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Quantitative Determination of Stress-Induced Myocardial Damage in Rats[1]

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MILLER, D. G. AND S. MALLOV. *Quantitative determination of stress-induced myocardial damage in rats.* PHARMAC. BIOCHEM. BEHAV. 7(2) 139–145, 1977. – The degree of myocardial damage produced in rats by exposing them to unsignalled, irregular foot-shock stress was determined in three ways: by measuring (1) enzymes (LDH, GOT and GPT) released into the circulation, (2) the rate of release of one of these enzymes (LDH) from isolated perfused hearts into the perfusate, and (3) the cardiac uptake, in vivo, of the radioactively labeled bone-seeking agents, technetium-99m-stannous pyrophosphate or technetium-99m-methylene diphosphonate. The latter two methods permitted quantitative determination of the degree of myocardial damage produced. Determination of cardiac technetium-99m uptake was found to be simple, quantitative, highly sensitive and truly indicative of cardiac damage, and therefore most suitable for studies of the effects of stress on cardiac injury.

Stress Heart Foot-shock Myocardium Cardiac injury Technetium-99m-stannous pyrophosphate Technetium-99-m-stannous methylene diphosphonate Enzymes Lactate dehydrogenase Glutamate oxalacetate transaminase Glutamate pyruvate transaminase Heart perfusion

THE FIRST substantial evidence that stress alone can produce myocardial damage in experimental animals was reported by Selye in 1962 [28]. This damage, produced in rats by restraint and demonstrated histologically, was transient, diffuse and did not occur in all of the animals. Swim stress in the rat has been reported to result in disruption of mitochondrial structure [18] but these results have been disputed [35,36]. Studies of myocardium of roosters [25] and male rabbits [37] stressed by crowding and of pigs stressed by shocks delivered by an animal prod during succinylcholine-induced paralysis [17] demonstrated that appropriate stressors can produce histological evidence of myocardial injury.

In none of these experiments, however, was the method of determination of the resultant injury sufficiently precise to permit quantitative studies of (a) the relation between the magnitude and/or duration of the stress and the degree of myocardial injury or (b) the degree of injury-reduction by the administration of various agents with respect to a given stress level. There are several ways of quantitatively assessing the degree of myocardial injury. One method involves the determination of the extent of release of certain intracellular enzymes from the myocardium. Measurement may be made of the activity of these enzymes in the serum [14, 23, 39]. However, since exercise causes the release of these enzymes from skeletal muscle [27], measurements of the enzymes in serum following stress that is accompanied by increased muscular activity may be subject to misinterpretation and may be of value only in preliminary screening of the effects of different levels of stress. Sobel [29,31] developed a sophisticated analysis of myocardial damage based on enzyme levels in serial blood samples, but this would be inapplicable for small animals. One also can measure the loss of myocardial enzymes from infarcted areas of the heart [19]. This technique is also difficult to apply to small animals, particularly when cardiac damage is limited.

We have employed the technique of determining the rates of release of certain myocardial enzymes from isolated perfused hearts of stressed animals as one way of quantitatively assessing the degree of myocardial injury produced by the stress. This avoids contamination with enzymes produced by tissues other than the heart.

A new method of assessing the degree of myocardial damage has been employed both experimentally [4, 5, 6, 40] and clinically [3, 15, 24, 25]. The radionuclide, technetium-99m, when bound to a bone-seeking agent, such as stannous pyrophosphate or stannous methylene diphosphonate, has been shown in studies on dogs [6,32], rabbits [9,13] and rats [1,20] to be sequestered in damaged or infarcted myocardium to a much greater degree than in normal hearts. We have found the ratio of injured/normal uptake of the tracer to reach values as high as 600 [22]. The increased uptake of radionuclide correlates with histologically assessed damage [4,32]. Because of its high sensitivity and quantitative indication of severity of damage, this agent appears to be well suited for use in stress research in which measurement of the degree of myocardial

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injury resulting from variations in environmental stimuli is desired.

We report here the results of our studies on the quantitation of the degree of myocardial injury in rats produced by variations in the parameters of unsignalled, irregular footshock stress, as determined by measurements of both cardiac enzyme release and radionuclide uptake.

