Levels of $F_2$-isoprostanes, $F_4$-neuroprostanes, and total nitrate/nitrite in plasma and cerebrospinal fluid of patients with traumatic brain injury

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Abstract
Several events occurring during the secondary damage of traumatic brain injury (TBI) can cause oxidative stress. $F_2$-isoprostanes ($F_2$-IsoPs) and $F_4$-neuroprostanes ($F_4$-NPs) are specific lipid peroxidation markers generated from arachidonic acid and docosahexaenoic acid, respectively. In this study, we evaluated oxidative stress in patients with moderate and severe TBI. Since sedatives are routinely used to treat TBI patients and propofol has been considered an antioxidant, TBI patients were randomly treated with propofol or midazolam for 72 h postoperation. We postoperatively collected cerebrospinal fluid (CSF) and plasma from 15 TBI patients for 6–10 d and a single specimen of CSF or plasma from 11 controls. Compared with the controls, the TBI patients exhibited elevated levels of $F_2$-IsoPs and $F_4$-NPs in CSF throughout the postsurgery period regardless of the sedative used. Compared with the group of patients who received midazolam, those who received propofol exhibited markedly augmented levels of plasma $F_2$-IsoPs, which were associated with higher $F_4$-NPs levels and lower total nitrate/nitrite levels in CSF early in the postsurgery period. Furthermore, the higher CSF $F_2$-IsoPs levels correlated with 6-month and 12-month worse outcomes, which were graded according to the Glasgow Outcome Scale. The results demonstrate enhanced oxidative damage in the brain of TBI patients and the association of higher CSF levels of $F_2$-IsoPs with a poor outcome. Moreover, propofol treatment might promote lipid peroxidation in the circulation, despite possibly suppressing nitric oxide or peroxynitrite levels in CSF, because of the increased loading of the lipid components from the propofol infusion.

Keywords: head injury, lipid peroxidation, sedative, outcome, nitric oxide

Introduction
Quantification of $F_2$-isoprostanes ($F_2$-IsoPs) derived from arachidonic acid, which is abundant in all cell types, by mass spectrometric methods, has been regarded as the most reliable approach for accessing lipid peroxidation in vivo [1]. In addition, $F_4$-neuroprostanes ($F_4$-NPs) formed from docosahexaenoic acid (DHA), which is enriched in neurons, has been considered a selective marker of neuronal oxidative damage [2]. $F_2$-IsoPs and $F_4$-NPs are initially generated in esterified form on phospholipids; however, on hydrolysis by enzymes with phospholipase A$_2$ activity, the free form is produced and then released into circulation or into surrounding body fluids [3]. Some of the isomers of $F_2$-IsoPs in the free form, particularly 15-$F_2$-IsoP, are known to exert the activities of vasoconstriction and platelet aggregation, which may contribute to the pathogenesis of diseases [4,5]. The gas chromatography/negative-ion chemical ionization mass spectrometry (GC/NICI-MS) method is the most sensitive and robust method to quantify free-form $F_2$-IsoPs and $F_4$-NPs levels in clinical specimen [6,7]. Using this unique method, we previously found that only the free forms of $F_2$-IsoPs and free $F_4$-NPs in cerebrospinal fluid (CSF) positively correlated with the greater extent of hemorrhage on hospitalization and poor outcomes 3-month postsurgery in patients with aneurysmal subarachnoid hemorrhage (aSAH), a common type of hemorrhagic stroke, despite observing elevated plasma levels of $F_2$-IsoPs and CSF levels of total nitrate ($NO_3^-$) and nitrite ($NO_2^-$) (total nitrate/nitrite), which are metabolites of NO and peroxynitrite (ONOO$^-$), in the aSAH patients [8,9].

Traumatic brain injury (TBI) is a major cause of mortality and disability in humans. Several comprehensive reviews have described the pathological events that occur when primary damage, such as formation of hematomas and diffuse axonal injury, is caused by direct mechanical injury to the brain tissue and that occur during the consequent secondary damage that develops at subsequent time points [10–13]. Many processes resulting from the secondary damage, such as ischemia–reperfusion injury, release of hemoglobin or free heme, mitochondrial damage, disruption of calcium homeostasis, and activation of inflammatory reactions, could lead to enhanced production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) or increased oxidative damage [10–15]. Several studies have indicated increased oxidative stress or elevated lipid peroxidation in TBI patients. However, most...
studies have employed indices that were inadequate to reflect oxidative damage in the brain or the entire body, such as the detection of antioxidant enzymes and antioxidants in erythrocytes and body fluids, or they have used unreliable markers of lipid peroxidation in vivo, such as the detection of malondialdehyde (MDA) by using the thiobarbituric-acid-reactive substances (TBARS) assay [16–20]. Although some studies have indicated increased F$_2$-IsoPs levels in the CSF or plasma of TBI patients, most researchers have used non-specific immunoassays to detect F$_2$-IsoPs [20–23]. Our preliminary study was the first to report the increased levels of free F$_2$-IsoPs and free F$_4$-NPs in the CSF of TBI patients by conducting GC/NICI-MS analysis [24]. Using the GC/NICI-MS method, Corcoran et al. examined levels of F$_2$-IsoPs, F$_4$-NPs, and isofuran in the CSF of TBI patients versus controls. However, they measured total (free plus esterified) levels of these markers, not the free form, and they only investigated the first-day levels of TBI patients. In addition, they did not collect plasma samples and did not analyze nitrate/nitrite levels in CSF or plasma [25]. Furthermore, the roles of different isoenzymes of nitric oxide synthase (NOS), nitric oxide (NO), and peroxynitrite (ONOO$^-$) were implicated in the pathogenesis of TBI, mostly using experimental animal models [26,27]. To date, only Uzan et al. have demonstrated elevated concentrations of total nitrate/nitrite in the CSF of TBI patients relative to those in controls [28].

