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EFFECT OF SUPEROXIDE DISMUTASE AND ACIDIFIED SODIUM NITRITE ON INFARCT SIZE FOLLOWING ISCHEMIA AND REPERFUSION IN DOGS

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Abstract : The effects of superoxide dismutase (SOD) alone or in combination with acidified sodium nitrite ($NaNO_2$), a liberator of nitric oxide were examined in dogs after ischemia and reperfusion. Animals were divided into five groups. Left anterior descending coronary artery was occluded for 90 min followed by 4 hours of reperfusion with or without therapeutic interventions given preceding reperfusion. Left ventricular end diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP) and ECG changes were monitored throughout the study. Area at risk was defined by Evans blue and area of infarction by incubation in triphenyltetrazolium. Myocardial tissue lipid peroxidation was measured in ischemic and non-ischemic zones. There was no evidence of infarction until ninety minutes of ischemia. Percentage area of necrosis vis-a-vis area at risk percentage necrosis in left vertricular mass was significantly low in animals treated with combination of SOD and $NaNO_2$ in comparison with isolated treatment with saline, SOD or $NaNO_2$. LVEDP increased significantly following ischemia and remained unchanged during saline reperfusion. Treatment with SOD, $NaNO_2$ in isolation or its combination significantly lowered LVEDP. Maximum increase in tissue lipid peroxidation was observed in saline and $NaNO_2$ treated animals. SOD alone or in combination with $NaNO_2$ significantly lowered the lipid peroxidation. The results clearly demonstrate that reperfusion can cause necrosis in ischemic myocardium. Combined treatment with SOD and $NaNO_2$ offers significant cardioprotection against oxidative stress.

Key words : superoxide dismutase acidified sodium nitrite ischemia reperfusion infarct size cardioprotection

INTRODUCTION

Large scale randomized trials in patients with acute myocardial infarction have shown that thrombolysis can reduce mortality. However, experimental studies show that reperfusion itself might kill myocytes which are reversibily injured at the end of ischemia (1, 2). Isehemia depletes antioxidant enzymes like superoxide

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dismutase, catalase and glutathione peroxidase and makes the myocardial cells vulnerable to oxidant induced damage (3, 4). Reperfusion in previously ischemic myocardium results in increased formation of superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) (5, 6). It has been suggested that oxygen free radicals can also damage endothelial cells at the time of reperfusion (7). Endothelial cells synthesise and secrete nitric oxide which is endothelium derived relaxation factor (8). It has been shown that acidified sodium nitrite at pH 2 gets converted into nitric oxide (9).

Studies to examine the role of free radical scavengers on infarct size, have shown confilicting results (10–15). The reasons for such discrepancies are unknown. At present there is no clear consensus on whether antioxidants given at the time of reperfusion are capable of reducing infarct size in experimental animals. The concept of reperfusion injury remains to be proven.

Therefore, the main objective of the present study was to re-examine the concept of reperfusion injury and to determine whether superoxide dismutase, acidified sodium nitrite, infused alone, or in combination, could offer any significant cardioprotection following ischemia and reperfusion.

METHODS

Animals, surgical preparation and instrumentation : In the present study, adult male mongrel dogs weighing 10 to 15 kg were used. Animals were anaesthetised with intravenous pentobarbital sodium (30 mg/kg) and ventilated with room air by INCO positive pressure ventilator. Ventilatory parameters were adjusted to maintain normal pH and satisfactory oxygenation. The chest was opened by left thoracotomy through fourth intercostal space and heart was suspended in precardial cradle. A polythene cathetar was inserted into left atrium for administration of drugs or saline. Another catheter (1.5 mm inner diameter) was placed in left ventricle through apex to record ventricular pressure changes (Grass, model 78 D) by using (Gould P 231D) pressure transducer. Heart rate and ST segment changes were recorded in limb leads on electrocardiograph.