METHOD

Male Sprague-Dawley rats weighing 300–350 g were placed in individual galvanized steel cages 30 x 23 x 16 cm with electrically isolated floors composed of 0.6 cm diameter steel rods placed 2.25 cm apart. At random intervals with a mean of 24 sec, an electric current of 1 sec duration was passed through the rods. In all experiments, the rats were subjected to unsignalled, irregular foot-shock stress, consisting of scrambled AC shocks that varied in intensity, in different experiments, from 0.3 to 1.2 mA, for periods of 0.5 to 12 hr. At the end of the stress period the animals were either immediately removed from the cages or allowed to remain for specific periods of time before being removed.

In order to carry out serum enzyme analyses, rats were anesthetized by the intraperitoneal administration of sodium pentobarbital and blood was withdrawn by syringe, after abdominal incision, from their inferior venae cavae. The sera were analyzed for lactate dehydrogenase (LDH), glutamate oxalacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) activities by the methods of Henry, *et al.* [16], and expressed as milli-International Units per ml (mIU/ml).

Isolated hearts, removed from control and stressed rats after the animals were anesthetized with pentobarbital, were perfused via the aortas as Langendorff preparations according to the method previously described [21] except that the perfusion fluid did not contain amino acids and was not recirculated after passing through the heart. Heart rates, flow rates and perfusion pressures were monitored during the perfusion, the flow rates being maintained at 10.5 ml/min for all hearts. After a 5 min washout period, determined with the aid of labeled inulin to be more than adequate for the elimination of residual blood, the perfusate was collected. In the initial experiments, 1.5 ml samples of perfusate were collected every 5 min from hearts of stressed and non-stressed rats for periods of 50 min. One ml aliquots were then analyzed for LDH activities. In subsequent experiments, the perfusate was collected until a total of 200 ml had passed through each heart. This perfusate was then assayed for total LDH activity.

For measurement of myocardial uptake of technetium-99m, in vivo, each rat was lightly anesthetized with sodium pentobarbital. Approximately 25 mCi of technetium stannous pyrophosphate (Tc-99m-PPi) or stannous methylene diphosphonate (Tc-99m-MDP) were injected into the right femoral vein. Each animal was again anesthetized exactly 100 min after the tracer administration and its heart was removed. The heart was trimmed free of extraneous tissue, perfused via the severed aorta through the coronary vessels with 10 ml of ice cold Krebs-Ringer bicarbonate buffer, blotted and weighed. It was then placed in a vial and its total radioactivity counted in a scintillation well counter. The radionuclide uptake was expressed as the % of the dose of administered tracer taken up by the heart/the % of body weight represented by the heart. This method of representing uptake corrects for variation in dose, heart weight and body weight.

RESULTS

Serum Enzyme Levels

Serum enzyme levels were measured in control rats and in rats stressed for 8 hr with a current intensity of 1 mA. The stressed animals were killed 4, 8, 12 and 16 hr after the termination of the stress. As shown in Table 1, serum activities of LDH, GOT and GPT of the stressed rats were elevated significantly above those of the non-stressed controls for at least 8 hr following termination of the stress. After 12 hr, serum LDH returned to control levels but serum GOT and GPT remained significantly elevated even after 16 hr.

Enzymes Released From Isolated Perfused Hearts

Hearts were removed from non-stressed control rats and

TABLE 1

EFFECT OF FOOT-SHOCK STRESS ON RATS ON SERUM ENZYME LEVELS

Hours after stress	Serum enzyme activity (mIU/ml) LDH	GOT	GPT
Controls (nonstressed)	290 ± 25* (18)	61 ± 3 (15)	12 ± 2 (18)
0	570 ± 57‡ (11)	182 ± 31‡ (12)	34 ± 1‡ (15)
4	443 ± 84† (4)	234 ± 70‡ (4)	52 ± 10‡ (4)
8	501 ± 95‡ (5)	251 ± 34‡ (6)	66 ± 9‡ (6)
12	301 ± 68 (5)	116 ± 17‡ (6)	38 ± 6‡ (6)
16	234 ± 20 (8)	111 ± 23‡ (8)	27 ± 5‡ (8)