Midazolam, a benzodiazepine, and propofol (2,6-diisopropylphenol) are the two common sedatives for treating TBI postsurgery. Both these drugs exert their sedative effect by acting as agonists for the $\gamma$-aminobutyric acid type A receptor [29,30]. Clinically used propofol is prepared in emulsion formulation that contains soya bean oil, glycerol, and egg phospholipids [30], which is the same as the intravenous lipid emulsion Intralipid used for neonatal parenteral nutrition [31]. Compared with midazolam, propofol provides the benefit of rapid recovery from sedation for patients after its use is discontinued, but it may also pose greater risks of hypotension and hypertriglyceridemia in patients [29,30]. Previous studies have shown that propofol could react with peroxynitrite in vitro [32,33] and could suppress the expression of the inducible form of NOS (iNOS) and the production of inflammatory mediators augmented by lipopolysaccharide in cultured cells or animals [34–36]. Because of its phenolic hydroxyl group, propofol also has been indicated to be an antioxidant [37,38]. However, studies demonstrating the suppression of lipid peroxidation by propofol have detected either MDA using the TBARS assay or 15-F$_2$-IsoP using immunoassays [34–41]. In addition, soybean triglycerides in Intralipid in the concentration of 20% contains more than half of esterified polysaturated fatty acids (PUFAs) [42]. High levels of lipid hydroperoxide, an intermediate from the lipid peroxidation process of PUFAs, have been indicated in Intralipid [31,43]. Therefore, the potential effect of the lipid components in the propofol infusion on the status of oxidative stress remains unknown.

In the current study, we measured the free form of F$_2$-IsoPs in both CSF and plasma, the free form of F$_4$-NPs in CSF, and total nitrate/nitrite of the daily specimens collected from TBI patients postoperatively for 6–10 d, depending on the conditions of the patients, and compared the results with the same markers in specimens from a group of controls. Since surgery is not performed for patients with mild TBI, CSF samples could not be obtained from that population. Thus, only samples from patients with moderate or severe TBI were studied. Furthermore, because propofol and midazolam are the two sedatives that are routinely used in the treatment of TBI patients during the first 72 h after surgery at Chang Gung Memorial Hospital, propofol or midazolam was given to the TBI patients in a randomized manner. The significance of differences in the levels of various oxidative stress markers between TBI patients and controls was first examined. The results between the 2 sedative groups among the TBI patients were then compared. Finally, we analyzed any significant correlation between the various oxidative stress markers with the grades of Glasgow Coma Scale (GCS) and Glasgow Outcome Scale (GOS) of the TBI patients.

Materials and Methods

Evaluation of clinical conditions and surgery for TBI patients

The clinical presentation of TBI patients on admission was graded according to the GCS, which is the sum of points evaluated for eye opening (1–4 points), verbal response (1–5 points), and motor response (1–6 points) [44]. TBI patients with the GCS scores of 14–15, 9–13, and ≤8 are routinely categorized as mild, moderate, and severe cases of TBI, respectively. Based on the findings of computed tomography (CT) before surgery, the location of hemorrhage occurred on the TBI patients is routinely classified into subdural hemorrhage (SDH), epidural hemorrhage (EDH), subarachnoid hemorrhage (SAH), intracerebral hemorrhage (ICH), and intraventricular hemorrhage (IVH). Surgery was carried out either by craniotomy or craniectomy to remove hematoma and conduct external ventricular draining for monitoring intracranial pressure (ICP) in the TBI patients. Patient outcomes were graded 3, 6, and 12 months postsurgery using the GOS, for which the following 5 categories are used: good recovery and resumption of normal life (G), independent lifestyle with moderate disability (M), severe disability and dependent for daily support (S), persistent vegetative state (V), and death (D) [45].

