ORIGINAL CONTRIBUTIONS

TIME-RELATIVE CHANGES IN THE ERYTHROCYTE ANTIOXIDANT ENZYME ACTIVITIES AND THEIR RELATIONSHIP WITH GLASGOW COMA SCALE SCORES IN SEVERE HEAD INJURY PATIENTS IN THE 21-DAY POSTTRAUMATIC STUDY PERIOD

CHANDRIKA D. NAYAK, DINESH M. NAYAK*, ANNASWAMY RAJA**, ANJALI RAO***

ABSTRACT

BACKGROUND: Reactive oxygen species are indicated to play a prime role in the pathophysiology of brain damage following a severe head injury (SHI). AIM: The current study was designed to understand the time-relative changes and relationship between erythrocyte antioxidant enzyme activities and Glasgow Coma Scale (GCS) scores of SHI patients in the 21-day posttraumatic study period. SETTINGS AND DESIGN: The study included 24 SHI patients and 25 age- and sex-matched normal controls (NC). Activities of superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GSH-Px) were assayed in these patients and controls. The GCS scores of these patients were also recorded for the comparative study. MATERIALS AND METHODS: Venous blood samples were collected on day 7 (D7) and D21 from SHI patients and NC for the assay of SOD, GR and GSH-Px activities. These changes were correlated with age and changes in GCS scores of patients. STATISTICAL ANALYSIS: A one-way analysis of variance (ANOVA) was used to compare mean values of each parameter between group 1 (NC), group 2 (D7 changes in SHI patients) and group 3 (D21 changes in SHI patients). ANOVA was followed by Bonferroni post hoc tests. The Pearson correlation was applied to correlate between the antioxidant parameters and age and GCS scores of these patients. RESULTS: A significant increase in erythrocyte SOD and GSH-Px activities was observed in group 3 as compared to groups 1 and 2. The increase in GSH-Px activity was significant in group 2 as compared to group 1. Although not significant, there was an increase in mean GR activity in groups 2 and 3 as compared to group 1. CONCLUSION: These findings indicate that SHI patients have shown significantly enhanced erythrocyte SOD and GSH-Px activities during the 21-day posttraumatic study period.

Key words: Erythrocytes, Glasgow Coma Scale, glutathione peroxidase, head injury, superoxide dismutase

Department of Biochemistry, Melaka Manipal Medical College (Manipal Campus), Manipal, Departments of *Pediatrics, **Neurosurgery and ***Biochemistry, Kasturba Medical College and Hospital, Manipal, India!

Correspondence:
Dr. Chandrika D. Nayak,
Department of Biochemistry, Melaka Manipal Medical College (Manipal Campus), Manipal University, Manipal - 576 104, Udupi, Karnataka, India. E-mail: chandrikadinesh@yahoo.com!

Excessive generation of reactive oxygen species (ROS) or an inadequate antioxidant capacity of the cell to neutralize these species results in a condition known as oxidative stress. Experimental evidences indicate several biological sources for ROS generation within the injured nervous system. ROS-mediated lipid peroxidation (LP) is believed to be a major cause for posttraumatic neuronal damage in head injury patients.[1] Experimental studies have demonstrated a significant rise in LP products in the brain tissue homogenates in the immediate post-head-injury period.[2,3] Brain is sensitive to LP because of its high concentration of polyunsaturated fatty acids, high oxidative metabolic activity, relatively low antioxidant activity and nonreplicating nature of neuronal cells.[4,5] Recent studies reveal that oxidative stress induces blood brain barrier (BBB) dysfunction.[6] The ROS and the cytotoxic LP metabolites generated at the site of injury can diffuse out and react with distant intra- and extracellular macromolecules.[7,8] Circulating red cells are mobile ROS scavengers and provide antioxidant protection to other tissues and organs.[9] Thus the erythrocytes act as mobile ROS scavengers and hence reflect the oxidative stress status of patients after a traumatic head injury. The Glasgow Coma scale (GCS) is the most common grading scale in neurotraumatology and is used to quantify the clinical severity of brain trauma. Its validity in providing strong predictive value to assess the functional outcome for traumatic head injury patients is well accepted in the Anglo-American literature.[10] Many serum and cerebrospinal fluid (CSF) markers of neuronal damage have been correlated with changes in GCS scores of head injury patients.[11,12] Erythrocyte thiobarbituric acid reactive substances (TBARS), reflecting oxidative damage, were reported to be significantly higher in severe head injury (SHI) patients than in normal controls (NC) throughout the 21-day study period, indicating prolonged severe oxidative stress.[13] Previous studies have evaluated the early oxidative damage in erythrocytes of severe head injury patients.[1,13] At present there are very few reports indicating changes in erythrocyte antioxidant enzyme activities in the late posttraumatic period of severe head injury. Thus the current study was designed with the aim to evaluate the alterations in erythrocyte superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GSH-Px) activities and to correlate these changes with the age and GCS scores of patients in the 21-day posttraumatic period of severe head injury.

