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Hypothermia Decreases Cerebrospinal Fluid Asymmetric Dimethylarginine Levels in Traumatic Brain Injury Children

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Abstract

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Objectives—Pathological increases in asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase (NOS) inhibitor, have been implicated in endothelial dysfunction and vascular diseases. Reduced NO early after traumatic brain injury (TBI) may contribute to hypoperfusion. Currently, methods to quantify ADMA in the cerebrospinal fluid (CSF) have not been fully explored. We aimed to develop and validate a method to determine ADMA in the CSF of a pediatric TBI population and to use this method to assess the effects of (i) TBI and (ii) therapeutic hypothermia (TH) on this mediator.

Design, Setting, Patients—An ancillary study to a prospective, phase II randomized clinical trial (RCT) of early hypothermia in a tertiary care pediatric intensive care unit for children with TBI admitted to Children's Hospital of Pittsburgh.

Interventions—None

Measurements and Main Results—A UPLC-MS/MS method was developed and validated to quantitate ADMA. A total of 56 samples collected over 3 days starting with injury onset were analyzed from the CSF of consented therapeutic hypothermia (n=9) and normothermia (n=10) children. Children undergoing diagnostic lumbar puncture (n=5) were controls. ADMA was present at a quantifiable level in all samples. Mean ADMA levels were significantly increased in normothermic TBI children compared to control ($0.19 \pm 0.08 \mu\text{mol/L}$ and $0.11 \pm 0.02 \mu\text{mol/L}$ respectively, $p=0.01$), and hypothermic children had significantly reduced mean ADMA levels ($0.11 \pm 0.05 \mu\text{mol/L}$) vs. normothermic ($p=0.03$) measured on day 3. Patient demographics including age, gender, and NO levels (measured as nitrite and nitrate using liquid chromatography coupled with Griess reaction) did not significantly differ between normothermia and hypothermia groups. Also, NO levels did not correlate with ADMA concentrations.

Conclusions—ADMA levels were significantly increased in the CSF of TBI children. Early hypothermia attenuated this increase. The implications of attenuated ADMA on NOS activity and regional cerebral blood flow after TBI by TH deserve future attention.

Keywords

traumatic brain injury; nitric oxide synthase; cerebrospinal fluid; asymmetric dimethyl arginine; therapeutic hypothermia; neuroprotection

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Traumatic brain injury (TBI) is the most common cause of death and disability in children in the United States. The mechanisms of injury include inflicted childhood neurotrauma in infants, pedestrian injuries in preadolescents, and motor vehicle accidents in adolescents. Of the affected patients, up to 48% are disabled by physical and cognitive deficits which represent a significant societal and public health problem (1). For patients who survive the initial injury, morbidity and mortality is often determined by secondary injury processes which are both clinically-apparent and currently underappreciated. Multiple mechanisms may play a role in the secondary insults after TBI including edema, excitotoxicity, calcium accumulation, cell death cascades, axonal injury, altered cerebral blood flow (CBF), increase in oxidative species, and enhanced inflammation among others (2). Novel therapies are needed to augment the intracranial pressure (ICP)-directed therapy that encompasses much of the current management of TBI. Mild to moderate hypothermia is a third tier therapy for refractory intracranial hypertension that has been evaluated as an early neuroprotectant.

However, multicenter and single center trials in both children and adults have failed to demonstrate a mortality benefit (3-7). Given that hypothermia has been shown to have differential effects on various secondary injury mechanisms in clinical TBI (8), additional studies to define how this therapy alters the natural history of the cerebrodynamics after TBI are required.