Inclusion and exclusion criteria

This study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Approval for studying humans was received from Institutional Review Board at Chang Gung Memorial Hospital. Written informed consent was obtained from all patients or their closest relative. The age of all patients was within the
range of 15–75 y. Non-TBI control patients were patients who underwent spinal surgery without central nervous diseases. Only patients with moderate or severe TBI who were admitted to the intensive care unit (ICU) of Chang Gung Memorial Hospital within 6 h of onset were included in this study, but cases caused by penetrating head injury were excluded. TBI patients who were brain dead at admission, had severe hypotension (systolic blood pressure < 90 mmHg) resulting from the injury, or had a condition requiring cardiopulmonary resuscitation were also excluded. In addition, patients who had taken anticoagulation drugs, such as heparin and warfarin, or had a history of allergic reactions to sedatives were excluded. All of the included patients had no history of major systemic diseases such as neurodegenerative diseases, cardiovascular diseases, and diabetes.

**Sedation management for the TBI patients**

Fresofol 1% containing 10 mg/mL of propofol (Fresenius Kabi Austria GmbH, Austria) and Midatin injection containing 5 mg/mL of midazolam (Nang Kuang Pharmaceutical Company, Taiwan) are the two sedatives routinely used at Chang Gung Memorial Hospital. Fresofol 1% is a milky white liquid that also contains soybean oil, glycerol, egg lecithin, oleic acid, and sodium hydroxide as inactive ingredients. The TBI patients were randomly assigned to propofol or midazolam groups in a double-blind manner. In the ICU, the TBI patients received one of the sedatives for 72 h after surgery by intravenous infusion to control ICP and prevent agitation of the patients. Patients were maintained with the dosage of 0.3–0.4 mg/kg/h for propofol and 0.03–0.2 mg/kg/h for midazolam.

**Collection and storage of specimen**

Single CSF samples were collected from the control patients at the time of spinal anesthesia, and single blood samples were collected into EDTA-containing Vacutainers by venipuncture. Regarding the specimens obtained from the TBI patients, CSF samples were collected through the external ventricular draining, whereas blood samples were collected through venipuncture. CSF and blood samples were collected once daily after surgery for up to 10 d, because the draining tube must be removed 10 d after surgery. However, serial samples were collected for less than 10 d, such as for 6 or 7 d, for some TBI patients because of early removal of draining tubes due to adequate recovery, drainage dysfunction, or unexpected shedding of draining tubes. All samples were centrifuged at 3000 rpm for 10 min using a clinical centrifuge within 1 h of collecting the samples. Supernatants from the CSF samples and plasma from the blood samples were aliquoted and stored in a freezer at −70°C immediately after centrifugation.

**Detection of free F₂-IsoPs in CSF and plasma**

The GC/NICI-MS procedures adopted in this study for sample purification and derivatization for detecting free F₂-IsoPs in human body fluids were conducted based on the method described by Milne et al. [7,46] with minor modifications [8,9,47,48]. Approximately 0.5 mL of CSF or plasma was mixed with 3 mL of ultrapure water containing the internal standard [²H₄]-15-F₂-IsoP. A hydrogen chloride solution was added to adjust the pH to 3, and the samples were subjected to two consecutive runs of solid phase extraction (SPE) using 6-mL disposable C₁₈ and silica SPE columns with 500 mg of sorbent (J. T. Baker) operated on a negative-pressure vacuum manifold column processor (J. T. Baker). The trace water in the final eluate from the C₁₈ column was dried briefly using 2 g of anhydrous sodium sulfate and the eluate was immediately transferred into the silica columns. F₂-isoPs in the final eluate from the silica columns were converted to pentafluorobenzyl (PFB) esters with PFB bromide and N,N′-disopropylethylamine. Subsequently, the analyte was purified using thin-layer chromatography (TLC). Silica on TLC plates (LK6B silica plates from Whatman) in the range of 1 cm above and below the position of the TLC standard (methyl ester of PGF₂α), which was visualized by exposing the standard to a phosphomolybdic acid solution on a separate plate, was scraped and extracted using ethyl acetate. The PFB esters of F₂-IsoPs in the analyte were further derivatized to ether derivatives by the reaction with N,O-bis(trimethylsilyl)trifluoroacetamide and dimethylformamide. The final analyte was dried under nitrogen gas and was dissolved in CaH₂-treated undecane. Finally, 2 µL of analyte in undecane was injected into a GC/MS instrument (6890 GC/5975 MS, Agilent) for detection under the NICI mode.

