THE IMPORTANCE OF GOOD ENDOCARDIAL REFLOW IMMEDIATELY AFTER REPERFUSION FOR MYOCARDIAL SALVAGE IN DOGS

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We studied the importance of reflow after reperfusion for myocardial salvage. In 19 open-chest dogs, the left anterior descending coronary artery was occluded for 3h and then reperfused. Non-radioactive colored microspheres were injected into the left atrium to measure regional myocardial blood flow (RMBF). Immediately after occlusion, RMBF was reduced to $23 \pm 2\%$ (of control) in the inner layer and $32 \pm 2\%$ in the outer layer. Five minutes after reperfusion, RMBF was increased to $170 \pm 20\%$ and $156 \pm 11\%$ of control in the inner and outer layers, respectively. One week later, RMBF in the inner layer was reduced to $63 \pm 4\%$ but it was not reduced ($100 \pm 6\%$) in the outer layer. There was a roughly positive correlation between the inner/outer flow ratio measured 5 min after reperfusion and myocardial creatine kinase activity. Myocardial necrosis determined by triphenyl tetrazolium chloride stain varied inversely with the inner/outer flow ratio. These results indicate that good reflow in the inner layer 5 min after reperfusion is a favorable indicator for myocardial salvage.

REPERFUSION therapy after myocardial infarction is essential in order to achieve successful myocardial salvage. There are reports showing that emergency coronary angioplasty is effective in establishing recanalization when thrombolysis has failed¹. However, there are numerous studies clearly demonstrating that reperfusion injury characterized by explosive cell swelling, myocardial hemorrhage, and arrhythmias has been caused by abrupt unrestricted reperfusion^{2,3}. As a result of these observations, several investigators prefer to recanal-

ize slowly or gently to avoid reperfusion injury⁴⁻⁶. There is an urgent need to determine the optimal method of reperfusion. We have, therefore, studied the relationship between regional myocardial blood flow after reperfusion and myocardial salvage in dogs.

MATERIALS AND METHODS

Animal preparation:

Nineteen mongrel dogs weighing 19.5 ± 1.1 kg (mean $\pm$ 1 SE) were anesthetized with pentobarbital intravenously (20 mg/kg initially, supplemental doses as required). Dogs were placed in the right decubitus position.

Key words:

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Fig.1. Schematic representation of the experimental protocol.
TTC: Triphenyl tetrazolium chloride stain

The chest was opened using sterile technique under positive pressure ventilation. The heart was suspended in a pericardial cradle. The tip of a multiple side-hole catheter was inserted into the left atrium from the appendage, in order to inject non-radioactive colored microspheres (E-Z trac, Los Angeles, CA, USA)? A catheter inserted into the right femoral artery and advanced into the thoracic aorta was used to collect arterial blood for flow determinations. A Swan-Ganz thermodilution catheter was used to measure cardiac output. The left anterior descending coronary artery was tied gently with elastic rubber thread for 3h and the thread was removed to reperfuse the artery. The elastic rubber thread occluded the artery without injuring the vascular wall. Lidocaine hydrochloride 20 mg was given intravenously 10 min prior to occlusion and also 10 min prior to release to prevent ventricular fibrillation. Three hours after reperfusion, the chest was closed and the dog was allowed to recover for 1 week. The chest was then reopened to measure regional myocardial blood flow.

Regional myocardial blood flow measurements:

Non-radioactive colored microspheres were injected into the left atrium 5 times

(control; 5 sec and 3h after occlusion; 30 min and 3h after reperfusion) in 8 dogs and 7 times (control; 5 sec and 3h after occlusion; 5 min, 15 min, 3h and 1 week after reperfusion) in 11 dogs. The microspheres (11.9 ± 1.9 microns) were of different colors (red, green, orange, black, blue, yellow and brown) and were obtained in vials containing approximately 20 million microspheres/ml. A 0.2 ml aliquot of a stock suspension of microspheres (4 million) was diluted in 10 ml of 5% glucose solution and flushed into the left atrium. A reference arterial blood sample was withdrawn from the thoracic aorta using a withdrawal pump (Model 915, Harvard Apparatus, Millis, MA, USA) at a rate of 9 ml/min. Blood was withdrawn for 90 sec and the microspheres were injected 30 sec after the initiation of blood withdrawal. Heart rate, arterial blood pressure, and cardiac output were measured each time microspheres were injected. Blood from a donor dog was transfused as required to replace blood lost. The results of arterial blood gas analysis were: PO_2 was 119 ± 9 mmHg, PCO_2 was 37 ± 2 mmHg, and pH was 7.411 ± 0.072 .

