Original Article

The alteration of serum bile acid profile among traumatic brain injury patients: a small-scale prospective study

Yuanrun Zhu,[1,†] Peidong Zheng,[2,†] Yajun Lin,[2] Juehan Wang,[2] Wendong You,[2,3] Yadong Wang,[1] Huiqing Zheng,[2] Liang Wen,[1,*] and Xiaofeng Yang[1,*]

[1]The First Affiliated Hospital, School of Medicine, Zhejiang University, 1367 West Wenyi Rd., Hangzhou, Zhejiang Province 310003, China
[2]Zhejiang University School of Medicine, 866 Yuhangtang Rd., Hangzhou, Zhejiang Province 310003, China
[3]The First Affiliated Hospital of Fujian Medical University, 20 Chazhong Rd., Fuzhou, Fujian Province 350000, China

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Traumatic brain injury is one of the major causes of morbidity and mortality worldwide. With the development of bile acids as a potential treatment, to identify the influence of traumatic brain injury on bile acid metabolism shows growing importance. This present study did a preliminary exploration of the bile acid profile alteration among traumatic brain injury patients. In total, 14 patients and 7 healthy volunteers were enrolled. The bile acid profile of the blood samples were detected by an Ultra-performance Liquid Chromatography Mass Spectrometer/Mass Spectrometer system. It was found that 6 bile acids were statistically decreased in traumatic brain injury patients comparing with healthy volunteers: glycocholic acid (median level 44.4 ng/ml vs 98.7 ng/ml, $p = 0.003$), taurocholic acid (median level 10.9 ng/ml vs 19.5 ng/ml, $p = 0.006$), glycoursodeoxycholic acid (median level 17.4 ng/ml vs 71.4 ng/ml, $p = 0.001$), ursodeoxycholic acid (median level <1 ng/ml vs 32.4 ng/ml, $p = 0.002$), taurochenodeoxycholic acid (median level <1 ng/ml vs 53.6 ng/ml, $p = 0.003$) and glycochenodeoxycholic acid (GCDCA, median level 160 ng/ml vs 364 ng/ml, $p<0.001$). In conclusion, traumatic brain injury events are able to induce bile acid metabolism alteration in plasma and might cause reduction in glycocholic, taurocholic, glycoursodeoxycholic, ursodeoxycholic, taurochenodeoxycholic and glycochenodeoxycholic acid levels.

***Key Words*:** **traumatic brain injury, bile acid, brain-gut axis, nutrition for neurological disease, prospective study**

Traumatic brain injury (TBI) is one of the major causes of morbidity and mortality worldwide.[1–3] For the past decades, researchers and clinical workers have made continuous efforts to improve the outcomes of TBI, and there have been a few achievements in the area, such as positive results of decompressive craniectomy.[4–6] However, apart from the achievements, it still lacks effective medication for TBI treatment currently[7] and results from previous clinical trials were mostly unsatisfying.[8,9]

During the recent exploration, bile acids and their potential effects on the central nervous system have been noticed. It has been noted that bile acids might be beneficial in both neurodegenerative diseases and acute brain injuries such as intracranial hemorrhage or ischemic stroke.[10–13] In 2016, a review mentioned that tauroursodeoxycholic acid (TUDCA) could prove promising in the treatment of TBI due to its anti-apoptotic and anti-inflammatory mechanisms.[14] Moreover, in 2017, an experimental study confirmed that administration of TUDCA could attenuate early brain injury in TBI mice model via AKT pathway activation,[15] and another study identified that bile acid transporter-expressing neurons in the hypothalamus were changed after TBI.[16] Although these experimental results were preliminary, they successfully linked bile acids with TBI treatment and indicated that bile acid metabolism might be a potential target in future TBI rehabilitation practice.