Coronary artery occlusion and reperfusion : Left anterior descending coronary artery was dissected free above the first diagonal branch and below the origin of left circumflex artery. Following completion of surgical preparation and instrumentation, 30 minutes were given for stabilization. The left anterior descending coronary artery was occluded abruptly by a vessel occluder for 90 min. Occluder was removed after 90 minutes to allow reperfusion for four hours. No attempt was made to resuscitate the animals which developed ventricular fibrillation during occlusion or reperfusion.

Experimental groups and treatment : Gr. I : Coronary artery was occluded for 90 min. Gr. II : (untreated saline reperfused) occlusion of coronary artery (90 min) was followed by reperfusion with normal saline (115 ml) through left atrium for 4 hrs. Gr. III : (SOD treated animals). Animals received loading dose of (26000 IU/kg)

bovine SOD (Sigma Chemicals, U.S.A) at the time of reperfusion upto 1 hr folowed by maintenance dose of 15000 IU/kg through left atrial line for 3 hrs. Gr. IV : (Acidified $NaNO_2$ treated animals). Infusion of acidified $NaNO_2$ (0.30 Mol/L HCl, pH 2) at the time of reperfusion for 4 hrs. Gr. V : (Acidified $NaNO_2$ + SOD treated animals) combined infusion of $NaNO_2$ and SOD at the time of reperfusion for 4 hrs. Dose of $NaNO_2$ and SOD were similar to Gr. III and Gr. IV.

Measurement of infarct size : At the end of 90 min of ischemia (Gr. I) and 4 hrs of reperfusion (Gr. II, III, IV and V), coronary artery was reoccluded. 15 ml of 5% Evans blue was infused via left atrium to stain the area perfused by patent coronary arteries. The area of risk was identified by negative staining. Animals were killed by injecting 2.56 M potassium chloride into left ventricle. Heart was excised from thorax, blotted dry and weighed. Heart was sliced parallel to atrioventricular groove in 1 cm thick sections. The unstained portions of myocardium (area at risk) was separated from the rest of myocardium and weighed. Myocardium at risk was again sectioned to 1 mm thickness and was incubated in 1% triphenyltetrazolium chloride (TTC) prepared in phosphate buffer (pH 7.4) for 30 min at 38°C to demarcate the infarcted portions of myocardium. Unstained portions by TTC were weighed, it was the area of infarction. Myocardial tissue from all groups were routinely processed by paraffin embedding technique by stardard procedures. Five micron thick sections were obtained using Riechert Autocut 2040 microtome and were stained by Haematoxylin – Eosin (H&E).

Tissue MDA (Malodialdehyde) concentration : Tissue MDA levels were estimated in area at risk and infarction by method of Kartha and Krishnamurthy (16). MDA concentration was expressed as nmol MDA/ gm of wet tissue after taking molar extinction coefficient of MDA as 1.5×10^5.

Statistical analysis : All values were presented as mean ± SEM. Comparisons were made by using unpaired student 't' test. Statistical significance was considered as $P<0.05$.

RESULTS

The data presented in Table I shows that increase in incidence of ventricular fibrillation was observed only in Gr. II and Gr. IV. Superoxide dismutase, a known free radical scavenger infused alone or in combination with acidified $NaNO_2$ prevented the reperfusion induced ventricular fibrillation. In animals, subjected only to 90 min ischemia without reperfusion (Gr.1), area at risk was 31.12 ± 3.34 without any evidence of infarction either by TTC staining or histologically (Table II and Fig. 1). In the untreated saline reperfused animals (Gr. II), the percent area at risk, percent necrosis in area at risk, percent left ventricular necrosis and viability of ischemic myocardium were 34.06 ± 3.26, 39.35 ± 7.58, 12.80 ± 2.08 and 60.64 ± 7.43 repectively (Table II). Isolated treatment with either SOD or $NaNO_2$ could not decrease the infarct size significantly in comparison with Gr.II. However, combined administration of SOD and $NaNO_2$ at the time of reperfusion (Gr. V) documented significant decrease in the area of necrosis (Table II) when compared with Gr. II (Table II). Effect of

ischemia and reperfusion on LVEDP and LVSP has been presented in Table III. LVEDP in all groups, 60 min in comparison to pre-CAO and 90 min of ischemia in comparison to 60 min was significantly higher. Reperfusion had no significant effect on LVEDP in untreated animals.