Cerebral ischemia is considered to be an important secondary injury mechanism after TBI. Early after TBI in children, cerebral ischemia occurs via decreased global or regional CBF. Nitric oxide (NO), constitutively synthesized from L-arginine by a group of isoenzymes, nitric oxide synthases (cNOS), plays a major role in the basal regulation of CBF due to its vasodilatory effects. Reduced NO production is suggested as a mechanism of the observed reduction of CBF after severe TBI (9, 10). An early decrease in NO is reported in animal models of TBI which may, in part, account for the low CBF during this period of injury (11, 12). Additionally, inducible form of NOS (iNOS) can produce NO in response to injuries, and may play a role in increasing CBF in delayed time periods after injuries (13). Although the mechanisms of altered NO production after TBI are not well understood, inhibition of NOS is implicated as one of the mechanisms of altered CBF.

Asymmetric dimethylarginine (ADMA) is a non-selective endogenous inhibitor of all forms of NOS. ADMA is released from proteins that have been post-translationally methylated and subsequently hydrolyzed by the protein arginine methyl transferase I (PRMT I), found mainly in endothelial and smooth muscle cells (Fig. 1). More than 90% of ADMA is metabolized by dimethylarginine dimethylamino hydrolase (DDAH) into citrulline and dimethylamine. Vascular endothelial cells are also capable of synthesizing ADMA (14). An increased concentration of ADMA reduces endothelial NO production (15). Furthermore, increased concentrations of ADMA in plasma have been linked to various diseases related to endothelial dysfunction including hypertension and coronary heart disease and neurodegenerative disease such as Alzheimer's disease (16-18). Recently, elevated ADMA concentrations in CSF have been linked to vasospasm and stroke severity in a small number of subarachnoid hemorrhage (SAH) patients and in adult ischemic stroke patients, respectively (19, 20). However, reports regarding the determination of ADMA in CSF of TBI victims, either adult or pediatric, are non-existent. Moreover, rapid high-through-put methods to quantify ADMA from biological fluids are limited especially from human CSF.

The chromatographic methods employed to determine ADMA in adult SAH and stroke patients may suffer from drawbacks including complicated sample preparation, long chromatographic run times, and monitoring analytes by limited sensitivity detectors. Therefore, rapid and accurate quantification of ADMA in the CSF of a pediatric TBI population was investigated in the context of relevant clinical variables. This method features polymeric mixed mode solid phase extraction of samples for cleaner extracts with reduced matrix effects, minimal sample volume requirement, and rapid chromatographic run time. Based on the evidence implicating elevated ADMA in vascular diseases and the potential efficacy of moderate hypothermia in TBI children, we aimed to 1) develop and validate a method to quantify ADMA in CSF from TBI children, 2) to determine whether CSF ADMA levels differ between TBI children and controls and whether or not the levels are increased or decreased by therapeutic hypothermia, and 3) to determine

whether NO, as measured by the nitrate/nitrite levels, are associated with ADMA concentrations in these patients.

Materials and Methods

Chemicals

ADMA was purchased from Cayman Chemical (Ann Arbor, MI). The deuterated internal standard (IS), ADMA-d₆ was synthesized on site according to published procedure (21). All other chemicals were of analytical grade or better.

Assay Validation

A solid-phase extraction method for the concentration and subsequent detection of ADMA and ADMA-d₆ was validated over a three day period using Waters Oasis 1cc MCX extraction cartridges (Waters Corporation, Milford, MA) followed by UPLC-MS/MS. The calibration curve range chosen was 75-3000 pg on column (1-40 ng/mL). Six replicates of low, medium, and high (90,650, and 1750 pg on column, respectively) quality controls (QCs) for 3 consecutive days were used for inter-day coefficient of variation assessment whereas 12 replicates in a single day were used for intra-day coefficient of variation assessment. All samples were run in duplicate and ADMA-d₆ was added to all samples followed by 1mL of 2% phosphoric acid in order to apply to the extraction cartridges. The cartridges were equilibrated with 2mL of 100% methanol followed by 2mL of ddH₂O. The calibration standards and QCs were then applied to the extraction cartridges and subsequently washed with 2mL of 2% formic acid and 2mL of 100% methanol. ADMA and ADMA-d₆ were then eluted from the cartridges with 2mL of 10% NH₄OH (in methanol). Samples were dried under N₂ at 37°C using a Techne Dri-Block DB-3 sample concentrator (Bibby Scientific Ltd., Burlington, NJ) and then reconstituted with 100µL of 70% acetonitrile containing 0.1% formic acid.

