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Antioxidants and intracerebral haemorrhage

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Abstract
Intracerebral haemorrhage (ICH), a serious cerebrovascular condition, is accompanied by a series of biochemical and pathological events constituting the release of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and thus altering the antioxidant mechanisms and antioxidant enzymatic levels. In studying these antioxidant cascades, cerebrospinal fluid (CSF) analysis offers the most innocuous tool as it is in close connection with the central nervous system (CNS). This study aimed at assessing the difference in the activities of Catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (Gred), glutathione transferase (GTS) activities in CSF samples of healthy and ICH patient. The level of oxidative damage was highlighted by malondialdehyde (MDA) as a parameter of lipid peroxidation. Total nitrite levels were also measured. During ICH, the decrease in the activity of SOD, CAT and GPx is accompanied by a significant increase in the activities of GST and Gred. Total nitrite and MDA levels both increased significantly. In present study we may conclude that ICH alters antioxidants levels and several of these parameters can be used as biomarkers of extreme cerebral oxidative stress.

Keywords: Intracerebral haemorrhage, cerebrospinal fluid, antioxidants, oxidative stress

1. Introduction
Intracerebral haemorrhage (ICH) is a severe cerebrovascular condition resulting in high mortality or major disability in adults. The pathological and biochemical mechanisms after ICH in brain parenchyma activates a sequence of adverse events resulting in secondary brain injury (SBI) and serious neurological discrepancies damaging proteins, lipids, carbohydrates, DNA and RNA (Belur *et al.*, 2013) [3]. In recent years, it has been recognized that oxidative stress plays an imperative role in SBI after ICH that results in irreversible disturbance in neurovascular components, followed by blood brain barrier disruption and fatal brain edema with immense brain cell death (Aronowski and Zhao, 2011) [2].

ICH patients' brain is extremely sensitive to reactive oxygen species (ROS) and reactive nitrogen species (RNS). The cellular oxidative damage caused by these reactive species can be effectively resisted and repaired by antioxidants. Rapidly after the initial cause of the blood extravasation, amongst the highly effective antioxidant interventions are enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) that neutralizes ROS via numerous mechanisms (Gilgun-Sherki *et al.*, 2001; Papacocea *et al.*, 2014) [5, 17]. However, the direct detection of several oxidative stress biological markers in the human brain is unrealistic; thus, for an oxidative stress biomarker to have clinical value it should accurately indicate the key source of ROS and must also have convenient specimen collection. Therefore, determining the activities of antioxidant enzymes in cerebrospinal fluid (CSF) along with their modulation is an indirect method of reflecting the brain oxidative stress and may provide a better protection in ICH patients (Mihai *et al.*, 2012) [15]. Antioxidative treatment aiming at reducing or preventing oxidative stress has facilitated new insights into ICH therapy. In the current paper, we studied the effect of ICH on various parameters: SOD, CAT, GPx, glutathione transferase (GTS), glutathione reductase (Gred), lipid peroxidation and total nitrite levels in control and ICH CSF samples.

2. Materials and Methods
2.1 Sample collection
The present study group included 25 patients (12 males, 13 females with age ranging from 32 to 67 years) with acute ICH. ICH was confirmed by computerized tomography scan or magnetic resonance imaging. 10 healthy control subjects without any nervous diseases, from whom CSF samples were procured via lumbar punctures for spinal anesthesia during other surgical interventions.

Elimination criteria included: the presence of intra-ventricular blood effusion and subarachnoid haemorrhage; haematological disorders; posttraumatic brain injuries; chronic lung and kidney diseases; intake of medications having ability of interfering oxidative equilibrium: non-steroidal anti-inflammatory drugs, chemotherapy, iron and derivatives, ascorbic acid, vitamin E and other antioxidants.

The CSF samples were collected by lumbar puncture and were frozen at –80°C in sterile Eppendorf tubes. Samples were examined in triplicates using spectrophotometric methods for the estimation of several antioxidant enzymes: Catalase (CAT), Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx), Glutathione Transferase (GTS), Glutathione Reductase (Gred) activities, Total nitrite and MDA levels (lipid peroxidation).

