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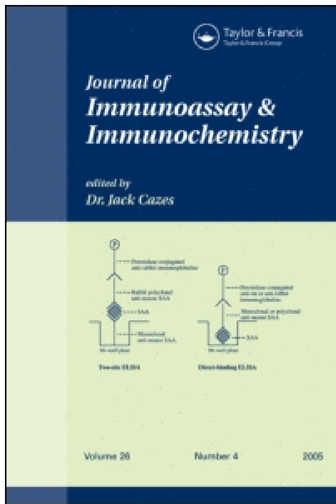

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Radioimmunoassay for Dog Myoglobin

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RADIOIMMUNOASSAY FOR DOG MYOGLOBIN

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ABSTRACT

A radioimmunoassay specific for dog myoglobin (MGB) has been developed. Antibody (Ab) to purified dog heart MGB was prepared in rabbits and the Ab was labelled with ^{125}I using the chloramine-T procedure. The assay is conducted at room temperature with addition of second Ab at 24 hr, the forward reaction being 95% complete at this point and irreversible. The assay is free of matrix affects. The working range is 20 to 360 ng/ml. Coefficients of variation for within run replicates are 10.5% (low) and 4.4% (high), and for between run replicates are 13.8% (low) and 7.6% (high). Serial measurement of serum MGB in dogs undergoing experimental myocardial infarction indicates that appearance time, peak time, and disappearance rate are respectively, 1.1 hr, 7.7 hr and 0.0025 min^{-1} for MGB and 2.6 hr, 13.35 hr, and 0.0018 min^{-1} for CK. (KEY WORDS: Myoglobin, Radioimmunoassay, Experimental infarction, Infarct size)

INTRODUCTION

Myoglobin is a low molecular weight (17,800 daltons) heme protein found as a normal constituent of human cardiac and skeletal muscle. It is released into the serum by necrosis of

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these muscles. Quantitation of serum myoglobin following myocardial infarction in humans was first attempted by Kagen et al (1) and has been accomplished with much greater sensitivity using radioimmunoassays (2,3,4,5).

There is presently widespread investigation of interventions to limit human myocardial infarct size (6) with the aim of improving prognosis. A variety of techniques for the non-invasive measurement of infarct size has been developed (7) best representative of which is the serial CK technique of Shell et al (8,9). The technique permits an estimate of infarct size by determination of cumulative CK release into serum during the course of an acute myocardial infarction. However a number of problems (10,11,12) prompted us to examine serial myoglobin determinations during the course of human myocardial infarction, with the expectation that an alternate technique for infarct size measurement might be developed. It then became necessary to investigate the kinetics of myoglobin release in experimental infarction in the dog as had been previously done for CK (8). While an antibody raised against human myoglobin has significant cross-reactivity with horse myoglobin (5), it has very low cross-reactivity with dog myoglobin. A specific radioimmunoassay for dog myoglobin was therefore required. Stone et al (13) have previously reported the development of a radioimmunoassay for dog myoglobin, and herein we

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report the development and optimization of our radioimmunoassay for dog myoglobin.

MATERIALS AND METHODS

Myoglobin Extraction and Purification

Dog heart myoglobin was prepared by a method similar to that of Yamazaki et al (14), as modified by Stone et al (2). Dog hearts (986 g) were homogenized with ice and water (2400 ml total) in a Waring blender. The pH was kept at 7.5 by adding 2 mol/l aqueous ammonia. The mixture was centrifuged at 2500 rpm for 30 minutes at 4°C and the supernatant poured off through six layers of gauze. Stepwise fractionation of the filtrate was carried out with 50% and with 60% ammonium sulfate, maintaining the pH at 7.5 with 2 mol/l aqueous ammonia. The precipitate after each fractionation was separated by centrifugation at 4000 rpm for 15 min. The supernatant was dialyzed for 24 hours against cold running tap water. The final volume of about 3.5 l was concentrated to 200 ml using Aquacide II-A (Calbiochem-Behring Corp., P.O. Box 12087, San Diego, California 92112). The concentrated solution was dialyzed for 48 hours against 0.005 mol/l Tris/HCl buffer, pH 8.5 with changes of buffer at 6, 12, and 24 hours. The final extract had a volume of 250 ml.

