Indian J Physiol Pharmacol 2004; 48 (2): 199–205

LIPID PEROXIDATION, HEMOLYSIS AND ANTIOXIDANT ENZYMES OF ERYTHROCYTES IN STROKE

SUDHA K.[a], ASHALATHA V. RAO[b] , SURYANARAYANA RAO[c] AND ANJALI RAO[d]*

[a] *Department of Biochemistry, Kasturba Medical College, Mangalore – 575 001*

[b] *Department of Biochemistry, Father Mullers Medical College, Mangalore – 575 001*

and

Departments of [c]Neurology and [d]Biochemistry, Kasturba Medical College and Hospital, Manipal – 576 104

(Received on June 2, 2003)

Abstract : Erythrocyte membrane lipid peroxidation and consequent percentage hemolysis and related antioxidant enzymes viz., superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase were determined in 16 cases of hemorrhagic stroke and 30 cases of thrombotic stroke. The results obtained were compared with 50 age and sex matched controls. 12 thrombotic stroke patients who showed symptomatic recovery after medication were considered for follow up. Lipid peroxidation and percentage hemolysis in patients with thrombotic stroke and hemorrhagic stroke was significantly elevated when compared to controls. Glutathione reductase and superoxide dismutase levels were found to be significantly reduced in thrombotic stroke and hemorrhagic stroke respectively, when compared to healthy subjects. There was no significant difference in the other parameters when compared to controls. In post treatment thrombotic stoke, catalase and glutathione reductase levels increased significantly and oxidative hemolysis decreased compared to their pretreatment values. Thus, our results indicate considerable oxidative stress in stroke.

Key words : stroke lipid peroxidation antioxidant enzymes

INTRODUCTION

Oxidative stress may be implicated in pathophysiology of a number of neurological diseases like epilepsy, tubercular meningitis, Alzheimer's disease (1, 2, 3). Among the various biochemical events associated with these conditions emerging evidence suggests increased free radical production and consequent lipid peroxidation as a main cause of cell death (4). These free radicals are scavenged in vivo by a wide range of antioxidants such as vitamins E and C, carotenoids, glutathione (GSH) and

*Corresponding Author

antioxidant enzymes like glutathione peroxidase (GP), superoxide dismutase (SOD), catalse (CT) and glutathione reductase (GR, 5).

Recent studies, primarily in animals suggest that vascular injury noted in acute hypertension, fluid percussion brain injury and ischemia may also be due to formation of free radicals (6, 7, 8). During ischemia, when oxygen supply is limited , calcium influx may activate phospholipase C which results in breakdown of membrane phospholipids (9, 10) or may convert xanthine dehydrogenase to xanthine oxidase in the cerebral blood vessels (11) leading to the formation of superoxide radicals or hydrogen peroxide.

There are several reports on free radical damage in cerebrovascular accidents (CVA) in humans (12, 13) but there is a paucity of data regarding the effect of free radicals on erythrocytes of stroke patients. Hence, the present study aims at exploring oxidative stress in erythrocytes of such patients.

METHODS

Blood samples were collected from stroke patients meeting following criteria:

(1) Intra cerebral hemorrhage or cerebral thrombosis diagnosed by brain CT.

(2) No blood in ventricular system or subarachnoid space on brain CT.

(3) No evidence of organic heart disease or other possible cause of cerebral embolism.

(4) Patients suffering from diabetes mellitus or having habits like cigarette smoking, alcoholism etc. were excluded from this study.

There were 46 patients in this study aged between 40 to 60 years, including 16 cases of intracerebral hemorrhage (males 10, females 6); 30 cases of cerebral thrombosis (males 21, females 9). Fifty age and sex matched healthy subjects formed the control group. 12 thrombotic stroke patients were considered for follow up studies after one month of treatment. These patients received drugs aspirin (150 mg/day) or clopidogrel (75 mg/day) or warfarin (5 mg/day).