MATERIALS AND METHODS

The present study was conducted over a 2-year time frame and included 24 patients (all males, mean age 29 ± 6 years) with SHI and postresuscitation GCS score of 8 or less (mean GCS score, 5.21 ± 1.5) at admission. SHI patients enrolled into the study and who died in the hospital during the 21-day posttraumatic study period were excluded from the study. Diagnostic computerized tomographic (CT) scans were done at admission to evaluate the extent of brain damage. The CT scan findings of the study patients are summarized in Table 1. Brain injury was the major medical problem in majority of the patients, while associated bone injuries and facial lacerations were found in a few of them. 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 head injury results in

Table 1: Clinical characteristics of severe head injury patients

Case No.	*Age (yr), Sex*	*Description of head injury*
1 !	37, M	CHI, Left acute frontoparietal SDH
2 !	27, M	OHI, Left parietal bone fracture, Right frontal and temporal lobe contusion, pneumocephalus with dif fuse axonal injury
3 !	22, M	CHI, posttraumatic SAH, diffuse axonal injury
4 !	38, M	OHI, Right frontal and temporal lobe contusion with pneumocephalus
5 !	24, M	OHI, Right frontal bone fracture with underlying brain contusion with primary brainstem contusion
6 !	30, M	CHI, SAH with diffuse cerebral edema and right parietal extradural hematoma
7 !	37, M	CHI, traumatic SAH with right clavicle fracture
8 !	26, M	CHI, diffuse axonal injury
9 !	30, M	CHI, diffuse axonal injury, deep seated parietal hematoma with posttra ımatic right III cranial nerve palsy
10 !	37, M	CHI, diffuse axonal injury, left temporal, midbrain, bilateral thalamic contusions with intraventricular hemorrhage
11 !	26, M	CHI, Right occipital and parietal laceration, bilateral basifrontal and anterior temporal contusions
12 !	35, M	CHI, diffuse axonal injury
13 !	23, M	CHI, fracture right temporal bone, counter coup with left temporal bone contusion
14 !	36, M	OHI, bilateral frontal bone fracture, right frontal EDH, Right frontal contusion with speck of pneumo cephalus in right posterior fossa
15 !	30, M	OHI, primary brain stem injury, cerebral contusions
16 !	22, M	OHI, left frontal bone fracture with pneumocephalus
17 !	23, M	CHI, right frontal and parietal linear fracture with traumatic SAH
18 !	24, M	CHI, Left temporoparieatal SDH with underlying contusion, right posterotemporal contusion with right ear bleed
19 !	37, M	CHI, speck of contusion in the left parietal region with cerebral edema and multiple facial lacerations and abrasions over the right frontal bone
20 !	30, M	CHI, right frontal EDH with right frontal contusion and pneumocephalus
21 !	32, M	CHI, brain stem contusion, left temporal contusion and with posttraumatic communicating hydrocephalus
22 !	23, M	CHI, left basal ganglion and internal capsule contusion and with right III nerve palsy
23 !	37, M	CHI, traumatic SAH, cerebral edema
24 !	30, M	CHI, acute SDH, right temporal EDH, traumatic SAH

*CHI - Closed head injury, EDH - Extradural hematoma, OHI - Open head injury, SAH - Subarachnoid hemorrhage, SDH - Subdural ! hematoma!