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Two columns, each containing 250 ml of Whatman DE-52, DEAE cellulose swollen in 0.05 mol/1 Tris/HCI buffer, pH 8.5 were poured and washed with 100 ml of the same buffer. Half of the extract was applied to each column and eluted with 0.05 mol/1 Tris/HCI buffer, pH 8.5. A very dark band formed at the top of the column and from it the reddish brown myoglobin slowly separated. Each column required about 3 1 of buffer for elution of the myoglobin. The eluate was concentrated with Aquacide II-A to 100 ml. This concentrate was dialyzed against 0.01 mol/1 phosphate buffer, pH 7. The final solution was passed through a Biogel P-10 column. The P-10 was swollen in 0.01 mol/1 phosphate buffer, pH 7.0, which was also used for elution. The myoglobin moved with the solvent front.

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The purified extract gave only one band on polyacrylamide disc gel electrophoresis. The absorption spectrum of the extract was very similar to that published for horse (14) and human (15) metmyoglobin. Maxima were at 280, 410, 505, and 635 nm. When a small amount of solid sodium dithionate ($Na_2S_2O_4$) was added to the cuvet and the mixture shaken to dissolve the solid, the 410 nm peak shifted to 434 nm. The protein concentration of the extract was measured by the method of Lowry et al (16) and by a modified micro Kjeldahl nitrogen analysis (17) using a standard solution prepared from bovine albumin (Sigma Chemical Co., P.O. Box 14508, St. Louis, Mo., 63178) with the phosphate buffer as

a blank. The myoglobin concentration by the Lowry technique was 6.8 ± 0.4 g/l (mean ± SD, a = 6), and by the micro Kjeldahl was 7.0 ± 0.7 g/l (mean ± SD, n = 3). A myoglobin concentration of 6.8 g/l was assigned to the standard solution and all results are in terms of this standard.

Antibody Preparation

Antimyoglobin antiserum was prepared in New Zealand white rabbits. Deep intramuscular injections of 300 μgm of myoglobin in complete Freund's adjuvent were made in the fore and hind legs. Booster doses of 300 μgm of myoglobin with adjuvent were given at three week intervals. Two weeks following the second booster dose, serum from the rabbit was tested by Ouchterlony type immunodiffusion. There was only a single band of reactivity against the immunizing antigen, and no precipitin lines were found against normal dog serum.

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Myoglobin Labelling

Myoglobin was labelled with ^{125}I by the chloramine-T procedure of Hunter and Greenwood (18). Five μgm of myoglobin in 20 μl of phosphate buffer (0.1 mol/l, pH 7.4) was added to 500 Ci of ^{125}I in 10 μl (100 Ci/l diluted with an equal volume of phosphate buffer, 0.4 mol/l, pH 7.4; Amersham-Searle, Arlington Heights, Ill 60005). 10 μl of chloramine-T (1g/l; British Drug Houses, Toronto, Ontario, M8Z 1K5) was added and

after 30 sec at room temperature, 50 μl of sodium metabisulfite (1 g/l) was added. After the addition of 10 μl of potassium iodide (1 mol/l), the labelled ligand was separated from free iodide by loading the solution onto a column of sephadex G-100, 9 ml volume, pre-equilibrated with phosphate buffer (0.01 mol/l pH 7.8, with added bovine albumin, 10 g/l). The iodinated myoglobin was diluted with the same buffer so that 100 μl contained sufficient myoglobin to yield about 10,000 cpm.

Goat antirabbit gamma globulin was obtained from Antibodies Incorporated, Box 442, Davis, California, 95616. Polyethylene glycol (MW 6000) was obtained from British Drug Houses, Toronto, Ontario, M8Z 1K5.

