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Therapeutic Hypothermia Preserves Antioxidant Defenses after Severe Traumatic Brain Injury in Infants and Children

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Abstract

Objective—Oxidative stress contributes to secondary damage after traumatic brain injury (TBI). Hypothermia decreases endogenous antioxidant consumption and lipid peroxidation after experimental cerebral injury. Our objective was to determine the effect of therapeutic hypothermia on oxidative damage after severe TBI in infants and children randomized to moderate hypothermia vs normothermia.

Design—Prospective randomized controlled study.

Setting—Pediatric ICU of Pittsburgh Children's Hospital

Patients—The study included 28 patients

Measurements and main results—We compared the effects of hypothermia (32-33°C) vs normothermia in patients treated in a single center involved in a multi-center randomized controlled trial of hypothermia in severe pediatric TBI (GCS score ≤ 8). The patients randomized to hypothermia (n=13) were cooled to target temperature within ~6h-24h for 48h and then re-warmed. Antioxidant status was assessed by measurements of total antioxidant reserve [AOR] and glutathione. Protein oxidation and lipid peroxidation were assessed by measurements of protein-thiols and F_2-isoprostane, respectively in ventricular CSF samples (n=76) obtained on day1-3 after injury. The association between GCS score, age, gender, treatment, temperature, time after injury, and CSF AOR, glutathione, protein-thiol, F2-isoprostane levels were assessed by bivariate and multiple regression models.

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Demographic and clinical characteristics were similar between the two treatment groups. Mechanism of injury included both accidental injury and nonaccidental injury. Multiple regression models revealed preservation of CSF antioxidant reserve by hypothermia ($p = 0.001$). Similarly, a multiple regression model showed that glutathione levels were inversely associated with patient temperature at the time of sampling ($p = 0.002$). F2-isoprostane levels peaked on day 1 after injury and were progressively decreased thereafter. Although F2-isoprostane levels were ~3-fold lower in patients randomized to hypothermia vs. normothermia, this difference was not statistically significant.

Conclusion—To our knowledge this is the first study demonstrating that hypothermia attenuates oxidative stress after severe TBI in infants and children. Our data also support the concept that CSF represents a valuable tool for monitoring treatment effects on oxidative stress after TBI.

Introduction

Mild to moderate therapeutic hypothermia (32-33 °C) has been used in clinical practice in pediatric patients with severe traumatic brain injury (TBI), specifically as a second tier therapy (1,2). Two recent studies including a multicenter trial have demonstrated safety of this treatment modality after TBI in children along with beneficial effects on intracranial hypertension (3,4). Neuroprotective effects of hypothermia have been shown in a variety of animal species and mechanisms of injury (5-9) and clinical trials have reported efficacy in both cardiac arrest in adults (10,11)and perinatal asphyxia in newborns (12). Several secondary injury mechanisms are favorably influenced by moderate hypothermia in experimental TBI (13,14) (15). However, the precise mode of neuroprotective action of mild-moderate hypothermia is not known.

Moderate hypothermia has been shown to have beneficial effects on oxidative stress in experimental models of TBI. Generation of hydroxyl radicals as analyzed by salicylate-trapping method was attenuated by moderate hypothermia after fluid percussion injury in rats (17). Furthermore, treatment of rats with moderate hypothermia after controlled cortical impact increased superoxide dismutase activity relative to values in injured normothermic animals (13). Similarly, therapeutic hypothermia has been shown to attenuate consumption of endogenous antioxidants and decrease lipid peroxidation in experimental temporary focal ischemia and cardiac arrest (14,15).

Assessment of the extent of oxidative stress in vivo is a complex task requiring employment of a battery of assays evaluating radical scavenging capacity and oxidation products of biomolecules. We previously presented evidence for free radical—mediated lipid peroxidation (by assessment of F2-isoprostanes) and protein oxidation (by assessment of protein thiol) and sustained decreases in total antioxidant reserve and glutathione (GSH) concentrations in cerebrospinal fluid (CSF) after severe TBI in infants and children. F2-isoprostanes are bioactive cyclopentanone prostaglandin-like compounds produced in vivo by free radical peroxidation of arachidonyl-containing lipids, and represent a reliable lipid biomarker of oxidative stress (16). Free radical attack on proteins results in oxidation of their sulfhydryl groups leading to decreased protein thiol concentrations. Our findings suggested that these CSF markers could be valuable to assess the effect of therapies on oxidative stress after TBI in patients.

Several studies suggest that CSF oxidation markers might be associated with outcome after TBI. Pilitsis et al., demonstrated that elevated levels of highly oxidizable polyunsaturated fatty acids in CSF were associated with worse outcome in adults with severe TBI (17). Enhanced lipid peroxidation, as assessed by CSF thiobarbituric acid reactive substances, was reported to correlate with the severity of head injury in adults with contusion (18). In a recent study, Darwish et al., showed that poor neurologic outcome was associated with increased levels of nitrotyrosine, a marker of protein damage by oxidative/nitrative stress, in CSF after TBI in

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adults (19). These studies indicate that CSF markers of oxidative stress may be useful in prognostication after TBI.

