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Effect of Glutathione on Canine Myocardial Ischaemia Without Reperfusion

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Abstract—The present study was to evaluate the effect of exogenous glutathione on myocardial damage resulting from permanent (no reperfusion) coronary ligation (3 or 6 h) in anaesthetized dogs. Haemodynamics, infarct size and myocardial glutathione content were determined. Erythrocyte superoxide dismutase (SOD) activity was also determined in coronary venous blood samples. Glutathione was administered by the intraperitoneal route, 100 mg kg^{-1} as initial dose given 5 min before coronary ligation, and successive doses of 25 mg kg^{-1} every 40 min throughout the study period. Saline-treated dogs showed myocardial infarction, a decrease in myocardial glutathione content, and a transient increase in SOD activity. Three hours occlusion in glutathione-treated dogs resulted in a small reduction of infarct size, and no changes in myocardial glutathione content and SOD activity. By contrast, administration of glutathione failed to reduce infarct size and failed to prevent myocardial glutathione decrease in dogs subjected to 6 h occlusion. These results indicate that exogenous glutathione is of minor beneficial effect for myocardial damage resulting from permanent coronary occlusion and suggest that endogenous glutathione has a limited role in protecting against myocardial ischaemia without reperfusion.

Myocardial ischaemia is associated with an enhanced production of toxic oxygen-derived free radicals, and with a loss of protective antioxidant mechanisms (Rao et al 1983; Burton et al 1984; Ferrari et al 1985). Free radicals may derive from the myocardium and also from extrinsic sources such as activated neutrophils. Studies have demonstrated that the administration of antioxidant enzymes (superoxide dismutase and catalase), free-radical scavengers and drugs interfering with leucocyte infiltration and activation exhibit myocardial protective effects in animal models of infarction (for review see Lucchesi & Mullane 1986; Werns & Lucchesi 1990).

Among the protective intracellular antioxidant systems, the glutathione redox pathway seems important in the myocardium (Curello et al 1985), as previously demonstrated in non-cardiac tissues. Furthermore, glutathione peroxidase appears to be more active than other antioxidant enzyme systems (Fantone & Ward 1982).

The role of the glutathione redox pathway in myocardial injury has been studied in the ischaemia-reperfusion model of infarction (Forman et al 1988; Singh et al 1989). However, myocardial injury by free radicals is not restricted to reperfusion models. Permanent coronary occlusion—which is relevant to the clinical setting—is also associated with a rapid leucocyte infiltration (Mullane et al 1984; Kolodgie et al 1985; McCluskey et al 1985) and the resulting infarction is as susceptible to pharmacological intervention as those tested in the ischaemia-reperfusion models (Kolodgie et al 1985; Alberola et al 1991).

Ischaemia without reperfusion decreases the myocardial content of reduced glutathione in dogs (Romero et al 1987), pigs (Singh et al 1989), and rabbits (Curello et al 1985). Additional reperfusion resulted in little (Curello et al 1985) or no (Singh et al 1989) further diminution of myocardial glutathione content. Drug-induced glutathione depletion renders the myocardium more susceptible to ischaemic damage (Blaustein et al 1989; Singh et al 1989). Furthermore, pharmacological intervention such as the administration of glutathione or *N*-acetylcysteine which enhance myocardial glutathione content are beneficial in animal models of infarction (Such et al 1986; Forman et al 1988; Blaustein et al 1989; Singh et al 1989; Alberola et al 1991).

In the present study, the role of exogenous glutathione in protecting against myocardial ischaemic injury has been evaluated in anaesthetized dogs subjected to either 3 or 6 h of permanent coronary occlusion. Glutathione was administered by the intraperitoneal route and the plasma glutathione and cysteine levels were followed for the duration of the experiment. Haemodynamics, infarct size, myocardial glutathione content and the superoxide dismutase activity in coronary venous blood samples were determined to investigate the effects of glutathione in this model.

Materials and Methods

Surgical preparation

Twenty-nine adult healthy mongrel dogs of either sex, 13–21 kg, were fasted overnight with free access to water. They were anaesthetized with 20 mg kg^{-1} intravenous thiopentone sodium (Pentothal, Abbott), and additional doses were given as needed throughout the experiment. The dogs were intubated and ventilated with room air via a respirator. Ventilation was adjusted to maintain arterial blood gases at physiologic levels. The lead II of the electrocardiogram (ECG) was obtained. Mean arterial blood pressure (MAP) was measured via a catheter inserted into the femoral artery and attached to a Gould-Statham P23ID transducer. ECG and MAP were recorded on a Mingograf 34 Elema-Shonader (Siemens) polygraph. A femoral vein was catheterized for fluid and drug administration. Thoracotomy was performed at the left fifth intercostal space and the heart suspended in a

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pericardial cradle. The left anterior descending coronary artery (LAD) was dissected free at the level of the first major diagonal branch and a ligature passed around it.