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Antibody Dilution

All reactions were performed in 10 x 75 mm glass tubes. Antibody was diluted with phosphate buffer (0.1 mol/l, pH 7.4, with normal rabbit serum, 100 ml/l and disodium EDTA, 0.05 mol/l) except for the 1:10 dilution which was prepared using only the phosphate buffer. There was therefore approximately the same concentration of rabbit serum in each of the dilutions of 1:10, 1:50, 1:100, 1:500, 1:1000, and 1:5000. 100 μl of each antibody dilution was placed in a series of tubes, and to each was added 100 μl of labelled myoglobin (about 10,000 cpm) and 100 μl of buffer. The tubes were incubated at $4^{o}C$ for three days and 100 μl goat antirabbit gammaglobulin (diluted 4-fold because of the

high rabbit serum concentration) and 100 µl polyethylene glycol (100 g/l) were added. The contents were vortexed, allowed to stand at room temperature for 5 min, and then centrifuged at 1500 x g for 30 min. The deposit, comprising the apparently bound fraction, was counted. Results were expressed in terms of the proportion of total counts that were bound (y'). An antibody dilution of 1:1000 corresponded to y' = 0.5 and this dilution was selected for reaction rate studies.

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Forward Reaction Rate and Temperature Effect

At the 1:1000 antibody dilution, 0-standard tubes were incubated at 4°C for 1, 3, 5, 12, 22, 48 and 96 hours before terminating the reaction by the addition of the second antibody. We found that the forward reaction was 95% complete by 24 hours. A second set of 0-standard tubes was incubated at room temperature for the above times. There was no difference in binding at this temperature and room temperature incubation was selected for routine assay.

Reversibility of Binding

We tested reversibility of binding by addition of 100 µl of 500 mg/l of unlabelled ligand (1000 times the highest analytical standard) to 0-standard tubes that had come to equilibrium with the first antibody and continued intubation for a further 24 hours. We observed no displacement of counts already bound. The first antibody was therefore effectively irreversible in its binding.

Assay Procedure

In the routine assay procedure, the first antibody dilution was 1:1000, and an aliquot of 100 µl was placed in each tube. Standards were prepared by diluting stock myoglobin in phosphate buffer (0.01 mol/l, pH 7.8, with added bovine serum albumin, 10 g/l) to give concentrations of 10, 20, 30, 50, 100, 200, and 300 µg/l. A 0-standard consisting of only phosphate buffer was included. 100 µl of standard or unknown serum, and 100 µl of labelled myoglobin (about 10,000 cpm) were added to each tube. Tubes were incubated at room temperature for 24 hours. Then 100 µl of goat anti-rabbit gamma globulin (diluted 20-fold) and 100 µl of polyethylene glycol (100 g/l) were added to each tube. The contents were vortexed, allowed to stand at room temperature for 5 minutes, centrifuged at 1500 x g for 30 min, and the deposit was counted. The antibody binding capacity (Ab) and the equilibrium constant (K) were determined from a Scatchard plot (19) with computer optimization of the upper and lower limits of counts bound (20) and the results were calculated from the expression $L = (Ab/y - [K^{-1}/(1-y)]$ where y is the bound/total counts and L is the total ligand concentration, labelled and unlabelled.

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Mass of Labelled Ligand

The mass of labelled ligand was measured by self-displacement in the assay (21) using increments of 1, 3 and 5 times the

regular mass. The estimate of regular labelled ligand mass was 38, 40, and 36 ng/tube respectively indicating that the antibody was not discriminating between labelled and unlabelled ligand.

Matrix-Accuracy-Recovery

We set up two sets of standard tubes and to each of one set we added 100 μl buffer and to each tube of the other, 100 μl of a serum sample containing 5 μg/l myoglobin. The displacement due to standards was uninfluenced by the presence of plasma. Two sera containing 1500 μg/l and 1420 μg/l respectively were assayed at dilutions of 1:5, 1:10, 1:20, and 1:40. The assayed results correspond to the dilutions used and there was no evidence of deviation from parallelism with the standard curve.