Random blood samples were collected in EDTA bottles from normal subjects and stroke patients. Sampling was performed between 24 to 48 hours of acute phase of completed stroke. Blood was centrifuged at 3000 g for 10 minutes. Plasma was separated, buffy coat was carefully removed and separated erythrocytes thrice with 0.01 M saline phosphate buffer pH 7.4 (containing 0.15 M NaC1), then diluted 1:2 with the same buffer and stored as 50% suspension at 4–5 °C. The hemoglobin content of the erythrocytes was determined by cyanmethemoglobin method. Erythrocyte enzymes were estimated in hemolysate prepared by the addition of 4 ml distilled water to 1 ml RBC suspension. The reaction mixture for GR estimation contained 1.6 ml of 0.067 mM phosphate buffer pH6.6, 0.12 ml of 0.06% NADPH, 0.12 ml of 1.15% oxidized glutathione (GSSG) and 0.1 ml of the hemolysate. The reaction mixture for GP activity measurement consisted of 50 mM phosphate buffer pH 7, 1 mM EDTA, 1 mM sodium azide, 0.2 mM NADPH, 1 mM GSH, 0.25 mM hydrogen peroxide (H_2O_2) and one enzyme unit of GR/ml reaction mixture. Both GR and GP activities were determined by recording the decrease in absorbance due to depletion of NADPH for a period of 5 minutes at 340 nm (14, 15). SOD was determined according to the method of Beauchamp and Fridovich (16) based on inhibition of nitroblue tetrazolium (NBT)

reduction. 0.1 ml of the hemolysate was added to a reaction mixture containing 5.6×10^{-5} M NBT, 1×10^{-2} M methionine, 1.17×10^{-6} M riboflavin in 3 ml 0.05 M phosphate buffer. The mixture was illuminated for 10 mins. and absorbance was read at 560 nm. CT activity in the hemolysate was determined by the method of Brannan et al (17), which is based on the disappearance of H_2O_2 in the presence of enzyme source at 26°C. 0.2 ml 0.05 M phosphate buffer, pH 7 containing 1.2 mM H_2O_2. At the end of 25th minute the reaction was stopped by the addition of 2.5 ml of peroxidase reagent containing peroxidase and chromogen system. The lipid peroxidation and oxidative hemolysis of RBC were determined by incubating 1 ml RBC suspension in 9 ml saline phosphate buffer containing 0.44 mol/l H_2O_2 at 37 °C for 2 hours (18, 19). 2.5 ml aliquots were withdrawn from the above mixture at 0 hour and at 2 hours, treated with 1 ml 28% TCA containing 0.1 M sodium meta arsenite and centrifuged. Then 3 ml of supernatant was boiled with 1 ml of 1% thiobarbituric acid (TBA) for 15 mins. Absorbance of pink chromogen was a measure of malondialdehyde (MDA) produced.

GR (EC No. 1.6.4.2) and GSSG were purchased from Sigma Chemical Co. USA, NBT and TBA were from Loba Chemicals, India. All other chemicals used were commercial products of highest purity.

Data was analysed statistically by Mann - Whitney U - test and Wilcoxon rank sum test. The difference of $P<0.05$ was considered significant.

RESULTS

RBC lipid peroxidation was significantly high in hemorrhagic stroke both at 0 hour and at 2 hours compared to controls. In thrombotic stroke lipid peroxidation was statistically significant only at 2 hours of incubation of RBC with H_2O_2 (Table I). The percentage hemolysis was significantly high both in thrombotic stroke and hemorrhagic stroke at 0 hour as well as after 2 hours of incubation of RBC with H_2O_2 when compared to normal (Table I). Both lipid peroxidation and oxidative hemolysis values of post treated patients did not show any statistical significance compared to their pretreated condition. However, the percent hemolysis values of post treated patients showed a tendency to decrease at both 0 hour and 2 hours of incubation of RBC with H_2O_2 when compared to their pretreated values (Table II).

The erythrocyte GR activity decreased in case of hemorrhagic stroke and thrombotic stroke compared to the normal subjects (Table III). A comparison of

TABLE I: Erythrocyte lipid peroxides and oxidative hemolysis in stroke (Mean±SEM).

	Hour	*Normal (n=47)*	*Thrombotic stroke (n=30)*	*Hemorrhagic stroke (n=16)*
MDA	0	1426±86	1682±155	1821±139[d]
(nmol/dl RBC)	2	3159±193	4663±252[a]	4943±336[a]
% Hemolysis	0	1.47±0.19	3.93±0.72[c]	4.53±1.24[d]
	2	8.18±0.79	18.96±2.73[b]	15.67±3.08[d]

[a]$P<0.0001$, [b]$P<0.001$, [c]$P<0.01$, [d]$P<0.05$ significantly different from normal.
n = sample size

TABLE II: Follow up studies of in vitro lipid peroxidation and percentage hemolysis in thrombotic stroke (Mean±SEM).