Patient Characteristics and CSF Collection

This was an ancillary study to a phase II, prospective, and randomized clinical trial (RCT) of early hypothermia in a tertiary care pediatric intensive care unit for children with TBI admitted to Children's Hospital of Pittsburgh, University of Pittsburgh Medical Center (22). Informed consent was obtained from parents of children suffering from TBI in accordance with the regulations of the Institutional Review Board at the University of Pittsburgh. Specifically, parents consented to the randomized intervention of hypothermia and to the collection of CSF samples. Children eligible to receive hypothermia had Glasgow Coma Scale (GCS) scores ≥ 8 after resuscitation or after secondary deterioration, and an external ventricular drain (EVD) was placed as part of their routine clinical care. All children in this study had deterioration from the initial admission GCS within 6 hr of insult. CSF was drained continuously at 3 cm above the midbrain, and all children received standard neurocritical care for intracranial hypertension (ICH) based on published guidelines (22). Specifically, stepwise therapies were administered, including sedation, neuromuscular blockade, hyperosmolar therapies, and barbiturates. A consecutive sample of CSF from 19 children (age 0.16 – 13yrs) with varying levels of TBI was chosen for analysis. At least one sample per patient was available (a total of 3 samples for all patients except for one patient

who had only 2 samples) during the time period between 0-24, 25-48, and 49-72 hr after injury. The sample collection hours ranged from 4-24 (median=14.5) hr for the day 1 samples, 26-48 (median=34) hr for day 2 samples, 50-71 (median=57) hr for day 3 samples since the onset of injury. Demographic data including age, gender, and mechanism of injury, GCS scores, and Glasgow Outcome Scale (GOS) scores at 6 months post-injury were also obtained. Upon entry into the trial, children were randomized to receive either hypothermia (n=9) or normothermia (n=10). Children randomized to receive hypothermia were cooled to 32-33°C for 48 h followed by a slow rewarming period as previously described (22).

CSF was collected daily from an EVD during the first three days after TBI. The samples were immediately centrifuged, aliquotted, and stored at -80°C until analysis. CSF specimens from children undergoing diagnostic lumbar puncture (n=5) to rule out infection were used as controls for this study. Patient information was available in 4 of the 5 subjects. All 4 were male, one was African American and 3 were Caucasians. Their ages ranged from 2 months-14 yr. Two were diagnosed with meningoencephalitis, one with pulmonary hypertension and another one with ALTE.

ADMA Quantification from CSF

Sample analysis was done using a TSQ Quantum Ultra Triple Quadrupole Mass Spectrometer (ThermoFisher Scientific, Pittsburgh, PA) coupled to an Acquity UPLC (Waters Corporation, Milford, MA). ADMA extraction from CSF was performed using the methods described in the assay validation section. A total of 200µL of CSF per sample was utilized. A total of 7.5µL of each sample was injected onto a 1.7µm Waters Acquity C₁₈ BEH Column, 100 × 2.1mm (Waters Corporation, Milford, MA). ADMA and ADMA-d₆ were both detected using positive electrospray ionization at m/z 203→46 and 209→70 daughter ion fragments, respectively. The mobile phases used for the detection of both compounds were 0.1% formic acid and 100% acetonitrile containing 0.1% formic acid, running at a flow rate of 0.3mL/minute. ADMA concentrations were quantified from the standard curve of the ratio of ADMA to internal standard peak area.