Myocardial sample:

After the final measurement the heart was removed. The heart weighed 146 ± 9 gm and

the left ventricle weighed 82 ± 6 gm ($56 \pm 1\%$ of the total heart weight). It was cut transversely in 1 cm-thick slices and each slice was divided equally into 12 sections; each section was further divided into inner and outer layers to yield 24 myocardial segments (Fig. 1). From the center of each segment, a piece of myocardium was obtained to measure myocardial creatine kinase (CK)⁸ and tissue water content⁹. The remainder of the myocardium was used for counting. A representative sample of non-occluded normal myocardial wall was obtained by removing a 2–3 gm piece of the basal posterior wall supplied by the left circumflex coronary artery; myocardial blood flow as well as myocardial CK and tissue water content were measured.

Microsphere counting:

The method for counting microspheres has been described elsewhere.⁷ Briefly, each myocardial segment was weighed and minced finely with scissors in a 50 ml tube. After adding 15 ml of 2M NaOH, the sample was agitated for 1 min with a vortex mixer and placed in a boiling water bath for 15 min. After being cooled to room temperature, the sample was placed in the vortex mixer for 1 min. The sample was then diluted to 25 ml with Tissue Digestive Reagent II (E-Z trac) and centrifuged at 3000 Hz for 30 min. The supernatant fluid was aspirated carefully so as not to disturb the centrifuged microspheres. The sample was resuspended in 4 ml of Tissue Microsphere Counting Reagent (E-Z trac). After vortex mixing, the mixture was transferred to a 15 ml tube; the larger tube was washed 3 times with 2 ml of Tissue Microsphere Counting Reagent and the washes were added to the 15 ml tube. The samples were again centrifuged for 15 min, and the supernatant fluid was then aspirated with caution, leaving exactly 0.05 ml of the sediment at the bottom of the tube. After vortex mixing, the solution was placed in a Fuchs-Rosenthal hemacytometer and the total number of microspheres was counted under a microscope at 200X magnification.

The method for counting microspheres in blood samples has been described elsewhere⁷ and will be described here briefly. After the blood samples were placed in a 50 ml conical

plastic tube and centrifuged for 30 min, the plasma was aspirated. The red cell sediment was diluted to 40 ml with Blood Hemolysis Reagent (E-Z trac) and centrifuged for 30 min. The supernatant fluid was aspirated carefully and 25 ml of distilled water was added. After being mixed and centrifuged for 15 min, the supernatant fluid was again aspirated. Five ml of 2M NaOH was then added and the sample was heated in a boiling water bath for 15 min. After being cooled, the sample was vortex mixed for 1 min. The digest was diluted with 25 ml of distilled water and the sample was centrifuged for 15 min. The supernatant fluid was aspirated and 4 ml of Tissue Microsphere Counting Reagent was added to the sediment and processed similarly to the myocardial samples. After the final centrifuge, the supernatant fluid was aspirated very carefully leaving exactly 0.1 ml at the bottom of the tube. After vortex mixing, the number of microspheres in the sample was counted as the myocardial samples had been in a Fuchs-Rosenthal hemacytometer.

RMBF was calculated using the following equation:

$$\text{RMBF (ml/min/gm)} = (\text{Cm/Cr}) \times k \times \text{Qr/wt},$$

where Cm = microsphere count of the myocardial sample,

Qr = withdrawal rate (ml/min) of the reference arterial blood (Qr = 9 in this experiment),

Cr = microsphere count of the reference arterial blood,

k = correction factor for the final centrifuged volumes (in this study, myocardial and blood samples were centrifuged to final volumes of 0.05 and 0.1 ml, respectively; accordingly, $k = 0.05/0.10 = 0.5$), and

wt = weight of myocardial sample (gm).

In this experiment, a transverse section of the heart was divided into 24 segments. Each segment weighed from 0.145 to 1.383 gm. This weight was considerably smaller than that illustrated in the E-Z trac manual (2–3 gm). By centrifuging to 0.05 ml in the final step, it was possible to measure regional myocardial blood flow in a small sample.