2.2 Methods and Data Analysis

For measuring SOD activity, a spectrophotometric method by Marklund *et al.* (1974) [13] was used. This method was based on the enzyme's ability to inhibit autoxidation of pyrogallol in the presence of ethylenediaminetetraacetic acid (EDTA). The change in absorbance was recorded at 420 nm at an interval of 30 sec upto 3 min. SOD activity was expressed as U/mL where one unit of SOD is defined as the amount of enzyme causing 50% inhibition of auto-oxidation of pyrogallol observed in the blank. GPx enhances the formation of glutathione and was spectrophotometrically assayed on the basis of the reduction of glutathione in the presence of excess Gred. The concomitant nicotinamide (NADPH) reduction was monitored at 340 nm for 3 min by using method by Mannervik (1985) [12]. Catalase, an antioxidant enzyme that detoxifies H_2O_2 to H_2O and O_2, was measured using method by Aebi (1983) [1].

Glutathione reductase catalyzes the NADPH- dependent reduction of oxidized glutathione (GSSG). Gred activity was determined *in vitro* in the presence of flavin adenine dinucleotide (FAD) and GSSG at 340 nm for 3 min using protocol by Beutler (1974) [4]. Glutathione transferase activity was determined by following the method by Habig (1974) [7]. The transfer of 4-chloro-1,2-dinitrobenzene (CDNB) on GSH was analyzed for 3 min at 340 nm. One unit of GTS is defined as the quantity of enzyme catalyzing the formation of a one mole of S- 2, 4 - dinitrophenyl glutathione per min. The GTS enzymatic activity was expressed as IU/ mg of protein.

Lipid peroxidation was measured in terms of malondialdehyde (MDA), a product of lipid peroxidation, estimated by thiobarbituric acid (TBA) reaction in which a coloured 1,2 adduct is formed whose absorbance is taken at 532 nm (Jentzsch *et al.*, 1996) [9]. MDA levels were expressed as µmol/mL. Total proteins were determined by a modification of Lowry's procedure as described by Markwell *et al.* (1978) [14]. Protocol by Green *et al.* (1982) [6] was used to determine Nitrite levels using Griess reactive. The data was expressed as means ± standard deviation (SD) and the differences between groups were tested using t-test and $p < 0.05$ was considered significantly different.

2.3 Ethical Issues

In each case, an informed consent was obtained from each patient prior the lumbar puncture. Anonymized CSF samples from remnants were collected in our Laboratory after the completion of diagnostic protocols, and were used for conducting this study. All experiments performed in this study involving human participants were in accordance with the ethical guidelines for biomedical research by ICMR. The study was approved by the Ethical committee of Elite Pathological Diagnostic Centre.

3. Results and Discussion

Oxidative stress shows an imperative role in SBI subsequently ICH (Robbins and Swanson, 2014) [18]. It defines a state of body when it replies to several harmful stimuli and produces large amounts of reactive free oxygen radicals i.e. reactive oxygen species (ROS), and reactive nitrogen radicals, called as reactive nitrogen species (RNS). Accumulation of ROS and RNS in the body or cells causes cell toxicity and eventually results in tissue damage. Antioxidant enzymes can help scavenge these free radicals and hence reduce/eliminate oxidative damage. Therefore, determining the activities of antioxidant enzymes in cerebrospinal fluid is an indirect method of reflecting the brain oxidative stress (Mihai *et al.*, 2012) [15].

Lipid peroxide is also an important product of brain damage. It is chiefly derived from the secondary products resulting from peroxidation of unsaturated fatty acid of membrane phospholipids and subsequently results in structural and functional injury to the cell membrane. All through ICH followed by an inflammatory response, neutrophils are stimulated and activated, hence leading to disruption of the respiratory chain, releasing large amounts of ROS and nitric oxide, and the excessive SOD consumption as well as lipid peroxidation (Yu *et al.*, 2014) [24]. Currently, lipid peroxides' (MDA) presence in the CSF is the most commonly used marker reflecting oxidative stress. In our study, MDA levels were statistically higher in the ICH patients (16.67 µmol/mL) compared to the healthy controls (6.15 µmol/mL) as depicted in Fig. 1a. Several clinical experiments and animal studies have depicted that MDA levels witness a rapid increase at early ICH stages, and these levels are closely associated with severity of clinical symptoms (Keep *et al.*, 2012; Wang *et al.*, 2012; Zeng *et al.*, 2008) [10, 23, 25].Catalases are tetrameric heme containing enzymes which directly dismutate H_2O_2 into water and oxygen. Catalase activity was found to be significantly higher in control CSF samples in comparison to the ICH ones (Fig. 1b). Wang *et al.* (2011) [22] reported that superoxide dismutase and catalase rapidly stabilizes the free radicals that result from the occurrence of ICH and is overexpressed in the initial 6 hrs. However, subsequently, various experiments reflect conflicting results of SOD levels. Hussein *et al.* (2012) [8] and Titova *et al.* (2008) [21] depicted high enzymatic activities of SOD and CAT even after 24 hrs, whereas others in contrast indicated a reduction in their levels. In the present study, the SOD activity was significantly lower in the ICH samples (2.73 U/ml) as compared to that in the control (6.76 U/ml) (Fig. 1c). This reduction in SOD activity depicted by our data can be attributed to excessive superoxide anion in CSF during ICH condition (Liu *et al.*, 2009) [11].

