Indian J Physiol Pharmacol 1998; 42(1) : 50–56

EFFECT OF N–ACETYLCYSTEINE ON MYOCARDIAL INFARCT SIZE FOLLOWING ISCHEMIA AND REPERFUSION IN DOGS

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(Received on June 30, 1997)

Abstract : The present study was designed to examine the role of N–acetylcysteine (NAC) on free radical mediated reperfusion injury in canine model. Forteen dogs underwent 90 min of left anterior descending coronary artery (LAD) occlusion followed by 4 h of reperfusion. Treated animals received loading dose of NAC (250 mg/kg) at the time of reperfusion upto 1 h followed by maintenance dose (70 mg/kg) for remaining 3 h through left atrial line. Infarct size, myocardial tissue lipid peroxidation, superoxide dismutase (SOD) and glutathione (GSH) levels were measured at the end of reperfusion in treated (n=7) and untreated animals (n=7). Left ventricular end diastolic pressure was significantly lower in treated animals compared to untreated group. SOD and GSH levels in myocardial tissue at risk and in infarcted zone were similar in both groups. However, in NAC treated animals the lipid peroxidation was significantly lower in comparison to untreated control animals. Infarct size in the area at risk, percent left ventricular necrosis and myocardial tissue preservation were not significantly different in treated and untreated animals. These results suggests that N–acetylcysteine infusion at the time of reperfusion following 90 min of ischemia and 4 h of reperfusion fails to offer significant cardioprotection against free radical damage but it can improve ventricular performance by decreasing pre load.

Key words : ischemia, reperfusion, N–acetylcysteine, superoxide dismutase, lipid peroxidation, infarct size, glutathione, cardioprotection

INTRODUCTION

Imbalance between oxygen supply and demand results in myocardial ischemia. Ischemia depletes antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase and makes the myocardial cells vulnerable to oxidant mediated damage (1, 2). Reperfusion might be a prerequisite for the salvage of ischemic myocardium. However, reperfusion in previously ischemic myocardium results in formation of superoxide radical (O_2^-), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) (3, 4). Studies examining the role of oxygen free radical scavengers on myocardial infarct size have shown conflicting results (5–11). N–acetylcysteine

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is a low molecular weight compound and precursor of glutathione (12). It has been suggested that N-acetylcysteine can replenish glutathione stores, increase superoxide dismutase activity, scavenge hydroxyl radicals and interferes with autocatalytic lipid peroxidation (13). Therefore, with the main objective of the study to examine the effects of N-acetylcysteine on infarct size, lipid peroxidation, superoxide dismutase and glutathione status following ischemia and reperfusion, this study was conducted.

METHODS

Male adult mongrel dogs (10 to 15 kg) were used in the study. Animals were anesthetised with intravenous pentobarbital sodium (30 mg/kg, bw) and were ventilated with room air by using INCO positive pressure ventilator. Chest was opened through fourth intercostal space and heart was suspended in pericardial cradle. A polythene catheter (1.5 mm inner diameter) was placed in the left ventricle through apex to record ventricular pressure changes on Grass (model 78 D) using gould P 231 transducer. Heart rate and ST segment changes were monitored in limb lead II ECG recorded on BPL electrocardiograph.

Coronary artery occlusion and reperfusion: Left anterior descending (LAD) coronary artery was dissected free above the first diagonal branch and below left circumflex artery. LAD coronary artery was occluded for 90 minutes with a vessel occluder. Following 90 min of occlusion, occluder was removed to facilitate reperfusion of the ventricle. No attempt was made to resuscitate the animals which developed ventricular fibrillation at the time of reperfusion.

Plan of experiment : Group I (Untreated control) : Seven animals underwent 90 min of LAD coronary occlusion and 4 h of saline infusion (115 ml, 0.9% Nacl) at the time of reperfusion through left atrium.

Group II (NAC treated) : In this group, duration of LAD occlusion and reperfusion was similar to Gr. I exept treatment with NAC. N-acetylcysteine (Sigma Chemical Company) was dissolved in 115 ml of saline. Seven animals received loading dose of NAC (250 mg/kg, bw) at the time of reperfusion upto 1 h followed by maintenance dose (70 mg/kg, bw) for remaining 3 h through left atrium.