Measurement of Nitrite and Nitrate

The nitrite and nitrate concentrations from CSF were measured using an ENO-20 system (Eicom Corporation, San Diego, CA), which is a combination of liquid chromatography and a Griess reaction (23). The analysis is achieved by combining a diazo coupling method with HPLC. Before measuring nitrate and nitrite levels, CSF samples were deproteinized by adding an equal volume of methanol (100µl) and then centrifuged at 10000 × g for 20min to avoid occlusion of a polystyrene polymer. Nitrite and nitrate were separated on a polystyrene column, and the nitrate was reduced to nitrite by passage through a cadmium column. Nitrite was mixed with a Griess reagent to form a purple azo dye. The absorbance of the product was measured at 540nm. Concentrations of nitrite and nitrate were measured separately based on their specific retention times and quantified by comparing the peak area with a standard one.

Statistical Analysis

Statistical analyses were performed using SPSS version 18 and SAS 9.2. The Student's *t*-test was used to compare the mean levels of ADMA, nitrate and nitrite levels between hypothermia and normothermia subjects. Fisher's exact test and Mann-Whitney test were completed to determine the differences between the treatment groups and demographic data specifically, age and gender. A repeated measures modeling with PROC MIXED in the SAS system was used to model repeated measures of ADMA data. A Pearson rank correlation test was applied to test the significant correlation between ADMA and nitrite + nitrate data groups. A *p* value < 0.05 was considered significant in all analyses.

Results

Calibration, Precision, and Accuracy of Assay

Linear calibration curves were obtained for ADMA over the range of 75-3000 pg on column. The lower limit of detection (LLOD), defined as a peak signal-to-noise ratio > 3, for this assay was 25 pg on column and the lower limit of quantification (LLOQ), defined as the lower limit of the calibration range, was 75 pg on column. The LLOQ was demonstrated to be reproducible (% relative standard deviation (RSD) = 4.8 %). The intra- and inter-day precision and accuracy were < 10% at all concentrations of ADMA (Table 1). Identical chromatograms were obtained from a medium QC (625 pg of ADMA on column) and a CSF sample of a pediatric TBI patient randomized to receive hypothermia. ADMA was observed at *m/z* of 203→46 with a retention time of 0.99 min.

Demographic and Clinical Data of TBI Patients

Demographic data of 19 TBI children are presented (Table 2). There were 9 females and 10 males with ages ranging from 0.16-13 years with a median age of 7 years. The initial GCS scores ranged from 3-15. No significant differences were observed with regards to age (*p*=0.74) and gender (*p*= 0.37) between normothermic and hypothermic groups (Table 3).

CSF ADMA Levels During the First 3 Days Following TBI

We assessed ADMA in a total of 56 CSF samples from children studied over the initial 3 days after TBI. The concentrations of ADMA in the controls, normothermic, and hypothermic groups ranged from 0.88-0.146, 0.069-0.437, and 0.062-0.287 $\mu\text{mol/L}$ respectively. The ADMA levels were significantly increased (~ 2 fold) in normothermic patients on all days compared to the control levels. As a representation, the mean ADMA concentrations in controls, normothermic, and hypothermic children on day 3 are shown in Figure 2. ADMA was increased about two-fold in normothermic TBI children compared to controls (*p*=0.01). In addition, in children treated with TH, CSF ADMA concentrations were approximately the same as the controls, and therefore significantly different from that of normothermic children (*p*=0.03). Inspection of the time course of CSF ADMA concentrations after TBI in normothermic and hypothermic groups revealed that the maximal ADMA concentration occurred early after injury (i.e. during the initial days after injury). In addition, significant decreases in CSF ADMA concentration were seen in hypothermic vs. normothermic groups on days 2 and 3 after injury (Figure 3). Statistical

analysis of longitudinal changes for within group comparisons showed that there was no significant decrease in ADMA in normothermic group over time, but there was significant decrease of ADMA in hypothermic group on day 3 compared to day 1 ($p < 0.05$). Because of the initial study design of the hypothermia trial (whereby a subset of children who were consented to receive hypothermia as late as 24h after TBI), the temperature ranged from 32.1-37°C for the first sample of CSF of the hypothermic group. However, all children who ultimately received hypothermia were cooled to desired temperature within the next 29-47h, at which phase we noted a significant difference in ADMA levels between normothermic and hypothermic groups. This difference persisted in the rewarming phase at day3 (48-72h).

