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Original Article

Erythrocyte indicators of oxidative changes in patients with graded traumatic head injury

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Context: Acute oxidative stress following a traumatic head injury (HI) has been implicated in inducing severe secondary brain damage and influencing the clinical outcome of HI patients. **Aims:** This study was performed to evaluate and compare the oxidative changes in patients with varying severity of HI in the early posttraumatic period using erythrocyte indicators. **Settings and Design:** Head injury patients were divided into two groups based on their Glasgow Coma Scale (GCS) scores recorded at admission to the hospital on the day of trauma itself. Accordingly, the study included 30 severe HI (SHI, GCS scores 8 or less) and 25 Mild HI (MHI, GCS scores more than 8) patients. Thirty age and sex-matched healthy individuals were included in this comparative study as controls. **Materials and Methods:** Blood samples were obtained from controls and HI patients (within 24 h of trauma onset). Erythrocyte oxidative changes were studied by estimating thiobarbituric acid reactive substances (TBARS), glutathione (GSH), superoxide dismutase (SOD) and glutathione reductase (GR). **Results:** Erythrocyte TBARS levels were significantly higher and GSH levels were significantly lower in SHI and MHI patients as compared to controls. The SOD activity was significantly increased only in SHI patients and remained unchanged in MHI patients as compared to controls. As compared to MHI patients, erythrocyte TBARS levels were significantly higher, GSH levels were significantly lower and SOD activity was markedly elevated in SHI patients. Erythrocyte GR activity did not show significant changes in both groups of patients as compared to controls. **Conclusion:** Oxidative stress is evident in both SHI and MHI patients in the early posttraumatic period as reflected by their erythrocyte indicators, but the severity of oxidative stress has varied relatively with the severity of head injury. The present findings provide indications that early oxidative changes could influence the neurological recovery of HI patients.

Key words: Erythrocytes, glutathione. head injury, lipid peroxidation, superoxide dismutase

The brain damage after a head injury (HI) occurs primarily at impact and secondarily with the onset of autodestructive processes. These changes produce gradual vascular and neuronal degeneration, which ultimately contribute to the destruction of the anatomical substrate of brain necessary for neurological recovery.[1] Reactive Oxygen Species (ROS) generated by endogenic metabolic pathways are found to be lethal when generated in excess.[2] To combat these potential toxins called oxidants, living organisms have been endowed with a rich defensive system known as antioxidants. Cumulative oxidative damage has been strongly implicated in neurodegeneration and neurological diseases.[3] Generation of ROS and oxidative stress are said to play an important role in the pathophysiology of traumatic brain injury (TBI).[4,5] Lipid peroxidation (LP), the damage of lipids induced by excess production of ROS, plays a crucial role in posttraumatic neuronal degeneration following a traumatic HI in humans.[6] Destructive oxidative events peak by 20 to 24 h after head trauma as reported from experimental HI.[7] Oxidative stress was evident in severe HI patients throughout the 21-day study period.[8,9] Early oxidative changes in the plasma, erythrocytes and CSF have shown a significant correlation with neurological outcome in HI patients.[6,10,11] High lipid content, high rate of oxygen-consuming metabolic activities and a paucity of antioxidant enzymes dissipating ROS contribute to the susceptibility of brain tissue to LP.[12,13] The ROS, which are short-lived are difficult to be measured directly in the brain tissue of humans after a traumatic HI. Circulating red cells can act as mobile scavengers of ROS and form an excellent material to study oxidative stress because of their easy availability, simple structure and relatively large amount of polyunsaturated fatty acids in their membranes.[14,15] The Glasgow coma scale (GCS) is the most common grading scale in neurotraumatology and is used to quantify the clinical severity of brain trauma.

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Its validity in providing strong predictive value to assess the functional outcome for traumatic HI patients is well accepted in the Anglo-American literature.[16] The current study has hence attempted to evaluate and compare the oxidative stress status in HI patients with graded traumatic HI using erythrocyte indicators.