Quantification of infarct size : At the end of 4 h of reperfusion, the left anterior descending coronary artery was completely re-occluded. 15 ml of 5% Evans blue dye was infused via left atrium to stain the area perfused by patent coronary arteries. The myocardial tissue at risk was identified by gross examination of the ventricle. It was the area which did not take the Evans blue stain. Animals were killed by injecting 2.56M potassium chloride directly into the left ventricle. Heart was excised from thorax rapidly and subsequently greater vessels above atrioventricular groove were removed. The heart was washed by normal saline, blotted dry and weighed. The heart was sliced parallel to atrioventricular groove to 1 cm thick sections. The unstained portions of myocardium (tissue at risk) was separated from rest of the myocardium and weighed. The separated portions of myocardium at risk were once again sectioned to 1 mm

thickness and incubated in 1% solution of triphenyl tetrazolium chloride (TTC) prepared in phosphate buffer (pH 7.4) for 30 min at 38°C to demarcate the infarcted portions of myocardium (14). In viable myocardium TTC is converted by dehydrogenase to a red formazan pigment that stains tissue dark red (15). The injured (necrotic) tissue of myocardium which did not take TTC stain was separated from ischemic myocardium and weighed. Thus, myocardial tissue unstained by Evans blue represented the zone at risk and unstained portions of myocardium by TTC stain showed the infarcted zone.

Biochemical estimations : Myocardial tissue peroxidation, GSH and SOD levels in tissue at risk and infarction were measured by standard methods (16–19). 2–thiobarbituric acid (TBA), purchased from Loba Chemicals was purified before assay. TBA solution (0.67%) was mixed with neutral alumina and was left overnight. Next day it was filtered by using Whatman filter paper. Values of TBA reactive materials were expressed in terms of n mol of MDA (malondialdehyde)/gm of wet tissue after taking molar extinction coefficient of MDA as 1.56×10^5 (17). Glutathione concentrations were expressed as µg/g of tissue protein. One unit of superoxide dismutase was defined as the amount of enzyme required to inhibit reduction of nitrobluetetrazolium (NBT) by 50% under specific conditions and results were calculated in terms of SOD units/mg of tissue protein. Protein was estimated by Lowry's method (20).

Histopathological analysis : Myocardial tissue at risk and infarction in both groups were routinely processed by paraffin embedding technique. Five micron thin sections were obtained by using Riechert Autocut 2040 microtome and were stained by Haematoxylin and Eosin. Degree of myocardial injury was assessed by studying parameters like inflammatory infiltrate, edema, fatty cell infiltration, hemorrhagic changes and development of contraction band necrosis in infarcted and non infarcted zones of myocardium. An average of 10 high power fields per slide were examined. A score, 0 for no change, +1 for mild, +2 for moderate and +3 for severe changes were assigned.

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

RESULTS

Table I shows that in NAC treated animals, LVEDP was significantly low after 4 h of reperfusion (11.62 ± 2.56) whereas LVSP did not change in comparison to untreated control group following ischemia and reperfusion (153.2 ± 2.9 vs 159.7 ± 3.2). Myocardial tissue MDA levels in zone at risk and infarction is presented in Fig. 1. It shows that MDA formation in the zone of infarction in comparison to zone at risk was significantly higher in untreated control animals (19.84 ± 1.78 vs 8.12 ± 0.1, $P<0.001$). NAC treatment at the time of reperfusion had significant effect on MDA levels in the zone of infarction when compared with control (14.31 ± 1.24 vs 19.84 ± 1.78, $P<0.001$). A significant decrease in SOD and GSH levels in the zone of infarction in

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

Experimental groups	*Left ventricular pressure (mm of Hg)*	*Pre CAO*	*Post CAO (min)*			*Post reperfusion (h)*			
			30	60	90	1	2	3	4
I (Untreated control)	LVSP	158.6 ± 2.8	154.72 ± 1.78	160.4 ± 3.34	156.7^{NS} ± 5.16	159.5^{NS1} ± 2.29	160.87^{NS1} ± 3.46	156.2^{NS1} ± 2.7	159.7^{NS1} ± 3.2
	LVEDP	2.37 ± 1.41	1.46 ± 1.1	8.31* ± 1.6	20.64** ± 2.4	14.32^{NS1} ± 3.8	16.4^{NS1} ± 2.6	17.68^{NS1} ± 1.86	15.86^{NS1} ± 3.6
II (NAC treated)	LVSP	156.3 ± 4.38	160.45 ± 3.34	156.4 ± 2.66	151.00^{NS} ± 3.2	152.24^{NS1} ± 2.8	156.6^{NS1} ± 2.42	157.38^{NS1} ± 3.7	153.2^{NS1} ± 2.9
	LVEDP	1.94 ± 0.64	2.4 ± 1.2	9.6* ± 1.44	19.2** ± 1.9	10.17*** ± 1.8	9.3*** ± 1.62	12.14*** ± 2.34	11.62*** ± 2.56

n =7 in each group, Data expressed as Mean ± SEM, CAO –Coronary artery occlusion
*P<0.001 versus Pre–CAO, **P<0.001 versus 60 min; ***P<0.001 versus 90 min.
NS – Not significant versus 60 min, NS1 –Not significant versus 90 min.

