

## Elevated Plasma Levels of Bilirubin in Patients with Neurotrauma Reflect its Pathophysiological Role in Free Radical Scavenging

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**Abstract.** Bilirubin, a powerful endogenous antioxidant, is one of the catabolites of heme oxygenases (HOs). In this study, the plasma bilirubin concentration was measured to establish bilirubin kinesis after traumatic brain injury (TBI). Furthermore, in *in vitro* studies, the free radical scavenging activity and antioxidant potency of bilirubin was also investigated at various concentrations, including physiological ones. Indirect plasma bilirubin was measured in 25 patients on days 1, 2, 3 and 4 after presentation with TBI. The ability of bilirubin to scavenge the hydroxyl (OH·) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, and its antioxidant potency, were also analyzed using electron spin resonance (ESR) and the bioantioxidant power (BAP) methods, respectively. Plasma bilirubin levels were significantly higher on days 2, 3 and 4 than on patient admission (day 1;  $p < 0.05$ ). ESR and BAP results revealed that bilirubin has direct OH· and DPPH radical scavenging activities and potent antioxidant effects *in vitro* at physiological concentrations. These data indicate that physiological concentrations of bilirubin have antioxidant properties and that it constitutes one of the biological defense mechanisms in neurotrauma patients.

Reactive oxygen species (ROS) are known to play an important role in a variety of neuropathological conditions (1-6). A myriad of evidence supports the contention that controlling free radical production is an important treatment strategy for many of these conditions (7-9). The pathophysiological steps in neuronal injury have gradually

been elucidated with the advent of molecular biology techniques. The brain is rich in polyunsaturated fatty acids, which render neuronal cells vulnerable to oxidative attack. The control of radical formation has been shown to be very important for neuroprotection (7-9) and, in animal experiments, antioxidants dramatically reduced cerebral damage (8-10). However, clear clinical corroboration of the animal evidence has been somewhat slow to emerge and results obtained have been ambiguous, in part because of the inherent difficulty in measuring both radicals and their quenching in humans (7, 11).

Heme oxygenases (HOs) are the rate-limiting enzymes in heme degeneration, catalyzing the cleavage of the heme ring to form ferrous irons, carbon monoxide (CO) and biliverdin. Biliverdin is rapidly metabolized to bilirubin, which is known to have powerful antioxidant properties (12-15). Heme oxygenase-1 (HO-1) is a highly inducible protein, activated in systemic inflammatory conditions by oxidative stress (16-23). The induction of HO-1 may be associated with cellular protection against oxidative stress by bilirubin (12-15, 17, 20, 24-36). Recent reports demonstrated that HO-1 is also expressed in various central nervous system diseases including stroke and trauma (6, 18, 19, 21, 23, 37). Thus, to investigate the role of HO-1 in traumatic brain injury (TBI) patients, the plasma bilirubin concentration was measured. In *in vitro* studies, the ability of bilirubin to scavenge hydroxyl (OH·) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and exert antioxidant effects within physiological concentrations were also investigated.

This protocol was approved by the Ethical Committee for Human Study of Showa University, Japan.

### Materials and Methods

**Subjects.** Twenty-five consecutive isolated TBI patients, admitted to Showa University Hospital (Japan) from 2002 to 2004, who had their blood collected over the 4-day period from the time of the brain insult, were enrolled in the present study. Patients with liver dysfunction complications were excluded.

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**Key Words:** Bilirubin, heme oxygenase, traumatic brain injury, antioxidant, bioantioxidant potential (BAP), electron spin resonance, ESR, hydroxyl radical.

Table I. Demographic characteristics of the study population (Mean±SE).

Parameters	Cases (n=25)	
Mean age±SE, years	51.7±4.2 (16-87)	
Gender (M/F)	(16/9)	
Mean GCS±SE	6.3±0.6	
GOS		
good recovery	5	
moderate disability	4	
severe disability	3	
vegetative state	2	
Dead	11	
Types of TBI		
		(M/F)
ASDH	15 cases	(9/6)
Contusion	3 cases	(1/2)
DAI	7 cases	(6/1)
Surgical case	14 cases	(8/6)

GCS, Glasgow Coma Scale; GOS, Glasgow Outcome Scale; ASDH, acute subdural hematoma; DAI, diffuse axonal injury.