With the development of bile acids as potential treatment for TBI, to identify the influence of traumatic events on bile acid metabolism shows growing importance. Details of how TBI affects bile acid might serve as guidance in future studies. In fact, there have been studies reporting the influence of ischemic stroke or Parkinson disease on bile acid profile in patients.[17,18] Relationships of serum bile acids and acute intracerebral hemorrhage have also been reported in a clinical study.[19] Comparing with these findings, data about whether TBI would affect bile acid metabolism remains inadequate. In our previous studies, we found that TBI would alter bile acid profile in animal model through dysbiosis of gut microbiota.[20] Moreover, by performing a retrospective clinical study, we indicated that bile acid alteration also existed in TBI patients.[21] However, this previous clinical report only analyzed the overall changes of bile acid in clinical samples with no detailed metabolism profile involved. Thus up to now, the influence of TBI on bile acid metabolism in real patients still lacks data.

In this study, we performed a single-center, small-scale, controlled analysis to explore the alteration of serum bile acid profile among TBI patients. As a preliminary exploration, we hope that this study could work as a fundament to large-scale studies of this topic in the future.

Materials and Methods

Subjects. This prospective study enrolled TBI patients and healthy volunteers between 2021-08-01 and 2021-09-30 in the First Affiliated Hospital, Zhejiang University School of Medicine. The inclusion criteria for patients or volunteers included: (1) Adult, (2) Agreed for blood sample collection and signed the consent form (for TBI patient without full capacity to make agreements, authorized personnel should sign the consent form). The exclusion criteria included: (1) Known history of other diseases which could still influence bile acid metabolism at the time of blood sample collection, (2) Blood sample unqualified for bile acid profiling, (3) For TBI patients, necessary

*To whom correspondence should be addressed.
E-mail: zjcswk@zju.edu.cn (XY); wenliang@zju.edu.cn (LW)
†These authors contributed equally to this work.

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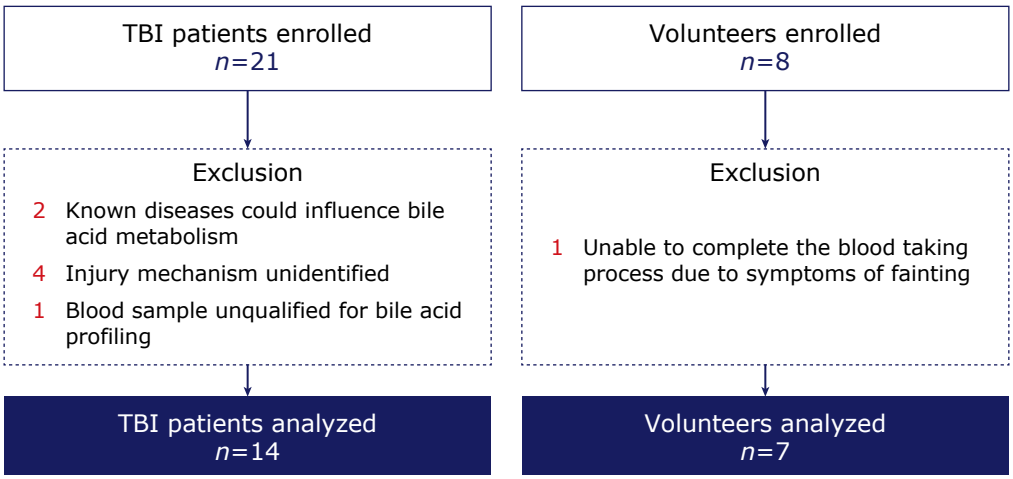

Fig. 1. Details of enrollment of patients and volunteers. A total of 7 TBI patients met exclusion criteria. Two patients were due to medical history of liver cirrhosis and chronic cholecystitis, 4 patients were due to unidentified injury mechanism, and 1 patient's blood sample did not pass the quality control process of bile acid profiling. One volunteer was excluded due to symptoms of fainting during blood taking.

medical record could not be provided (for example, the patient was unable to recall the injury mechanism and no witness was available either). The patients' enrollment was completed in neurosurgery ward and no outpatients were included. The detailed process of patients' and volunteers' enrollment is shown in Fig. 1.