	Hour	*Before treatment (n=12)*	*After treatment (n=12)*	*P*
MDA	0	1820±187	2165±193[b]	NS
(nmol/dl RBC)	2	4531±443	5230±530[d]	NS
% Hemolysis	0	5.96±1.71	4.68±0.67[a]	NS
	2	23.76±6.87	22.47±4.26[c]	NS

[a]P<0.0001, [b]P<0.001, [c]P<0.01, [d]P<0.05 significantly different from normal.
n = sample size

TABLE III: Comparison of erythrocyte antioxidants enzymes in patients with stroke (Mean±SEM).

	Normal	*Thrombotic stroke*	*Hemorrhagic stroke*
GR	1.08±0.07	0.69±0.08[a]	0.71±0.15
(µmol NADPH Oxidised/min/gHb)	n = 47	n = 29	n = 16
GR	7.94±0.47	8.14±0.89	9.76±1.16
(µmol NADPH Oxidised/min/gHb)	n = 43	n = 27	n = 14
SOD	5235±239	4523±336	4016±350[b]
(U/gHb)	n = 46	n = 30	n = 16
Catalase	148550±16686	118104±9355	125496±13641
(U/gHb)	n = 50	n = 23	n = 14

[a]P<0.0001, [b]P<0.05 significantly different from normal.
n = sample size

TABLE IV: Comparison of erythrocyte antioxidant in thrombotic stroke before and after treatment (Mean±SEM).

	Before treatment	*After treatment*	*P*
GR (µmol NADPH Oxidised/min/gHb)	0.68±0.1	1.08±0.16	<0.05
GP (µmol NADPH Oxidised/min/gHb)	7.23±0.79	12.05±1.97	NS
SOD (U/gHb)	4181±409	4857±255	NS
Catalase (U/gHb)	117792±34240	228019±40672	<0.0001

n = sample size, NS = Not significant.

reductase levels between thrombotic stroke and corresponding post treatment cases showed a significant increase in the latter (Table IV). GP activity remained unaltered both in hemorrhagic and thrombotic stroke compared to control values (Table III). SOD activity was significantly low in hemorrhagic stroke when compared to healthy controls (Table III). Though, the enzyme activity was low in thrombotic

stroke compared to normal subjects it was not statistically significant. In both types of stroke, the CT activity showed a tendency to decrease when compared to controls (Table III). On the contrary, the enzyme activity in the follow up patients was significantly higher when compared to the diseased condition (Table IV).

DISCUSSION

Free radical formation and subsequent oxidative damage in the form of lipid peroxidation has been implicated in the injury that occurs to the brain tissue in response to ischemia and reperfusion insults. It has been suggested that lipid peroxidation may be an important pathophysiological event in CNS ischemia or trauma (20). Ischemia induced by occlusion of both common carotid arteries showed a significant increase in thiobarbituric acid reactive substances (TBARS) in rat brain (21). The present study on RBC of stroke patients showed a similar result. The lipid peroxidation increased at 0 hour as well as after 2 hours of incubation of RBC with H_2O_2 in both thrombotic stroke and hemorrhagic stroke patients. The above finding is justified by Satoh (22) and Polidori et al (12) who observed significantly increased serum MDA levels in stroke compared to controls. Lipid peroxidation products in CSF were significantly high in acute phase of stroke patients as compared to normal subjects (13).

Any free radical formed under ischemic condition could contribute to cellular damage by reacting with phospholipid membrane and disrupting cellular transport process (23). Lipid peroxidation of the membrane causes RBCs to lose their ability to change shape and squeeze through the smallest capillaries and eventually lead to hemolysis. Results of the present study show a significant increase in percentage hemolysis both at 0 hour and at 2 hours of incubation of RBC with H_2O_2 in both types of stroke.

Superoxide anion, which is believed to be one of the initiators of free radical reactions plays an important role in the pathogenesis of vasogenic edema in cerebral ischemia (24). Hence, the decrease in activity of the enzyme may be one of the events in early phase of stroke patients, in the present study. Earlier studies indicate that products of membrane lipid peroxidation and other oxidants like H_2O_2 may react with SOD resulting in oxidative modification thereby causing loss of enzyme activity (25). There is a report showing 55–68% decline in SOD activity in rat brain used as a model for cerebral ischemia produced by middle cerebral artery occlusion compared to control brain (26). Intra-arterial injection of SOD to the ischemic brain tissue improves neuronal functions in rats, subjected to transient global cerebral ischemia (27).