To date there has not been a study assessing the effects of hypothermia on oxidative stress after clinical TBI in either adults or children. Therefore, in the present study we tested the hypothesis that therapeutic hypothermia attenuates oxidative damage as assessed by markers of lipid peroxidation, protein oxidation, and antioxidant status (reduced glutathione and total antioxidant reserve [AOR]) in CSF after severe TBI in infants and children.

Methods

Patient Selection and Data Collection

We examined the effect of moderate therapeutic hypothermia on oxidative stress in CSF from subsets of patients (n=28) enrolled at our center in two concurrent randomized controlled trials assessing the effect and safety of moderate therapeutic hypothermia in severe TBI (GCS [Glasgow Coma Scale] score ≤ 8) in infants and children. The general paradigm for patients treated with hypothermia involved cooling to 32–33°C (within either 6 h or 24h following injury) for 48 h and then gradual re-warming. The details of the study protocols and results of these trials on clinical outcome have been previously reported (3). Briefly, once the patient was randomized to normothermia or hypothermia, a temperature control unit with a rectal probe was used for surface cooling or warming as needed. Temperature was maintained by means of a rectal probe at 32 to 33°C for hypothermia and at 36.5 to 37.5°C for normothermia for the 48-hour study period. To prevent shivering, which could make cooling difficult, sedation and paralysis were used before the initiation of cooling (hypothermia group) and during the study period in both groups. Patients randomized to normothermia were maintained at 36.5 to 37.5° C throughout the study period and were passively warmed if their initial presenting core temperature was less than 36°C. After 48 hours of cooling, rewarming occurred by passively warming the patient 1°C every 3 to 4 hours so as to reach normothermia (36.5°C) within 12 to 18 hours (3). The predetermined entry criteria, in addition to closed head injury and a GCS score of ≤ 8, were age of 0 – 156 months (multicenter trial) or 0 –18 years (single center trial). Patients were excluded from the study if they had a normal initial CT scan (no blood, fracture, swelling, and/or shift), GCS score of 8 with a CT scan with only minimal abnormal findings, prolonged hypotension (>15 min) defined as a mean blood pressure less than 5th percentile for age, failure to obtain informed consent within 6 h of injury (multicenter trial), failure to obtain informed consent within 24 h of admission to Children's Hospital of Pittsburgh (single center trial), brain dead clinically, penetrating cerebral injury, coagulopathy; PT > 16 and PTT > 40, or pregnancy. This study was approved by the Institutional Review Board of the Children's Hospital of Pittsburgh, and informed consent was obtained from parents for sample collection. CSF samples (n=76) were centrifuged for 10 min at 5000 x *g* and stored at -70°C until the time of analysis. Demographic and clinical parameters are seen in Table1.

All patients with severe TBI admitted to the Children's Hospital of Pittsburgh were treated with ventricular catheter insertion, and CSF was drained continuously. All patients were also intubated and mechanically ventilated to $PaCO_2$ of 35–38 mm Hg. They received sedation with narcotics (fentanyl) and neuromuscular blockade with vecuronium bromide to maintain their intracranial pressure (ICP) and cerebral perfusion pressure (CPP) in the age-appropriate target range in accordance with the guidelines for management of severe pediatric TBI (1). Barbiturates and mechanical ventilation to $PaCO_2$ < 35 mm Hg were used as second tier therapies as needed for refractory intracranial hypertension.

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Chemiluminescence measurements of total antioxidant reserve

Total AOR in CSF was assayed by chemiluminescence produced in the presence of luminol and a source of peroxyl radicals, as described by Tyurina *et al*. (20). A water-soluble azo-initiator, AAPH, was used to produce peroxyl radicals at a constant rate. Oxidation of luminol (400 μM) by AAPH-derived peroxyl radicals was assayed by monitoring the chemiluminescence response. The reaction was initiated by addition of AAPH. A delay in the chemiluminescence response, which is caused by interaction of endogenous antioxidants with AAPH-derived peroxyl radicals, was observed upon addition of CSF. Based on the known rate of peroxyl radical generation by AAPH, the amount of peroxyl radicals scavenged by endogenous antioxidants was determined. A Microlite ML 1000 microtiter plate luminometer (Dynatech Labs, Chantilly, VA, U.S.A.) was used for these determinations.

Fluorescence assay of protein sulfhydryls and glutathione

CSF protein sulfhydryls (Prot-SH) and glutathione concentrations were measured by fluorescent assay using using ThioGlo-1 (Convalent Associates, Inc., Woburn, MA), a maleimide reagent that produces a highly fluorescent product upon its reaction with sulfhydryl groups, as described previously (21). A Cytofluor 2350 fluorescence plate reader (Millipore Corporation, Marlborough, MA, U.S.A.) was used to detect fluorescence using excitation and emission wavelengths of 388 nm and 500 nm, respectively. The data acquired were exported from the spectrophotometer using Cytofluor software.