Nitrite and Nitrate Levels Over First 3 Days Following TBI

We determined the nitrite and nitrate levels in CSF samples ($n=52$). The average nitrite, nitrate, and nitrite + nitrate concentrations on days 1 to 3 after injury in normothermic and hypothermic children are shown in Figure 4. The mean value of combined nitrite and nitrate was highest on day 1 and gradually decreased over the 3-day monitoring period. However, the decrease was not statistically significant. Also, we did not observe a statistically significant difference in nitrite, nitrate, and nitrite + nitrate levels between normothermic and hypothermic children. The reference value of the ventricular CSF from control patients was not available. The results of the correlation analysis between ADMA and nitrite + nitrate levels are presented in Table 4. There was no correlation between ADMA and nitrite + nitrate levels in both groups.

Discussion

This study demonstrated that ADMA can be detected over a linear concentration range via UPLC-MS/MS from every sample of severe TBI children with precision, accuracy, and minimal sample requirement. In addition, we demonstrated that ADMA levels were significantly increased in children with TBI compared to controls. Interestingly, children who received early hypothermia had significantly lower levels of ADMA in their CSF as compared to those who remained normothermic. However, CSF nitrite/nitrate levels were similar between the two groups. The ADMA concentrations measured from control and TBI children were higher than that were previously reported in adults. This observation is consistent with the reports that in children ADMA biosynthesis is higher than in adults and diminishes considerably with age (24). There is also a 6-fold range in CSF ADMA values for patients with TBI which could be attributed to the different types of brain injury that the children are afflicted with. These results provide the first clinical evidence of elevated ADMA in a pediatric TBI population and that TH attenuates the rise in CSF ADMA levels. Our results are also consistent with a previous study that has reported elevated ADMA levels in the CSF of adult SAH patients using a similar analytical method (25).

Measurement of ADMA from biological fluids is of particular interest as it may reliably identify pathological conditions associated with NO-related vascular dysfunction. Among the various analytical methods currently available, LC-MS/MS is being widely accepted for its specificity, reliability, and less laborious sample preparation for the quantitative analysis of the NO family of compounds (26). Previously, numerous studies have utilized mass

spectrometric methods to measure ADMA from human plasma (21, 27, 28). However, data on CSF ADMA concentrations using such sensitive methods are scant. Moreover, in acute neurological diseases, plasma ADMA concentrations may not adequately reflect cerebral concentrations considering the fact that arginine can cross the blood-brain barrier via the specific system y^+ cationic amino acid transporter (29). Also, ADMA concentrations in CSF of patients with Alzheimer's disease and SAH patients do not correlate with plasma concentrations (18, 19). Therefore, it is expected that assessment of CSF is more suitable to estimate brain levels particularly in TBI, where extracerebral injury is common. The analytical method we employed to quantitate ADMA from CSF offers several of the advantages previously reported by Martens-Lobenhoffer et.al. (25). These advantages include less laborious sample preparation, short chromatographic run times, and specified detection as compared to previously published methods (18, 19, 30).

Neuronal injury after TBI is associated with proteolysis which may release ADMA. The elevation in ADMA levels may occur via two mechanisms 1) increased methylation of L-arginine by upregulation of PRMTs and 2) decreased hydrolysis of ADMA by DDAH. Oxidative stress and inflammation which typically contribute to secondary neuronal cell injury after TBI are known to upregulate PRMTs and inhibit DDAH (31, 32). Our current results are consistent with these previous preclinical observations by demonstrating that normothermic children with TBI have significantly elevated CSF ADMA levels after injury which may occur as a result of any of the aforementioned mechanisms.