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Specificity

Dog hemoglobin at a concentration of 10 mg/l did not displace labelled myoglobin in the assay.

Precision

Quality control material at low and high levels was prepared from pooled dog plasma, stored at $-70^{o}C$ in small aliquots and assayed in each of 29 batches over a 5 month period. Within-run precision data was obtained with replicates of low and high levels run 9 times at intervals during the same assay run. Precision data are shown in table 1.

TABLE 1

Precision Data

	Within Run		Between Run (5 mo.)	
	low	high	low	high
n	36	29	28	28
$\bar{x}$, ng/ml	43	212	43	206
SD, ng/ml	4.5	9.4	5.9	15.5
CV, %	10.5	4.4	13.8	7.6

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Experimental Myocardial Infarction

Mongrel dogs weighing 19-32 kg (mean 25 kg) were subjected to myocardial infarction by a previously reported protocol (22). A silk snare was placed about the left anterior descending (LAD) coronary artery and 5-7 days later this was pulled tight and secured. 3 ml blood samples were obtained from a jugular vein catheter q½hr x 8 hr, q1hr x 22 further hr, and twice more prior to sacrifice at 48 hr. Sera were removed and kept frozen at -20°C, until the completion of sampling when they were thawed and myoglobin concentration was determined on each sample in duplicate as a batch for each dog. The control myoglobin was subtracted from each value, which was then plotted on a log y-axis against time on the x-axis (fig. 1). Each sample was also assayed for CK content and the serial CK values were similarly plotted (fig. 1). CK determinations were done using

the SKI bulk reagent modification of the Rosalki method (23) at $37^{o}C$ (Smith, Kline and French Canada, Ltd., 300 Laurentian Blvd., Montreal, Quebec, H4M 2L6).

RESULTS

The serial myoglobin results are presented for the 5 dogs in table 2 and figure 1. Myoglobin elevation in the serum began at 1.10 ± 0.22 hr (mean ± SD) following LAD ligation (table 3). Peak serum myoglobin level occurred at 7.60 ± 0.82 hr, with a return to baseline values within 48 hr in 4 of the 5 dogs. The downslope of the curve of serial serum myoglobin values was 0.002513 ± 0.000147 min^{-1}. Myoglobin appearance and peak times were significantly earlier than those of CK, and the disappearance rate was more rapid (table 3). By 48 hr post ligation, CK levels remained elevated 1.5-8 times baseline in 4 of the 5 dogs.