TABLE I SYSTEMIC HEMODYNAMICS AND REGIONAL MYOCARDIAL BLOOD FLOW DURING OCCLUSION AND REPERFUSION

	Control	Occlusion		Reperfusion				
		5 sec	3 hr	5 min	15 min	30 min	3 hr	1 week
Systemic hemodynamics								
Heart rate (beats/min)	157±5	161±6	161±5	157±16	160±10	161±4	161±4	158±9
Mean aortic pressure (mmHg)	112±3	113±3	113±3	105±3	102±4	114±4	109±4	93±4
Cardiac output (l/min)	2.52±0.15	2.52±0.12	2.41±0.18	2.68±0.19	2.48±0.19	2.08±0.23	2.30±0.21	2.40±0.17
%PVC (%)	0	0	2±1	6±5	19±7	18±7	41±10	0
Regional myocardial blood flow								
Non-occluded area (normal myocardium)								
Inner layer (% of control)	100	99±5	100±7	101±11	105±8	73±7	104±6	103±7
Outer layer (% of control)	100	109±8	96±5	116±13	107±11	83±10	112±8	119±16
Inner/Outer ratio	1.13±0.05	1.05±0.10	1.19±0.10	0.91±0.12	1.01±0.10	1.18±0.14	1.11±0.09	0.95±0.13
Occluded area (risk area)								
Inner layer (% of control)	100	23±2**	36±5	170±20	128±12	78±6**	68±5**	63±4**
Outer layer (% of control)	100	32±2	48±4	156±11	117±7	123±14	88±6	100±6
Inner/Outer ratio	1.10±0.03	0.59±0.05 [§]	0.84±0.10 [§]	1.07±0.12	1.24±0.20	0.91±0.07 [§]	0.92±0.06 [§]	0.62±0.05 [§]
%PVC=PVC/(PVC+NSR)×100 PVC: extrasystole per min NSR: Sinus rate per min								
Mean ± standard error								
Difference between inner and outer layers								
Difference between control								
** p<0.01								
§ p<0.001

Fig.2. An example of RMBF and myocardial CK activity determined in the inner layer of a transverse section.

The regional myocardial blood flow was expressed as the percentage of each pre-occlusion value (% of control). We defined it as the "risk area" provided that regional myocardial blood flow 5 sec after occlusion was reduced to 75% or less.

Myocardial CK, tissue water content, triphenyl tetrazolium chloride (TTC) stain:

Myocardial CK was measured according to Shell et al⁸ and expressed as a percentage of a non-occluded normal myocardial sample obtained from the posterior wall at the base. Myocardial tissue water content (% [H₂O]) was measured using the following equation:

$$\% [\text{H}_2\text{O}] = \frac{[(\text{wet weight}) - (\text{dry weight})]}{(\text{wet weight})} \times 100,$$

where wet weight represents the weight of the myocardium measured immediately after sampling and dry weight represents weight measured 12h after drying in an oven heated to 100 °C⁹.

The transverse section of the heart facing the section used for microsphere counting was stained with TTC to measure infarct size¹⁰ (Fig. 1). TTC stain was graded as follows:

- 0: All myocardium was stained red indicating that there was no necrotic area;
- +1: Myocardium which was visualized as white was less than 1/5;
- +2: Myocardium which was visualized as

white was less than 2/5;

+3: Myocardium which was visualized as white was less than 3/5;

+4: Myocardium which was visualized as white was less than 4/5;

and

+5: All myocardium was visualized as white indicating that it was all necrotic.

Reproducibility:

In four separate experiments, we evaluated the effect of microsphere injection on myocardial hemodynamics as well as the reproducibility of the colored microsphere technique for determining regional myocardial blood flow. The electromagnetic flowmeters were placed at the left anterior descending artery and left circumflex artery. A catheter-tip manometer (Millar) was inserted into the left ventricle. Coronary flow was increased by isoproterenol (5 µg/min intravenously) or reduced by propranolol (4 mg intravenously) in order to observe reproducibility over a wide flow range. Two species of colored microspheres (4 million of each) were injected simultaneously into the left atrium to determine whether the RMBF measured with each color were the same.