The potential deleterious role of ADMA in vascular diseases such as ischemic stroke and SAH has been documented (20, 33). Increased CSF ADMA concentrations may inhibit NO synthase activity and reduce NO production. Decreased production of NO is a source of endothelial dysfunction after cerebral ischemia and TBI (34, 35). Previously, two studies reported that nitrite levels in a small number of adult SAH patients were negatively correlated, albeit a weak to moderate relationship, with elevated ADMA levels and with the degree of vasospasm (19, 25). Decreased NO availability may result in increased production of free radicals, vasoconstriction, platelet aggregation, and leukocyte adhesion on the endothelial surfaces (20). These processes may in turn aggravate ischemia and reduce CBF. A similar scenario may occur in TBI. TBI causes an early reduction in CBF. In experimental TBI studies, constitutive NOS activity decreases and NO levels decrease rapidly which may in part account for the low CBF. L-arginine administration during this early post-injury period improves CBF (36) and restores NO levels possibly through increasing activity of endothelial NOS. In adults, CSF NO concentrations peaked between 20-42 h after injury. The microdialysate NO levels were highest within the first 24 h and gradually decreased over the 5 days post-injury, and there was a significant relation between regional CBF and NO levels (9). Another study reported that NO levels in adult CSF peaked at 20-28 h and then gradually decreased over 74 h post-injury (37). However, we did not observe a significant decline in overall NO levels over time in our pediatric population. To date, reports regarding ADMA levels in TBI patients and concurrent measurements of ADMA and NO are unavailable. Our study was limited by small sample size and short time frame for ADMA and NO measurements. It still remains to be demonstrated how ADMA and NO synthesis are affected after TBI.

The use of moderate hypothermia in the treatment of TBI is unsettled. Animal models of TBI have shown that mild or moderate hypothermia decreases mortality, excitotoxicity, disruption of blood-brain barrier, and improves behavioral outcome (38-40). However, clinical trials have been disappointing. This may be related to the increase in side effects, especially during rewarming. Alternatively, hypothermia may attenuate both detrimental and beneficial effects. The basic mechanisms through which hypothermia protects the brain are multifactorial. Hypothermia has been shown to promote ubiquitin recovery in experimental models of ischemia, therefore, preventing proteolysis (41). We speculate that the protein preservation through ubiquitin recovery by preservation of DDAH activity may be responsible for reduced ADMA levels in hypothermic TBI patients. Additionally, moderate hypothermia reduces brain oxidative stress and inflammation (42, 43) thereby potentially reducing ADMA levels by downregulation of PRMTs and upregulation of DDAH activity.

We anticipated an increase in NO levels concomitant with the decrease in ADMA levels after hypothermia treatment, but we observed no significant difference between the two groups. Previously it was reported that CSF nitrate and nitrite concentrations were higher in hypothermic adult TBI patients than in normothermic patients over time, although there were some imbalances in injury severity between groups in that study (44). They did not, however, report concurrent ADMA measurements. In our pediatric study population, therapeutic hypothermia did not appear to influence CSF NO levels over the 3 day study period even though ADMA levels were significantly reduced. It is also possible that hypothermia directly attenuates both ADMA and NO synthesis, and a direct inhibition of NO synthesis by hypothermia could blunt any impact of increased levels of ADMA. Thus, effects of hypothermia may be complex. Additionally, the method to estimate the endogenous NO production has limitations despite its utilization in several clinical studies. Little is known about the blood-brain barrier integrity after TBI in humans and the rate of elimination of nitrite and nitrate from the CSF space. We previously reported differential effects of mild hypothermia on various CSF mediators in infants and children after severe TBI (45-47). Given the small sample size and short time frame of NO assessment in our study, it is unclear whether ADMA affects regulation of NO production in pediatric TBI.