Determination of F2-isoprostane (8-epi-PGF2α)

CSF F2-isoprostane content was measured by a commercial enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) with a detection limit: 2 pg/ml.

Statistical Analysis

Data are shown as mean ± standard error of mean. Demographic and clinical data were compared by Students t test. The association between GCS score, age, gender, treatment (randomization to hypothermia or normothermia), time after injury, and CSF F2-isoprostane, prot-SH, glutathione, AOR levels were assessed by bivariate and multiple regression models. Because there are multiple observations per person, standard regression models violated the assumption of independence of observations. Therefore, Generalized Estimating Equation models, regression models that control for the correlation within individuals, were used. Some of the study patients randomized to therapeutic hypothermia did not reach target temperature at the time of CSF sampling. Similarly, some patients randomized to normothermia were hypothermic on admission. To assess the impact of actual core temperature on CSF F2-isoprostane level, we used a second model that included the actual core temperature at the time of CSF sampling instead of randomization to hypothermia or normothermia. In each of the models, the beta-coefficient represents the average increase or decrease in CSF biochemical marker levels for a one unit increase in continuous variables (e.g., temperature) or the difference in the average CSF biochemical marker level between the two groups for dichotomous variables.

Results

Patient Demographics

Demographic and clinical parameters of TBI patients are shown in Table 1. Age ranged from 2 months to 16 years. There were 13 patients randomized to hypothermia and 15 patients randomized to normothermia. Initial GCS score ranged between 3 and 8. Mechanism of injury included both accidental injury and child abuse. Five patients randomized to hypothermia and 8 patients randomized to normothermia received barbiturates. One patient randomized to

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hypothermia and 2 patients randomized to normothermia underwent decrompressive craniotomy. There was no statistically significant difference between the groups for demographic and clinical characteristics.

Total antioxidant reserve

For all biochemical analysis, mean sample collection times were not different between hypothermia and normothermia groups (10.4 ± 4.8 h vs 12.4 ± 7.3 h on day 1; 42.5 ± 3.2 h vs 40.8 ± 4.4 h on day 2, 62.6 ± 5.4 h vs 66.9 ± 4.1 h on day 3, Mean ± SD). The luminol-enhanced chemiluminescence assay revealed a reduction in total antioxidant reserve in patients randomized to normothermia vs hypothermia (117.9 ± 8.34 vs 90.23 ± 5.96 on day 2 after TBI) (Fig. 1). Bivariate and multiple regression models revealed a highly significant effect of hypothermia on CSF total antioxidant reserve independent of age, gender and initial GCS score ($p = 0.002$ and $p = 0.001$) (Table 2). Sustained depletion of total antioxidant reserve was suspected because the greatest decrease was observed on day 3, compared with day 1 and 2 (Fig. 1). There was an inverse relationship between temperature and CSF total antioxidant reserve after injury ($p = 0.022$) (Table 3).

Glutathione

GSH levels progressively decreased after the peak on day 1 similar to that previously reported (Fig. 2). Bivariate and multiple regression models revealed a tendency for higher GSH levels in CSF with hypothermia ($p = 0.097$ and $p = 0.090$) (Table 2). There was an inverse relationship between temperature and glutathione concentration in CSF after injury ($p = 0.002$) (Table 3).

Protein sulfhydryl oxidation

CSF concentration of protein sulfhydryls was about 3-fold lower on d 2 in patients randomized to hypothermia than patients randomized to normothermia (9.45 ± 1.41 vs 26.59 ± 7.21 nmol/mg protein) (Fig. 3). Bivariate and multiple regression models revealed a tendency for higher CSF protein sulfhydryl levels with hypothermia ($p = 0.063$ and $p = 0.079$) (Table 2). In general there was an inverse relationship between temperature and CSF protein sulfhydryl levels at all times after injury, but this was not statistically significant ($p = 0.104$) (Table 3)..

F2-isoprostane

F2-isoprostane levels progressively decreased after the peak on day 1 in patients randomized to normothermia similar to that previously reported (Fig. 4). Bivariate and multiple regression models did not reveal a significant effect of hypothermia (Table 2) despite 3.6-fold higher CSF F2-isoprostane levels in patients randomized to normothermia on d 1 after TBI (65.70 ± 24.83 pg/mL) compared with patients randomized to hypothermia (18.23 ± 1.84 pg/mL) (Fig. 4). At all times after injury, there was no significant temperature effect on CSF F2-isoprostane levels ($p = 0.104$) (Table 3).

Discussion

We have previously shown that severe TBI in infants and children is accompanied by marked and progressive compromise of antioxidant defenses and free radical-mediated lipid peroxidation (21). Here we present data demonstrating a beneficial effect of hypothermia on oxidative stress in the same setting. Our clinical data are consistent with results in experimental trauma models (13,22) in that hypothermia attenuates consumption of endogenous antioxidants. In addition, we have shown for the first time that hypothermia tended to decreased protein oxidation. We observed an early peak in F2-isoprostane levels, consistent with our previous study in infants and children (21), suggesting the possible need for early application of therapies targeting some aspects of oxidative stress after TBI, such as lipid peroxidation.