brain injury by breaking or piercing the tough ! skull. A closed head injury occurs due to an ! impact to the head from an outside force ! without damaging or fracturing the skull. On ! admission to the intensive care unit, patients ! were subjected to a standard management ! protocol. Those individuals with symptoms ! and signs of raised intracranial pressure (ICP) ! or CT scan showing evidence of cerebral ! edema were managed with either mannitol, ! lasix and occasionally with CSF drainage. The ! entire study group was not on any ventilator ! assistance or on any sedatives. Patients with ! oxygen saturation of less than 90% by pulse ! oximetry or an arterial blood gas analysis ! revealing PO_2 less than 70 mmHg were ! excluded from the current study. None of the ! patients received corticosteroids or any form of ! antioxidant medication during the study period. ! Patients who were febrile or having other ! features of septic shock - like poor peripheral ! perfusion, hemodynamic instability in the ! absence of hemorrhage and lab investigations ! showing evidence of sepsis as evidenced by ! leukocytosis, toxic granulations, bandemia and ! blood culture proven were excluded from the ! study due to the fact that these conditions could ! contribute to the fluctuations in the parameters ! studied. Twenty-five age- (mean age 28 ± 5 ! years) and sex-matched healthy individuals ! were selected as NC for this comparative ! study. The selected NC group individuals !

had no history of recent illness or pathological ! disturbances relating to the current study or ! affecting the parameters studied.

Approval for the present study was obtained ! from our Institutional Ethical Committee. Venous ! blood samples were collected from patients in ! Ethylenediamine tetraaceticacid (EDTA)!tubes ! on day 7 and 21 of the posttraumatic period. Blood samples were centrifuged at 3,000 x ! *g* for 10 min. Plasma and buffy coat were ! carefully removed, and the separated packed ! cells (erythrocytes) were washed thrice with ! cold physiological saline, pH 7.4 (sodium ! phosphate buffer containing 0.15 $molL^{-1}$! NaCl). The packed cells were suspended in ! an equal volume of physiological saline to ! prepare 50% cell suspension at 4°C to be used ! immediately. Appropriately diluted hemolysates ! were prepared from the erythrocyte suspension ! by the addition of distilled water, for the assay ! of SOD, GR and GSH-Px activities.

Assay of SOD (EC 1.15.1.1)

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.[14] 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. Hemoglobin ! was estimated by the method of Tentori and ! Salvati.[15]!

Assay of GR (EC 1.6.4.2)

Erythrocyte GR activity was estimated by the ! procedure of Horn and Burns.[16] This enzyme ! catalyzes the reduction of oxidized glutathione ! (GSSG) to Reduced glutathione (GSH) in the ! presence of reduced 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 µmol ! of NADPH oxidized per minute in the reaction ! mixture.

Assay of GSH-Px (EC 1.11.1.9)

GSH-Px activity in the erythrocyte hemolysate ! (0.1 ml) was assayed by the method of Paglia ! and Valentine.[17] All the hemoglobin in the lysate ! was converted to stable cyanmethemoglobin ! by adding Drabkin's reagent. The rate of ! oxidation of GSH by H_2O_2, catalyzed by GSH-! Px, was measured. The GSSG formed by the ! action of GSH-Px was further reduced by GR ! added to the assay mixture. The decrease in ! absorbance at 340 nm due to the depletion of ! NADPH for a period of 5 min was recorded. ! Nonenzymatic oxidation of GSH was measured ! in a simultaneous assay system, without the ! hemolysate. The difference between the two ! systems gave the enzyme activity, which was ! expressed as units/ g hemoglobin, where units ! represented the number of µmol of NADPH ! oxidized per minute in the reaction mixture.

All reagents used were of analytical reagent grade. GSH, GSSG, GR and NADPH were obtained from Sigma Chemicals, St. Louis, MO.

Assessment of GCS scores

GCS scores of SHI patients as assessed by the neurosurgeon were noted at the time of blood sampling at admission and on days 7 and 21 of the posttraumatic period.

Statistical analysis

The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS/PC; SPSS, Chicago, USA). A one-way analysis of variance (ANOVA) was used for determining the significance of changes in the erythrocyte SOD, GR and GSH-Px activities between group 1 (NC), group 2 (D7 changes in SHI patients) and group 3 (D21 changes in SHI patients). ANOVA was followed by Bonferroni's post hoc tests. The Pearson correlation was applied to correlate the changes in antioxidant enzyme activities on D7 and D21 with the age and GCS scores of these patients. All the values are expressed as mean (n) ± standard deviation (SD), and a '*P*' value of ≤0.05 was considered statistically significant.