All stressed rats received unsignalled, irregular foot-shock ofr 8 hr with a 1 mA current.
Numbers in parentheses are number of rats.
*Mean ± SE.
†Significantly different form controls ($p<0.05$).
‡Significantly different from controls ($p<0.01$).

from rats that had been subjected to a 12 hr stress, and perfused. Current intensities of 0.3, 0.5, 0.8 and 1.2 mA were employed. The results are given in Table 2. As the current was increased, so was the release of LDH from the isolated perfused hearts into the collected perfusate. LDH levels in the perfusates were statistically significantly above the control levels when rats were stressed with currents of 0.8 and 1.2 mA. Figure 1 shows a typical effect of subjecting rats to a 12 hr stress with a current of 1.2 mA.

TABLE 2

EFFECT OF FOOT-SHOCK STRESS OF RATS ON LDH RELEASE BY ISOLATED PERFUSED HEARTS

Shock Current (mA)	Number of Rats	Total LDH Output (mIU)	p value
0	45	2536 ± 80*	
0.3	5	2715 ± 343	NS
0.5	12	2859 ± 249	NS
0.8	27	3104 ± 204	<0.005
1.2	10	3302 ± 318	<0.001

All stressed rats received irregular, unsignalled foot-shock for 12 hr.

*Mean ± SE.

Technetium–99m Uptake In Vivo

Cardiac uptake of Tc-99m-MDP was determined in rats stressed at a current of 1 mA for periods of 0.5, 1, 2, 4 and 12 hr in order to determine the relationship between duration of stress and severity of myocardial damage as judged by tracer uptake. The results, shown in Fig. 2, indicate a linear relationship between hr of stress and tracer uptake by the heart, in vivo, when determined immediately after cessation of the stress. The relationship may be expressed by the equation, $Y = 3.4X + 3.3$, where Y = tracer uptake and X = hr of stress. The correlation coefficient, $r = 0.82$. After 0.5 hr of stress, tracer uptake was slightly but significantly greater than that of controls ($p<0.025$). After 1 hr or more, the increases were greater and highly significant ($p<0.01$).

Since it had been reported that cardioversion by electric shock was capable of producing direct damage to the heart [10,11], it was possible that the cardiac damage we observed was due to the electric current passing through the heart, rather than to central nervous system mediated-stress. To determine if this were so, 40 rats were divided into 4 groups of 10 animals each. Each rat in Groups A and B was injected IP with 90 mg of sodium barbital, a dose which produced anesthesia for the full 12 hr period of stress. The feet of the rats were taped to the bars of the cage floor to insure electrical contact. Rats in Group A were shocked as usual, while those in Group B were not shocked. The animals in Group C were unanesthetized, non-stressed controls, while those in Group D were subject to the 12 hr stress, but were not given barbital. Tc-99m-PPi was injected 8 hr after termination of the stress to allow the animals to regain full consciousness before being injected. It had been observed, as indicated in a later experiment, that cardiac tracer uptake 8 hr after termination of a 12 hr stress was significantly above that of controls.

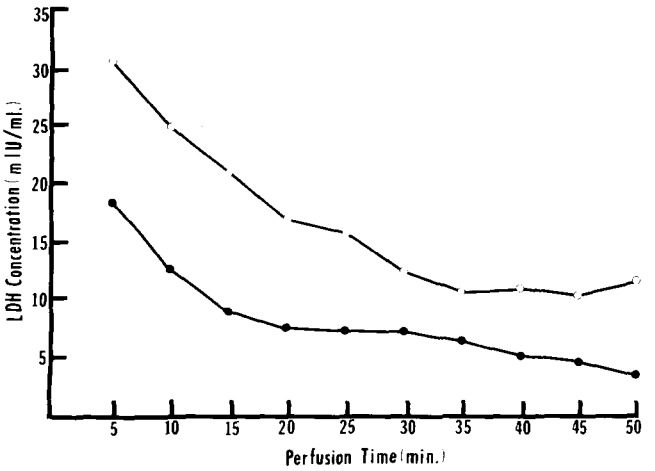

FIG. 1. Effect of foot-shock stress of rats on rate of LDH release from isolated perfused hearts of these animals; typical example. One rat was stressed for a period of 12 hr with a 1.2 mA current; the other was placed in a similar cage but not shocked. Open circles = stressed rat. Closed circles = non-stressed control.

