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Antioxidant and Neuroprotective Effects of L-arginine Administration After Traumatic Brain Injury and Hemorrhagic Shock in Rats

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ABSTRACT

AIM: To investigate the effect of fluid resuscitation and L-arginine administration on oxidant status markers, blood gases, lactate and apoptosis in the brain tissue of a rat model of TBI with hemorrhagic shock.

MATERIAL and METHODS: A total of 60 rats were divided into six groups: control, isotonic saline-treated, 7.5% NaCl-treated (hypertonic saline), L-arginine-treated (100 mg/kg), saline + L-arginine-treated and 7.5% NaCl + L-arginine-treated groups. Closed head contusive weight-drop injuries were performed with hemorrhagic shock in all of the groups. Mean arterial pressure (MAP), pulse rate, lactate, malondialdehyde (MDA), total antioxidant capacity (TAC) and apoptosis were investigated.

RESULTS: In a total of 48 rats, MAP levels remained higher than 60 mmHg for 3 hours in all of the treatment groups. The highest MAP values in each group were recorded. Higher MDA and lower TAC levels were observed in the control group than in all of the treatment groups (all $p<0.05$). The number of apoptotic cells was highest in the control group and lowest in the L-arginine group.

CONCLUSION: L-arginine administration may be an alternative treatment option for individualized fluid resuscitation in patients with TBI and hemorrhagic shock.

KEYWORDS: Antioxidant, Hemorrhagic shock, Resuscitation, Traumatic brain injury

ABBREVIATIONS: ANOVA: Analysis of variances, **INOS:** Inducible nitric oxide synthetase, **LR:** Lactated Ringer, **MAP:** Mean arterial pressure, **MDA:** Malondialdehyde, **NO:** Nitric oxide, **SBP:** Systolic blood pressure, **TAC:** Total antioxidant capacity, **TBARS:** Thiobarbituric Acid Reactive Substances, **TBI:** Traumatic brain injury

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INTRODUCTION

Traumatic brain injury (TBI) is a disruption of brain function and/or structure resulting from external physical force, including sudden acceleration or deceleration, impact, blast waves or penetration by a projectile (25). The leading cause of potentially preventable death in TBI patients is uncontrollable posttraumatic massive hemorrhage and related severe hemorrhagic shock (19). TBI can occur as a primary injury via direct mechanical damage to neurons, blood vessels or glial cells or as a secondary injury associated with metabolic responses, including hypoxia, space-occupying lesions, oxidative stress, inflammation, apoptosis, edema, reperfusion injury, mitochondrial dysfunction and ischemia (9). Trauma and hypovolemia can induce multiple local and systemic compensatory mechanisms, including activation of the sympathetic nervous system, inflammatory and immunological responses, hormonal and metabolic responses, cardiac adaptations and electrolyte changes that act to maintain oxygenation and tissue blood flow (3). The basic management principles involve bleeding control, fluid resuscitation, re-establishment of tissue perfusion and the prevention or minimization of additional damage to vital organs (18). Despite advances in animal and human studies on the management of trauma-related fluid resuscitation, no consensus has been reached on the optimal fluid strategy. However, due to the complexity and variability of clinical conditions, resuscitative interventions should be individualized according to various factors, including the type of bleeding (controlled or uncontrolled), the mechanism of injury (blunt or penetrating) and severity of injury, the availability of resources (blood products or colloids against crystalloids), the timing of fluid administration (prehospital, presurgical, postsurgical or intensive care), concomitant comorbidities and the patient's clinical condition (4). Crystalloids, including isotonic or hypertonic saline or Lactated Ringer's (LR) solution, are widely used as initial resuscitation fluid for traumatic patients. Despite its easy availability and inexpensiveness, aggressive crystalloid treatment tends to cause hemodilution, decreased oxygen carrying capacity and delivery and other harmful effects, including coagulation, brain and pulmonary edema, inflammation, electrolyte abnormalities and acidosis (13). Hypertonic saline is utilized in patients with TBI due to its effect in increasing blood pressure and reducing intracranial pressure (16).