Glutathione peroxidase levels were significantly reduced ($p<0.05$) from 291.36 U/ml in control to 129.03 U/ml in ICH group (Fig. 2a). The existing data illustrated that subsequent the brain injury, astrocytes release high quantities of ascorbate which is then transformed to dehydroascorbate (DHHA) in the presence of reactive oxygen species, and further DHHA causes GPx inhibition (Nicolae *et al.*, 2013) [16]. Zhou *et al.* (2013) [26] illustrated that GPx is inactivated on contacting MDA and other lipid peroxidation products. These both could be the possible reasons for decreased Glutathione peroxidase activity.

Both Gred and GTS activities were found to be significantly

higher in the ICH group as compared to control (Fig. 2b; Fig. 2c). As a result of oxidative stress, the intracellular glutathione levels decrease and thus stimulatory effects Gred activity. Additionally, Gred undergoes inactivation by the oxidation of two susceptible sulfhydryl groups. The increase in GST activity in ICH group can be attributed to the oxidative aggression that almost doubles GTS activity. This upsurge might be a long-term adaptive reaction of the cerebral tissue (Aronowski and Zhao, 2011) [2]. Total nitrite levels were also significantly higher in ICH patients (2.99 ng/ml) than control (16.87 ng/mL) (Fig. 2d). This parameter reflects NO generation, secondary to the activation of N-methyl-d-aspartate receptors (Sehba *et al.*, 2011) [19]. Once NO is synthesized it generates ONOO- which is a highly destructive compound for both astrocytes and neurons (Siuta *et al.*, 2013; Wang *et al.*, 2011) [20, 23].

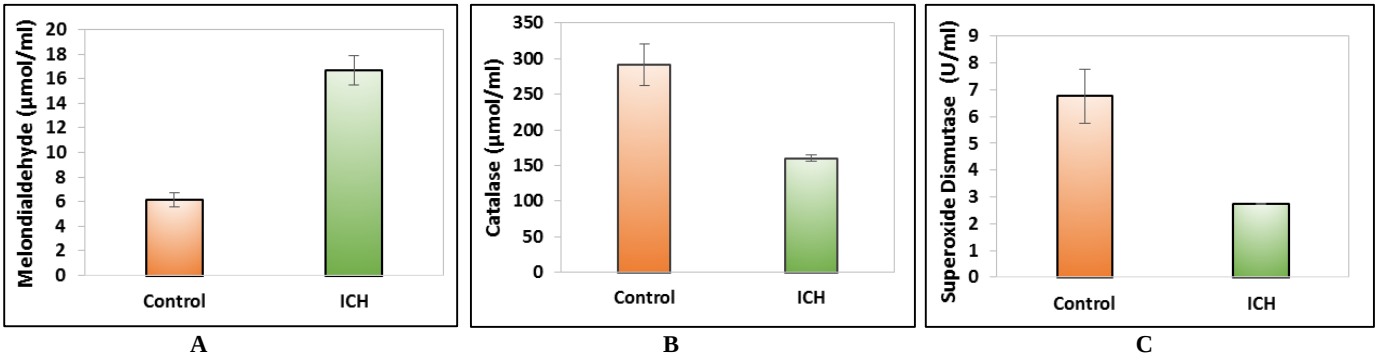

Fig 1: Concentrations of (A) Melondialdehyde (µmol/mL) (B) Catalase activity (µmol/mL) (C) Superoxide Dismutase activity (U/ml) in control and Intracerebral Haemorrhage (ICH) CSF samples.

