct size was highly correlated with risk size in group 2, but not, however, in group 1 (Fig. 4-B).

2. Allopurinol study

The aim of the second study was to determine whether allopurinol pretreatment could reduce myocardial reperfusion injury using this RF diversion model (group 2 vs group 3), which was demonstrated for RBF to be well controlled, as already presented.

There was no significant difference in heart rate, mean aortic blood pressure nor rate pressure product during the experiment between groups 2 and 3 (Table I).

Figure 5-A shows that there was no significant difference in coronary circulation between the 2 groups. Both groups using RF diversion had well-controlled and severe myocardial ischemia. Fig. 5-B also shows that risk size was about 18% LV in both groups, but that the infarct/risk ratio in group 3 was significantly smaller than in group 2 ($89.4 \pm 11.4\%$ vs $48.9 \pm 12.7\%$; $p < 0.01$). Infarct size in group 3 was smaller than, but was not statistically different from, that in group 2.

Fig. 6-A shows the relationship between infarct/risk ratio and RBF. Infarct size was significantly limited in group 3 under similar RBF. A close correlation between infarct and risk size was obtained for both groups (Fig. 6-B). Furthermore, the regression line in group 3 was significantly shifted down compared with that in group 2 ($p < 0.01$), although the hemodynamics and RBF were not significantly different between the 2 groups as mentioned earlier. Thus, it is suggested that allopurinol pretreatment limits the infarct size after ischemia and reperfusion, even under very severe ischemia.

DISCUSSION

Myocardial infarct size is related not only to the risk size and the myocardial metabolic demand, but also to RBF to the ischemic region in the canine heart⁸. In the critical examination of a drug's effect on the canine infarct size, it is necessary to analyze the relation of infarct size to these variables. However, there have been few investigations in which this precise analysis has been under-

taken. Furthermore, there exists a species difference in CCF^{10,11}. In the pig and rabbit in which CCF is negligible, it is possible to conclude the beneficial effect of intervention by calculating only the average infarct size. On the other hand, the collateral circulation of the canine heart is relatively rich and the quantity varies, ranging from very low to near normal, which produces a wide variation of RBF even if the coronary artery is occluded under the same conditions. In consequence, a complicated analysis is needed for the evaluation of a drug's effect on infarct size in a canine experiment. Most previous studies have used a canine heart for experimentation³⁻⁷ because a canine heart is economical and it is easy to obtain many cardiac parameters as compared with the pig or rabbit. Therefore, we need a canine ischemic heart model in which it is feasible to evaluate the effect of intervention.

We were able to make a canine ischemic heart model in which RBF was almost constant for any given CCF by RF diversion. Our first study showed that RF diversion produced a well-controlled and severe ischemia in the canine heart. Independent of CCF, RBF was in an almost fixed range of from 0 to 15% CBF by RF diversion. Therefore, the infarct/risk ratio was constant for group 2 because infarct size is inversely correlated with RBF, a reflection that infarct size in group 2 was highly correlated with risk size ($r=0.92$). Therefore, the canine ischemic model with RF diversion should be considered very useful for infarct size study, as it should be easy to evaluate a drug's effect on infarct size under constant RBF.

RF collection has been used to assess CCF for a long time, although CCF is probably overestimated because collateral resistance is higher than the resistance of atmospheric pressure when the retrograde flowing blood is drained²⁶. Wyatt et al¹² proposed the load line method using RF diversion to estimate CCF and showed that it was well correlated with CCF calculated by the labeled microspheres method. Yoshida et al^{13,14} showed that the accuracy of Wyatt's load line method was doubtful for estimating CCF by comparison with the RF collection method, the labeled microspheres method and the ¹³³Xe clearance method, and proposed the modified load line method, which was well

correlated with the most accurate ¹³³Xe clearance method. This method seems to be useful because CCF can be calculated using only simple parameters, that is, RF, peripheral coronary pressure and mean aortic blood pressure, and does not need radioactive substances.

Oxygen derived free radicals have been proposed as major contributors to reperfusion injury of the ischemic heart!² One potential source of free radicals in reperfused myocardium is the xanthine-xanthine oxidase system.³ The onset of acute myocardial ischemia results in the rapid breakdown of high energy phosphates, and consequently causes the accumulation of inosine and hypoxanthine. At the same time, xanthine dehydrogenase is converted to xanthine oxidase, which uses molecular oxygen as an electron acceptor to produce superoxide radicals during the oxidation of hypoxanthine. The high intracellular concentration of hypoxanthine and the increased xanthine oxidase activity in ischemic myocardium may produce superoxide radicals with the introduction of oxygen at reperfusion. These reactive free radicals are considered to initiate lipid peroxidation that can disrupt cell membranes and organelles leading to myocardial cell death!⁶ Therefore, the xanthine oxidase inhibitor, allopurinol, could possibly limit infarct size by inhibiting this process in ischemic reperfusion myocardium.