The demographic characteristics of the study population are presented in Table II. The male/female ratio in this series was 16:9 and the mean age was 51.7±4.2 (SE) years (range 16 to 87 years). The severity of consciousness on admission was assessed according to the Glasgow Coma Scale (GCS), prior to intubation and sedation. The mean consciousness score was 6.3±0.6 (SE). These patients were classified into 3 groups (severe group: 3-5 /GCS; moderate group: 6-8 /GCS; mild group: >8 /GCS).

The TBI classifications of the group were as follows: 15 with acute subdural hematoma (ASDH) and cerebral contusion, 7 with diffuse axonal injury (DAI) and 3 with cerebral contusion. Surgical decompression was performed in 14 patients within 24 hours of injury.

The outcome of each patient was assessed at 1 month after admission by the Glasgow Outcome Scale (GOS). At this assessment period, GOS scores were as follows: 5 with good recovery (GR), 4 with moderate disability (MD), 3 severe with disability (SD), 2 with vegetative state (VS) and 11 dead (D). The patients were reclassified into a good outcome group (GR and MD/GOS) and a poor outcome group (SD, VS and D/GOS) (Table I).

**Measurement of plasma bilirubin.** From day 1 to day 4 inclusive, 5-ml aliquots of blood were collected in the morning. Indirect bilirubin concentrations in the plasma were measured for 4 consecutive days using an enzyme assay (38).

#### In vitro ESR study

**Materials.** 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma Chem. Co. Ltd., St. Louis, MO, USA); 5, 5-dimethyl-1-pyrroline-1-oxide (DMPO) (Dojin Ltd., Kumamoto, Japan); diethylenetriamine pentaacetic acid (DETAPAC) (Wako Pure Chem Ind. Ltd., Osaka, Japan); bilirubin (Sigma Chem. Co. Ltd.).

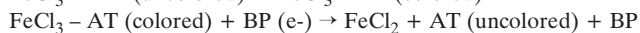
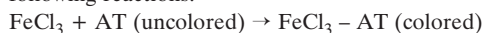
**Assay of OH· radical scavenging activity of bilirubin.** The hydroxyl radical scavenging activity of bilirubin was determined at 25°C, using ESR spectroscopy (JEOL JES REIX, X band, 100 kHz modulation frequency). Instrument settings: center field, 335.0±5

mT; microwave power 5 mW ; modulation amplitude, 0.1 mT; time constant, 0.1 sec; scanning time, 2 min.

Hydroxyl radical (OH·) was produced by Fenton reaction in a system containing a total reaction volume of 200 µL [1 mM FeSO<sub>4</sub> (0.2 mM DETAPAC) 50 µL, 92 mM DMPO 20 µL, sample 50 µL, 0.1 M phosphate buffer (PB) 50 µL, 1 mM H<sub>2</sub>O<sub>2</sub> 30 µL]

**Assay of DPPH radical scavenging activity of bilirubin.** To analyze DPPH radicals by ESR spectrometer, 100 µL of a 30 mM DPPH ethanol solution and 100 µL of a suspension of bilirubin or α-tocopherol were placed in a test tube and mixed for 10 sec. The mixture was transferred to a special flat cell and ESR parameters were set as follows: magnetic field 339.6±10 mT, Mn 553, field modulation D.79 x 0.1, time constant 0.3 sec, sweep time 3 min, power 10, gain x 1000. After 60 sec, the signal intensity was evaluated from the peak height of the third DPPH radical signal.