The basic settings for the GC/MS detection procedure were as described by Milne et al. [7,46], but with a few modifications and additional details that have been described in our previous publications [9,47,48]. The GC/MS instrument was equipped with a 15-m capillary column (DB-1701, Agilent). The Drilled Uniliner Liner with Press-Tight seal and deactivated wool (Restek) was installed in the GC inlet. The flow rate of the carrier gas (helium) and reagent gas (methane) was set at 55 mL/min and 2 mL/min, respectively. The temperature of the GC column was initially programmed to increase at a rate of 18°C/min from 190°C to 300°C and then to be maintained at 300°C for 2 min during the acquisition of chromatograms. Subsequently, a second holding at 280°C for 20 min was carried out, at which point the detector was turned off. The second holding of the column temperature could eliminate interference caused by the retention of unknown substances in the samples [47,48]. Moreover, the electron multiplier (EM) gain in the setting of “MS Instrument Parameters” was elevated to enhance the relative abundance or instrument sensitivity, which could markedly enhance the baseline-corrected peak height of signals while only slightly increasing background noise [48]. The sum of basal EM voltage after tuning and EM voltage added by the method setting was kept in the range of 1700–1800 V. The mass-to-charge ratio (m/z) values adopted to detect the carboxylate ions of F₂-IsoPs in the samples and the internal standard [²H₄]-15-F₂-IsoP added to the samples under the selected ion monitoring
mode were \( m/z \) 569.4 and \( m/z \) 573.4, respectively. The baseline-corrected peak heights of the single peak representing the major isomers of \( F_4^{-}\)-IsoPs and that of the internal standard were obtained to quantify the amount of endogenous \( F_2^{-}\)-IsoPs.

**Detection of free \( F_4^{-}\)-NPs in CSF**

The method for detecting free \( F_4^{-}\)-NPs using GC/NICI-MS has been described in our previous publications [9, 47, 48], which was modified from the methods described by Roberts et al. [49] as well as Arneson and Roberts [50]. The overall sample processing steps were similar to those for \( F_2^{-}\)-IsoPs analysis, and \([^{2}H_6]^{-}\)-15-\( F_2^{-}\)-IsoP was also used as the internal standard. The major differences were the solvents employed for washing the silica columns and wider scraping range during TLC purification (1 cm below and 3 cm above the TLC standard) for \( F_2^{-}\)-NPs analysis. For GC/NICI-MS analysis, the settings were also similar to those for \( F_2^{-}\)-IsoPs analysis, except \( m/z \) 593.5 was applied to detect endogenous \( F_4^{-}\)-NPs and the temperature was maintained at 280°C for 30 min after acquiring the data [47]. Moreover, in vitro oxidation of DHA in air was conducted and the oxidized DHA was purified following the procedures for the samples. Each day, an appropriate amount of purified DHA was analyzed with the samples. This strategy facilitated consistent identification of the range of \( F_4^{-}\)-NPs for quantifying the peak area at \( m/z \) 593.5, and was first implemented in our previous study [9].

**Analysis of total nitrate/nitrite in CSF and plasma**

Total nitrate/nitrite levels in the CSF and plasma samples were detected using the Nitrate/Nitrite Fluorometric Assay Kit and the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical), respectively. The samples were centrifuged using a filter unit (Vivaspin 500, GE) with a 10 000-MW cutoff at 13,000 g (after washing the filter with an assay buffer). Subsequently, the lower filtrate of the samples was used for the assay. Ultrapure water and sterile containers were employed to minimize possible background interference from bacteria. To detect the total nitrate/nitrite levels in CSF, the assay reagents were mixed with standards or samples in 96-well black polystyrene plates (Corning). Seven nitrate standards were prepared with standards or samples in 96-well black polystyrene plates (Nunc). Each well contained 40 μL of plasma for each reaction. After reacting with the nitrate reductase, the samples or standards were further mixed with Griess reagents. The absorbance of the final product was detected at 540 nm using the VersaMax ELISA microplate reader (Molecular Devices).

**Statistical analysis and data presentation**

The mean, peak, first-day, and mean of first 2 days’ mean (first-2) levels during the postoperative period were calculated for all of the measured parameters for the TBI patients. Statistical analysis was performed using the SPSS 12.0 software (SPSS Inc.). Two-tailed t-test was conducted to compare the age between controls patients and TBI patients included in this study. A non-parametric Mann–Whitney \( U \) test was conducted to examine the differences between two groups for all of the measured markers of oxidative stress, to minimize the influence of extreme values from highly variable clinical data. A non-parametric Spearman’s correlation analysis was performed to examine the significance of the correlation. Differences were considered statistically significant where \( P < 0.05 \). The data are presented as box plots, which were generated using SigmaPlot (Systat Software, Inc.). Each box on the graph ranges from the 25th percentile to the 75th percentile of the data population. The line within the box marks the median. The whiskers above and below the box indicate the 90th percentile and 10th percentile, respectively. The dots outside the boxes show the outliers. The whiskers and outliers are unable to be computed by the SigmaPlot when there are less than nine data points.

**Results**

**Patient information**

Table I presents a summary of the information of the TBI patients in this study. The age distribution of the patients was 16–75 (47.8 ± 21.8) y for controls and 24–68 (45.8–15.5) y for TBI patients. There was no difference in age between these two groups. Among the 15 TBI patients, eight were administered propofol and seven were administered midazolam. The 3-, 6-, and 12-month GOS differed slightly for a few patients. Regarding types of hemorrhage, 11 patients had ICH. Three patients (TBI2, TBI7, and TBI10) expired at discharge after the period of specimen collection but within 14–22 d of hospitalization. Three patients (TBI1, TBI6, and TBI9) had the GOS of “G”, which was reflected by their shorter stay at the ICU and shorter hospitalization period.