TABLE II : Infarct size, % left ventricular necrosis and % ischemic myocardial preservation following NAC treatment.

Experimental groups	*% Zone at risk*	*% zone of infarction in area at risk*	*% left ventricular necrosis*	*% ischemic myocardial preservation*
I (Untreated control)	32.83 ± 3.34	42.31 ± 9.44	13.47 ± 3.82	58.97 ± 8.29
II (NAC treated)	35.57 ± 2.97^{NS}	37.35 ± 4.26^{NS}	8.76 ± 2.65^{NS}	66.23 ± 5.27^{NS}

n=7 in each group
Values are Mean ± SEM, NS : Not significant versus Gr. 1

TABLE III : Changes in histopathological parameters following ischemia and reperfusion.

Histopathological parameters	*Zone at risk*		*Zone of infarction*	
	control (Gr. I)	*NAC treated (Gr. II)*	*Control (Gr. I)*	*NAC treated (Gr. II)*
Inflammatory infiltrate	0	0	+3	+2
Edema	+1	+1	+3	+3
Fatty cell infiltration	0	0	+2	+2
Hemorrhagic changes	0	0	+3	+3
Contraction band necrosis	0	0	+3	+3

comparison to zone at risk was observed in untreated control animals (Fig. 2). In NAC treated animals, the GSH and SOD levels in the zone of infarction were not statistically different than controls (Fig. 2). NAC infusion at the time of reperfusion could not prevent ischemic myocardium from becoming necrotic whether calculated as percentage of necrosis in the zone at risk or percentage of the total weight of the ventricle (Table II). Further, percent preservation of ischemic myocardium in treated and untreated groups were similar. Analysis of histopathological parameters presented in Table III shows that the degree of inflammatory infiltrate, fatty cell infiltration, hemorrhage and development of contraction band necrosis was same in two groups.

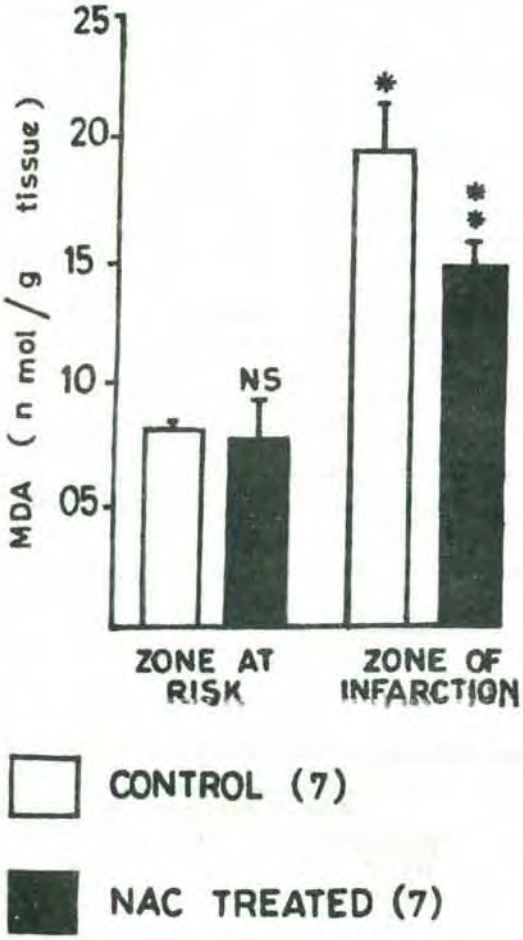

Fig. 1 : Tissue MDA levels in the zone at risk and infarction. Figures in paranthesis show the number of animals used in the study. Values are mean ± SEM.*P < 0.001 vs zone at risk, **P<0.001 vs control; NS – Not significant versus control.