Clinical parameters and laboratory tests. Collected clinical data included gender, age, injury mechanism, surgery type, days from TBI event, GCS (at the time of blood sample collection), antibiotics application (at the time of blood sample collection), nutrition support type (at the time of blood sample collection), and medical history. No personal information is traceable in this report, and the study is in keeping with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. The study had been approved by the ethics committee of our medical center before its conduction and the approval number was 2021IIT975 (2021-07-22).

All blood samples were collected between 6 a.m. and 8 a.m. before breakfast for both TBI patients and healthy volunteers. Fasting was required after 10 p.m. on the night before the blood sample collection. All blood sample collection procedures were completed by a same neurosurgery nursing team (including blood samples of volunteers), so as to maintain consistency in practice details. Blood samples were centrifuged and plasma was obtained before undergoing low-temperature transportation to laboratories.

The bile acid profiling protocol was similar to our previous study analyzing bile acid metabolism in experimental TBI models.[20] It was slightly changed since the LC-MS system was different from the previous study. The procedure is briefly introduced as follows.

For sample preparation, a volume of 100 µl of plasma was thoroughly mixed with 400 µl of pre-cooled methanol by 1-min vortex, and then centrifuged at 12,000 rpm, 4°C for 10 min. The supernatant was then evaporated to dryness and re-dissolved in 100 µl of solvent. Finally, the supernatants were injected into the UPLC-MS/MS system.

For UPLC-MS/MS detection, a Thermo Scientific Ultimate 3000 coupled to a Thermo Scientific TSQ Quautiva liquid chromatograph-triple quadrupole mass spectrometry (Thermo Fisher Scientific, Waltham, MA) was used. Samples (5 µl) were separated by Thermo Hypersil GOLD C18 column (100 × 2.1 mm, 1.9 µm) (Thermo Fisher Scientific) at 50°C. Mobile phases consisted of A (water with 0.1% HCOOH, v/v) and B (acetonitrile with HCOOH, 1:1, v/v). Ion source condition: vaporizing temperature 350°C and ion transfer tube temperature 300°C. Multiple reaction monitoring (MRM) was used for quantification of screening fragment ions. Peak determination and peak area integration was performed with Thermo Scientific Xcalibur software (Thermo Fisher Scientific). The UPLC-MS/MS experiments in this study were supported by LC-BIO (Hangzhou, China).

Statistical analysis. Statistical analysis was carried out using SPSS ver. 19.0 (IBM Corp, Armonk, NY). Continuous variables are given as means and SD if normally distributed and as medians if not normally distributed. Shapiro-Wilk test is performed to determine whether the distribution type is normal or not. Categorical variables are given as numbers and percentages. To compare continuous variables, Student's t test (if normally distributed) or Mann–Whitney U test (if not normally distributed) were performed. Statistical significance was determined when $p<0.05$. Hierarchical clustering and linear normalization in heat map were performed using Heatmap Illustrator (HemI 1.0.3.7, CUCKOO workshop).[22]

Results

Clinical characteristics of TBI patients and healthy volunteers. In this study, a total of 21 blood samples were finally analyzed, which were obtained from 7 healthy volunteers and 14 TBI patients. The 7 volunteers include 3 females and 4 males, whose ages vary from 22 to 79. No relevant medical history or traumatic events existed among the volunteers. The 14 TBI patients include 7 males and 7 females, whose ages vary from 23 to 77. The reasons causing TBI events included 4 major types: Fall, fall from height, traffic accident and blunt hit. Eleven of these patients underwent at least one neurosurgery procedure before blood sample collection. The surgeries included decompressive craniectomy, intracranial pressure monitor implantation, external ventricular drain, hematoma evacuation and ventricular-peritoneal shunt. Six patients were accepting antibiotic treatment and 9 patients were supported by enteral nutrition production instead of normal food at the time of blood sample collection. Three patients had relevant medical history including acute ischemic stroke, traumatic events other than TBI and abdominal surgery history. Detailed clinical characteristics of all 21 TBI patients and healthy volunteers are shown in Table 1.