TABLE I: Effect of reperfusion in the different experimental animals.

Experimental groups	*Number of animals included in study*	*Animals died during coronary artery occlusion*	*Animal died at the time of reperfusion*	*Survival number%*	
I. (90 min CAO)	5	NIL	–	5	100*
II. (Untreated saline reperfused)	10	NIL	5†	5	50
III. (SOD treated)	5	NIL	NIL	5	100*
IV. (Acidified $NaNO_2$ treated)	10	NIL	5†	5	50^{NS}
V. (Acidified $NaNO_2$ +SOD treated)	5	NIL	NIL	5	100*

†Animals died due to ventricular fibrillation.
*P<0.001 versus Gr. II, NS – Not significant versus Gr. II

TABLE II: Effect of different treatments on area at risk, area of necrosis in area at risk, left ventricular necrosis and preservation of ischemic myocardium in experimental animals.

Experimental groups	*% area at risk*	*% area of necrosis in area at risk*	*% left ventricular necrosis*	*% preservation of ischemic myocardium*
I. (90 min CAO)	31.12 ± 3.34	NIL	NIL	NIL
II. (Untreated saline reperfused)	34.06 ± 3.26	39.35 ± 7.58	12.80 ± 2.08	60.64 ± 7.43
III.(SOD treated)	30.19 ± 3.78^{NS}	28.04 ± 5.32^{NS}	8.31 ± 1.08^{NS}	72.30 ± 4.95^{NS}
IV. (Acidified $NaNO_2$ treated)	32.00 ± 2.18^{NS}	31.14 ± 3.40^{NS}	12.85 ± 0.57^{NS}	61.89 ± 1.19^{NS}
V. (Acidified $NaNO_2$ +SOD treated)	33.42 ± 2.56^{NS}	19.20 ± 2.18*	5.63 ± 0.3*	81.96 ± 1.15*

n=5 in each group, Data are mean ± SEM.
*P<0.001 versus Gr. II, NS–Not significant versus Gr. II

TABLE III : Left ventricular end diastolic pressure (LVEDP) and left ventricular systolic pressure (LVSP) preceding and following ischemia and reperfusion.

Experimental groups		*Pressure (mm of Hg)*							
		Pre CAO	*Post CAO (min)*			*Post reperfusion (h)*			
			30	*60*	*90*	*1*	*2*	*3*	*4*
I (90 min CAO)	LVEDP	1.90±0.56	2.26±1.71^{NS}	8.44±0.70*	20.51±0.84**				
	LVSP	160.8±0.85	156.72^{NS}±5.18^{NS}	150.81±2.43^{NS}	151.00±3.12^{NS}				
II. (Untreated saline reperfused)	LVEDP	2.41±1.61	1.76±1.07^{NS}	7.11±1.09*	19.88±3.3**	13.1±1.47^{NS1}	12.32±4.24^{NS1}	13.05±2.23^{NS1}	11.62±4.71^{NS1}
	LVSP	164.4±0.45	160.88±1.89^{NS}	155.54±1.89^{NS}	154.66±2.17^{NS}	155.49±4.08^{NS1}	149.43±3.03^{NS1}	155.99±4.08^{NS1}	148.10±2.74^{NS1}
III. (SOD treated)	LVEDP	0.81±0.1	1.24±0.2^{NS}	10.40±2.40*	23.46±7.64**	4.43±1.3***	4.26±1.9***	2.13±1.3***	1.06±1.06***
	LVSP	160.8±4.3	152.70±3.28^{NS}	151.19±2.70^{NS}	142.67±5.41^{NS}	152.24±3.28^{NS1}	152.24±2.9^{NS1}	147.39±3.62^{NS1}	153.21±2.9^{NS1}
IV. Acidified $NaNO_2$ treated	LVEDP	1.5±1.10	2.91±1.06^{NS}	8.31±2.62*	20.76±3.76**	4.43±1.3***	4.26±1.9***	2.13±1.3***	1.06±1.06***
	LVSP	148.49±4.84	150.00±2.4^{NS}	151.71±4.71^{NS}	155.73±2.61^{NS}	165.32±3.37^{NS1}	154.66±3.36^{NS1}	162.13±3.98^{NS1}	155.72±7.22^{NS1}
V. Acidified $NaNO_2$ +SOD treated	LVEDP	0.19±0.11	1.87±0.19^{NS}	7.42±2.32*	18.8±2.65**	7.68±2.39***	6.4±2.02***	10.24±3.25***	10.24±2.5***
	LVSP	156.72±3.97	158.82±2.82^{NS}	160±3.92^{NS}	152.33±5.22^{NS}	152.53±3.19^{NS1}	161.06±4.88^{NS1}	155.73±4.01^{NS1}	164.27±5.16^{NS1}