Materials and Methods

Subjects

Patients were divided into two groups according to the severity of HI using their Glasgow Coma Scale (GCS) scores recorded at admission to the Neurosurgery intensive care unit (ICU) of the hospital on the day of HI i.e. Severe HI (SHI: GCS score 8 or less) and mild HI (MHI: GCS score more than 8) groups. Accordingly, this study included 30 SHI patients and 25 MHI patients. Brain injury was the major medical problem in the majority of the patients, while associated bone injuries and facial lacerations were found in a few of them. Patients with polytrauma, severe posttraumatic hypotension, hypoxic condition (PaO_2 < 70 mm Hg) and those who developed sepsis were excluded from the current study due to the fact that these conditions could contribute to the fluctuations in the parameters studied. Immediately following admission to the ICU, patients were subjected to a standard management protocol of mechanical ventilation, craniotomy and measures to reduce cerebral edema/raised intracranial pressure as indicated. None of the patients received corticosteroids or any form of antioxidant medication. Diagnostic computerized tomographic (CT) scans were done at admission to evaluate the extent of brain damage. A conscious effort was made to include only those subjects who had obvious impact on the cranium with a resultant open/closed injury. An open HI results in the exposure of brain and its meninges due to the fracture of skull bones, while a closed HI occurs due to a rapid bouncing back and forth movement of the brain inside the cranium as a result of external impact on the head during injury. The clinical and demographical data of study patients are presented in Table 1. Road traffic accidents have been the leading cause of head trauma in both groups of patients. Among the 30 SHI patients, 21 had closed head injury and nine had open head injuries. Among the 25 MHI patients, 19 had closed head injury and six had open head injuries. Patient outcome was evaluated at 90 days after trauma using the extended Glasgow Coma Outcome Scale (GCOS-E) on which a score of 8 indicates minimal or no neurological deficits and one indicates death. The outcome of SHI and MHI patients were further divided into two groups each. The unfavorable outcome (UO) group included patients with severe neurological deficits having a GCOS-E score of 2 to 4. The favorable outcome (FO) group included patients with mild or no neurological deficits having a GCOS-E score of 5 to 8. For patients who were discharged from the hospital before Day 90, the last observation record was carried forward. Out of the 30 SHI patients, 25 had unfavorable outcome and five, favorable outcome. Among the 25 MHI patients, only three had unfavorable outcome and 22 had favorable outcome. The CT scan findings revealing the different types of neurotrauma in the study groups are in Table 1. Thirty randomly selected age (mean age 29 ± 10 years) and sex (25 males, five females) matched healthy individuals were considered as controls (C) for this comparative study. Care was taken to select only those individuals with no history of recent illness or pathological disturbances related to the current study or any condition that would affect the parameters studied. They were not on any kind of prescribed medication or dietary restrictions.

Sample collection

The present study was conducted over a two-year timeframe and was approved by our Hospital ethical committee. Venous blood samples were obtained from the femoral vein of patients in EDTA vacutainers on admission to the Neurosugery ICU of our institute, which was within 24 h of the onset of head trauma (median time, 13 ± 2.8 h after the onset of trauma). It was ensured that the blood samples were collected before any surgical interventions were done. Blood samples were centrifuged at 3000×*g* for 10 min. Plasma and buffy coat were carefully removed and the separated packed cells (erythrocytes) were washed

Table 1: The clinical and demographical characteristics of head injury patients (mean ± SD)

	Severe head injury patients (n = 30)	Mild head injury patients (n = 25)
Age (years)	31 ± 13	36 ± 12
Sex (males/ females)	26:4	23:2
Glasgow coma scale scores at admission	4.87 ± 1.5	11.6 ± 2.2
Number of patients with different types of neurotrauma(Diffuse axonal injury: Acute subarachnoid/subdural hemorrhage: Contusions)	11:11:8	0:6:10
Number of patients with different causes of head injury (Road traffic accidents: Fall from height: fall of heavy objects on head)	24:6:0	20:3:2
Number of patients with different types of head injury (closed head injury: open head injury)	21:9	19:6
Number of patients with different neurological outcomes (unfavorable outcome: Favorable outcome)	25:5	3:22

thrice with cold physiological saline, pH 7.4 (sodium phosphate buffer containing 0.15 mol/L NaCl). The washed erythrocytes were used for the estimation of thiobarbituric acid reactive substances (TBARS) and glutathione (GSH) levels. The packed cells were also suspended in physiological saline to prepare 50% cell suspension at 4°C for immediate assay. Appropriately diluted hemolysates were then prepared from the 50% cell suspension by the addition of distilled water, for the assay of superoxide dismutase (SOD) and glutathione reductase (GR) activities. Special chemicals were obtained from Sigma, St Louis, MO. All other reagents used were of analytical grade.

Biochemical estimations

Estimation of TBARS

Packed cells were used for the evaluation of LP by estimating the TBARS levels.[17] Fatty acid peroxidation products react with TBA to form colored adducts which have a maximum absorbance at 532 nm. TBARS levels were expressed as nmol/g hemoglobin. Hemoglobin concentration was estimated by the cyanmethemoglobin method.[18]

Estimation of GSH

Reduced GSH levels were estimated by the colorimetric method of Beutler *et al.*[19] The sulfydryl group of reduced GSH reduces the disulfide chromogen 5,5' dithiobis (2-nitrobenzoic acid) (DTNB) to an intense yellow-colored compound, the absorbance of which was measured at 412 nm. Glutathione content was expressed as mg/g hemoglobin.