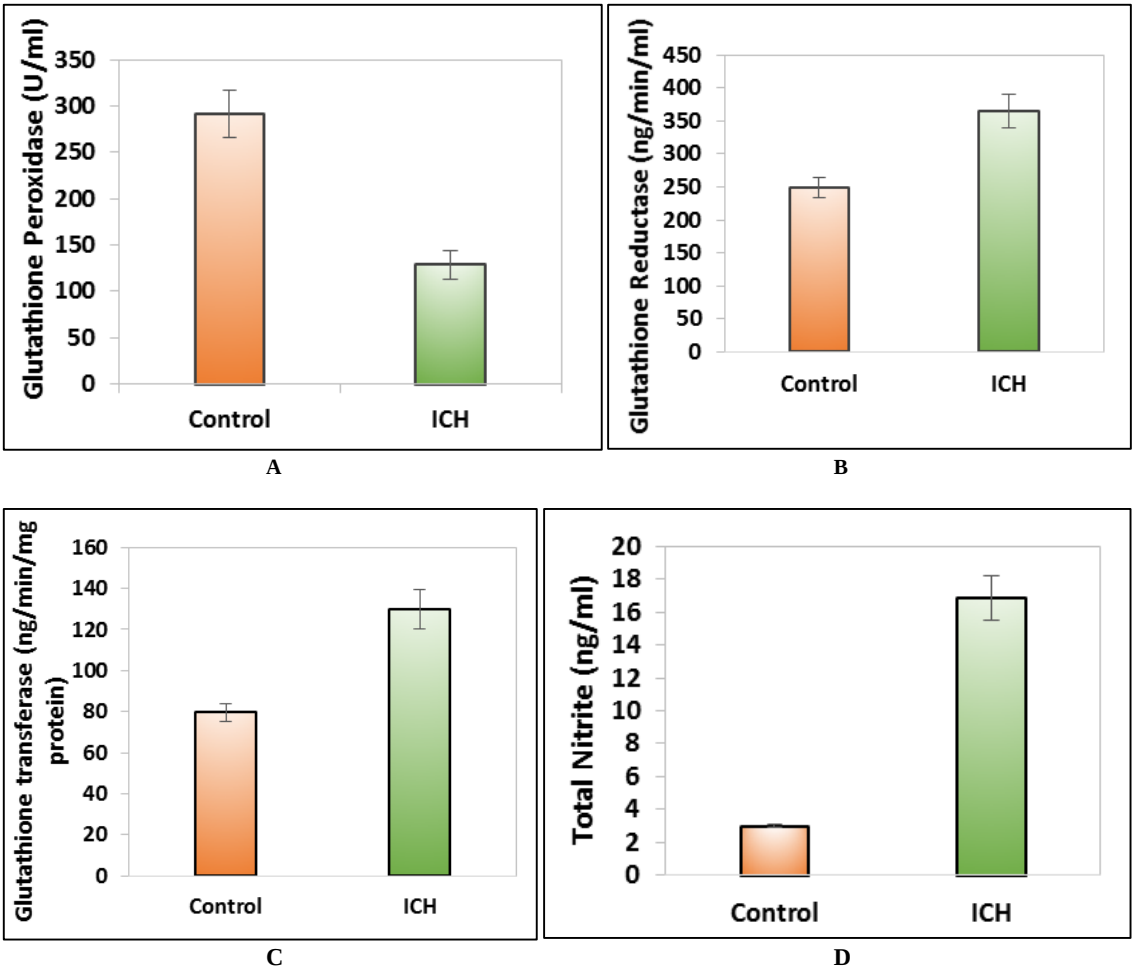

Fig 2: Concentrations of (A) Glutathione Peroxidase activity (U/ml) (B) Glutathione Reductase activity (ng/min/ml) (C) Glutathoione Transferase activity (ng/min/mg protein) (D) Total Nitrite levels (ng/ml) in control and Intracerebral Haemorrhage (ICH)

4. Conclusions

During intracerebral haemorrhage, the decrease in the activity of SOD, CAT and GPx is accompanied by a significant increase in the activities of GST and Gred. This process takes place due to high oxidative stress induced by increased nitrite and MDA levels. As long as oxidative stress deteriorates the clinical outcome in ICH patients, we recommend using certain antioxidant enzymes as biomarkers of extreme cerebral oxidative stress.

5. Acknowledgments

A. K. planned and designed the study, conducted biochemical

experiments and analyzed data, and performed statistical analysis thereafter. A.K. drafted the original manuscript and approved the final manuscript after reviewing by other authors. J. K. contributed to initial conception and designing of the study, analyzed the data, revising and reviewing the manuscript. P. S. reviewed, supervised and approved the final draft of this manuscript as submitted. Every author has contributed sufficiently in this study, meeting the authorship criteria, and each one has reviewed and approved this final version of manuscript.

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