Bile acid alterations in plasma of TBI patients. Bile acids with concentration higher than 1 ng/ml in at least one of samples

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Table 1. Detailed clinical characteristics of TBI patients and healthy volunteers

Patient/ Volunteer No.	Gender	Age	Injury mechanism	Surgery	Days from TBI event	GCS (E + V + M)	Antibiotics application (1: Yes, 0: No)	Nutrition type (1: Enteral nutrition productions, 2: Normal food)	Medical history
TBI1	Female	53	Fall	NA	3	15	0	2	NA
TBI2	Female	63	Traffic accident	DC	10	1 + T + 1	1	1	AIS
TBI3	Female	53	Traffic accident	DC & ICP monitor implantation	159	1 + T + 2	1	1	NA
TBI4	Male	66	Traffic accident	DC and VP shunt (secondarily)	152	4 + T + 3	1	1	NA
TBI5	Male	66	Fall from height	DC	91	2 + T + 3	0	1	NA
TBI6	Female	52	Fall	NA	1	3 + 3 + 5	0	2	NA
TBI7	Male	59	Blunt hit	SDH evacuation	35	15	1	2	Other trauma events
TBI8	Female	56	Traffic accident	ICP monitor implantation	22	3 + T + 4	0	1	NA
TBI9	Male	77	Fall	SDH evacuation & ICP monitor implantation	13	1 + T + 1	0	1	NA
TBI10	Male	67	Traffic accident	DC	58	4 + T + 4	0	1	NA
TBI11	Female	48	Fall	NA	2	15	0	2	NA
TBI12	Male	23	Fall from height	EVD & VP shunt (secondarily)	310	4 + T + 2	1	1	NA
TBI13	Female	54	Traffic accident	Intracranial hematoma evacuation & ICP monitor implantation	17	4 + 2 + 5	0	1	Laparoscopic surgery
TBI14	Male	47	Fall	DC	53	15	1	2	NA
CON1	Male	30	NA	NA	NA	15	0	2	NA
CON2	Female	61	NA	NA	NA	15	0	2	NA
CON3	Male	32	NA	NA	NA	15	0	2	NA
CON4	Male	41	NA	NA	NA	15	0	2	NA
CON5	Male	31	NA	NA	NA	15	0	2	NA
CON6	Female	22	NA	NA	NA	15	0	2	NA
CON7	Female	79	NA	NA	NA	15	0	2	NA

DC, decompressive craniectomy; ICP, intracranial pressure; VP, ventricular-peritoneal; SDH, subdural hematoma; EVD, external ventricular drain; AIS, acute ischemic stroke.

among TBI and healthy groups were analyzed, which included 15 different types: glycocholic acid (GCA), taurocholic acid (TCA), glycoursodeoxycholic acid (GUDCA), glycochenodeoxychlolic acid 3-sulfate (GCDCA-3-S), glycodeoxycholic acid 3-sulfate (GDCA-3-S), glycohyocholic acid (GHCA), ursodeoxycholic acid (UDCA), taurochenodeoxycholic acid (TCDCA), glycolithocholic acid 3-sulfate (GLCA-3S), glycochenodeoxycholic acid (GCDCA), cholic acid (CA), glycodeoxycholic acid (GDCA), chenodeocycholic acid (CDCA), glycolithocholic acid (GLCA), and deoxycholic acid (DCA). Among these bile acids, 6 types showed statistical decrease in TBI patients comparing with healthy volunteers: GCA (median level 44.4 ng/ml vs median level 98.7 ng/ml, $p = 0.003$), TCA (median level 10.9 ng/ml vs median level 19.5 ng/ml, $p = 0.006$), GUDCA (median level 17.4 ng/ml vs median level 71.4 ng/ml, $p = 0.001$), UDCA (median level <1 ng/ml vs median level 32.4 ng/ml, $p = 0.002$), TCDCA (median level <1 ng/ml vs median level 53.6 ng/ml, $p = 0.003$) and GCDCA (median level 160 ng/ml vs median level 364 ng/ml, $p<0.001$). Rest 9 types of bile acids were found to be statistically similar between TBI patients and healthy volunteers. The distribution of bile acid concentrations in plasma is presented as box plot in Fig. 2.