RESULTS

Table 2 depicts the mean ± SD of the changes in activities of erythrocyte SOD, GR and GSH-Px compared between group 1, group 2 and group 3; and the GCS scores of group 2 and 3. Changes in the mean SOD activity compared between the three study groups were statistically significant ($P < 0.001$, F = 7.3). The SOD activity in group 3 was significantly increased as compared to group 1 ($P < 0.001$) and group 2 ($P = 0.036$). The alterations in the mean GR activity compared between groups were not statistically significant ($P = 0.06$, F = 2.9). The changes in erythrocyte GSH-Px activity compared between the study groups were statistically significant ($P < 0.0001$, F = 19.76). The GSH-Px activity in group 3 was significantly increased as compared to group 1 ($P < 0.001$) and group 2 ($P < 0.005$). The increase in GSH-Px activity of group 2 as compared to group 1 was also statistically significant ($P = 0.008$). There was no correlation between age and the antioxidant parameters in the study patients. Erythrocyte SOD and GSH-Px activity did not correlate with the GCS scores in groups 2 and 3. However, erythrocyte GR activity correlated negatively with GCS scores in both group 2 (r = -0.470, $P = 0.027$) and group 3 (r = -0.495, $P = 0.043$).

Table 2: Glasgow Coma scale scores of severe head injury patients and the comparison of erythrocyte antioxidant enzyme activities of patients and controls

	Group 1 (n=25)	*Group 2 (n=24)*	*Group 3 (n=24)*
Erythrocyte superoxide dismutase activity [U(g Hb)$^{-1}$]	2469.04 ± 1005.79	2824.53 ± 1153.55	3832.62 ± 1010.29***†
Erythrocyte glutathione reductase activity [U(g Hb)$^{-1}$]	1.27 ± 0.67	1.68 ± 0.53	1.46 ± 0.54
Erythrocyte glutathione peroxidase activity [U(g Hb)$^{-1}$]	4.49 ± 1.13	5.99 ± 2.09**	7.73 ± 1.5***†
Glasgow Coma scale scores of patients		7.1 ± 3.8	8.4 ± 4.2

Changes in GCS scores of SHI patients during the study period and the changes in activities of erythrocyte antioxidant enzymes SOD, glutathione reductase (GR) and glutathione peroxidase (GSH-Px) compared between group 1 (normal controls, NC), group 2 (D7 changes in SHI patients) and group 3 (D21 changes in SHI patients). Enzyme activity is expressed as units per gram hemoglobin [U(g Hb)$^{-1}$]. Values are expressed as mean (n) ± standard deviation (SD). '*P*' values by Bonferroni's post hoc tests (ANOVA). ***$P < 0.001$, **$P < 0.01$ *vs.* group 1; ††$P < 0.01$, †$P < 0.05$ *vs.* group 2.

DISCUSSION

Oxidative stress is stated to be an intrinsic component of the neurological sequel of traumatic head injury.[18] ROS generation and their appearance in the brain extracellular space during brain injury is well established with experimental evidence.[19] Oxidative stress has been found to cause BBB injury and dysfunction.[6] Previous experiments have shown that ROS, like superoxide, traverse the erythrocyte membrane with ease[20] and that the RBC also act as mobile ROS scavengers providing antioxidant protection to other tissues and organs.[9] Earlier studies indicate increased oxidative stress in the rest of the body during a selective head injury.[21] Several previous investigators have measured LP in plasma and erythrocyte membranes of head injury patients as a measure of oxidant stress.[1,13,22] A significant reduction in erythrocyte LP levels, reflecting adaptation to chronic oxidative stress, and a relatively significant clinical recovery trend towards the end of posttraumatic study period has been reported earlier.[13] The current study was hence attempted to evaluate the changes in the erythrocyte antioxidant enzyme activity in SHI patients in the 21-day study period.