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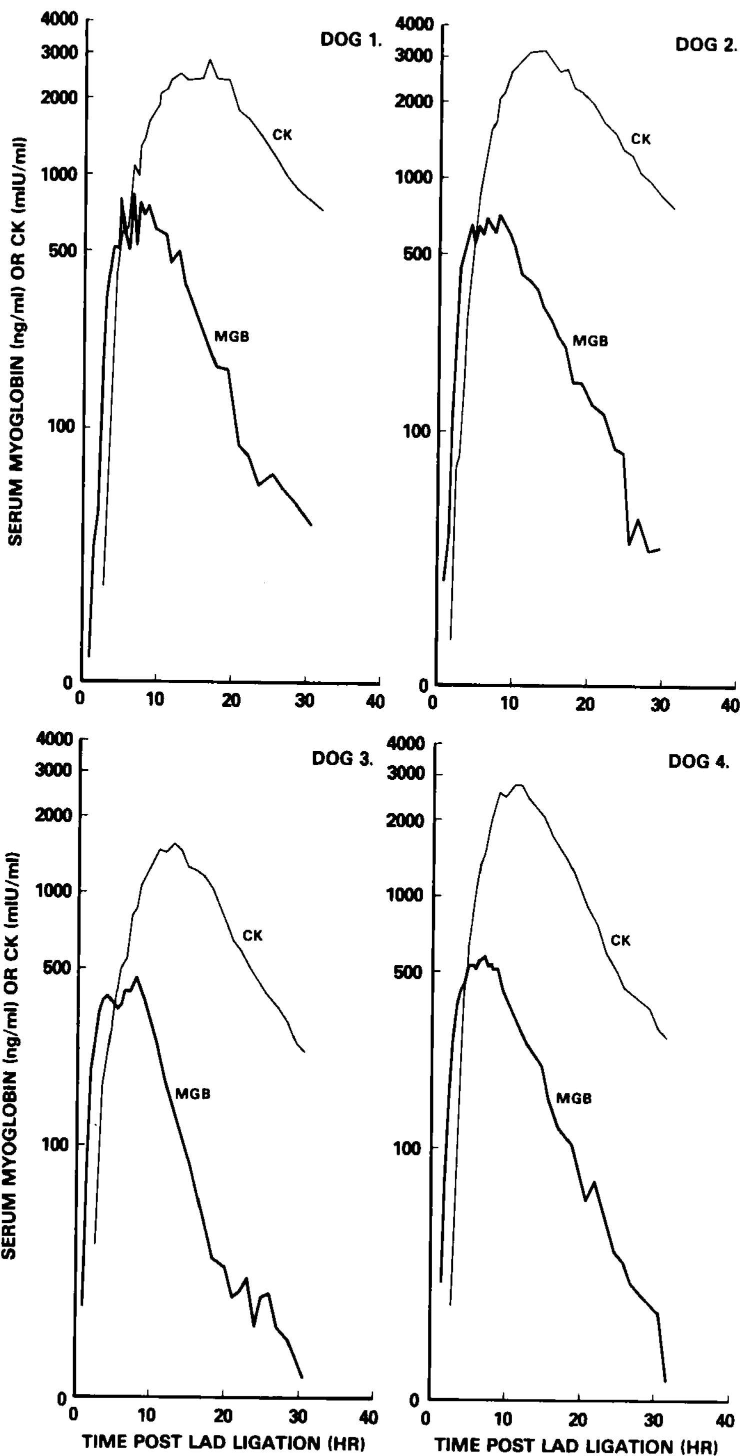
DISCUSSION

We have described a radioimmunoassay for dog myoglobin which is readily carried out at room temperature, having a high degree of sensitivity, and which is free of matrix effects through a range of dilutions necessary to make serial determinations of serum myoglobin in dogs undergoing experimental myocardial infarction. Within-run and between-run precision is satisfactory.

The initial data indicate that following experimental coronary ligation, myoglobin quickly enters the serum, rises to a peak, and



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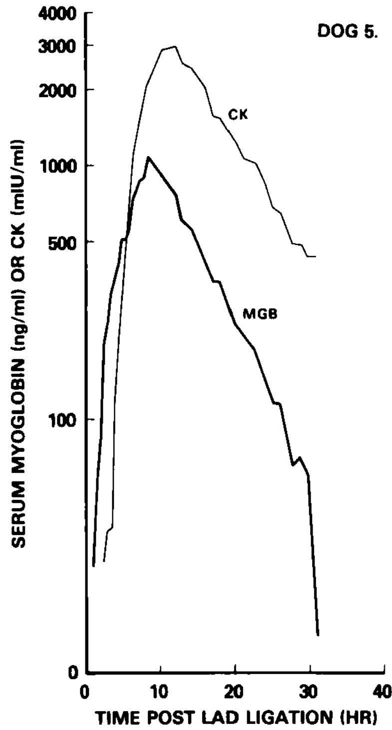

FIGURE 1. Serial serum myoglobin and CK values in dogs undergoing ligation of left anterior descending coronary artery.