**Levels of the various markers (TBI patients versus controls)**

The levels of various markers in all TBI patients during the postoperative period were expressed in different values and were first compared with those in the control groups. As shown in Figure 1, the mean, peak, first-day, and mean of first 2 days’ levels of plasma \( F_2^{-}\)-IsoPs, CSF \( F_2^{-}\)-IsoPs,
and CSF F$_2$-NPs were significantly higher in the TBI group than the levels in the control group. For the total nitrate/nitrite levels in CSF, only the mean and peak levels were elevated in the TBI group. However, the plasma levels of total nitrate/nitrite in the TBI group did not differ significantly from that in the control group.

**Comparison of the levels of the various markers in TBI patients (propofol group versus midazolam group)**

To further examine whether propofol exerted any effect on the levels of the various oxidative stress markers in the TBI patients, the mean, peak, first-day, and mean of first 2 days' levels of plasma F$_2$-IsoPs, CSF F$_2$-IsoPs, CSF F$_2$-NPs, and CSF nitrate/nitrite were compared between the propofol and midazolam groups. Plasma nitrate/nitrite levels were not further compared because the values were not different from controls (Figure 1A). Figure 2 shows the box plots for the comparison. The mean, peak, first-day, and first 2 days’ levels were all markedly augmented in the propofol group for plasma F$_2$-IsoPs levels (Figure 2A), but no difference was observed between the propofol and midazolam groups for CSF F$_2$-IsoPs levels (Figure 2B). Moreover, the first-day and mean of first 2 days’ levels of CSF F$_2$-NPs were also higher in the propofol group (Figure 2C), whereas the mean of first 2 days’ level of total nitrate/nitrite in CSF was lower in the propofol group (Figure 2D).

By observing the differences between the two groups, we further analyzed the significance of the difference between either the propofol or the midazolam group and the controls separately, although the sample size became smaller for each group. The differences between controls and the propofol group remained significant, but the elevation in the midazolam group became non-significant for plasma F$_2$-IsoPs levels (Figure 2A). For the CSF levels of F$_2$-IsoPs and F$_2$-NPs, the levels in either group remained significantly increased compared with that of controls except first-day levels of F$_2$-IsoPs in the midazolam group (Figures 2B and 2C). The increase in the peak levels of CSF nitrate/nitrite in both groups remained significant, but the propofol group was not different from controls for the first-day levels, and the mean of first 2 days’ levels became significantly augmented in the midazolam group (Figure 2D).

**Correlations between the CSF levels of F$_2$-IsoPs and the TBI patients' GCS or GOS**

Since CSF levels of F$_2$-IsoPs in TBI patients were higher (Figure 1) and there was no difference between the propofol group and the midazolam group (Figure 2B), we further examined the correlation between CSF levels of F$_2$-IsoPs and clinical conditions for all TBI patients. Due to the limitation of a small patient number, further correlation analysis for the midazolam or the propofol group for various markers separately might not be appropriate. Table II shows the results of the Spearman’s correlation analysis for the CSF levels of F$_2$-IsoPs and the GCS or the 3-, 6-, and 12-month GOS grades for the TBI patients. The GOS grades were recorded for statistical analysis (“G” = 1, “M” = 2, “S” = 3, “V” = 4, and “D” = 5), so that a positive and significant correlation inferred that higher oxidative stress marker levels were associated with worse outcomes. There was no significant correlation for GCS on admission. Regarding the GOS, only the first-day and peak values of F$_2$-IsoPs levels correlated positively with the 6- and 12-month GOS grades, whereas the correlations with the 3-month GOS grades were non-significant.

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**Table I. Information of TBI patients.**