To confirm the results, we also plotted a heat map of all bile acid concentrations using linear normalization and clustered the bile acid types. As a result, GCA, TCA, GUDCA, UDCA, TCDCA, and GCDCA are clustered as bile acids with decrease in TBI patients. The heat map is shown in Fig. 3.

Discussion

In this study, by comparing TBI patients with healthy volunteers, we identified 6 bile acids which were statistically decreased after traumatic events: GCA, TCA, GUDCA, UDCA, TCDCA, and GCDCA.

These results are partly consistent with what we found in our previous experimental study. Mice experiments showed that TCA, TCDCA, and UDCA were decreased in TBI mice comparing with sham groups in plasma, while GCA, GUDCA, and GCDCA were not abundant enough for analysis. On the other hand, CA, CDCA, and DCA were found to be reduced by TBI events among mice but unchanged among human patients in this study.

Among bile acids decreased after TBI, some have been reported to possess potential biological functions according to pre-clinical experiments. TCDCA was demonstrated to affect functions of monocytes and dendritic cells in a previous study. To be more specific, TCDCA is able to promote monocyte differentiation and to induce a dendritic cell phenotype with suppressed IL-12 secretion in response to LPS or LPS–interferon-γ stimulation. This known function indicates that TCDCA might have a role in immune response to inflammation.[23] UDCA and GUDCA have also been investigated in multiple pre-clinical models. One recent systematic review summarized findings from 36 articles, and concluded the biological functions of UDCA and GUDCA.[24] In neurodegenerative models UDCA was reported to reduce cell apoptosis, reactive oxygen species (ROS) and tumor