**Assay of antioxidant activity of bilirubin.** The antioxidant activity of bilirubin was measured by the BAP (bioantioxidant power) test performed with FRAS4 (Health & Diagnostics Limited Co., Italy). Ten µL of bilirubin (0.05, 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2 mg/dl) was dissolved in colored solution, previously prepared by mixing FeCl<sub>3</sub> with a thiocyanate derivative. After a short incubation (5 min) at 37°C, such a solution loses color and the intensity of this chromatic change is directly proportional to the ability of bilirubin to reduce, during the incubation, ferric ions to ferrous ions, according to the following reactions:



where: AT (uncolored) = a thiocyanate derivative (uncolored); FeCl<sub>3</sub> - AT (colored) = the colored complex of ferric chloride with the thiocyanate derivative; BP (e<sup>-</sup>) = a molecule of the bilirubin barrier with reducing/electron donating/antioxidant activity against ferric ions; BP = the oxidized form of BP (e<sup>-</sup>); FeCl<sub>2</sub> = the ferrous chloride obtained by the reducing activity of BP (e<sup>-</sup>).

Photometric reading was employed to assess the intensity of decoloration. A lyophilized human control serum with known antioxidant activity (µmol/l) was used to periodically calibrate the FRAS4 system.

**Statistical analysis.** The plasma bilirubin concentration at different time-points was compared with repeated ANOVA. Significant differences for these analyses were defined as *p*<0.05.

## Results

**Plasma bilirubin concentrations in TBI patients.** The mean plasma bilirubin levels on day 1 were 0.52±0.24 (SD) mg/dl (range 0.2 to 0.9 mg/dl). The plasma bilirubin concentrations were significantly elevated from days 2 to 4, as compared to day 1. The level was highest on day 2 (0.92±0.30mg/dl) and decreased on days 3 and 4. (Figure 1).

**Direct OH· scavenging activity.** The ESR study demonstrated that the formation of a hydroxyl radical-DMPO spin adduct was significantly inhibited by 100 µg/ml bilirubin and was completely inhibited by 1.7 mg/ml bilirubin (Figure 2).

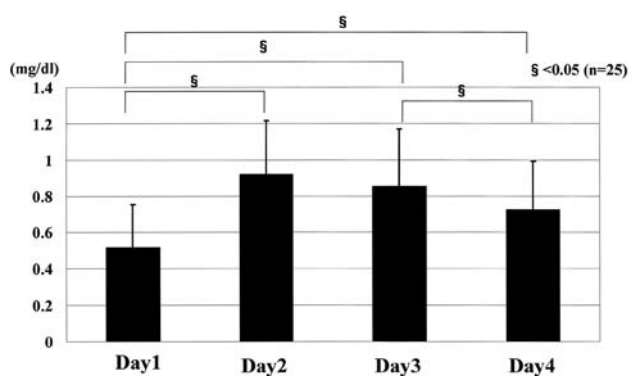


Figure 1. Time-course of plasma bilirubin concentration after TBI. Plasma bilirubin concentration was significantly increased on day 1. On days 2 and 3, plasma bilirubin concentrations gradually decreased. § $p < 0.05$ . Bars: SD

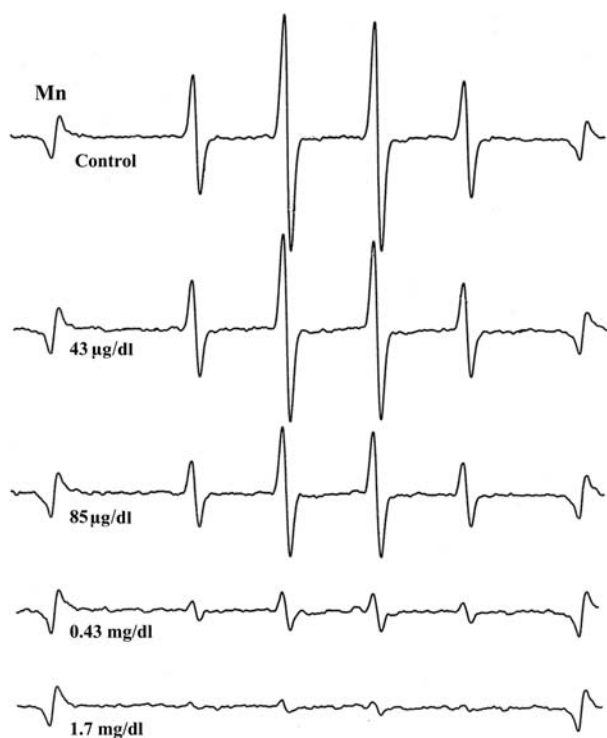


Figure 2. Hydroxyl radical ( $OH^\cdot$ ) scavenging ability of bilirubin. Bilirubin efficiently scavenged  $OH^\cdot$ , produced by the Fenton reaction.