Although this possibility has been investigated for years, a controversy still exists³⁻⁷. These disagreements are considered to be derived from differences in experimental protocol and the lack of precise analysis of infarct size determinant factors, particularly RBF, as mentioned above.

Reimer et al⁵ concluded that allopurinol did not limit infarct size in the canine heart with 40 min ischemia followed by 96 h reperfusion, although they noticed that CCF was lower in allopurinol treated dogs than in controls. Chambers et al³ and Werns et al⁴ reported that allopurinol can limit infarct size in an acute ischemic reperfusion canine model. They used 60 min ischemia followed by 4 h reperfusion, and 90 min ischemia followed by 6 h reperfusion. However, these positive studies did not analyze the correlation between infarct size and CCF. Our positive study does analyze the correlation

between infarct size and RBF using a canine heart with a long duration of ischemia (90 min) and a relatively short duration of reperfusion (4 h). In order to avoid a complicated analysis, we used the RBF controlled canine ischemic heart model with RF diversion. We also tried to create the same conditions of the infarct size determinants of rate pressure product, risk size, and RBF in both the control and allopurinol treated groups. Since the infarct size in our allopurinol pretreated dogs was significantly smaller than in the controls, we conclude that the xanthine oxidase inhibitor, allopurinol, can reduce ischemic reperfusion injury of the canine heart, and that free radicals via xanthine oxidase might be related to such injury.

One of the reasons why our result contradicts the report of Reimer et al⁵ is possibly because of differences in the duration of reperfusion, the duration of ischemia and the method of measuring the area of necrosis. The duration of reperfusion in this and previous positive studies^{3,4} was 4 to 6 h compared to the 96 h in Reimer's negative study.⁵ Miura et al⁷ reported that allopurinol pretreatment limited the infarct size after 24 h of permanent coronary occlusion in a canine model, but not after 48 h, and concluded that allopurinol delayed, but did not prevent, infarction in a permanent occlusion model. In Richard's negative study of a 96 h reperfusion model, the investigators speculated that the beneficial effect of allopurinol might only last for up to 6h of reperfusion and the initially salvaged tissue might undergo necrosis later in the reperfusion phase ("delayed reperfusion injury").²⁴ It is possible that the plasma level of allopurinol might be lower than the therapeutic level in the later reperfusion period²⁴ or, alternatively, that free radicals might continue to be produced in the later period. The duration of free radical production from xanthine oxidase during reperfusion has not been shown precisely. Furthermore, neutrophils and mitochondria have also been considered to be other sources of free radicals in reperfused myocardium² and the durations of their free radical production have not been identified. Therefore, these various different sources and the durations of their free radical production should be considered.

Although the duration of ischemia has

been considered one of the reasons for the controversy⁹, it did not prove to have an effect on infarct size limitation in nearly identical studies for 40 min and 90 min occlusion.^{5,24}

There are 2 different methods of evaluating necrotic tissue. Infarct size limitation was observed when the infarct size was determined by TTC staining^{3,4} whereas there was no beneficial effect when using the histological technique.⁵ It is possible that TTC staining might underestimate the necrotic tissue for the complex interdigitation between necrotic and viable areas⁸.

As to the mechanism of allopurinol's protective effect on myocardium, the prevention of purine loss¹⁷ and the increase of collateral flow¹⁸ have been proposed as well as the inhibition of oxygen-derived free radicals.^{1,3} However, metabolites of purines are thought to be washed out by reperfusion⁶ and our result, which supports the speculation of Akiyuki et al⁶ shows that the allopurinol effect is independent of CCF. The major sources of free radicals in reperfused myocardium have been considered to be xanthine oxidase^{1,3} and the activated neutrophils.¹⁹ Cronstein et al²⁰ demonstrated that adenosine reduced the neutrophil adherence and cytotoxicity to endothelial cells, and also reduced free radical production by the neutrophils. Puett et al²¹ showed that oxypurinol, an active metabolite of allopurinol, did not limit myocardial infarct size, but that neutrophil infiltration in the border zones of the treated group was reduced. They also speculated that oxypurinol might increase the circulating adenosine level by xanthine oxidase inhibition.

A species difference exists in xanthine oxidase activity in the heart^{22,23,25}. The canine heart contains high enzyme activity, but pig and rabbit hearts lack this enzyme. Eddy et al²³ reported that its quantity in the human heart was in trace amounts. In their study, the hearts investigated were of patients of cardiomyopathy, ventricular septal defect, and organ donors, not of healthy individuals. On the other hand, recent evidence suggests that allopurinol may inhibit myocardial injury independent of a xanthine oxidase mediated mechanism²⁵. Further study is needed if allopurinol is to be used in human myocardium to limit infarct size.

In conclusion, the canine ischemic heart model with RF diversion is useful for the estimation of a drug's effect on infarct size, and allopurinol pretreatment limits ischemic reperfusion injury of the canine heart.

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