Assay of SOD

Inhibition of the reduction of nitroblue tetrazolium (NBT) by superoxide radicals, generated by the illumination of riboflavin in the presence of oxygen and electron donor methionine was used as the basis for the assay of SOD activity.[20] A chloroform ethanol extract was prepared from the hemolysate and the supernatant obtained was used for the assay. The solution was illuminated for 10 min. The absorbance was then read at 560 nm. Controls with and without NBT were included in the assay. One unit of SOD activity was defined as that producing 50% inhibition of NBT reduction. Values were expressed as units of enzyme activity/g hemoglobin.

Assay of GR

Erythrocyte GR activity was estimated by the procedure of Horn and Burns.[21] This enzyme catalyzes the reduction of oxidized glutathione (GSSG) to GSH in the presence of reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). The rate of formation of GSH was measured by following the decrease in absorbance of the reaction mixture at 340 nm as NADPH is converted to $NADP^+$. The decrease in the absorbance at 340 nm for a period of 5 min was recorded and the activity was expressed as units/g hemoglobin where units represented the number of micromol of NADPH oxidized per minute in the reaction mixture.

Statistical analysis

The significance of changes in erythrocyte indicators of oxidative stress among the study groups were done by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison post hoc test using the Statistical Package for Social Sciences (SPSS/PC; SPSS, Chicago, IL). Data were expressed as mean ± standard deviation and $P \leq 0.05$ was accepted as statistically significant.

Results

Table 2 illustrates the comparison of the changes in erythrocyte TBARS, GSH levels and SOD and GR activities between controls, SHI and MHI patients. Erythrocyte TBARS levels were significantly increased in both SHI and MHI patients ($P < 0.001$) as compared to controls with the magnitude of increase being significantly higher in SHI patients ($P < 0.043$) as compared to MHI patients. Erythrocyte GSH levels were significantly decreased in SHI ($P < 0.001$) and MHI patients ($P < 0.01$) as compared to controls with a more pronounced decrease in SHI patients as compared to MHI patients ($P < 0.031$). Erythrocyte SOD activity was significantly elevated only in SHI patients as compared to controls ($P < 0.001$) and MHI patients ($P < 0.01$). However, the SOD activity in MHI patients was not different from that of controls. Erythrocyte GR activity in SHI patients showed a trend towards decrease in enzyme activity as compared to controls and MHI patients, although this was statistically not significant. The GR activity in MHI patients however, remained comparable to that of controls.

Discussion

Experimental evidence indicates that ROS-induced LP caused by the excessive release of glutamate, intracellular calcium overload and activation of arachidonic acid cascades play a principal role in neurodegenerative processes after the initial brain injury.[22] Increased formation of LP products have been observed in areas of contusions in mice brain after a head injury.[23,24] Experimental data from the work of Shohami *et al.*, indicates that even when the injury was selectively delivered to the brain, the other organs too, have been affected by the prevailing oxidative stress.[7] Oxidative stress in the CNS has been reflected as increased oxidative stress in the erythrocytes also.[25] Cytotoxic LP metabolites

Table 2: Comparison of erythrocyte indicators of oxidative stress in head injury patients and controls

	Controls (C, n = 30)	Severe head injury patients (SHI, n = 30)	Mild head injury patients (MHI, n = 25)
Erythrocyte thiobarbituric acid reactive substances levels (nmoles/g Hb)	5.06 ± 1.02	7.16 ± 1.34***†	6.40 ± 0.80***
Erythrocyte glutathione levels (mg/g Hb)	2.67 ± 0.80	1.70 ± 0.29***†	2.10 ± 0.57**
Erythrocyte superoxide dismutase activity (U/g Hb)	2658.92 ± 835.06	3648.76 ± 948.94***††	2797.29 ± 1212.73
Erythrocyte glutathione reductase activity (U/g Hb)	1.24 ± 0.69	0.94 ± 0.30	1.29 ± 0.88

Erythrocyte indicators reflecting oxidative damage and antioxidant status in severe (SHI) and mild head injury (MHI) patients in the early posttraumatic period (Day 1) of head injury as compared to controls (expressed as Mean ± SD). *P*-values by Bonferroni's multiple comparison post hoc test (ANOVA). ***$P < 0.001$, **$P < 0.01$ vs. controls (C). ††$P < 0.01$, †$P < 0.05$ vs. MHI patients

like 4-HNE diffuse from the site of production and react with distant intra- and extracellular macromolecules.[26,27] Thus in the present study, the increase in erythrocyte TBARS levels in SHI and MHI patients could be due to the damage caused by the LP products diffusing into the plasma from the CNS. The extent of LP has shown positive correlation with the severity of HI in guinea pigs.[28] In line with this report, in the current study we have also observed that the erythrocyte TBARS levels were significantly higher in SHI patients indicating severe oxidative stress in them as compared to MHI patients.