L-arginine functions as a second messenger in a wide variety of physiological processes, including the control of cerebral blood flow, interneuronal communications, synaptic plasticity, memory formation, receptor functions, intracellular signal transmission and release of neurotransmitters (6). L-arginine is a precursor of NO, which is a molecule that is a potent vasodilator causing an increase in blood flow to vital organs and blood tension and modulates the aggregation of leukocytes and platelets by reducing their adhesion to the endothelium (11). Studies have shown that the administration of L-arginine following brain trauma increases NO levels, decreases contusion volume, reduces neurological and cognitive deficits and improves cerebral blood flow during the early responses to trauma (6). To date, few studies have investigated the potential role of L-arginine therapy with different fluid treatment protocols on cellular oxidant status and apoptosis in traumatic patients with hemorrhagic shock.

The aim of the present study was to investigate the effect of normotensive fluid resuscitation treatment with various fluids and the administration of L-arginine on oxidant status markers, blood gases, lactate and apoptosis in the brain tissue of a rat model of TBI with hemorrhagic shock.

MATERIAL and METHODS

Animals and Groups

Herein, animal care and all of the experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. All of the experimental procedures were approved by the Animal Care and Use Committee of Mersin University (013/17). Sixty adult male Wistar albino rats weighing 250-350 g were involved in this study. All of the animals had free access to standard rodent chow and water. The rats were randomized into six groups as follows (10 animals per group):

1. Control group. Animals underwent the TBI procedure and were monitored for 3 hours without fluid resuscitation. At the end of this time period, blood and brain tissue samples were taken after sacrifice.
2. Isotonic saline group. Animals underwent TBI and were immediately resuscitated with normal saline (1 mL/kg/min). After monitoring for 3 hours, blood and brain tissue samples were obtained, and the animals were sacrificed.
3. Hypertonic saline (7.5% sodium chloride) group. Animals underwent TBI and were immediately resuscitated with hypertonic saline (1 mL/kg/min). Subjects were monitored for 3 hours until they were sacrificed for sample acquisition.
4. L-arginine group. Animals underwent TBI and were treated with L-arginine (100 mg/kg). Rats were sacrificed after 3 hours of monitoring, and samples were obtained.
5. Isotonic saline + L-arginine group. Rats underwent TBI and were administered isotonic saline (1 mL/kg/min) and L-arginine (100 mg/kg) treatment. After monitoring for 3 hours, blood and brain tissue samples were obtained, and the animals were sacrificed.
6. Hypertonic saline + L-arginine group. Animals underwent TBI and were treated with hypertonic saline (1 mL/kg/min) and L-arginine (100 mg/kg). Again, monitoring was performed for 3 hours, and the animals were sacrificed to obtain blood and brain samples.

TBI Model and Formation of Hemorrhagic Shock

All of the animals were anesthetized with 15 mg/kg xylazine and 25 mg/kg ketamine combination (intraperitoneally) and were allowed to breathe room air. The animals were exposed to closed head trauma via the method described by Marmarou et al. as follows: a blunt object weighing 500 g was allowed to drop freely from 1 m height through a copper tube on to a metal disc over the skull of the animal (15). After the induction of head trauma, the metal disc was removed, and all of the animals were placed into the supine position. Under sterile