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Possible mechanisms of beneficial effect of hypothermia on oxidative stress after TBI

Mild-moderate hypothermia has been shown to have neuroprotective effects in experimental and clinical brain injury resulting from trauma, cardiac arrest and ischemia (10,11,14,15,22, 23). The precise mode of neuroprotective action by mild-moderate hypothermia is not known. Most likely, mild-moderate hypothermia exhibits multiple and synergistic effects on brain metabolism however it does not indiscriminately slow down all biochemical cascades. There is controversy regarding effects of mild-moderate hypothermia on cerebral energy metabolism likely due to differences in experimental insults and species studied. Mild-moderate hypothermia has been shown to decrease the global cerebral metabolic rate for glucose and oxygen but maintain a slightly better energy level by reducing ATP breakdown (24,25). Beneficial effects of mild-moderate hypothermia on energy balance and production of reactive oxygen species in mitochondria have also been reported after experimental ischemia reperfusion injury outside the CNS and in retina (26-29). However, classic studies in cerebral ischemia failed to show an effect of mild-moderate hypothermia on energy metabolite levels (30).

Mild-moderate hypothermia reduces the increase in intracellular calcium levels, which is linked to excitotoxicity and oxidative stress, after experimental cerebral ischemia and TBI (31-33). The majority of the free radicals that are produced after brain injury are generated by enzymatically catalyzed mechanisms —such as nitric oxide synthase (NOS)- and by deregulated electron transporters in mitochondria (34-38). Therefore we speculate that beneficial effects of hypothermia on oxidative stress after TBI might be explained by prevention of mitochondrial failure and reduction in excitotoxicity. Moderate hypothermia has been shown to attenuate increases in CSF acetylcholine levels (39) and brain interstitial levels of glutamate and aspartate seen after fluid percussion injury (22). Although, the latter finding was not consistently observed across experimental TBI models (40,41). Moderate hypothermia has also been shown to attenuate increases in CSF glutamate levels in adult severe TBI victims (23).

TBI-induced oxidative stress is importantly linked to inflammatory response. Several components of the local inflammatory response to cerebral contusion are also favorably affected by therapeutic hypothermia in experimental TBI, as evidenced by reductions in neutrophil accumulation (42-44), interleukin-1 (IL-1) mRNA upregulation (45,46) and inducible NOS activity (47). Increases in cytokines after severe TBI, however, are not consistently attenuated by therapeutic hypothermia in adults (23,48,49). Similarly, macrophage accumulation as assessed by CSF quinolinic acid levels was not attenuated by moderate hypothermia in adult severe TBI victims (50).

A beneficial effect of hypothermia on oxidative stress has been shown in experimental studies outside the CNS with reduction in lipid peroxidation in ischemic kidney and liver tissue (51, 52). Antioxidant supplementation with hypothermia had additive protective effects against lipid peroxidation in these experiments. Similarly, our data shows that hypothermia only partially restored antioxidant defenses. Given that mild-moderate hypothermia has shown variable or partial beneficial effects (ICP vs outcome) in clinical TBI (4,23,53,54), supplementation with antioxidants may represent a valuable adjunct to hypothermia in the treatment of TBI victims.

Clinical implications

To date, there have been no studies assessing the effect of therapeutic hypothermia on oxidative stress after TBI in either adults or infants and children. Two large clinical trials in TBI using antioxidants (PEG-SOD and tirilazad) showed largely negative results (55,56). Although these were seminal clinical studies in this area of investigation, they focused exclusively on

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therapeutic effects on clinical outcome. Neither study assessed whether or not oxidative stress was favorably influenced by the treatment. Furthermore, these two agents appear to have very limited brain penetrating ability (57). Our findings suggest that quantification of biomarkers of oxidative stress and antioxidant status of CSF may provide a valuable tool for monitoring treatment effects of antioxidant strategies or other therapies such as hypothermia, anti-excitotoxic, anti-apoptotic and anti-inflammatory agents after TBI.

We have previously shown gender differences in response to beneficial effects of therapeutic hypothermia on oxidative stress in adults after severe TBI (58,59). Current study does not show a gender difference in oxidative stress response to hypothermia in infants and children. This may suggest a powerful effect of sex steroids on this specific parameter that would manifest after puberty.

Direct assessment of relationship of biochemical markers with temperature

In this study we also carried out a separate analysis to directly assess the relationship between markers of oxidative stress and patient temperature at the time of CSF sampling. This second approach demonstrated highly significant preservation effect of hypothermia on antioxidants in CSF—confirming our initial analytical approach. Given that hypothermia took time to induce and that some patients can present with mild hypothermia, this additional statistical approach is, we believe, important to provide a more complete picture of the relationship between markers of oxidative stress and temperature.