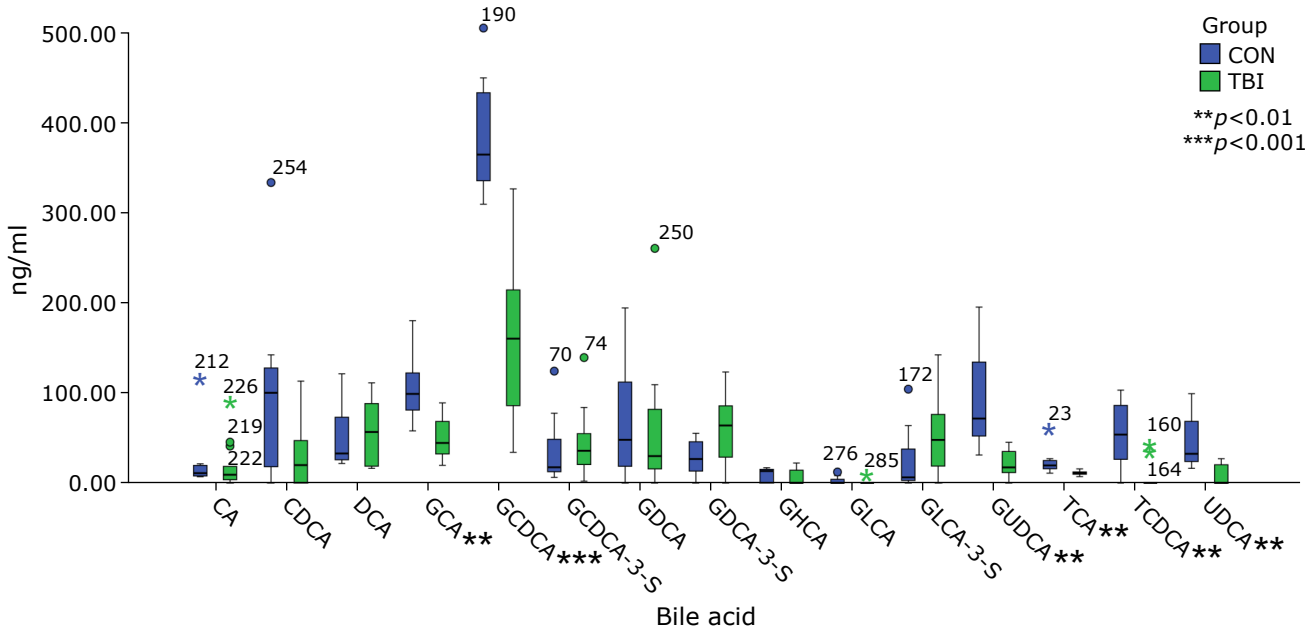

Fig. 2. The distribution of bile acid concentrations in plasma among TBI patients and healthy volunteers. Comparing with healthy volunteers, TBI patients had lower levels of GCA, GCDCA, GUDCA, TCA, TCDCA, and UDCA in plasma. The box plot showed the median, the first and third quartile levels of bile acids as well as outliers (presented as circles and stars). Of note, the outliers are defined as higher than third quartile plus 1.5 inter quartile range (Q3 + 1.5IQR) or lower than first quartile minus 1.5 inter quartile range (Q1 – 1.5IQR). These values are reserved in the analysis.