NO produced by multiple isoforms of NOS in many different cell types in the brain has complex roles in the pathophysiology of TBI. A triphasic change in the concentration of NO has been reported after TBI (12). The activity of endothelial NOS (eNOS) and neuronal NOS (nNOS) is believed to cause the initial peak in NO. NO produced by eNOS is thought to have the beneficial effect by maintaining CBF by vasodilation, while that produced by nNOS may have predominantly detrimental effects after TBI (48, 49). Following TBI, glutamate-induced excitotoxicity and generation of oxygen free radicals is thought to be mediated via NO formed by nNOS (50). Increased nNOS expression and function may contribute to the concomitant excitotoxic neuronal death after TBI. On the contrary, both detrimental and beneficial effects have been attributed to iNOS. In experimental TBI early after injury, iNOS may be detrimental via formation of peroxynitrite leading to neuronal death. Beneficial neuroprotective effects have been also reported for iNOS. Transgenic mice that were deficient in iNOS were reported to have impaired long-term functional outcome compared to wild-type mice (51) and using EPR spectroscopy, iNOS was found to be

responsible for over 60% of the NO detected in brain at 72 hours after injury (52). Further investigation is required to determine the predominant source of NO in the CSF after TBI.

ADMA regulation is also complex. ADMA can regulate its own degradation in endothelial cells via stimulation of DDAH-2 gene expression by NO (53). L-arginine administration during early post injury time period improves CBF, CO₂ reactivity, and tissue NO levels possibly via activation on iNOS (35, 54). The increased NO may then activate DDAH-2 enhancing ADMA degradation. The intracellular ADMA that escapes metabolism by DDAH can block NOS activity and limit the cellular uptake of L-arginine. However, L-arginine may also be diverted to other competing pathways such as arginase or PRMT. Future studies to elucidate the temporal alterations in NO pathway intermediates in addition to ADMA and NO are necessary to determine the sequence of mechanisms of altered ADMA and NO and their resultant effects on vascular regulation after TBI. The precise role of ADMA in pediatric TBI population remains an area for future investigation.

This study has certain limitations. First, the assessment of important clinical indices including CBF, ICP and CCP were not documented in this study population. The correlation of ADMA levels with these indices would have provided more insight into the impact of altered ADMA levels in normothermic and TH TBI children. Second, an assessment of L-arginine which was not determined in the current study, would have provided more insight into NOS function than ADMA itself. It has been demonstrated that TBI alters L-arginine levels and therefore it is possible that CSF L-arginine depletion may account for the observed altered ADMA levels (55, 56). Finally, the control subjects in this study were diagnosed with meningoencephalitis and pulmonary hypertension which potentially could have an influence on ADMA levels. Future studies in larger populations of patients to allow for stratification of these important factors are needed.

Conclusions

An ADMA assay has been established and validated. ADMA was increased in CSF in normothermic children with severe TBI compared to controls. The increase was sustained over 3 days following injury. Hypothermia prevented the increase in CSF ADMA on days 2 and 3 compared to ADMA levels in normothermic children. CSF nitrite/nitrate levels were similar between the two groups. The role of ADMA is thus unclear but the important role of NO after TBI suggests that ADMA merits future study in TBI.

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Figure 1.
Overview of the biochemical pathways for generation and degradation of ADMA.

Figure 2.

The mean levels of ADMA in control, normothermic, and hypothermic children on day 3 since the onset of injury. *denotes significant statistical difference by independent t-tests ($p < 0.05$).

Figure 3.

The overall profile of ADMA levels in normothermic and hypothermic TBI children.

*denotes significant difference ($p < 0.05$) between normothermia and hypothermia groups on days 2 and 3 since the onset of injury by repeated measures modeling with post hoc analysis.