Developmental Ramifications

Experimental studies in developmental studies of TBI support the notion that immature brain is particularly vulnerable to oxidative stress due to compromised antioxidant status (60). Our prior study supported this conclusion and showed profound antioxidant depletion lasting several days after severe TBI (21). Thus oxidative stress mechanisms might be an attractive target for therapeutic interventions in developmental CNS injury.

Limitations of the study

Despite the relatively small sample size and considerable variability in patient age, mechanism of injury, and treatment, statistical significance was achieved in a multivariate analysis between TBI patients randomized to hypothermia vs normothermia for AOR and glutathione. Although this study represents the largest biochemical study of oxidative stress markers in infants and children in a randomized trial of hypothermia after severe TBI, future studies with larger sample size are needed. Despite 3-fold lower value of F2-isoprostane in patients randomized to hypothermia vs normothermia, we were unable to show a significant attenuation of lipid peroxidation by hypothermia. Sample size estimates suggest that 36 patients per group would be needed to appropriately assess for an effect of hypothermia on this parameter accepting a power of 0.8. Similarly, the small number of patients with GCS 3-4 in both groups also limits our ability to assess the effect of hypothermia in most severely injured patients. While assessment of ventricular CSF provides great promise, it reflects changes across the entire brain and not simply in the injured tissue. It is likely that biochemical alterations seen with hypothermia may in part be from healthy brain tissue.

Barbiturates may reduce the overall cerebral metabolism and may therefore change the biochemical response. Although there was no statistically significant difference between the groups for barbiturate use ($p = 0.48$), a greater number of patients in the normothermia group received barbiturates vs hypothermia group. This suggests that other therapies, such as barbiturates, might be utilized more frequently for ICP control in the absence of hypothermia. In additional statistical analysis, barbiturate use did not have consistent effect on the CSF oxidative stress markers assessed in this study. Further studies with a larger sample size,

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including a comprehensive analysis of biochemical data as it relates to outcome, mortality, mechanism of injury (accidental trauma versus child abuse), age, gender, other therapies (such as barbiturate use and decompressive craniotomy) are needed. Asessment of the relationship between markers of oxidative stress and associated mechanisms of secondary damage such as apoptosis and excitotoxicity (61,62) could also be revealing.

Finally although we cite these limitations, we have also reported, in the same patient population, that hypothermia failed to attenuate the marked increases in CSF cytokine levels after TBI (63). Taken together our findings mirror those observations in experimental models of TBI and support a differential effect of mild-moderate hypothermia across injury mechanisms. Future research in this area might improve our understanding of mechanisms of neuroprotection by hypothermia in TBI and enable us augment its beneficial effects by targeted combination therapies.

Conclusions

Moderate therapeutic hypothermia preserves antioxidant defenses after severe TBI in infants and children. Our data support the concept that CSF represents a valuable tool for monitoring treatment effects on oxidative stress after TBI.

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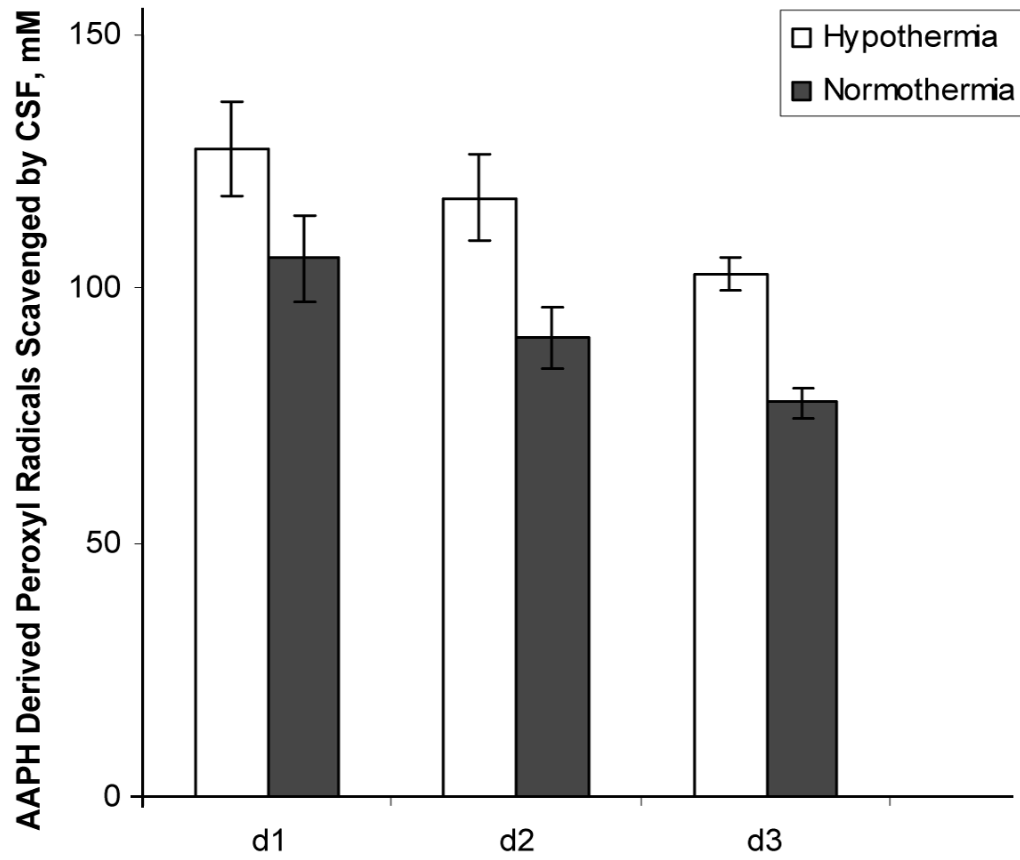