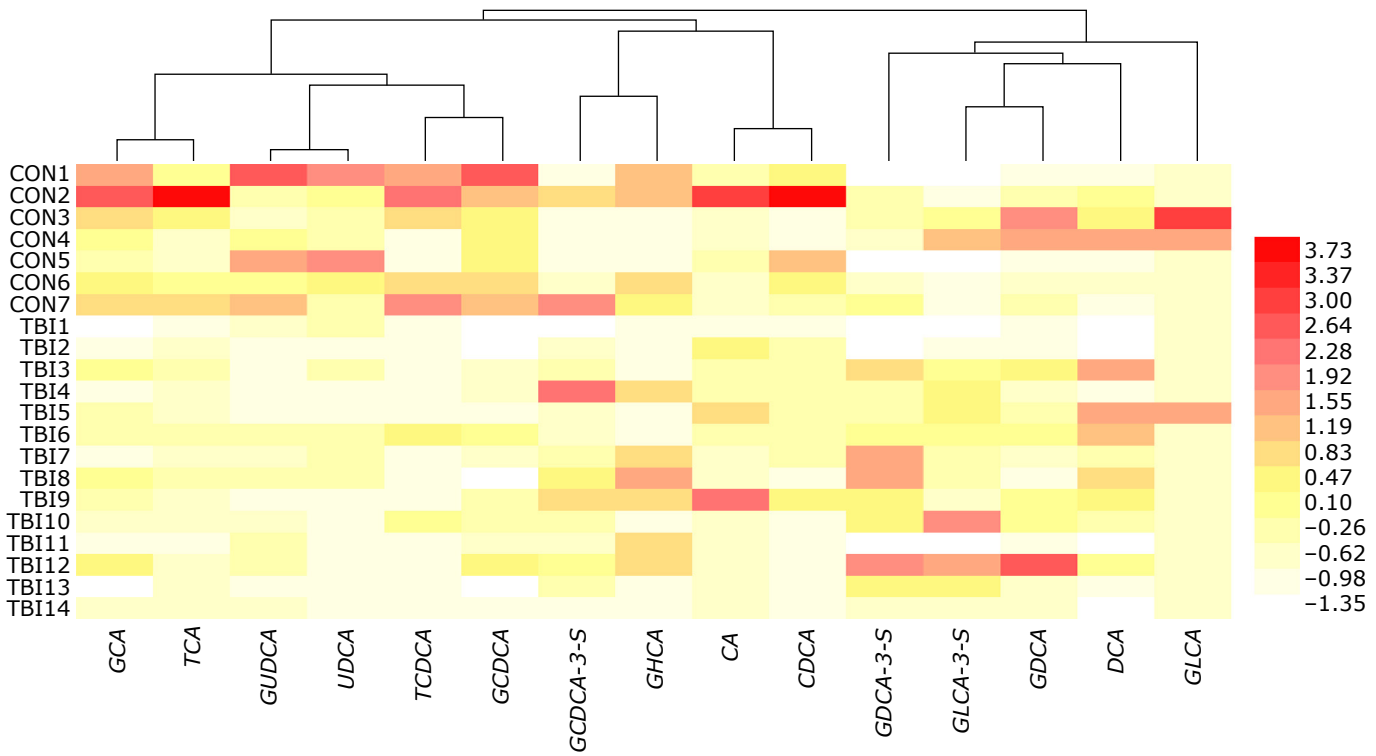

Fig. 3. Heat map showing detailed bile acid alteration in plasma of TBI patients. Hierarchical clustering indicates that GCA, TCA, GUDCA, UDCA, TCDCA, and GCDCA are most likely decreased among TBI patients, which is consistent with box plot results in Fig. 1. The sample IDs of patients and volunteers showed in this heat map are consistent with those in Table 1.

necrosis factor (TNF)-α production and GUDCA might reduce cytochrome c peroxidase production. In other models such as neuropsychiatric, UDCA was able to reduce nitric oxide (NO) and interleukin (IL)-1β production and GUDCA down-regulated lactate dehydrogenase, TNF-α and IL-1β production. Comparing with TCDCA, UDCA, and GUDCA, GCA is less reported in immunological studies. However, it is related to TBI more directly. In 2021, researchers reported that GCA was able to

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function as a biomarker in TBI rat models to estimate the injury time.[25]

Apart from pre-clinical results, there also have been clinical studies that connecting the decreased bile acids with neurological diseases.[17,26,27] In schizophrenia patients, TCDCA and other two bile acids were reported to be significantly decreased in plasma, and might be able to function as biomarkers. Previous studies have not reported relationship between TCDCA and TBI. Our results indicated that TCDCA was also decreased after TBI events, which was similar to the schizophrenia situation. In stroke patients, GCDCA along with L-methionine, homocysteine, glutamine and uric acid were found to be increased in blood, which was opposite to the results in TBI patients in our study.

Of note, one of the most well studied bile acids in TBI models was TUDCA. As mentioned in the introduction part, TUDCA was found to have potential treatment value in pre-clinical experiments for TBI. However, in our study, TUDCA was unable to be analyzed since the concentrations in plasma were not detectable in all blood samples. It is not clear whether this is due to study design such as fasting protocol or the characteristic of TUDCA itself. TUDCA is relatively low in human bile and is traditionally acquired from bear bile.[28]

The differences between the results in this study and previous results in mice TBI models are also worth noticing. The two studies were carried out with highly similar methods. The results were partially consistent but presented obvious differences. Several bile acids such as CA, CDCA, and DCA were not reduced in human plasma as they did in mice models. Also in mice models, TUDCA was able to be analyzed and showed reduction. These differences reminded us that the results in pre-clinical studies were important in guiding clinical research but should be interpreted with caution.

As a preliminary analysis, our study has a couple of limitations. First of all, the amount of patients and volunteers is small, which causes the distribution of bile acid concentrations to be abnormal. The small scale could lead to a reduction of the statistical effect. Of note, in box plot a few values are identified as 'outliers', which could also be partially caused by the small sample size. And since no medical reasons to rule out these 'outliers', they are reserved for the analysis. Secondly, as a single-center study, selection bias is difficult to avoid. For example, most of the patients in our study are injured due to fall or traffic accidents, violence caused by blunt hit was seen in only one patient and penetration wound was seen in none. Last but not least, due to high percentage of anti-biotic application, our study didn't analyze fecal bile acid profile since the influence of systematic anti-biotic application on gut microbiota is hard to ignore.

In conclusion, traumatic events are able to induce bile acid metabolism alteration in plasma among TBI patients and might cause reduction in GCA, TCA, GUDCA, UDCA, TCDCA, and GCDCA levels. Large-scale study is critical to obtain in-depth understanding of bile acid metabolism of TBI patients in the future.

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Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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