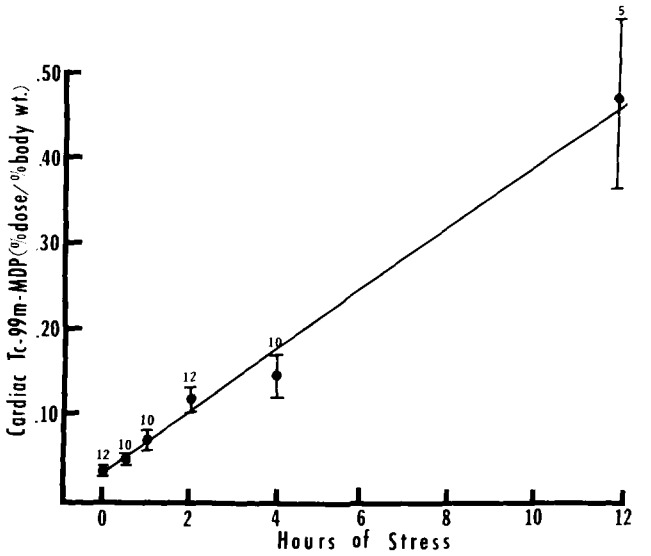

FIG. 2. Effect of duration of stress on myocardial uptake of Tc-99m-MDP. Vertical lines are SE's. Numbers above bars are numbers of animals.

As shown in Fig. 3, there were no significant differences in tracer uptake between non-stressed controls, anesthetized stressed rats and anesthetized non-stressed rats. On the other hand, without anesthesia, the 12 hr stress caused a significant increase in tracer uptake in comparison to the controls ($p<0.005$). Thus, the electric current per se did not affect tracer uptake and therefore presumably caused no direct injury to the heart.

While it was apparent that increased myocardial uptake of technetium-99m following stress occurred only in a conscious animal, it was desirable to attempt to separate the effect of the pain-induced psychological stress from that of the muscular exercise that occurred during the moments when current was applied to the cage floor.

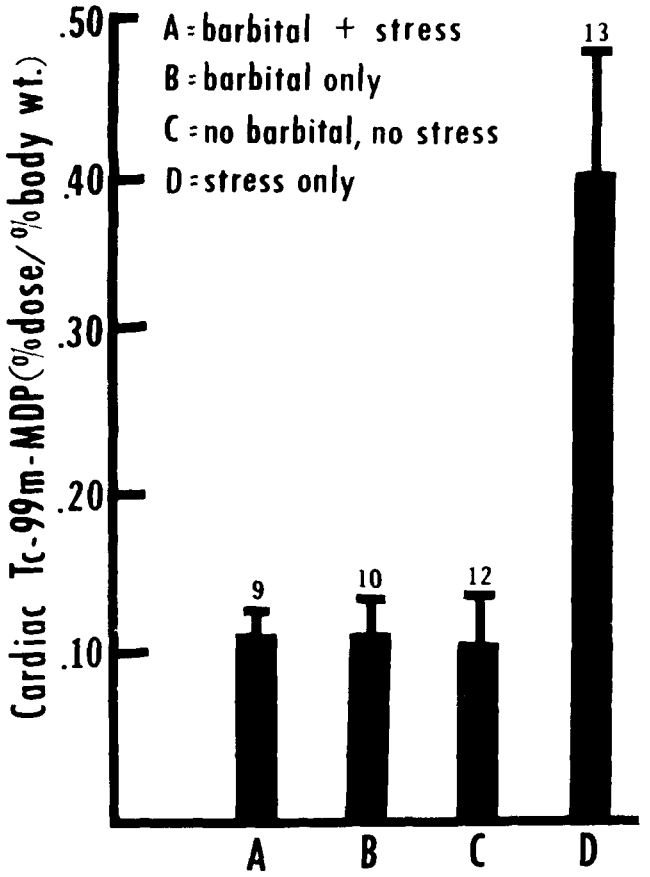

FIG. 3. Effect of barbital administration to rats on myocardial uptake of Tc-99m-PPi 8 hr after exposure of rats to a 12 hr stress. Vertical lines are SE's. Figures above bars are numbers of animals.