Figure 1.

Effect hypothermia on CSF antioxidant reserves after TBI. Hypothermia preserved antioxidant reserves after TBI compared with normothermia.

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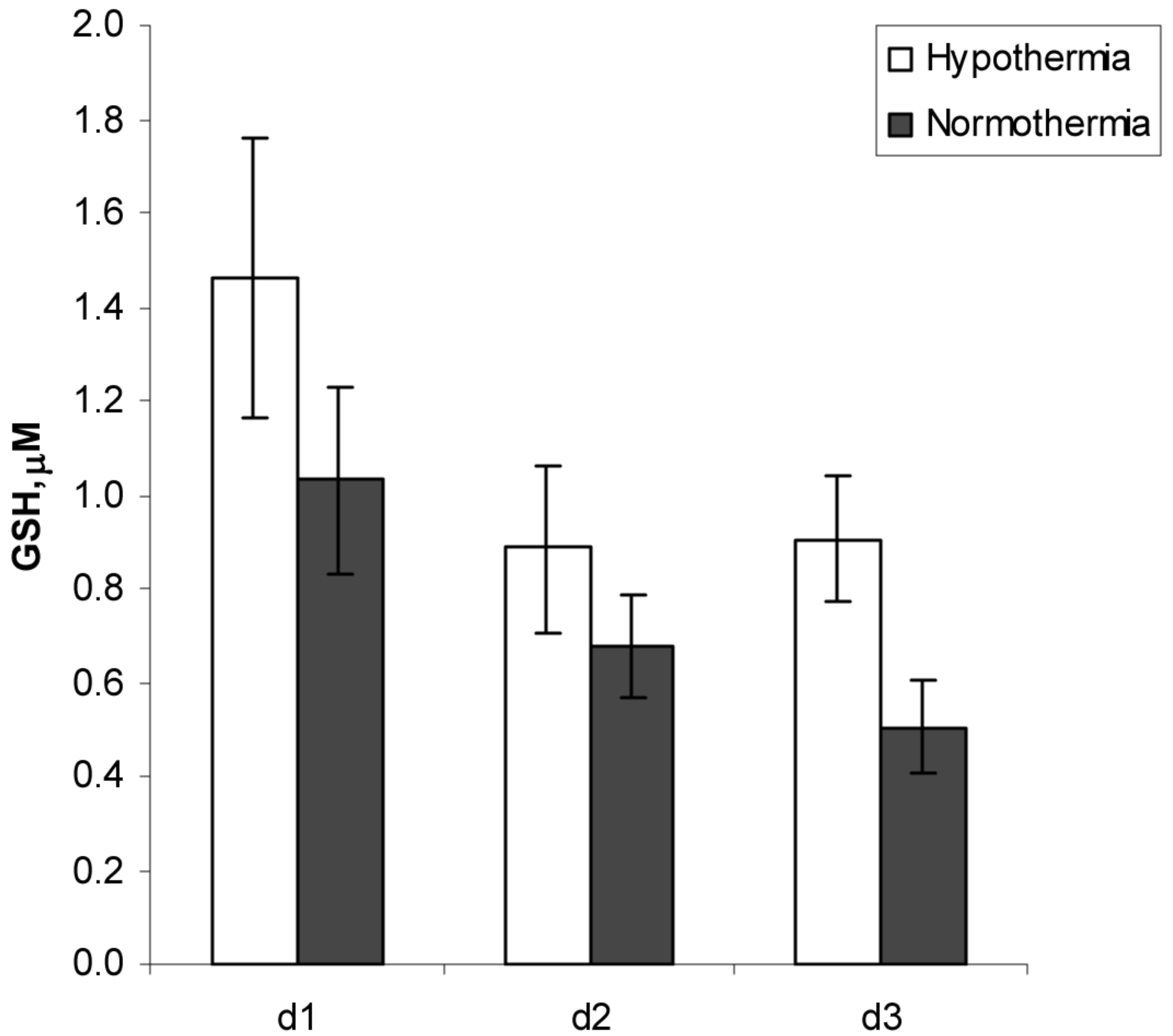

Figure 2.
Effect of hypothermia and time on CSF GSH levels after TBI.