N=5 in each group. Data expressed as mean ± SEM

CAO – Coronary artery occlusion, *P<0.001 versus Pre-CAO, **P<0.001 versus 60 min of ischemia, ***P<0.001 versus 90 min of ischemia.

NS – Not significant versus pre-CAO, NS1 – not significant versus 90 min of ishemia.

Treatments with SOD, $NaNO_2$ alone or in combination significantly lowered the LVEDP after reperfusion in comparison with Gr. IV (Table IV). Alone or combined infusion of SOD with $NaNO_2$ significantly decreased the MDA levels in comparison

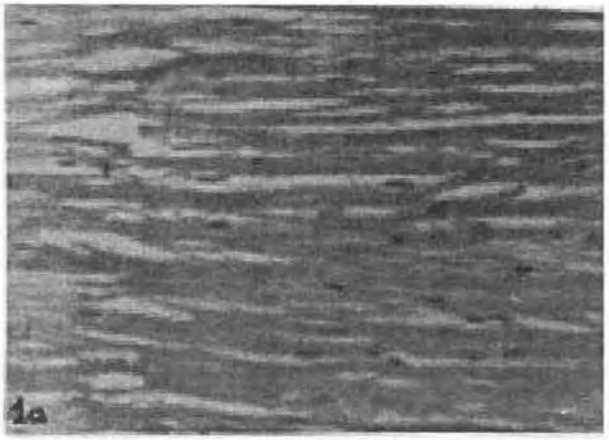

Fig.1 (a): Photomicrograph showing normal myocardium (H.E × 400).

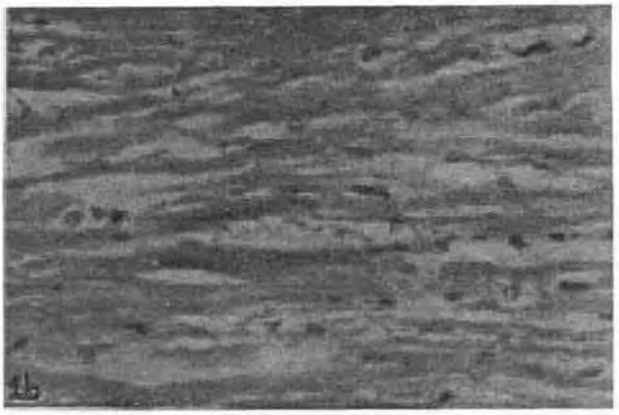

Fig.1 (b): Photomicrograph showing myocardial edema interstitial inflammatory cell infiltrate after 90 min of ischemia (H.E × 400).

the pressure at the end of 90 min of ischemia. LVSP remained unchanged in all groups. Lipid peroxidation presented as nmol MDA/gm of wet tissue weight was highest in the area of necrosis in Gr. II and with saline treated animals (Gr. II). Fig. 1 shows the histological changes following 90 min of ischemia. Increase in the number of inflammatory cells, mild edema was

TABLE IV: Malondialdehyde (MDA) levels in area at risk and in area of necrosis following ischemia and reperfusion.