Statistics:

All values are expressed as the mean ± 1 SE. The statistical significance of the differ-

Fig.3. RMBF during occlusion and after reperfusion.

Fig.4. Relation between myocardial CK activity and RMBF measured 5 min after reperfusion in the inner layer.

ence was evaluated using Student's *t* test for unpaired samples.

RESULTS

There were no significant changes in heart rate, aortic blood pressure, or cardiac output after coronary occlusion and reperfusion over a period of 1 week (Table I).

Ventricular extrasystoles occurred 3h after

occlusion, increased markedly 3h after reperfusion, and had disappeared after 1 week. There was no correlation between ventricular extrasystoles (% PVC, Table I) and RMBF at reperfusion.

Regional myocardial blood flow at the basal posterior wall before occlusion ranged from 0.60 to 1.38 ml/min/gm (0.99 ± 0.06) in the inner and from 0.51 to 1.46 ml/min/gm (0.83 ± 0.06) in the outer layer. The flow

Fig. 5. Relation between myocardial CK activity in the inner layer and inner/outer flow ratio measured 5 min after reperfusion.

ratio between inner and outer layers (I/O ratio) ranged from 0.77 to 1.42 (1.13 ± 0.05) before occlusion. RMBF of these non-occluded normal myocardium determined 1 week later showed no significant difference from these values (inner layer $103 \pm 7\%$ and outer layer $119 \pm 16\%$ of pre-occlusion value, I/O ratio 0.95 ± 0.13). Also, there were no significant changes in RMBF after coronary occlusion and reperfusion in these non-occluded normal myocardium (Table I). RMBF in occluded myocardium determined 5 sec after occlusion was reduced to 75% or less of the pre-occlusion value in 3 to 6 of the 24 segments (Fig. 2). The average values of RMBF after coronary occlusion and reperfusion in these segments are shown in Table I. Five seconds after occlusion, RMBF in the inner layers of these areas at risk were reduced to $23 \pm 2\%$ of control; this was greater than the reduction in the outer layers ($32 \pm 2\%$). RMBF recovered slightly during the 3h after occlusion (inner layer: 36 ± 5 , $p < 0.05$; outer layer: $48 \pm 4\%$, $p < 0.01$; Fig. 3). Five minutes after reperfusion, RMBF overshot the control value, reaching $170 \pm 20\%$ in the inner and $156 \pm 11\%$ in the outer layers. These marked flow increases disappeared gradually over the interval from 30 min to 3h after reperfusion. After these transient increases, RMBF in the outer layer was greater than in the inner layer (Fig. 3).

One week after reperfusion, RMBF returned to normal ($100 \pm 6\%$) in the outer layer; however, in the inner layer, it failed to recover to the control value ($63 \pm 4\%$). Accordingly, the I/O ratio decreased gradually over time: 0.92 ± 0.06 at 3h after reperfusion and 0.62 ± 0.05 one week after reperfusion; these values were significantly less than the control value (1.10 ± 0.03 , $p < 0.001$). There was no area where RMBF was reduced to zero during reperfusion.

Myocardial CK was markedly depleted in both the inner ($36.9 \pm 3.1\%$ of non-occluded myocardium) and outer ($53.3 \pm 3.2\%$) layers. There was a statistically significant direct positive correlation between myocardial CK and RMBF in the inner layer measured 5 min after reperfusion ($r = 0.376$, $p < 0.05$, Fig. 4). There was a better positive correlation between myocardial CK (inner layer) and the I/O ratio 5 min after reperfusion (Fig. 5), indicating that good flow recovery 5 min after reperfusion in the inner layer is conducive to protecting myocardial CK release. Myocardial CK in the outer layer also correlated directly with the I/O ratio 5 min after reperfusion ($r = 0.433$, $p < 0.01$). Myocardial tissue water content ($\% [H_2O]$) in the area at risk (inner layer: $81.0 \pm 0.2\%$, outer layer: $79.7 \pm 0.2\%$) was significantly increased ($p < 0.001$) over that in the normal myocardium (inner layer: $78.1 \pm 0.2\%$; outer

Fig. 6. Relation between TTC stain and inner/outer flow ratio measured 5 min after reperfusion.