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TABLE 2

Serial Serum Myoglobin (MGB) following Coronary Artery Ligation

Sample No.	Dog 1		Dog 2		Dog 3		Dog 4		Dog 5	
	Time(hr)	MGB(ng/ml)	Time	MGB	Time	MGB	Time	MGB	Time	MGB
1	0	13	0	7	0	10	0	10	0	14
2	1.0	26	1.0	36	1.0	33	1.0	13	1.0	40
3	1.5	49	1.5	48	1.5	107	1.5	39	1.5	74
4	2.0	59	2.0	120	2.0	212	2.0	96	2.0	97
5	2.5	196	2.5	269	2.5	268	2.5	186	2.5	198
6	3.0	333	3.0	438	3.0	339	3.0	293	3.0	258
7	3.5	425	3.5	519	3.5	388	3.5	386	3.5	323
8	4.0	524	4.0	608	4.0	398	4.0	441	4.0	365
9	4.5	323	4.5	661	4.67	384	4.5	473	4.5	421
10	5.0	817	5.0	569	5.0	373	5.0	543	5.0	518
11	5.5	589	5.5	657	5.5	352	5.5	454	5.5	515
12	6.0	513	6.0	609	6.0	369	6.0	524	5.92	567
13	6.5	843	6.5	695	6.5	418	6.5	565	6.45	743
14	7.08	538	7.0	675	7.0	414	7.0	587	6.9	818
15	7.5	780	7.5	623	7.5	450	7.43	544	7.5	886
16	8.0	697	8.0	723	8.0	472	8.0	548	8.0	908

17	8.67	768	8.5	672	8.5	424	8.5	522	8.5	1087
18	9.75	615	9.5	621	9.05	400	9.0	523	10.55	917
19	10.25	601	10.0	555	9.75	344	9.75	417	12.18	777
20	11.0	585	11.0	433	11.0	248	11.0	342	13.22	618
21	11.67	462	12.0	409	12.0	187	11.92	298	14.3	588
22	12.75	508	13.33	372	13.0	156	12.84	269	16.3	422
23	13.67	364	14.0	321	14.0	121	13.85	244	17.25	361
24	14.67	320	15.0	283	15.0	99	14.8	223	18.25	360
25	15.67	262	16.0	246	16.0	76	15.9	161	20.25	247
26	17.75	186	17.05	227	16.95	61	16.75	135	21.33	229
27	19.25	183	18.0	164	18.25	46	17.75	124	22.75	197
28	20.75	100	19.0	164	20.04	43	18.75	115	24.1	160
29	22.0	91	20.5	138	21.04	35	20.75	72	25.25	130
30	23.6	73	22.0	129	22.00	36	22.0	85	26.83	129
31	25.6	79	23.5	96	23.1	40	23.33	64	27.75	80
32	27.0	70	24.66	94	24.08	29	24.67	49	28.75	85
33	28.67	63	25.66	46	25.0	35	25.75	46	29.7	75
34	30.5	55	26.82	56	26.0	36	27.0	39	31.0	28
35	47.25	28	28.16	44	27.16	29	28.25	36	46.9	77
36			29.66	45	28.33	27	29.25	35	48.6	34
37			31.4	45	29.5	24	30.25	33		
38			47.5	10	30.58	22	31.5	22		
39			48.5	20	47.4	13	47.5	10		
40					49.25	10	49.5	10		

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TABLE 3

Comparison of Myoglobin and CK Kinetics

Dog	Time of First Elevated Value(hr)		Time of Peak Value(hr)		Disappearance			
	MGB	CK	MG	CK	MGB		CK	
					min^{-1}	r^2	min^{-1}	r^2
1	1.00	3.00	6.50	16.50	.002369	.96	.001781	.99
2	1.00	2.00	8.00	14.00	.002364	.98	.001418	1.00
3	1.00	2.50	8.00	13.00	.002679	.94	.002462	.98
4	1.50	3.00	7.00	11.00	.002640	.99	.002044	.99
5	1.00	2.50	8.50	12.25	.002514	.99	.001529	.98
mean	1.10	2.60	7.60	13.35	.002513		.001847	
± SD	0.22	0.41	0.82	2.07	.000147		.000420	
p	$p < 0.001$		$p < 0.001$		$p < 0.001$			

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within 48 hours returns to baseline values. The assay may now be used to evaluate the kinetics of myocardial myoglobin release and distribution during experimental myocardial infarction as has been done for CK (8). This investigation will be directed toward the development of a technique for infarct size measurement which may have advantages over the widely used serial CK technique.

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