At physiological rates of H_2O_2 generation, the GP/reduced glutathione (GSH)/GR system is more important in catabolizing H_2O_2 in RBC. Glutathione reserves can be depleted by oxidative stress and such depletion in brain can cause neurological deficits. In the present study, an apparent increase in erythrocytic GP and a significant decrease in GR activity demonstrate a possible depletion of GSH within the erythrocytes of patients with stroke. Supporting this view, one of the

earlier reports indicates decreased necrotic and apoptotic cell death in ischemic brains of transgenic mice overexpressing human GP (28). Crack et al (29) also suggest that GP contributes to neuroprotection in SOD transgenic mouse. GSH depletion results in cell death, leading to increased hemolysis. Moreover, the RBC CT levels in stroke also showed a tendency to decrease when compared to controls. An issue of potential interest of this study is the fact that both ischemic and hemorrhagic stroke patients exhibited almost the same level of oxidative damage despite the different nature of neurological lesion.

In the present study, 12 thrombotic stroke patients who were treated with aspirin/clopidogrel/warfarin and who showed clinical recovery over a period of one month were considered for follow up studies. The concentration of lipid peroxides in follow up patients remained significantly elevated compared to control group both at 0 hour and 2 hours. The studies of El Midaoui et al (30) and Nielsen et al (31) demonstrated the protective effect of aspirin in oxidative damage caused by free radicals. 5–amino salicylic acid acted as an antioxidant drug in patients with inflammatory bowel disease but did not in those with rheumatoid arthritis (31). Therefore antioxidant action of aspirin may also be tissue specific. Lipid peroxidation values of the post treated cases did not show significant change compared to their pretreatment condition in the present study. However, the percentage hemolysis of post treated patients, showed a tendency to decrease both at 0 hour and at 2 hours of incubation of RBC with H_2O_2 when compared to their pretreatment values.

The erythrocyte antioxidant enzymes namely, GR, SOD and CT have increased in follow up cases compared to their pretreatment condition. Further, the increase in GR and CT values are statistically significant compared to the pretreatment values. GR level which has significantly decreased at the onset of stroke, has increased to normal range after treatment. This increase in GR probably has resulted in the regeneration of GSH which could have eventually decreased percentage hemolysis in follow up patients.

On the whole, it can be proposed that the antioxidant defence capacity of RBC which had decreased at the onset of stroke has increased to the normal range after treatment. The present study clearly supports the fact that erythrocytes of stroke patients are under oxidative stress.

REFERENCES

1. Sudha K, Rao AV, Rao A. Oxidative stress and antioxidants in epilepsy. *Clin Chim Acta* 2001; 303: 19–24.
2. Sudha K, Rao AV, Rao SN Rao A. Oxidative stress and antioxidants in tubercular meningitis. *Ind J Clin Biochem* 2002; 17(1): 34–41.
3. Maier CM, Chan PH. Role of superoxide dismutases in oxidative damage and neurodegenerative disorder. *Neuroscientist* 2002; 8(4): 323–334.
4. Halliwell B, Gutteridge JM, Cross CE. Free radicals, antioxidants and human disease. Where are we now ? *J Lab Clin Med* 1992; 119: 598–620.
5. Halliwell B, Gutteridge JM, Cross CE. The antioxidants of human extracellular fluids. *Arch Biochem Biophys* 1990; 280: 1–8.
6. Kontas HA, Wei EP, Christmas CW, Levassens JE, Povlishock JT, Ellis EF. Free oxygen radicals in

cerebral vascular responses. *Physiologist* 1983; 26: 165–169.

7. Traystman RJ, Kirsch JR, Koehler RC. Oxygen radical mechanisms of brain injury following ischemia and reperfusion. *J Appl Physiol* 1991; 71: 1185–1195.

8. Mrsulja BB, Stanimirovic D, Micic DV, Spatz M. Excitatory amino acid receptors, oxidoreductive processes and brain oedema following transient ischemia in gerbils. *Acta Neurochir Suppl Wien* 1990; 51: 180–182.

9. Wieloch T, Siesjo BK. Ischemic brain injury: the importance of calcium lipoytic activities of fatty acids. *Pathol Biol* 1982; 30: 269–277.