(A) Duplicate measurement of a single sample

(B) Simultaneous measurement of RMBF using two different microspheres

Fig. 7. Reproducibility of the non-radioactive colored microsphere.

layer: $77.6 \pm 0.2\%$). The average grade by TTC stain was 2.40 ± 0.26 in the inner layer and 1.69 ± 0.24 in the outer layer, indicating that the inner layer was more necrotic than the outer layer ($p < 0.01$). In both layers, the infarcted area determined by TTC stain was smaller provided that the I/O ratio 5 min after reperfusion was increased (Fig. 6). This result indicates that if the myocardium had good flow in the inner layer 5 min after reperfusion, the necrotic area was limited.

Accuracy of the method:

The reproducibility and adequacy of the colored microsphere technique for measuring RMBF was evaluated in 4 separate experiments.

1) Effect of sample weight on reproducibility

Single samples removed from a normal heart were divided into 2 pieces. Each piece was placed in a separate tube in alkaline solution and centrifuged. The number of microspheres (per unit of weight) in each of

the 2 pieces was counted and the results are shown in Fig. 7A (left side). Microsphere counts were measured in 24 myocardial segments from a single transverse section in a normal heart. Each sample weighed from 0.145 to 0.806 gm. A larger myocardial sample (approximately 2 gm) was obtained from the basal posterior wall of the same heart. The microsphere counts per unit of weight of the smaller 24 samples were expressed as a percentage of the larger sample in order to determine whether or not smaller myocardial samples resulted in larger errors. These smaller 24 samples were further divided into three groups according to the weight of the sample. In the smallest (0.145 to 0.299 gm), the microsphere count was $103 \pm 3\%$ (76 to 128%, $n=28$); in the middle-sized samples (0.300 to 0.399 gm), it was $94 \pm 3\%$ (70 to 124%, $n=32$); in the largest samples (0.400 to 0.806 gm), it was $93 \pm 3\%$ (76 to 129%, $n=22$). Thus, there were no significant differences according to the weight of the samples.

2) Simultaneous determination of RMBF using two different kinds of microspheres

RMBF was measured in duplicate by injecting two kinds of microspheres simultaneously into the left atrium in order to ascertain the reproducibility of RMBF determinations. RMBF was reduced and increased using propranolol and isoproterenol in order to evaluate reproducibility over a wide flow range. RMBF ranged from 0.52 to 7.99 ml/min/gm, and the correlation between the two simultaneous determinations is shown in Fig. 7B. The results indicate good reproducibility.

3) Effects of microsphere injection on systemic hemodynamics and coronary blood flow

The microsphere solution contains a small amount of Thimerosal (a bacteriostatic agent) and Tween 80 (a dispersing agent). These agents may change the hemodynamics upon injection. The double-dosed colored microsphere (8 million) injection served to determine the effect of the injectate on the hemodynamics of the system. We found that there were no significant changes in heart rate, aortic blood pressure, left ventricular end-diastolic pressure, left ventricular maximum (or minimum) positive (or negative) dp/dt, or coronary flow as a result of injection of the microspheres.

DISCUSSION

In this study we found that RMBF, which was markedly reduced during occlusion, returned to its pre-occlusion value in the outer layer one week later. In the inner layer, it remained at 63% of the pre-occlusion value. Also, good flow recovery 5 min after reperfusion in the inner layer was an important indicator for myocardial salvage.

The non-radioactive colored microsphere technique has certain technical advantages and disadvantages. Hale et al⁷ in comparison of the present method with the radioactive microsphere technique, concluded that the colored microsphere method is a feasible alternative. Since the radioactive microsphere technique is not acceptable in our institution, only the reproducibility of the colored microsphere technique was evaluated in this paper. We found that we can count microspheres with considerable accuracy when the myocardial sample is larger than 0.145 gm. We were able to measure regional myocardial blood flow with considerable reproducibility as shown in Fig. 7. One of the advantages of the present method over the radioactive microsphere technique (which is, of course, subject to radioactive decay) is that we can measure RMBF over a 1-week period. However, the present method is rather complicated and may result in large errors unless considerable skill is exercised in handling the myocardial samples during centrifuging, aspirating the supernatant fluid, and counting under the microscope.