**DPPH radical scavenging activity.** The typical ESR spectra of DPPH radicals, as well as the radical scavenging effects of bilirubin and  $\alpha$ -tocopherol on DPPH radicals, are shown in Figure 3. Bilirubin exhibited a concentration-dependent ability to trap stable DPPH radicals (5-25  $\mu$ M) (Figure 3).

**Antioxidant activity of bilirubin.** The dose-response characteristics of the antioxidant of bilirubin activity were examined

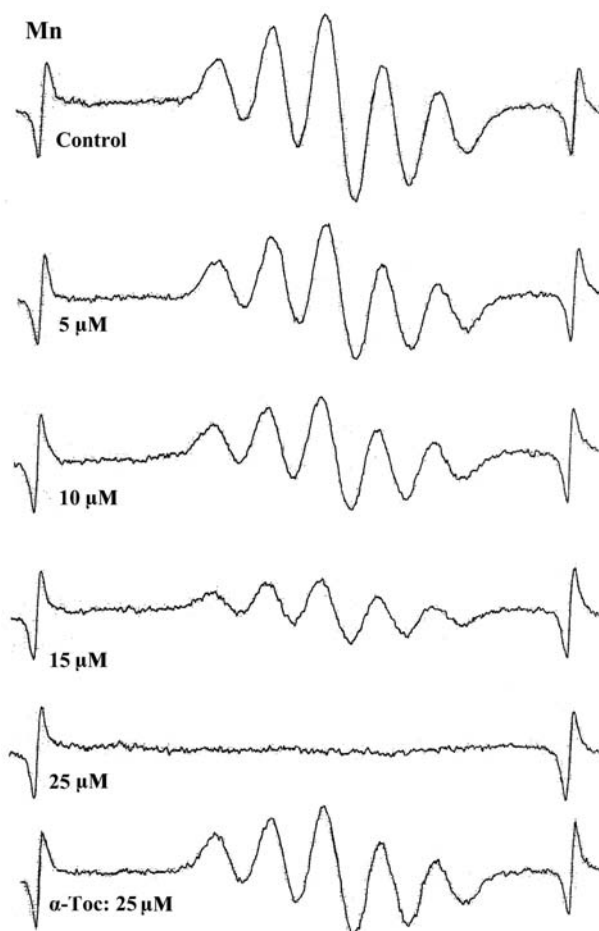


Figure 3. DPPH radical scavenging activity of bilirubin. The ESR spectra of DPPH after addition of 0, 5, 10, 15 and 25  $\mu$ M bilirubin.

by BAP test. This revealed that bilirubin has antioxidant activity at physiological concentrations. In healthy adults, the optimal value of the BAP test is in excess of 2200  $\mu$ mol/l, which equates to approximately 1.5 mg/dl of bilirubin (2314  $\mu$ mol/l) (Figure 4).

## Discussion

Heme oxygenases (HO) catalyze the cleavage of the heme ring to form ferrous iron, carbon monoxide and biliverdin (12, 13). Biliverdin is rapidly reduced by biliverdin reductase to bilirubin such that, in intact tissues, biliverdin rarely accumulates and the primary physiological product of HO is generally thought to be bilirubin (12, 13). Two principal forms of HO have been distinguished and molecularly cloned (39). HO-1 is an inducible enzyme, also designated as heat shock protein-32, and its protein synthesis is elicited by multiple stimulants (especially those associated with red