Experimental protocol

After completing the instrumentation, the dogs were allowed to stabilize and baseline haemodynamic measurements were obtained. Dogs were subjected to either 3 (7 control and 5 treated) or 6 h (11 control and 6 treated) of permanent coronary occlusion. The assignment of animals to each group was made at random. Treated animals received glutathione (Boehringer Mannheim) 100 mg kg^{-1} as a loading dose given intraperitoneally over 5 min in a volume of 1 mL kg^{-1}, followed by additional doses of 25 mg kg^{-1} every 40 min until the end of each experiment. Control dogs were similarly treated except that they received 0·9% NaCl (saline). Five minutes after finishing the first glutathione or saline administration, the coronary ligature was tied and left permanently occluded. Haemodynamic measurements were obtained immediately before and after the first glutathione or saline administration, and at various times after coronary ligation. At the end of each experiment, methylene blue (1 mL kg^{-1} of a saturated solution) was injected into the left atrium for the determination (by defect staining) of the area at risk. Immediately thereafter the animals were killed, the heart rapidly excised, and the atria and right ventricle removed.

Infarct size

The left ventricle was weighed, sliced transversely into sections around 0·5 cm thick, and incubated with nitroblue tetrazolium in phosphate-buffered saline (pH 7·4) for 20 min. Necrotic zones inside the area at risk were demarcated by the absence of nitroblue tetrazolium staining while viable myocardium stained bright red. The slices were carefully dissected into areas of infarction, area at risk and remaining left ventricle, and weighed. Infarct size was expressed as a percent of the area at risk and as a percent of the total left ventricle. Previous in-vitro studies (Singh et al 1989) had demonstrated that the histochemical reaction used to evaluate infarct size was unaffected by the addition of glutathione.

Myocardial glutathione content

In a separate series of experiments, 82 dogs were anaesthetized and surgically prepared as previously described in this study. Dogs were randomly distributed into three groups: sham-operated animals (31 dogs subjected to similar surgical manipulation as the other groups except for coronary occlusion); control group (26 saline-treated dogs undergoing coronary occlusion); and a treated group (25 dogs receiving glutathione and subjected to coronary occlusion). For each group, animals were randomly distributed into five subgroups (pre-occlusion, 10, 90, 180 min, and 360 min post-occlusion). The number of animals in each subgroup is indicated in Table 3. Transmural punch biopsies (about 200 mg) were taken from the centre of the region of left ventricle supplied by the LAD, and the animals were killed immediately thereafter. Biopsies were obtained at the end of the stabilization period (pre-occlusion) and at various times after sham-occlusion or coronary occlusion, as indicated above. Biopsy specimens were immediately frozen in liquid nitrogen and the glutathione content measured according to the method of Brigelius et al (1983). Results are expressed in μmol g^{-1}.

Superoxide dismutase activity in coronary venous blood

In other experiments, 20 dogs were anaesthetized and prepared as already described. The coronary vein adjacent to the LAD was catheterized to obtain venous blood samples at various times during the experiment. Erythrocyte superoxide dismutase (SOD) activity was measured in these samples by the method of McCord & Fridovich (1969). Activity is reported in units (g haemoglobin)$^{-1}$. Determinations of SOD activity were carried out at the end of the stabilization period (pre-occlusion value), 10 min post-occlusion, and 3 h post-occlusion, in animals randomly distributed into three

Table 1. Time course of heart rate (HR, beats min^{-1}) and mean arterial pressure (MAP, mmHg) in dogs receiving saline (control) or glutathione (first dose of 100 mg kg^{-1}, i.p., 5 min before coronary ligation, followed by successive doses of 25 mg kg^{-1}, i.p., every 40 min throughout the study period).