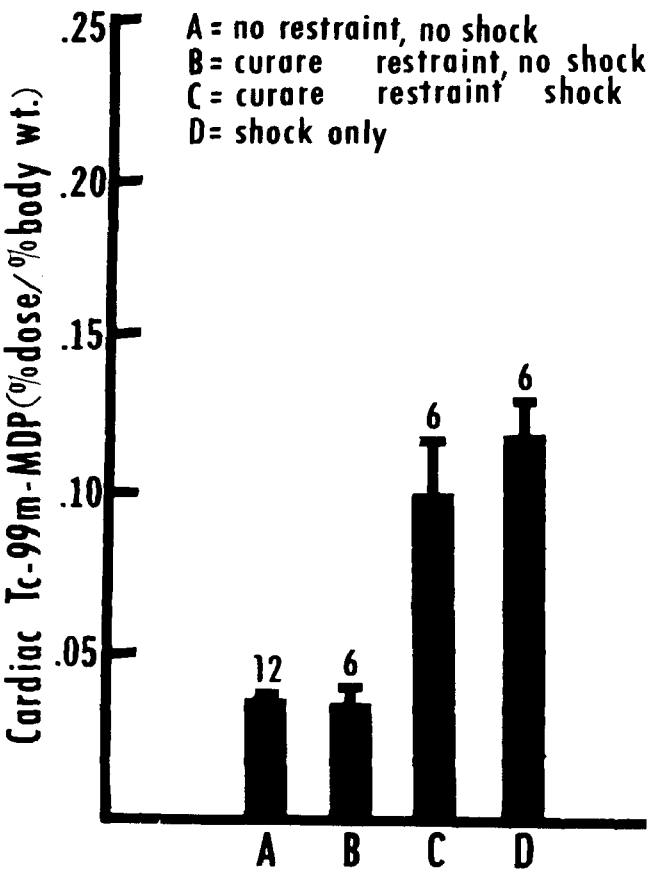

FIG. 4. Effect of curare and physical restraint on myocardial uptake of Tc-99m-MDP by rats subjected to a 2 hr stress. Vertical lines indicate SE's. Figures above bars are numbers of animals.

Garbus, *et al.* [12] and Tomanek and Banister [36] suggested that myocardial damage may occur in rats as a consequence of running wheel exercise. Twelve animals were divided into 2 groups. Each animal was injected subcutaneously with 0.15 mg/kg of d-tubocurarine in a solution of 0.5 ml volume. This dose was determined to be sufficient to minimize motion without interfering with respiration. The rats were placed individually in small restraining cages in such a way that the hind feet could be extended and taped to the bars of the cages in which the animals were stressed. The rats in the first group were stressed for a period of 2 hr, while the rats in the second group were similarly restrained but not shocked. Myocardial uptakes of tracer by rats in these two groups were determined and the results compared to those obtained with non-stressed controls and with unrestrained, untreated rats stressed for 2 hr. As shown in Fig. 4, there was no significant difference between curare-treated, restrained rats stressed for 2 hr and unrestrained, untreated rats stressed for 2 hr. In both cases, the cardiac tracer uptake was significantly greater than in the non-stressed controls. On the other hand, the administration of curare and restraint, without shock, produced no increase in myocardial Tc-99m-MDP over controls.