Experimental groups	*MDA(nmol/gm of wet tissue)*	
	Area at risk	*Area of infarction*
I. (90 min CAO)	7.8 ± 3.24	–
II.. (Untreated saline reperfused)	5.12 ± 2.22	20.90 ± 1.72
III. (SOD treated)	9.40 ± 2.16^{NS}	16.14 ± 1.20*
IV. (Acidified $NaNO_2$ treated)	8.3 ± 1.3^{NS}	19.26 ± 1.16^{NS}
V. (Acidified $NaNO_2$+ SOD treated)	7.82 ± 2.78^{NS}	15.18 ± 1.08*

n=5 in each group, Data presented as mean ± SEM

*P < 0.001 versus Gr. II, NS – Not significant versus Gr. I

observed after 90 min of ischema (Fig. 1b) whereas reperfusion of ischemic myocardium resulted in severe edema, myocardial cell separation, haemorrhage and contraction band necrosis (Fig. 2 a, b).

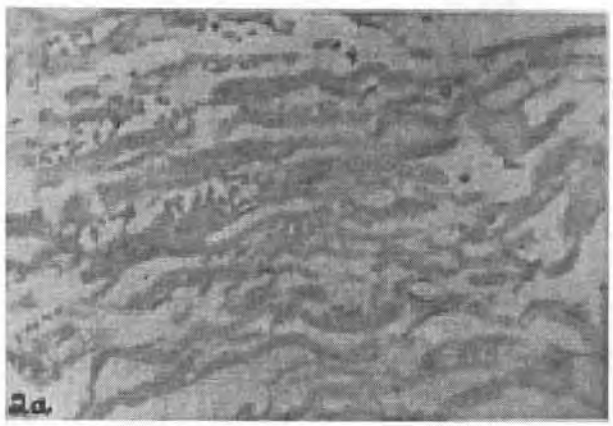

Fig.2 (a): Photomicrograph showing interstitial hemorrhages in the myocardium after 90 min ischemia and 4 hrs reperfusion (H.E × 800).

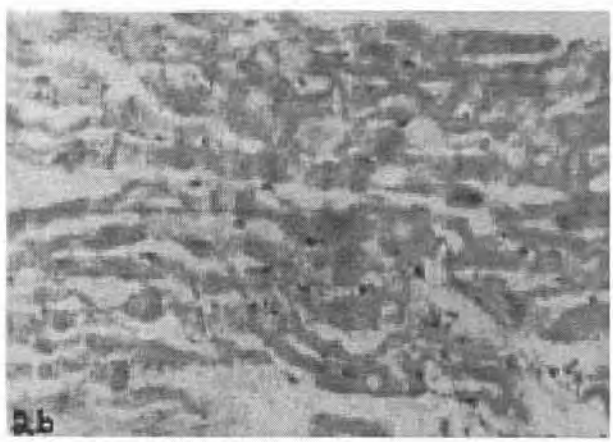

Fig.2 (b): Photomicrograph showing marked separation of myocardial fibres, inflammatory cell infiltrate and contraction band necrosis of myocardium (H.E × 800).

DISCUSSION

Myocardial ischemia occurs whenever there is imbalance between oxygen supply and demand. Early reperfusion of the myocardium might be necessary for the survival of ischemic tissue however, reperfusion can also be detrimental by causing irreversible damage to ischemic myocardium known as reperfusion injury (1–4). Release of toxic oxygen free radicals have been implicated in the development of reperfusion injury (4–6). To prove the existence of lethal reperfusion injury, it is necessary to show that cells are killed by reperfusion after ischemia.