10. Nemoto EM, Shiu GK, Nemmer JP, Bleyaert A. Fatty acids in the pathogenesis and therapy of ischemic brain injury. *J Cereb Blood Flow Metab 1982; 2: S 59–S 61.*

11. Kinuta Y, Kimura M, Itokawa Y, Ishikawa M, Kikuchi H. Changes in xanthine oxidase in ischemic rat brain. *J Neuro Surg* 1989; 71: 417–420.

12. Polidori MC, Cherubini A, Stahl W, Senin U, Micocci P. Plasma carotenoid and malondialdehyde levels in ischemic stroke patients: relationship to early outcome. *Free Radic Res* 2002; 36(3): 265–268.

13. Selakovic VM, Jovanovic MD, Jovicic A. Changes of cortisol levels and index of lipid peroxidation in cerebropinal fluid of patients in the acute phase of completed stroke. *Vojnosanit Pregl* 2002; 59(5): 485–491.

14. Horn HD, Burns FH. In: Bergmeyer HV ed. Methods of Enzymatic analysis. Academic Press, New York 1978: 875.

15. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* 1967; 70: 158–169.

16. Beauchamp C, Fridovich 1. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* 1971; 44: 276–287.

17. Brannan TS, Maker HS, Raes IP. Regional distribution of catalase in the adult rat brain. *J Neurochem* 1981; 36(1): 307–309.

18. Stocks J, Dormandy TL. The autooxidation of human red cell lipids induced by hydrogen peroxide. *Br J Haematol* 1971; 20: 95–111.

19. Kartha VN, Krishnamurthy S. Effect of hypervitaminosis A on hemolysis and lipid peroxidation in rat. *J Lipid Res* 1978; 19: 332–334.

20. Horakova L., Lukovie L., Uraz V, Stole S. Time course of lipid peroxidation during incomplete ischemia followed by repurfusion in rat brain. *Physio Bohemoslov* 1990; 39: 513–517.

21. Ito H, Torii M, Suzuki T. A comparative study on lipid peroxidation in cerebral cortex of stroke prone spontaneously hypertensive and normotensive rats. *Int J Biochem* 1993; 25: 1801–1805.

22. Satoh K. Serum lipid peroxides in cerebro vascular disorders determined by a new colorimetric method. *Clin Chim Acta* 1978; 90: 37–43.

23. Hess ML, Okabe E, Kontos HA. Proton and free radical interaction with the calcium transport system of cardiac sarcoplasmic reticulum. *J Mol Cell Cardiol* 1981; 13: 767–772.

24. Chan PH, Fishman RA, Wesley MA, Longar S. Pathogenesis of vasogenic edema in focal cerebral ischemia. Role of superoxide radicals. *Adv Neurol* 1990; 52: 177–183.

25. Lee MH, Park JW. Lipid peroxidation products mediated damage of superoxide dismutase. *Biochem Mol Biol Int* 1995; 35: 1093–11102.

26. Mischowiz SD, Melamed E, Pikarsky E, Rappaport ZH. Effect of ischemia induced by middle cerebral artery occlusion on superoxide dismutase activity in rat brain. *Stroke* 1990; 21: 1613–1617.

27. Roda JM, Carceller F, Pajares R, Diez-Tejedor E. Prevention of cerebral ischemia repurfusion injury by intra aterial administration of superoxide dismutase in the rat. *Neurol Res* 1991; 13(3): 160–163.

28. Ishibashi N, Prokopenko O, Weisbrot LM, Reuhl KR, Mirochnitchenko O. Glutathione peroxidase inhibits cell death glial activation following experimental stroke. *Brain Res Mol Brain Res* 2002; 109(1–2): 34–44.

29. Crack PJ, Taylor JM, de Haan JB, Kola I Hertzog P, Jannello RC. Glutathione peroxidase - 1 contributes to the neuroprotection seen in the superoxide dismutase- 1 transgenic mouse in response to ischemia/reperfusion injury. *J Cereb Blood Flow Metab* 2003; 23(1): 19–22.

30. El Midaoui A, Wu R, de Champlain J. Prevention of hypertension, hyperglycemia and vascular oxidative stress by aspirin treatment in chronically glucose-fed rats. *J Hypertens* 2000; 20: 1279–1281.

31. Nielsen OH, Achnfelt Ronne I. Involvement of oxygen derived free radicals in the pathogenesis of chronic inflammatory bowel disease. *Klin Wochenschr* 1991; 69(21–23): 995–1000.