Tracer studies in rats [1,20] have reported that myocardial accumulation of technetium-99m-bone seeking agents occurs when the tracer is injected up to 4 days after the infarct-producing event. Afterwards, no image can be seen in scintigrams. After stress, the increased uptake of tracer observed in the preceeding experiments might be due to altered coronary blood flow, as, for example, in the case of temporary hyperemia. If this occurs, a delay of several hr, after which there is a return of the cardiovascular system to resting levels, should result in a return of myocardial Tc-99m-PPi or Tc-99m-MDP uptake to control levels. Thus, Tepperman and Tepperman [34] found that exhaustive exercise resulted in blood lactate increases that were followed by return to normal levels in 1 hr. Barnard, *et al.* [2] reported that rat heart rates returned to near resting levels 1 min after exhaustive exercise and Williams, *et al.* [38] found that rat blood pressures returned to baseline values 4 hr after a maximum blood pressure response to foot shock was observed.

A group of 20 rats, therefore, was stressed for 2 hr and another group of 20 stressed for 12 hr. Tc-99m-MDP was injected into 10 animals from each group 8 hr after the end of the period of stress. The remaining rats were injected with Tc-99m-MDP 30 hr after the stress was terminated. If the previously shown increase in myocardial uptake was due to simply an alteration in the state of the circulation, a decline in tracer uptake to control or nearly control levels would be expected to occur 8 hr after termination of the

stress, particularly after a stress of only 2 hr duration. Furthermore, it would be expected that 30 hr after termination of the stress there would be no significant increase in tracer uptake above control values, regardless of whether the duration of the stress was 2 hr or 12 hr.

As shown in Fig. 5, an exponential decrease in cardiac uptake of tracer by 12 hr stressed animals occurred as the delay between the end of the stress and Tc-99m-MDP administration was increased. The curve extrapolated to control values after a delay period of about 4 days. After an 8 hr period of delay before Tc-99m-MDP injection, tracer uptakes by hearts of both 2 and 12 hr stressed rats were still significantly above control values ($p<0.005$). At this time, it is presumed that any temporary alteration in coronary circulation would have disappeared. Even after a 30 hr delay period, cardiac tracer uptake by 12 hr stressed animals was still significantly greater than that by non-stressed controls ($p<0.001$).

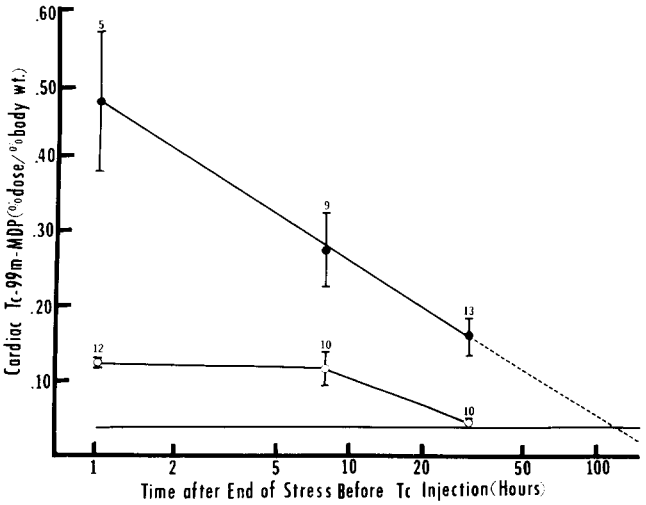

FIG. 5. Effect of delay after stress before Tc-99m-MDP injection. Rats were stressed for either 2 or 12 hr. Vertical lines are SE's. Figures above bars are numbers of animals. Long horizontal line at bottom is uptake by hearts of non-stressed controls. Closed circles = 12 hr stress. Open circles = 2 hr stresss.

DISCUSSION

We have presented two methods for the assessment of stress-induced myocardial damage. The time course of serum enzymes following stress (Table 1) is not, of itself, evidence of myocardial damage. However, it is consistant with the time course of myocardial Tc-99m-MDP uptake following stress, suggesting that at least some of the enzymes released into circulation were from damaged myocardium. Determination of enzyme released from isolated perfused hearts is a somewhat more difficult and less sensitive procedure than is measurement of cardiac uptake, in vivo, of administered Tc-99m-bone seeking agents. However, as demonstrated in our experiments, it can provide quantitative measurements of the small degrees of cardiac injury provoked by different levels of stress in experimental animals.