| Patient # | Age | Days at ICU | Conditions of hemorrhage | Sedative | Days of hospitalization | 3-mo GOS | 6-mo GOS | 12-mo GOS |
|-----------|-----|-------------|--------------------------|----------|------------------------|---------|---------|----------|
| TBI 1     | 35  | 12          | SAH                      | Propofol | 4                      | 10      | G       | G        |
| TBI 2     | 68  | 8           | ICH, SDH, SAH            | Propofol | 10                     | 15      | D       | D        |
| TBI 3     | 67  | 6           | ICH, SDH                 | Propofol | 16                     | 29      | S       | S        |
| TBI 4     | 68  | 11          | SAH                      | Midazolam | 19                     | 34      | M       | M        |
| TBI 5     | 52  | 10          | ICH, EDH                 | Propofol | 16                     | 43      | M       | M        |
| TBI 6     | 35  | 9           | ICH, SDH                 | Midazolam | 7                      | 16      | G       | G        |
| TBI 7     | 26  | 5           | ICH, SDH                 | Midazolam | 14                     | 14      | D       | D        |
| TBI 8     | 28  | 4           | SDH                      | Midazolam | 18                     | 46      | S       | M        |
| TBI 9     | 37  | 11          | ICH, SDH                 | Propofol | 7                      | 19      | G       | G        |
| TBI 10    | 50  | 6           | ICH, IVH, SAH            | Midazolam | 22                     | 22      | D       | D        |
| TBI 11    | 52  | 8           | ICH                      | Propofol | 7                      | 68      | S       | S        |
| TBI 12    | 24  | 9           | ICH, EDH                 | Propofol | 7                      | 38      | M       | M        |
| TBI 13    | 56  | 8           | ICH, SDH                 | Midazolam | 8                      | 23      | S       | S        |
| TBI 14    | 55  | 6           | ICH, SDH                 | Midazolam | 6                      | 25      | V       | S        |
| TBI 15    | 34  | 4           | EDH                      | Propofol | 31                     | 86      | V       | S        |

Classification on the conditions of hemorrhage in TBI patients are described in “Materials and Methods” section. Days of hospitalization included the time stayed at ICU and that in ward before patient discharge. Midazolam or propofol was administered to TBI patients after surgery for 72 h. GOS of patients was graded at 3, 6, and 12 months (mo) postsurgery. Three patients, TBI2, TBI 7, and TBI 10, expired when they were discharged from the hospital. SDH, subdural hemorrhage; EDH, epidural hemorrhage; SAH, subarachnoid hemorrhage; ICH, intracerebral hemorrhage; IVH, intraventricular hemorrhage; G, good recovery and resumption of normal life; M, independent lifestyle with moderate disability; S, severe disability and dependent for daily support; V, persistent vegetative state; D, death.
The alterations in the daily levels of various oxidative stress markers in TBI patients

The changes in CSF F$_2$-IsoPs, plasma F$_2$-IsoPs, CSF F$_4$-NPs, total nitrate/nitrite in CSF, and total nitrate/nitrite in plasma in the TBI patients over the postoperative period for up to 10 d are shown with the individual values in the control group in Figure 3, Figure 4, Supplementary Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1080363, Supplementary Figure 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1080363, and Supplementary Figure 3 to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1080363, respectively. The TBI patient data in these figures are presented in four panels according to the 12-month GOS classification and the sedatives applied to the patients. Fluctuations in the various markers exhibited no discernable pattern, indicating that the changes were affected by multiple events. The peak values for various markers in individual TBI patient were present at different days. Consequently, data analysis based on daily mean values over different TBI patients would be highly variable and therefore not feasible.

In agreement with the results of correlation analysis shown in Table II, there was overall greater CSF levels of F$_2$-IsoPs in the group of TBI patients with the 6- or 12-month GOS grades of “S,” “V,” or “D” (Figures 3C and 3E) than the group with “G” or “M” grades (Figures 3B and 3D). Such differences were especially evident for the midazolam group between those with good outcomes (Figure 3D) and those with poor outcomes (Figure 3E). On the other hand, it appeared that the results of higher plasma levels of F$_2$-IsoPs in the propofol group compared with those the midazolam group (Figure 2A) coincided with a substantial increase in the values at early time points in most patients in the propofol group (Figures 4B
and 4C) regardless of the status of outcome. The fluctuations were in general less for the midazolam group (Figures 4D and 4E), and the values were close to the values of controls (Figure 4A).

Discussion

In this study, we conducted a comprehensive investigation on changes in the levels of free $F_2$-IsoPs in CSF and plasma, free $F_4$-NPs in CSF, and total nitrate/nitrite in CSF and plasma to evaluate the status of lipid peroxidation and nitric or peroxynitrite production in TBI patients over a postoperative period. Although elevated level of these markers (except for total nitrate/nitrite in the plasma) in the TBI patients were found when comparing all TBI patients with the control group regardless the sedative used, we showed that postsurgery propofol infusion for 72 h markedly enhanced the levels of $F_2$-IsoPs in plasma and $F_4$-NPs in CSF, but decreased that of total nitrate/nitrite in CSF in the TBI patients, especially at early time points. Furthermore, the mean and peak levels of $F_2$-IsoPs in CSF were significantly correlated with worse 6-and 12-month GOS grades in the TBI patients.

This is the first study to investigate the effect of propofol on free $F_2$-IsoPs levels in human plasma and CSF and $F_4$-NPs in CSF of TBI patients using the GC/NICI-MS method. In contrast to the findings suggesting that

![Figure 2. Comparison on the levels of different markers between the propofol group and the midazolam group. Levels between the group of propofol (P) and the midazolam group (M) are compared in box plots. *Significant difference between two groups at the level of $P < 0.05$. **Significant difference between two groups at the level of $P < 0.01$. +Significant difference between control and the indicated group at the level of $P < 0.05$. aSignificant difference between control and the indicated group at the level of $P < 0.01$.]