Erythrocytes are very susceptible to oxidative damage due to high degree of polyunsaturated fatty acids in them and the high concentration of intracellular oxygen and hemoglobin, whose redox chemistry is known to produce oxyradicals.[23] Hence by evolution they have become highly specialized cells to handle the threat of ROS at all times with high activities of antioxidant enzymes SOD, GSH-Px and catalase compared to other cells of the body. They also have a rich pool of the nonenzymatic antioxidant GSH, which is preserved in its reduced state by the activity of GR using NADPH. Basal level of antioxidant enzyme activity is maintained at all times, yet cells are said to have ways to amplify these activities to counter sudden increases in ROS.[24] Erythrocyte antioxidant enzyme activities in New Zealand white rabbits were significantly increased on chronic exposure to ROS and lipid peroxides.[25] Finnish Landrace sheep with a genetic lesion causing decreased erythrocyte GSH levels due to restricted cysteine transport across the erythrocytes were shown to exhibit resistance to oxidant challenge due to the adaptive induced higher levels of antioxidant enzymes in their erythrocytes.[26] SOD is said to be a substrate-inducible enzyme, and its increase is said to be indicative of increased generation of superoxide radicals.[27] Activation of SOD in erythrocytes is regarded as an induced compensatory adaptive response to excessive accumulation of ROS in patients with chronic obstructive pulmonary diseases[28] and in patients with allergy to pollen or house dust mite.[29] Comhair *et al.*[30] have provided *in vitro* experimental evidence for an eightfold increase in GSH-Px mRNA in bronchial epithelial cells after exposure to ROS and attributed this to the gene expression by the influence of redox status. GSH-Px has been said to be the principal antioxidant enzyme in erythrocytes to detoxify H_2O_2. It has been stated that cells elevate their catalase and GSH-Px activities relative to the increase in SOD activity in response to an increase in superoxide radicals.[24] GSH-Px is an 'oxidative stress'-inducible enzyme playing a significantly important role in the peroxyl-scavenging mechanism and in maintaining functional integration of the cell membranes.[31] We observed a significantly higher activity of erythrocyte GSH-Px and SOD on D21 in SHI patients as compared to NC, with a significant

increase on D21 as compared to D7 also. ! The rise in SOD and GSH-Px activities could ! be due to their induction to counter the effect ! of prevailing oxidative stress during the ! posttraumatic period of head injury.

Although the alterations in the mean values ! of GR activity on D7 and D21 compared ! between SHI patients and NC groups were ! not significant, these patients have shown an ! increase in the mean GR activity throughout ! the posttraumatic period as compared to NC. Furthermore, GR activity showed negative ! correlation with GCS scores of patients during ! the entire study period. GR plays the role of ! a crucial second line of antioxidant defense ! by regenerating GSH inside a cell. Additional ! studies at the molecular level of GR regulation ! and correlation of GR changes with changes in ! GSH levels need to be done to understand the ! changes in GR activity in head trauma.

Age and GCS scores are recognized among ! the strongest predictors of clinical outcome ! in patients with severe head injury.[1] While ! certain studies have demonstrated a positive ! correlation between age and oxidative ! parameters in head injury patients,[32] this ! finding has not been uniformly reproducible.[1] ! Our data are in agreement with the previous ! report,[1] that there is a lack of correlation ! between age and the antioxidant parameters ! studied. There was a lack of correlation ! between SOD, GSH-Px activities and GCS ! scores in SHI patients during the study period. Further studies correlating these antioxidant ! activities with other standard clinical variables ! need to be carried out before assigning a ! prognostic role for these parameters in severe ! head trauma in humans.

CONCLUSION

In the present study, we have observed a ! significant increase in the erythrocyte SOD and ! GSH-Px activities of SHI patients in the 21-day ! posttraumatic study period. The changes in ! erythrocyte antioxidant enzyme activities did not ! correlate with the GCS scores of patients except ! for GR, which showed negative correlation with ! GCS scores during the study period. The results ! of our study further contribute to the previous ! literature supporting the severe oxidative ! stress hypothesis in traumatic head injury. The ! increased activities of antioxidant enzymes ! may be a compensatory regulation in response ! to the prevailing oxidative stress. The results ! reflect the necessity of designing antioxidant ! therapeutic strategies for these patients in the ! early posttraumatic period. However, further ! studies correlating these biochemical changes ! with the other standard clinical variables need to ! be carried out before assigning a prime role for ! these parameters in the prognosis of traumatic ! head injury. Augmenting antioxidant therapy as ! a secondary therapy to the prevailing therapies ! in the treatment of head injury patients also ! remains plausible.

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