D'Agostino [7] and D'Agostino and Chiga [8] reported that mineralization of mitochondria in heart occurs as a result of cardiac surgery or of focal necrosis produced by steroid administration. The crystalline structure of these deposits resembled hydroxyapatite. Shen and Jennings [30] reported similar calcium deposition in irreversibly injured ischemic myocardium. On the basis of these findings, Bonte, *et al.* [3] proposed the use of the recently developed [33] Tc-99m-tagged bone-seeking agents as a means of identifying myocardial infarcts. While these agents did accumulate in infarcted heart, more recent experiments by Dewanjee, *et al.* [9] suggested that Tc-99m bone-seekers accumulate not in mitochondria but in the soluble protein fractions of cells in infarcted myocardium, perhaps binding to certain intracellular macromolecules. In spite of the lack of agreement concerning the mechanism of accumulation, it is generally agreed that uptake of Tc-99m bone-seekers by heart is a highly sensitive and accurate measurement of myocardial injury.

Experiments in this laboratory [22] have shown that the procedure of inducing stress we have described results in both histological evidence of myocardial damage and significant accumulation of Tc-99m, even when the duration of stress is as short as 2 hr. The development of the technetium tracer has made it feasible to carry out extensive examination of behavioral variables that may influence the production of stress-induced myocardial injury. Our experiments show that such studies are now possible.

We have found [22] that concentrations of both Tc-99m-PPi and Tc-99m-MDP in the heart are the same after 12 hr of stress, suggesting an equivalent uptake of the two agents by injured myocardium. However, the normal heart sequesters very little of either agent and the tracer in the myocardium appears to be in equilibrium with that in the blood. Therefore, since Tc-99m-MDP is cleared more rapidly from the blood, the ratio of Tc-99m-MDP in stress/control hearts is higher than that of Tc-99m-PPi. For this reason, Tc-99m-MDP appears to be a better agent for the detection of extremely slight injury following low levels of stress. By perfusing hearts before counting their radioactivity, control activity is brought to a very low and uniform level, without washout of the activity in the cells of stress-injured hearts.

Our experiments with Tc-99m-MDP indicate that an increase in the duration of a fixed level of stress may result in a proportional increase in the degree of myocardial injury. Stress for as short as 1 hr, during which time approximately 150 shocks were administered to the animals, resulted in a detectable increase in myocardial tracer uptake. That the increased Tc-99m-MDP uptake was not merely due to the effect on the heart of the electric current itself, was shown by our experiments with anesthetized and unanesthetized rats. A fully conscious animal was required for the effect to occur. It was demonstrated, further, that the increased cardiac uptake of tracer was not due to damage induced by exercise. When muscular activity was minimized by the administration of curare and physical restraint, the increase in cardiac tracer uptake still occurred even though the curare-restraint procedure alone produced no increase in tracer uptake by the heart. The persistence of increased concentrations of tracer in the heart 8 and 30 hr after termination of the stress, as well as the previously shown histological evidence of damage, supports the hypothesis that accumulation of tracer in the hearts of stressed animals is an indication of permanent myocardial injury

and not merely of temporary changes in blood flow or capillary permeability.

The isolated perfusion technique examined the single stress parameter, current intensity, finding a significant myocardial response at 0.8 mA and above. On the basis of these results, a 1.0 mA current was selected for the radionuclide uptake study, and two further stress parameters were examined: duration of stress and time delay after the end of stress. Since the stress parameters were not held constant across procedures, the two procedures are not directly comparable, except at a single set of stress parameters: 1 mA, 12 hr stress and 0 hr delay after stress. When the myocardial response is examined at this set of parameters, the stress-to-control ratios are 1.25 for the isolated perfused hearts (by interpolation) and 13.15 for the Tc-99m-MDP uptake, illustrating the higher sensitivity of the latter procedure over the former.

Isolated perfusion as a method of quantifying stress-induced myocardial damage is, however, a unique application of this technique and may have use under certain circumstances. On the other hand, the studies described suggest that Tc-99m bone-seeking agents, such as Tc-99m-PPi or Tc-99m-MDP can be employed widely for studies on modification of stress-produced myocardial injury by appropriate environmental changes. The procedure is simple, rapid and highly sensitive and achieves a precision not possible by other means.

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