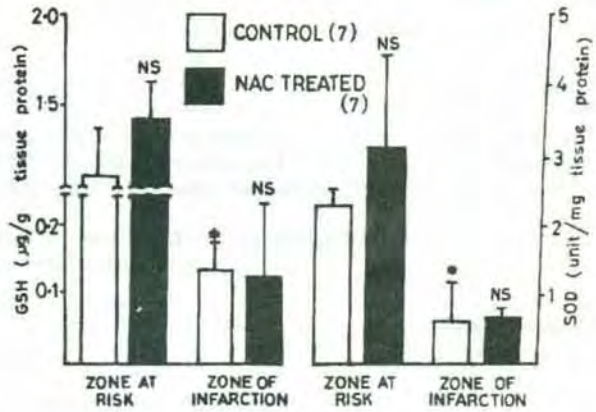

Fig. 2 : Tissue GSH and SOD levels in the zone at risk and infarction. Figures in parenthesis show the number of animals used in the study. Values are mean ± SEM. *P<0.001 vs zone at risk, NS – Not significant versus control.

DISCUSSION

The univalent reduction of molecular oxygen leads to the formation of oxygen free radicals (3). The free radicals can alter cellular functions by oxidation of cellular lipids, proteins and nucleic acids (3). Reperfusion of ischemic myocardium following prolonged ischemia is associated with impairment of mechanical functions and increased formation of free radicals (2). The consequences of reperfusion include the

conversion of reversibly injured myocyte to irreversibly injured myocytes known as reperfusion injury (1, 2). Number of studies have examined the effect of free radical scavengers on infarct size. While there are many positive studies, there are as many negative reports which failed to document beneficial effects of free radical scavengers (5–11). The reasons for such diversities are unknown.

In the present study, N–acetylcysteine given at the time of reperfusion failed to reduce infarct size in comparison to untreated control animals. However, a significant reduction in LVEDP which represents pre load, was observed in NAC treated animals. On the contrary, LVSP remained unchanged in both groups. It might have been due to localised cardiac muscle damage in the set up of experimentally induced myocardial infraction. Some studies have suggested that endogenous glutathione is a important enzyme in protecting ischemia induced reperfusion injury (21, 22). On the other hand, Forman et al (7) showed that N–acetylcysteine does not limit infarct size after 90 min of ischemia and 24 h of reperfusion and concluded that free radicals are not primary mediators of myocardial reperfusion injury. In order to evaluate the role of free radicals in reperfusion injury estimation of lipid peroxidation, a known marker of free radical injury was carried out apart from measuring endogenous antioxidants like glutathione and superoxide dismutase. Marked increase in lipid peroxidation and reduction in SOD and GSH content in necrotic myocardium in untreated animals following 90 min of ischemia and 4 h of reperfusion in our study suggests the involvement of free radicals in reperfusion injury. We have earlier shown that reperfusion of ischemic myocardium results in reperfusion injury (23). Glutathione peroxidase is more active enzyme than catalase in protecting the cell from oxidant mediated damage (24). N–acetylcysteine is precursor of glutathione and can increase cytoplasmic superoxide dismutase activity, scavenges hydroxyl free radicals, decreases lipid peroxidation and can inhibit neutrophil mediated free radical production (12, 13, 24, 25). In the present study failure of N–acetylcysteine in limiting infarct size, increasing glutathione and superoxide dismutase levels in necrotic myocardium following ischemia and reperfusion suggests one of the following possibilities: 1) Failure of NAC to reach to the ischemic zones of myocardium, 2) NAC when used alone cannot provide sufficient cardioprotection against the attack of free radicals. In our present study, N–acetylcysteine significantly decreased the lipid peroxidation in treated animals. Therefore, failure of NAC in limiting infarct size can not be ascribed to dosage used or to the permeability of the drug. Reduction of lipid peroxidation in treated animals shows that NAC treatment to some extent was effective against free radical attack.

Several mechanisms have been suggested for the formation of free radicals in a biological system. It includes xanthine oxidase (6), activated netrophils (26), direct donation of electron from myocardial electron transport chain (27), catecholamine oxidation (28), cyclo-oxygenase and lipoxygenase enzymes (29). Therefore, infusion of an individual antioxidant might not be beneficial against multifactorial free

radical attack. We have earlier shown that combination of scavengers can offer significant cardioprotection (23).

In conclusion, the results of the present study shows that N-acetylcysteine treatment at the time of reperfusion fails to offer cardioprotection against oxidant mediated free radical induced cardiac damage. N-acetylcysteine might play a significant role in improving ventricular performance by decreasing pre load.

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