In the present study, RMBF was measured 5 min after reperfusion. Myers et al¹⁴ occluded the coronary artery for 2h in dogs with chronically implanted flowmeters. They found that after reperfusion, coronary flow was increased by 383% after 1 min, 121% after 1h, and 68% after 24h. Cobb et al¹⁵ measured flow after a 2h occlusion and found that the flow increase lasted only 15 min. Accordingly, determination of RMBF 5 min after reperfusion was considered to be appropriate for evaluating the characteristic flow change caused by reperfusion.

A surprising result of our study was that

RMBF in the outer layer returned to almost normal values 1 week after reperfusion even though myocardial necrosis was considerable. Chu and Cobb¹² measured RMBF in outer and inner layers after reperfusion and demonstrated that, for given degrees of infarction, RMBF was significantly higher in the reperfused myocardium than it was in non-reperfused myocardium. Their results, as well as ours, indicate that RMBF cannot be used as an index of myocardial necrosis after reperfusion. However, there was a rough positive correlation between myocardial necrosis and RMBF at inner and outer layers when measured 5 min after reperfusion. This indicates that good reflows at the inner and outer layers are important indicators for myocardial salvage. Since I/O ratio 5 min after reperfusion also correlated with TTC stain, a greater reflow at the inner layer at reperfusion is favorable for myocardial salvage.

Kloner et al¹⁶ described a "no-reflow phenomenon" to illustrate flow reduction in the necrotic myocardium during reperfusion. They concluded that explosive cell swelling increases perivascular compression and reduces flow. However, it may be erroneous to describe the flow upon reperfusion as "no-reflow" because, in our study, as well as in all of the above reports, there is an abrupt flow increase upon reperfusion which overshoots the control RMBF. This increased flow disappears within 30 min to 3h after reperfusion and finally the flow is reduced 1 week after reperfusion in the endocardial layer. Accordingly, the flow change during reperfusion should be described as a "reduced-reflow phenomenon".¹⁷

In the present study, we have focussed on the role of flow during reperfusion on myocardial salvage rather than RMBF reduction during occlusion, because it is self-evident that the duration of coronary occlusion and the amount of residual flow during occlusion are both very important determinants of myocardial necrosis.¹³ We found that myocardial CK depletion was less and the necrotic area determined by TTC stain was less provided there was a prominent flow increase in the endocardial layer 5 min after reperfusion. Okamoto et al⁵ reported that gentle reperfusion is superior to sudden total reperfusion in promoting functional re-

covery and reducing infarct size; Yamazaki et al⁴ showed that staged reperfusion is superior to sudden reperfusion in maintaining ventricular function. These studies suggested that reperfusion injury plays a considerable role in reducing myocardial salvage at reperfusion. However, numerous studies¹⁸⁻²¹ as well as the present study stress the beneficial effect of total reperfusion for myocardial salvage. In a recent study,⁸ the effects of residual coronary stenosis on myocardial salvage were clarified in dogs. In that study, the infarct size as determined by TTC stain and myocardial CK depletion was compared in two groups: 1 hr occlusion followed by total reperfusion (no stenosis) for 1 week vs 1 hr occlusion and reperfusion with 99% residual coronary stenosis without contrast washout delay for 1 week. The infarct size was significantly smaller in the no stenosis group. The same study also demonstrated that reperfusion 7 hrs after occlusion caused a marked reduction in myocardial necrosis when compared with infarct size in a group of dogs where occlusion persisted 1 week. Since the duration of occlusion was only 3 hrs in the present study, it may be reasonable to anticipate a large amount of myocardium still salvageable. Prompt and good reflow may be favorable for myocardial salvage. This concept may be supported by several investigators,¹⁹⁻²¹ who have shown that hyperemic flow increase at reperfusion is beneficial for functional recovery of the salvageable myocardium. Heyndrickx et al¹⁹ first described a transient functional recovery during reactive hyperemia (functional rebound). Akaishi et al²⁰ showed that post-ischemic hypercontraction was present when there was reactive hyperemia upon reperfusion and the recovery of regional function was impaired if the reactive hyperemia was prevented by a critical coronary stenosis. Stahl et al²¹ stressed the beneficial effects of reactive hyperemia in promoting functional recovery of stunned myocardium. All of these reports indicate that good flow upon reperfusion is important and a good sign for myocardial salvage. In conclusion, we have found that good reflow in the endocardial layer after 5 min of reperfusion is a favorable indicator for myocardial salvage.

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