		Pre-occlusion		Post-occlusion			
		Baseline	After 1st dose	10 min	90 min	180 min	360 min
Occlusion (3 h)							
Control (n=7)	(HR)	194±23	190±25	199±19	197±26	184±24	
	(MAP)	127±8	124±5	122±6*	124±9	125±10	
Glutathione (n=5)	(HR)	196±11	195±10	192±26	199±23	199±32	
	(MAP)	121±16	104±23*	97±32*	107±29	109±33	
Occlusion (6 h)							
Control (n=11)	(HR)	186±22	188±21	182±27	186±21	188±38	179±32
	(MAP)	121±15	121±14	119±15*	122±20	119±21	113±14
Glutathione (n=6)	(HR)	200±22	188±22	180±29	195±21	186±36	185±46
	(MAP)	112±17	102±19*	99±16*	114±15	105±15	96±22

Data are means±s.d. *$P<0·05$ (Student's t-test) compared with baseline values.

Table 2. Size of infarct in dogs receiving saline (control) or glutathione (dose as in Table 1).

		Area at risk as % of left ventricle	Infarct size as % of area at risk	Infarct size as % of left ventricle
Occlusion (3 h)				
Control	(n=7)	23±3	88±4	21±2
Glutathione	(n=5)	21±2	81±5*	17±1*
Occlusion (6 h)				
Control	(n=11)	26±6	87±7	23±6
Glutathione	(n=6)	24±2	86±6	20±4

Data are means±s.d. *$P<0.05$ (Mann-Whitney U-test) compared with control.

groups: sham-operated (3 dogs instrumented but not subjected to coronary occlusion), control (8 dogs undergoing coronary occlusion but not treated) and treated (9 dogs receiving glutathione and subjected to coronary occlusion).

Plasma glutathione and cysteine levels

In separate experiments, 3 dogs were anaesthetized and instrumented as described. The animals received glutathione in a dosage schedule as mentioned in this study. Arterial blood samples were obtained before and at different time intervals after the first glutathione administration. Plasma levels of glutathione and cysteine were determined according to the methods of Brigelius et al (1983) and Gaitonde (1967), respectively, and the results are expressed in mmol L^{-1}.

Statistical analysis

Data are presented as the mean ± s.d. Parametric (analysis of variance and Student's *t*-test) or non-parametric (Mann-Whitney U-test) statistics were applied as indicated. Probability (*P*) values of less than 0·05 were considered as significant.

Results

Haemodynamics

Saline-treated animals showed no significant changes in heart rate (HR) or MAP, except a small but significant decrease in MAP 10 min post-occlusion. The administration of glutathione resulted in no changes of the HR, but MAP decreased after the loading dose and remained significantly diminished 10 min post-occlusion. Then, MAP returned to pre-drug values. Results are shown in Table 1.

Infarct size

Values for the area at risk did not significantly differ between groups. This indicates that any reduction in infarct size could not be attributed to differences in the amount of tissue rendered ischaemic. In dogs subjected to 3 h coronary occlusion, glutathione produced a significant limitation of the infarct size expressed as a reduction in the weight percentage of the necrotic area with respect to either the left ventricle or the area at risk (Table 2). In contrast, administration of glutathione to dogs undergoing 6 h of coronary occlusion did not result in a reduction of infarct size.

Myocardial glutathione content

The level of glutathione in the myocardium of sham-operated animals was maintained for the duration of the experiment (Table 3). By contrast, glutathione levels decreased after coronary occlusion in control, saline-treated, dogs. In the glutathione-treated animals, myocardial glutathione content remained similar to pre-occlusion values for up to 3 h of ischaemia, but a significant decrease was noticed 6 h post-occlusion. The difference in myocardial glutathione content between control and glutathione-treated dogs reached statistical significance only at 3 h post-occlusion.

SOD activity in coronary venous blood

The results are shown in Table 4. SOD activity rose significantly in control (saline-treated) dogs 10 min post-occlusion. This was not observed in dogs treated with glutathione.

Plasma glutathione and cysteine

Fig. 1 shows the time course of the plasma levels of glutathione and cysteine after administration of a loading dose of 100 mg kg^{-1} (i.p.) followed by successive 25 mg kg^{-1} (i.p.) doses every 40 min throughout the study period. Plasma glutathione levels rose at 5 min, reached a peak at 20 min, and descended to a plateau above pre-drug values for the rest of the experiment. By contrast, plasma cysteine levels rapidly rose to a level maintained until the end of the experiment.