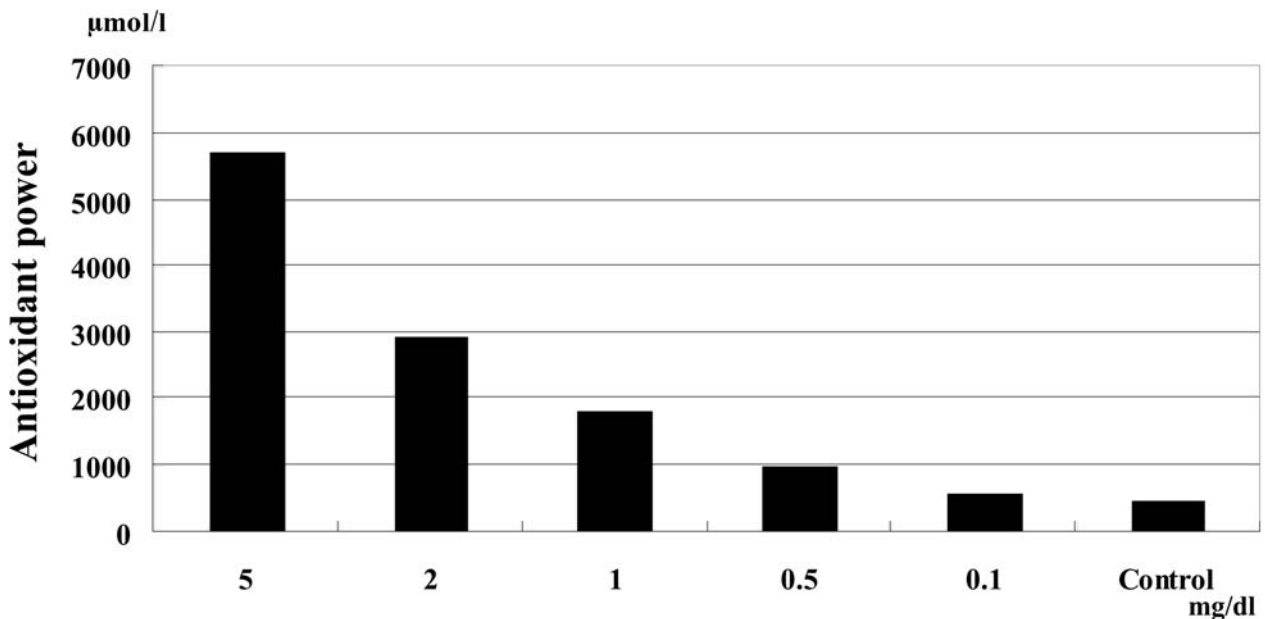


Figure 4. Antioxidant potency of bilirubin. Physiological concentrations of bilirubin have strong antioxidant potency.

blood cell damage, such as heme and other porphyrins). HO-1 is concentrated in tissues such as the spleen and liver, which degrade heme from aged red blood cells. By contrast, HO-2 is constitutive and is most prevalent in the testis and also in the brain, where it accounts for the vast majority of HO activity. Evidence suggests that HO-2 localized to selective neuronal populations plays a major role in neuromodulatory activities. Both its RNA and protein selectively concentrate in particular neuronal populations, although most, if not all, neurons possess HO-2. In 1997, the existence of a third HO isoform, HO-3, was described in the rat (40). In that study, HO-3 was reported to be the product of a single transcript of ~2.4 kb encoding a protein of ~33 kDa. Its transcript, the product of a single-copy gene, was found in various organs including the spleen, liver, kidney and brain and, therefore, displayed a tissue expression pattern similar to that of the HO-2 transcript. The predicted amino acid structure of HO-3 differs from HO-1 and HO-2, but is closely related to HO-2 (~90% identical). Bilirubin is regarded as a waste product, yet has been known for some time to possess antioxidant properties. This peculiar feature of bilirubin was investigated by Stocker and colleagues, who showed that physiological levels of the bile pigment suppress the oxidation of lipid membranes to a greater extent than  $\alpha$ -tocopherol, which hitherto had been regarded as the best antioxidant against lipid peroxidation (12). In the plasma, bilirubin is normally present in either free form or else bound to serum albumin in a bilirubin-albumin complex (12). Interestingly, free

bilirubin protects low-density lipoproteins from oxidation more efficiently than albumin-bound bilirubin. It has also been reported that both exogenous and endogenously generated bilirubin can effectively prevent the endothelial cell death mediated by hydrogen peroxide and peroxynitrite, respectively (31, 41).