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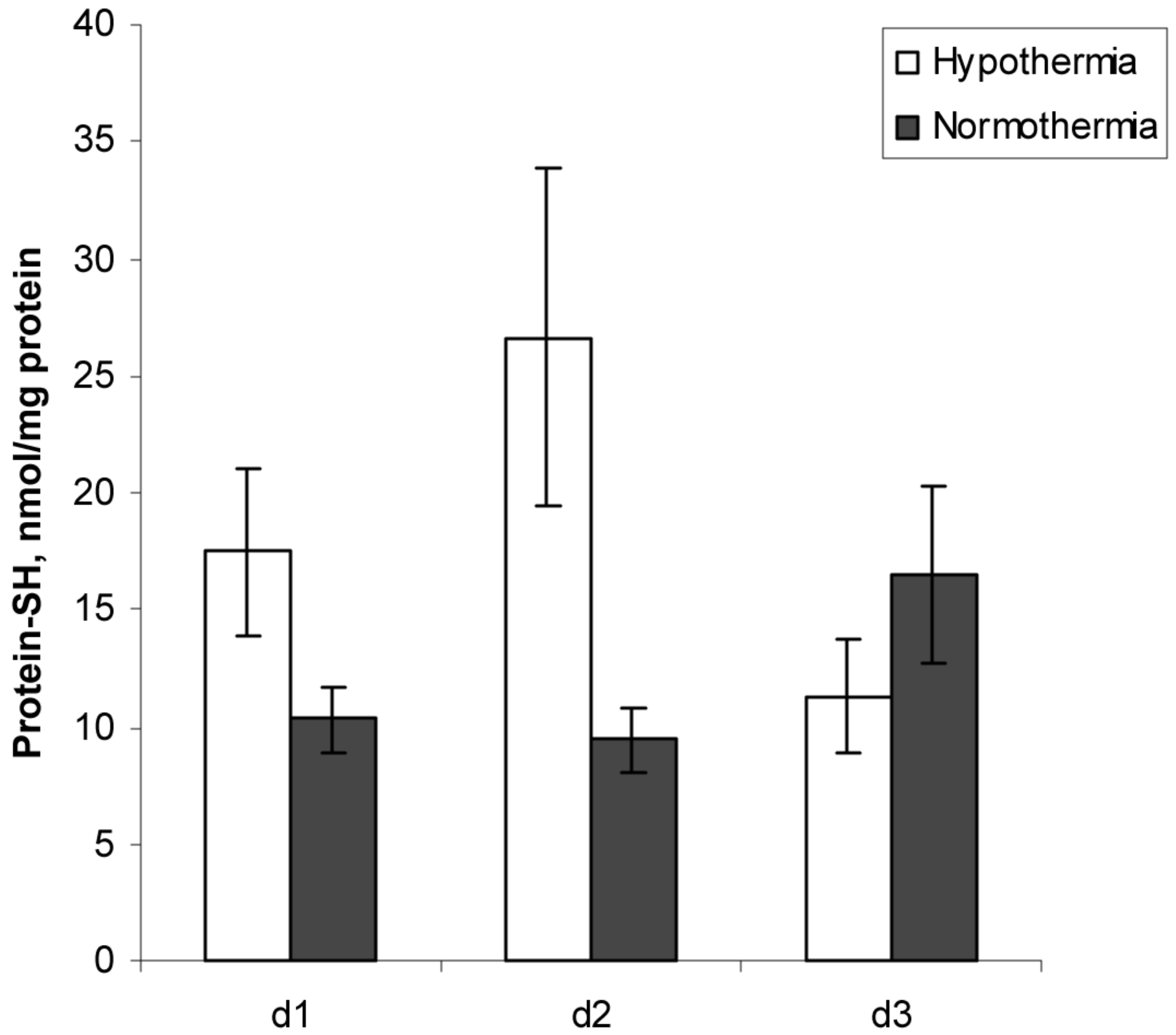

Figure 3.
Effect of hypothermia and time on CSF Protein sulfydryl levels after TBI.