Discussion

Coronary occlusion and the ensuing ischaemia reduces myocardial glutathione content as found in this (Romero et al 1987; this study) and other (Curello et al 1985; Röth et al 1985; Singh et al 1989) laboratories. On the other hand, glutathione depletion enhances myocardial susceptibility to

Table 3. Glutathione (μmol g^{-1}) in myocardial biopsies obtained from dogs undergoing permanent coronary occlusion.

		Time after occlusion			
Group	Pre-occlusion	10 min	90 min	180 min	360 min
Sham	0·94±0·09 (8)	1·06±0·15 (6)	0·82±0·42 (4)	0·93±0·30 (5)	0·88±0·06 (8)
Control	0·94±0·15 (5)	0·53±0·28* (6)	0·67±0·09* (5)	0·44±0·24* (6)	0·37±0·10* (4)
Glutathione	0·96±0·16 (4)	0·71±0·21 (4)	0·82±0·56 (4)	1·01±0·33# (4)	0·58±0·28* (5)

Data are means±s.d. The number of experiments is given in parentheses. *$P<0.05$ compared with pre-occlusion values and #$P<0.05$ compared with the corresponding control values by Mann-Whitney U-test.

Table 4. Erythrocyte superoxide dismutase activity (units (g haemoglobin)$^{-1}$) in blood samples from the coronary vein draining the ischaemic zone in glutathione-treated dogs (dosing schedule as in Table 1) subjected to 3 h occlusion.

Group	Pre-occlusion	Post-occlusion 10 min	Post-occlusion 3 h
Sham	2797±753 (3)	2911±758 (3)	2928±813 (3)
Control	2859±645 (8)	3910±1017 (8)*	3183±584 (6)
Glutathione	2979±1518 (9)	3582±1216 (9)	3430±1777 (9)

Data are means±s.d. Number of experiments is given in parentheses. *$P<0.05$ (Student's t-test) compared with pre-occlusion value.

the ischaemic injury (Blaustein et al 1989; Singh et al 1989). Therefore, systemic administration of glutathione in this and other (Singh et al 1989) studies was intended to augment myocardial glutathione and thereby limit myocardial damage.

The plasma half-life of glutathione is very short (Ammon et al 1986). High glutathione levels may be maintained in plasma by continuous intravenous infusion (Singh et al 1989). Alternatively, we decided to administer glutathione by the intraperitoneal route, giving a loading dose followed by successive doses at constant time intervals during the study. This multiple dosing schedule produces an initial peak, corresponding to the post-occlusion, followed by a stable lower concentration of plasma glutathione for the remainder of the study. Parallel determinations of plasma cysteine showed a different pattern with the concentration of this amino acid rapidly increasing and remaining high throughout the study. In the model of permanent coronary occlusion the access of drugs to the ischaemic myocardium is restricted to the collateral blood flow since reperfusion is not permitted. In the present study, glutathione was administered before coronary ligation; however, administration of drug before coronary occlusion is not achievable in the clinical setting.

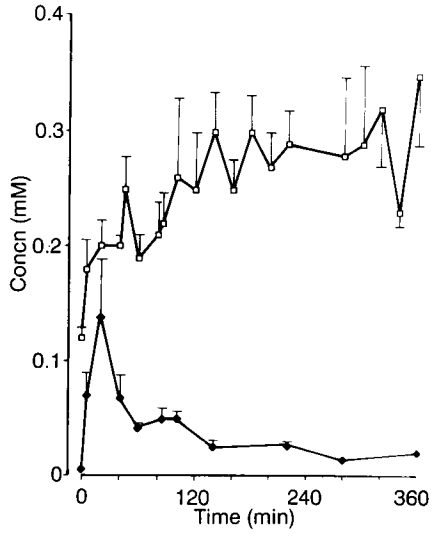

FIG. 1. Time course of plasma cysteine (□) and glutathione (◆) concentrations in anaesthetized dogs receiving glutathione as follows: an initial dose (time 0) of 100 mg kg^{-1} (i.p.) followed by successive 25 mg kg^{-1} (i.p.) doses every 40 min throughout the study period. Points are the mean of 3 experiments with vertical lines indicating s.d.