In the present study, we provide the first demonstration that plasma bilirubin concentrations are significantly elevated in neurotrauma patients, which may indicate that the bilirubin and HO pathways are functionally involved in TBI. We have previously reported plasma bilirubin concentrations in patients with hemorrhagic stroke (14). In intracerebral hemorrhage (ICH) patients, the plasma bilirubin concentration is highest on day 2, whereas it peaks on day 1 in subarachnoid hemorrhage (SAH) patients. A plausible explanation for this difference is that SAH may induce severe diffuse primary brain damage at the time of onset, whereas ICH is more likely to induce local, site-specific lesions. The transient elevation of the bilirubin concentration on day 2, in both ICH and neurotrauma patients, may suggest secondary brain damage. These temporal patterns of bilirubin level presumably directly reflect certain features of each disease.

The production of bilirubin in plasma appears to be attributable to systemic oxidative stresses caused by vascular and brain damage, which occur after TBI *via* HO pathway activation. The pathophysiology of neurotrauma is evidently more complicated than that of cerebral infarction. Although HO-1-associated signaling mechanisms have yet

to be elucidated in humans, recently, the first human case of HO-1 deficiency was reported (35). This patient had been suffering from persistent hemolytic anemia characterized by marked erythrocyte fragmentation and intravascular hemolysis, with a paradoxical increase in serum haptoglobin and low bilirubin (0.1-0.3 mg/dl). On histological examination, extended endothelial cell injury, evidently caused by oxidative stress, was observed. Chin *et al.* reported that the bilirubin level increases in patients with obstructive sleep apnea-hypopnea syndrome and that this elevation protects against disorders related to hypoxemia (36). High serum bilirubin is also associated with decreased risk of early familial coronary artery disease (24, 43). Carbon monoxide (CO), the other heme metabolite produced by HO-1, is considered to be involved in vasodilatation and to play a protective role in vascular remodeling, based on its ability to inhibit endothelin-1 and platelet-derived growth factor- $\beta$  (44).

In this study, we demonstrated the direct OH $\cdot$  and DPPH scavenging and potent antioxidant activities of bilirubin, all of which occur at physiological concentrations. Both OH $\cdot$  and DPPH are major free radicals with important roles in neuronal injury. In whole blood, there are a myriad of substances with either oxidant or antioxidant effects. The antioxidant capacity of normal serum is generally defined as being in excess of 2200  $\mu\text{mol/L}$ , as determined by the BAP method. The antioxidant potency of 1 mg/dl bilirubin is 1820  $\mu\text{mol/L}$  and that of 0.5 mg/dl of bilirubin is 997  $\mu\text{mol/L}$ . Notably, in the current study, plasma bilirubin was elevated by 0.40 mg/dl on day 1 after TBI. From our data, it is, therefore, reasonable to hypothesize that the elevation of bilirubin after human TBI confers an advantage in responding to oxidative stress. Recently, it has been reported that in stroke patients, the degree of blood antioxidant activity is associated with the volume of ischemic cerebral infarction and the degree of neurological impairment that ensues (42).

When considered in the context of other data, the results of this study provide important evidence that activation of HO pathways constitutes a key biological self-defense mechanism that is related to both the pathophysiology and outcome of TBI. Further clinical and basic studies are certainly warranted to more fully elucidate the importance of this process in patients afflicted with neurotrauma.

## Conclusion

The present, albeit preliminary, investigation provides evidence that the potent antioxidant, bilirubin, is induced after TBI, in a manner which is directly related to the intensity of oxidative stress. We propose that measurement of plasma bilirubin may be employed as a marker of both oxidative stress and antioxidant potency in patients with TBI.

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