In the present study, 90 min of ischemia failed to produce any qualitative or quantitative evidence of necrosis (Fig. 1a and b). It was observed that following ischemia for ninety min cell were still viable. Reperfusion in previously ischemic myocardium, resulted in hemorrhage, myocardial cell separation, severe edema and contraction band necrosis, which are irreversible changes (Fig. 2a and b). Therefore, it is evident that reperfusion after ninety min of ischemia causes irreversible damage to myocytes.

It has been suggested that univalent reduction of molecular oxygen results in production of reactive oxygen species i.e. oxygen free radicals (6). Superoxide dismutase is one of the protective enzymes to prevent damage by free radicals (5). In canine model, myocytes and endothelial cells are affected whenever the duration of ischemic injury exceeds 60 min (17). Therefore, an important aspect of reperfusion induced injury which might affect the endothelium, decrease in the

endothelium derived relaxing factor (7). Acidified sodium nitrite at pH 2 forms nitric oxide which has identical biological poperties to EDRF (9). Nitric oxide is a potent vasodilator and is thought to be EDRF itself (8, 9). Further, nitric oxide has been shown to inactivate superoxide radicals (18).

In the present study, analysis of myocardial tissue showed that isolated infusion with saline, $NaNO_2$ or SOD resulted in relatively larger infarcts in comparison with the group which received combined infusion of SOD and $NaNO_2$ preceding reperfusion. Combined treatment prevented significant amount of ischemic tissue from becoming necrotic to a greater extent whether calculated as a percentage of area at risk or as percentage of the total weight of the ventricle (Table II). A number of studies have examined the role of SOD and other antioxidants in limiting infarct size following ischemia and reperfusion (10–15). The results were conflicting (10–15). The reasons for the observed discrepancies are not known. Failure of SOD in limiting infarct size in the present study might have been due to many factors. In the present study, animals received loading dose of SOD (25000 IU/kg) preceding reperfusion upto an hr followed by 15000 IU/kg for remaining 3 hrs. Therefore, failure of SOD to limit infarct size cannot be ascribed to insufficient dosage. Previous studies have reported beneficial effects of SOD by using less dosage (13, 14,). Another possibility can be due to failure of exogenously administered SOD to get across the cell membrane. SOD is a dimeric protein having molecular weight of 32,000 daltons (19). Although, SOD under normal circumstances may not enter the cell completely but in stressful conditions like ischemia, it can enter into endothelial cells via pinocytotic activity or into the interstitium because of ischemia induced increased vascular permeability. In the present study, lipid peroxidation, a marker of free radical mediated injury, was significantly low in SOD treated animals in comparison with untreated group. It shows that adequate tissue levels of SOD were available throughout the reperfusion.

Left ventricular and diastolic pressure which represents preload, was maximum after 90 min of ischemia and remained unchanged during period of reperfusion in saline treated animals (Table III). Markedly improved left ventricular performance was observed in animals treated either with NaNO , SOD or that which received combined infusion of $NaNO_2$ and SOD. It was probably due to free radical scavenging property of superoxide dismutase and vasodilatory actions of acidified sodium nitrite, a releaser of nitric oxide.

Myocardial tissue lipid peroxidation, presented as malondialdehyde (MDA), increased after reperfusion in saline treated animals. Whereas, in other groups, except in $NaNO_2$ treated animals, the MDA formation was significantly low (Table IV). The observed decrease in MDA formation might have been due to free radical scavenging actions of SOD and nitric oxide liberated from acidified $NaNO_2$. Nitric oxide has been shown to inactivate superoxide radicals (18).

In conclusion, the presented results demonstrate that reperfusion in previously ischemic myocardium can cause injury *de novo*. Combination of superoxide dismutase and acidified sodium nitrite reduces

infarct size and lipid peroxidation after ischemia and reperfusion. There might be different mechanisms by which superoxide dismutase and acidified sodium nitrite, a liberator of nitric oxide can offer cardioprotection, probably by a direct action either on endothelium or on myocardial cells or both.

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