Previous studies have shown that exogenous glutathione is effective in increasing tissue glutathione content in isolated preparations (Tsan et al 1989) and in ischaemia-reperfusion models (Blaustein et al 1989; Singh et al 1989). The precise mechanism by which exogenous glutathione enhances glutathione tissue content is unknown. Extracellular breakdown and internal resynthesis has been suggested (Tsan et al 1989). We have not determined other metabolites of glutathione but cysteine is an effective precursor of the intracellular glutathione synthesis (Estrela et al 1983). Alberola et al (1991) reported that administration of *N*-acetylcysteine (150 mg kg^{-1}, i.v., 2 min before ligation) to dogs subjected to 3 h of permanent coronary occlusion maintained myocardial glutathione content, i.e. no depletion of myocardial glutathione was observed, while plasma cysteine concentration reached values similar to those found after glutathione administration in the present study. These findings suggest that the intraperitoneal administration of glutathione, although not commonly described in the literature, may be a valid approach to preserve myocardial glutathione content under ischaemic conditions as discussed below.

Sham-operated animals maintained myocardial glutathione content throughout the study period. Dogs subjected to coronary ligation showed a lower myocardial content of glutathione at 3 and 6 h post-occlusion. Administration of glutathione reversed this situation when the period of ischaemia was 3 h. By contrast, the decrease in myocardial glutathione content 6 h post-occlusion was not reversed in glutathione-treated dogs. These biochemical results correlate with infarct size data. Thus, administration of glutathione produced a small but significant reduction of infarct size in dogs subjected to 3 h occlusion but failed in those animals undergoing 6 h occlusion. However, plasma glutathione and cysteine levels were similar at 3 and 6 h after coronary occlusion. This suggests that in this situation, glutathione is merely delaying inevitable cellular death and that glutathione is unable to penetrate into the myocardium at the site of injury. Thus, even in the glutathione-pretreated animal, reperfusion may be of importance in preserving the myocardium and this should be performed before 6 h. Presumably, the degree of irreversible damage caused to the myocardium by 6 h of permanent coronary occlusion impedes the protective effect afforded by the glutathione treatment. In fact exogenous glutathione increased myocardial glutathione and reduced infarct size in pigs subjected to 45 min coronary occlusion followed by 2 h reperfusion (Singh et al 1989), whereas *N*-acetylcysteine (a glutathione precursor) neither increased myocardial glutathione nor reduced infarct size after 90 min occlusion plus 24 h reperfusion in dogs (Forman et al 1988).

It appears that certain alterations relevant to free radical-induced myocardial injury occur early after coronary occlusion and are of a transient nature (Becker & Ambrosio 1987). In this study, erythrocyte SOD activity was determined in blood samples obtained from the local coronary vein draining the ischaemic zone. The early release of superoxide radical to coronary venous blood (Rao et al 1983; Davies 1989) may transiently enhance erythrocyte SOD activity as found in the control (saline-treated) dogs at 10 min but not

after 3 h of coronary occlusion. This early increase in SOD activity was not observed in the glutathione-treated group which may be related to a direct scavenging effect of sulphydryl compounds. A similar time course of events has been found with *N*-acetylcysteine in the same model of myocardial ischaemia (Alberola et al 1991).

The beneficial effect of exogenous glutathione was apparently not due to changes in the balance between oxygen demand and supply to the myocardium. Intraperitoneal administration of glutathione produced only a small and transient decrease in blood pressure after the loading dose but sustained haemodynamic changes were absent. Continuous intravenous infusion of glutathione producing a maintained high plasma level had no effect on heart rate, left ventricular pressure or myocardial contractility (Singh et al 1989). Although regional myocardial blood flow was not measured in this study, several reports show that other sulphydryl compounds did not alter coronary collateral blood flow (Mitsos et al 1986; Forman et al 1988; Alberola et al 1991).

Thus, maintenance of myocardial glutathione may be a mechanism underlying the protective effect resulting from administration of glutathione to dogs with myocardial ischaemia. This beneficial effect of glutathione is attributable to its antioxidant properties (Flohe & Gunzler 1976) including its ability to inhibit lipid peroxidation (Weiss 1986). However, it should be emphasized that exogenous glutathione achieved only a small limitation of the infarct size produced by 3 h of permanent coronary occlusion, and none at 6 h. This is in contrast with findings in the ischaemia-reperfusion model (Singh et al 1989); it is possible that the administered glutathione was merely delaying, rather than limiting, myocardial necrosis. Further research should be carried out but present findings indicate that exogenous glutathione is of minor beneficial effect for myocardial damage resulting from permanent coronary occlusion and suggest that endogenous glutathione has a limited role in protecting against myocardial ischaemia without reperfusion. This reinforces the idea that substantial differences exist in the pathogenic mechanisms and pharmacological treatment of myocardial ischaemia with or without reperfusion.

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