Figure 4.
The mean levels of nitrite (A), nitrate (B), and the nitrite + nitrate (C) in normothermic and hypothermic children

Table 1
Intra-day and inter-day precision and accuracy with solid phase extraction

	Amount (pg on column)		% R.S.D.	% Bias
	Added	Observed (mean $\pm$ S.D.)		
Intra-day reproducibility Quality controls				
ADMA	90	82.3 $\pm$ 5.6	6.8	10.3
	625	623.7 $\pm$ 34.4	5.5	-0.02
	1750	1700.0 $\pm$ 61.4	3.6	-2.9
Inter-day reproducibility Quality controls				
ADMA	90	84.8 $\pm$ 7.3	8.6	-5.8
	625	645.5 $\pm$ 14.5	2.3	3.3
	1750	1802.3 $\pm$ 66.6	3.7	3.0
Standards				
ADMA	75	72.5 $\pm$ 3.5	4.8	-3.3
	100	103.4 $\pm$ 7.4	7.2	3.4
	250	249.8 $\pm$ 9.3	3.7	-0.01
	500	507.6 $\pm$ 17.7	3.5	1.5
	750	745.9 $\pm$ 12.3	1.7	-0.6
	1000	996.7 $\pm$ 12.4	1.2	-0.3
	1500	1497.4 $\pm$ 7.9	0.5	-0.2
	2000	2003.6 $\pm$ 13.9	0.7	-0.2
	3000	2818.3 $\pm$ 136.5	4.8	-6.1

Table 2
Demographics and clinical characteristics of 19 pediatric TBI patients

Patient ID	Age (years)	Gender	Mechanism of Injury	Group	Initial GCS Score	GOS Score
1	10	Female	bike vs. car	Hypothermic	5	3
2	5	Male	abuse	Hypothermic	6	3
3	12	Male	MVC	Hypothermic	3	5
4	8	Male	MVC	Hypothermic	7	U
5	7	Male	pedestrian vs. car	Hypothermic	6	3
6	0.7	Male	abuse	Hypothermic	13	D
7	2	Male	3 story fall	Hypothermic	9	2
8	7	Female	MVC	Hypothermic	7	2
9	3	Female	television fell on	Hypothermic	10	1
10	3	Male	pedestrian vs. car	Normothermic	7	1
11	2	Male	2 story fall	Normothermic	8	3
12	9	Male	fell off of bike	Normothermic	15	1
13	8	Female	MVC	Normothermic	14	1
14	0.2	Female	abuse	Normothermic	3	2
15	0.16	Female	abuse	Normothermic	11	3
16	9	Female	pedestrian vs. car	Normothermic	8	1
17	11	Male	recreational	Normothermic	7	U
18	13	Female	pedestrian vs. car	Normothermic	8	2
19	10	Female	MVC	Normothermic	4	2
20	14	Male	meningoencephalitis	Control	U	U
21	0.15	Male	pulmonary hypertension	Control	U	U
22	12	Male	meningoencephalitis	Control	U	U
23	0.2	Male	ALTE	Control	U	U
24	U	U	U	Control	U	U

MVC, motor vehicle collision; GCS, Glasgow Coma Scale; GOS, Glasgow Outcome Scale; G, good; MD, moderate disability; SD, severe disability; D, death; U, unavailable.

Table 3
Effect of demographic covariates on hypothermic and normothermic groups (n=19)

Characteristics	Group				p value
	Hypothermic (N=9)		Normothermic (N=10)		
	N (%)		N (%)		
Gender ^a	Male	6 (66.7)	4 (40)	0.37	
	Female	3 (33.3)	6 (60)		
Age ^b	Mean (SD)	Range	Mean (SD)	Range	0.74
	6.1 (3.7)	0.7-12	6.5 (4.7)	0.16-13	

^a Fisher's exact test;

^b Mann-Whitney test

Table 4
The correlation between ADMA and nitrite + nitrate levels in TBI patients

		Nitrite + Nitrate
Normothermic	ADMA	0.35 (0.07)
	Nitrite	
Hypothermic	ADMA	0.02 (0.92)
	Nitrite	

The values stated first in each cell signify the Pearson's product moment coefficient correlation (r) between the variables and the values in parenthesis signify the probability of the relationship between the variables.*