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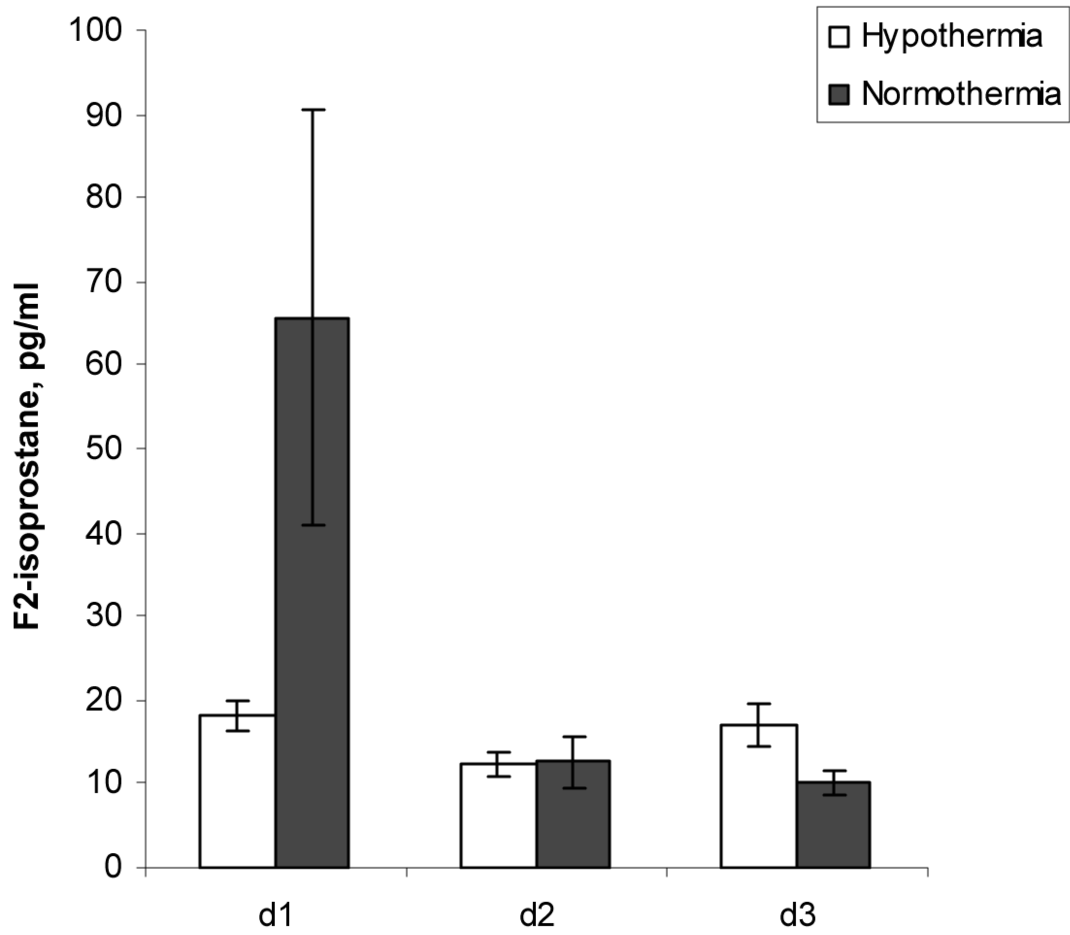

Figure 4.
Effect of hypothermia and time on CSF F2-isoprostane levels after TBI.

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Table 1

Clinical and Demographic characteristics of patients

Characteristic	*Hypothermia (n=13)*	*Normothermia (n=15)*
Age	6.8 ± 5.1	5.1 ± 5.4
Gender (male)	9 (69)	8 (53)
Glasgow Coma Scale Score	6.3 ± 1.3	6.0 ± 1.3
Mechanism of injury		
Motor vehicle injury	7 (54)	10 (66)
Inflicted TBI	2 (15)	4 (27)
Fall	3 (23)	1 (7)
Other	1 (8)	0 (0)
Barbiturate use	5 (39)	8 (53)
Decrompressive craniotomy	1 (8)	2 (13)

Plus-minus values are means ± SD. Percentages are shown in parenthesis.

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Table 2

Effect of hypothermia treatment on CSF biomarkers of oxidative stress

CSF biomarker	Univariate model		Multivariate model	
	β	*p* value	β	*p* value
Total Antioxidant Reserve				
Treatment (reference = NT)	-25.1	0.002	-25.4	0.001
Time (Days 1, 2, 3)	-14.5	<.0001	-14.3	<.0001
Age	0	0.79	0	0.473
Gender	-13.4	0.115	-9.5	0.18
GCS	-6.4	0.385	-10.92	0.112
Glutathione (GSH)				
Treatment (reference = NT)	-0.3	0.097	-0.4	0.09
Time (Days 1, 2, 3)	-0.3	<.0001	-2.9	<.0001
Age	0	0.911	0	0.705
Gender	-0.1	0.79	0.1	0.784
GCS	0.1	0.645	0.1	0.741
Protein sulfhydryls				
Treatment (reference = NT)	-6.1	0.063	-6	0.079
Time (Days 1, 2, 3)	0.1	0.969	0.2	0.873
Age	0	0.563	0	0.753
Gender	-4.6	0.151	-3.9	0.231
GCS	-0.7	0.878	-2.6	0.494
F_2 -isoprostane				
Treatment (reference = NT)	13.3	0.099	-15.6	0.183
Time (Days 1, 2, 3)	-14.7	0.029	-15	0.023
Age	0	0.913	0	0.636
Gender	3.6	0.668	4	0.605
GCS	5.4	0.381	11.7	0.188

GCS, Glasgow Coma Scale Score; NT, normothermia

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Table 3

Association of CSF metabolites with temperature.

Metabolite	r	p
Total antioxidant reserve	-0.3	.022
Protein sulfhydryls	-0.2	.104
Glutathione	-0.3	.002
F2-Isoprostane	-0.2	.104