Reports have indicated that there is a significant decrease in low molecular weight antioxidants in brain tissue after closed HI in rats.[29] Excitatory glutamate release in the CNS has been reported to cause a decrease in GSH synthesis and hence aggravate oxidative stress in CNS.[30-33] Glutathione is used to scavenge reactive nitrogen species generated during tissue injury and inflammation.[34] Glutathione is consumed during oxidative stress to regenerate the oxidized forms of non-enzymic antioxidants, vitamins E and C. Previous workers have reported a significant decrease in vitamin C content of brain, heart, lung, liver and intestinal tissues in rats following a closed HI.[7] Paolin *et al.*, have reported a significant decrease in plasma vitamin E levels in the early posttraumatic period in traumatic HI patients.[6] Although we have not estimated vitamin E and C levels in the blood in the current study, it can be hypothesized that a decrease in erythrocyte GSH levels observed in patients of the present study could be due to its consumption to regenerate vitamins E and C.

Superoxide radical is assumed by many investigators to figure prominently in the avalanche of events that are triggered by the traumatic HI episode.[35] Direct proof for superoxide radical generation and its appearance in the brain extracellular space during fluid percussion brain injury in fixed brain of cats has been given by Kontos *et al*.[36] Endothelial cells, activated neutrophils, macrophages and microglia acting at the site of HI act as potent sources of superoxide generation.[24] Release of catecholamines in the ischemic zone of the brain after a HI and their increased oxidation by monoamino oxidase are shown to be potential sources of superoxide generation.[37,38] Superoxide, which enters the extracellular space of brain, is believed to survive longer because of the lower concentrations of SOD in the extracellular space.[39] Lynch and Fridovich by their experiment using the anion channel blockers have shown that superoxide radical traverses the red cell membrane with surprising ease.[40] Thus in the present study we hypothesize that superoxide generated due to the impact of HI in the CNS, could be picked up by erythrocytes, which act as mobile free radical scavengers.[15] We propose that a stronger oxidant stimulus, severity of injury and the protective mechanism of the body to respond to the prevailing acute severe oxidative stress could have led to the activation of existing apoforms of SOD in erythrocytes of SHI patients. However the normal SOD activity in erythrocytes of MHI patients could indicate relatively milder oxidative stress in them.

Kamencic *et al.*, have reported a significant decrease in GR activity and increase in oxidative protein damage at the segments of the spinal cord in rats subjected to spinal cord compression injury.[41] In the present study we have observed a trend of decrease in erythrocyte GR activity only in SHI patients as compared to controls. A low GR activity indicates poor regeneration of GSH. Normal erythrocyte GR activity in erythrocytes of MHI patients could have helped them to regenerate GSH to a better extent, which further justifies the findings of this study, where erythrocyte GSH levels in MHI patients were significantly higher than those in SHI patients.

Outcome evaluation in the current study has revealed that a majority of SHI patients have had an unfavorable outcome (25 out of 30 patients). On the other hand, most of the MHI patients have shown a favorable outcome (19 out of 25 patients). Thus, the difference in severity of outcomes can be attributed to the relative severity of oxidative insult in the SHI and MHI patients as shown by the changes in their erythrocyte markers of oxidative insult.

Conclusion

The present study has attempted to use the erythrocyte indicators of oxidative damage and antioxidant defense to study the significant oxidative changes in the systemic circulation of SHI and MHI patients in the early posttraumatic period. The biochemical findings reveal that the oxidative stress has varied relatively with the

severity of injury as assessed by the initial GCS scores of these HI patients. The study also reflects the influence of oxidative changes in traumatic HI on the long-term neurological outcome in these patients. Further studies correlating these biochemical changes with the other standard clinical variables need to be conducted before assigning a prime role for these parameters in the prognosis of traumatic head injury.

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Accepted on 13-09-2007
Source of Support: Nil, **Conflict of Interest:** None declared.