Table II. Correlations between clinical conditions of TBI patients and CSF levels of $F_2$-IsoPs.

| Clinical conditions | Correlation coefficient (P values) for different values |
|---------------------|--------------------------------------------------------|
|                     | Mean         | Peak         | First        | First-2       |
| GCS                 | −0.047 (0.868) | 0.014 (0.959) | −0.043 (0.878) | 0.070 (0.803) |
| 3-month GOS         | 0.472 (0.075) | 0.434 (0.106) | 0.204 (0.465) | 0.135 (0.632) |
| 6-month GOS         | 0.583 (0.023)* | 0.586 (0.022)* | 0.272 (0.327) | 0.207 (0.459) |
| 12-month GOS        | 0.612 (0.015)* | 0.588 (0.021)* | 0.197 (0.481) | 0.129 (0.647) |

The correlation coefficients for the association between the patients’ GCS or GOS grades and mean, peak, first, or mean of first 2 days’ levels of $F_2$-IsoPs in CSF of TBI patients during the postoperative period were obtained using the non-parametric Spearman’s correlation analysis.

*Statistically significant at $P < 0.05$.  
propofol is a potential antioxidant for suppressing lipid peroxidation [37,39–41], we found that propofol infusion enhanced the lipid peroxidation levels in plasma and CSF of TBI patients (Figure 2). Despite using an immunoassay kit for detection, Ballester et al. also reported higher levels of 15-F₂-IsoP and total nitrate/nitrite in blood drawn from coronary sinus of patients who were administered propofol while undergoing coronary artery bypass graft surgery, compared with patients who received sevofluorane [51]. Since high levels of lipid hydroperoxide have been detected in Intralipid [31,43], we speculated that lipid hydroperoxide was also present in the lipid emulsion of the propofol infusion, and peroxy radical or alkoxy radical derived from the peroxy catalyzed by iron ions might occur in the blood system to augment the levels of esterified F₂-IsoPs on the lipoproteins of TBI patients undergoing propofol treatment. This might subsequently lead to increased levels of free F₂-IsoPs in plasma catalyzed by the platelet-activating factor acetyl hydrolase [52]. If such an effect could cause falsely increased levels of free F₂-IsoPs, then the effect would be even greater when measuring the esterified or total (free plus esterified) forms. Florian and Pawelczyk showed that levels of free F₂-IsoPs in plasma, detected by immunoassay, were elevated following infusion of Intralipid and heparin, which was used to activate lipoprotein lipase for the formation of non-esterified fatty acids from Intralipid, in humans [53,54], although the condition was not exactly the same as when Intralipid was infused alone. On the other hand, whether the increased loading of lipid hydroperoxide in the circulation could pass through the blood–brain barrier and promote lipid peroxidation in the brain tissue was uncertain, but it might occur during TBI due to the non-specific damage to the brain. Moreover, it has been indicated that Intralipid infusion in neonates could increase pulmonary vascular resistance [55] or promote vasoconstriction of peripheral arteries [56]. The more elevated levels of first-day or first 2 days’ F₄-NPs in CSF, but not those of F₂-IsoPs in CSF, in the propofol group (Figures 2B and 2C) could be attributed to DHA being more susceptible to lipid peroxidation than arachidonic acid [49], or F₄-NPs being a more sensitive marker for transient ischemia–reperfusion injury in the brain due to increased levels of vasoconstrictive F₂-IsoPs in the cerebral circulation. However, the tendency of higher F₂-IsoP levels in the propofol group with good outcomes (Figure 3B) than those in the midazolam
group with good outcomes (Figure 3D) at early time points suggest that propofol effect might also exist for CSF F₂-IsoPs levels to a certain extent, although not so obvious. In some investigations on the effect of propofol in which animal models were used, pure propofol without the lipid emulsion was employed [34,39], but some studies investigated the effect of clinically used propofol in animals by administering lipid emulsion to controls [40,41]. This could explain the inconsistency in the conclusion regarding the effect of propofol. However, it would be impossible to investigate the effect of propofol in TBI patients by injecting lipid emulsion to controls or the midazolam group in a clinical study.

Regarding the comparison of the levels of lipid peroxidation between the controls and all TBI patients, the mean, peak, first-day, and mean of first 2 days’ levels of free F₂-IsoPs in plasma and CSF as well as free F₄-NPs in CSF were markedly elevated in the TBI patients (Figure 1). However, the differences in the levels of F₂-IsoPs in plasma were largely attributed to the effect of propofol, which was supported by the observation that the levels in the midazolam group were not different from that of controls (Figure 2A). The conclusion would be misleading by considering plasma F₂-IsoPs as one of sensitive markers for indicating oxidative damage in TBI patients when conducting a study without considering the sedatives used. By contrast, although the first-day and mean of first 2 days’ levels were higher in the propofol group, the marked increase in CSF F₄-NPs levels in either the propofol or the midazolam group in all measurements (Figure 2C) indicated specific enhancement of oxidative damage to DHA in the brain of TBI patients.

Although NO can be metabolized to nitrite, hemoglobin converts NO to nitrate. Moreover, nitrate can be from dietary sources and can be further reduced to nitrite by bacteria in the body. However, nitrite can also be reduced back to NO under hypoxic conditions by heme-containing proteins [57]. Peroxynitrite or the conjugated acid peroxynitrous acid can be decomposed into nitrate and nitrite in aqueous solutions [59]. Therefore, detection of total nitrate/nitrite levels is not able to distinguish the contribution of NO from that of peroxynitrite. Hypoxic conditions and hemorrhagic conditions in the brain following TBI could also contribute to fluctuations in the CSF levels. Several reports have
examined the relationship between total nitrate/nitrite levels in CSF or cerebral microdialysis and conditions of TBI patients [28,60–62], but it would be too simplified by only inferring changes in NO production. Uzan et al. showed that total nitrate/nitrite levels in CSF of patients with severe TBI were elevated during the first 74 h posttrauma [28], but they did not indicate the sedatives used. Although there was a trend of increased CSF nitrate/nitrite levels in TBI patients in this study, it was not as obvious as that for F$_2$-IsoPs or F$_4$-NPs in CSF, and therefore it did not appear to be critical for evaluating oxidative stress in TBI patients. On the other hand, the lower mean of first 2 days’ levels of total nitrate/nitrite in CSF in the propofol group (Figure 2D) could be attributed to the scavenging of peroxynitrite or suppression of the expression of iNOS by propofol, as reported in the literature [32,34].

The peak and mean levels of CSF F$_2$-IsoPs correlated with poorer 6- and 12-month GOS grades, implying that the extent of lipid peroxidation in the entire brain during the postsurgery period was inversely associated with the patients’ recovery. By contrast, we found that there was no correlation between other oxidative stress markers and GOS (data not shown), although the analysis might be influenced by the propofol effect. Nevertheless, we could not observe any possible trend for the associations between high CSF levels of F$_2$-NPs in either the propofol or the midazolam group and worse outcomes (Supplementary Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1080363). Such findings therefore differ from our previous studies on aSAH, in which peak, mean, and first-day CSF F$_4$-NPs levels were strongly correlated with worse GOS grades [8,9]. Although damage to DHA in neural cells in the brain, particularly the neurons, following TBI was demonstrated by increased CSF F$_4$-NPs levels, widespread and non-specific injury to different types of brain cells, as well as many other events leading to damage of vascular cells or inflammatory responses, would more likely be reflected by the extent in the augmentation of F$_2$-IsoPs levels in CSF, which therefore would be more relevant to the long-term outcome. However, one drawback of this study was limited patient number. Consequently, it was not appropriate to perform correlation analysis for different markers for the midazolam group and the propofol group separately to exclude the possible effect of the lipid emulsion in propofol infusion on the assessment of oxidative stress status. Corcoran et al. also employed the GC/NICI-MS method and they reported that the total levels of isofurans and F$_4$-NPs in CSF specimens collected from TBI patients within 24 h of injury were higher than that in a group of controls, but the level of total F$_2$-Isop was unaltered [25]. However, we have previously questioned the relevance of measuring the total form of these lipid peroxidation products, instead of the free form, in CSF [48]. Information on the sedatives used in that study was lacking, and the correlation between the levels of the studied markers and the patients’ GOS grades was not analyzed. Moreover, our results were obtained through investigating not only the first-day levels, but also those over a postsurgery period, which would be more representative. The findings of obvious elevation of CSF F$_2$-IsoPs levels and its correlation with worse outcome in this study is therefore distinct from the conclusion obtained by Corcoran et al. [25].

In conclusion, free F$_2$-IsoPs in CSF detected by GC/NICI-MS are likely the most reliable markers for assessing the extent of oxidative damage or oxidative stress in TBI patients and for predicting the long-term outcome of TBI patients independent of the propofol effect. Although the increased CSF F$_4$-NPs levels suggest neuronal oxidative damage to DHA in the brain following TBI, CSF F$_2$-IsoPs levels would be more representative of the contribution of overall oxidative damage from non-specific and complex damaging processes during TBI. Furthermore, the results of enhancing the extent of TBI-induced lipid peroxidation by propofol infusion indicate that caution is warranted regarding the use of propofol, although such an effect could be attributed to the preparation of lipid emulsions. Using plasma F$_2$-IsoPs levels, which might further compound falsely increased levels if esterified or total forms are measured, in investigating oxidative stress and TBI in patients receiving propofol might result in misleading conclusions.

Declaration of interest

Authors report no declaration of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Supplementary Figure 1–3 to be found online at http://informahealthcare.com/doij/10.3109/10715762.2015.1080363.