conditions, a 22-gauge heparinized cannula was inserted into the femoral artery of each rat to initiate hemorrhagic shock and monitoring. For fluid resuscitation, a 26 gauge cannula was inserted into the saphenous vein. Hemorrhagic shock was induced via volume-controlled withdrawals of blood (2 ml of blood per 100 g of body weight, slowly drawn throughout at least 30 minutes). The catheter was then connected to a pressure transducer with a data acquisition system to monitor the mean arterial pressure (MAP). Free drainage was performed from the femoral artery (accompanied by continuous monitoring), and MAP was expected to decrease below 40 mmHg to ensure the development of hemorrhagic shock. When the MAP was below 40 mmHg, free drainage from the femoral artery was terminated, and monitoring was continued. After the formation of hemorrhagic shock, the treatment protocols were intravenously administered with regard to treatment plans, with a MAP target of 60 mm Hg. For a total of 180 minutes, MAP, systolic and diastolic blood pressure, pulse rate and respiratory rate were continuously monitored and recorded at 15-min intervals. The base deficit and pH values of each rat were recorded at 0, 60, 120 and 180 minutes. At the end of the experimental procedures, a total of 48 surviving rats were sacrificed with the use of guillotine decapitation after collecting all of the necessary blood samples, and the rats remained under anesthesia. Blood samples were centrifuged at 4,000 rpm for 15 min to obtain serum, which was stored at -80 °C until measurements were performed. After decapitation, the brains of the rats were removed from the skull and fixed in 10% buffered formaldehyde solution.

Biochemical Analyses

Malondialdehyde (MDA) levels were spectrophotometrically determined at 532 nm by using a Thiobarbituric Acid Reactive Substances (TBARS) Assay Kit. The reference range for MDA was 1.86–3.94 μM/L. Serum TAC levels were measured via a Cobas Integra 800 autoanalyzer by using an automated kit. This technique is based on the reduction of the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; ABTS) radical. The change in absorbance value at 660 nm was related to the total antioxidant level of the sample (mmol Trolox equivalent/L). The measurement of blood lactate was performed with an enzymatic calorimetric technique on a Cobas Integra 800 autoanalyzer. The reference range was 4.5–19.8 mg/dL for blood lactate.

Histopathological Assessment

For the histopathological examinations, all of the tissue samples were fixed in 10% buffered formaldehyde solution for 1 day, embedded in paraffin and routinely processed for light microscopic examination. Four-millimeter-thick serial sections of brain tissues were stained with hematoxylin-eosin and examined by a pathologist who was blinded to the groups and experimental material. The TUNEL method was applied to determine apoptotic cells in tissues by using the In Situ Cell Death Detection apoptosis kit. Meningeal and parenchymal inflammatory cell infiltration, hemorrhage and apoptotic cell counts were recorded, and all of these parameters were scored according to the study by Stern et al., as follows: 0 = absent, 1 = mild, 2 = moderate and 3 = severe (22).

Statistical Analyses

All of the analyses were performed by using SPSS v21. For the normality check, the Shapiro–Wilk test was used. Data are shown as the mean ± standard deviation as descriptive statistics for arterial pressure, pulse rate, blood gas analysis, MDA, blood lactate and TAC values at each time point. Frequency and percentage values were provided for the qualitative variables. Normally distributed variables were analyzed with the use of one-way analysis of variances (ANOVA), and the Dunnett T test method was used to compare the groups in the post hoc analysis. Chi-square tests were used to compare the distributions of the categorical variables. Furthermore, p values of < 0.05 were accepted as the level of statistical significance.

RESULTS

Forty-eight surviving rats were included in the study. Nine rats survived in each of the isotonic, isotonic + L-arginine and hypertonic + L-arginine treatment groups. Three rats died in the hypertonic group, four rats died in the L-arginine group and two rats died in the control group during TBI and/or hemorrhagic shock formation procedures. At the beginning of the procedure, there were no significant differences among the rat groups in terms of body weight (overall mean: 270 ± 22.02, $p>0.05$). The mean pulse rate, MAB and blood gas results of the rats at 0, 60, 120 and 180 minutes are shown in Table I. The mean pulse rate at 120 minutes in the hypertonic group was significantly higher than that in the control group, isotonic + L-arginine group and hypertonic + L-arginine group ($p=0.025$, 0.01 and 0.01, respectively). Significant differences were found in the treatment groups compared to the control group in terms of MAP values at 60, 120 and 180 minutes (all $p<0.001$). The highest MAP values were determined in the isotonic and L-arginine groups at 60 minutes, in the isotonic + L-arginine and hypertonic + L-arginine groups at 120 minutes and in the hypertonic and isotonic groups at 180 minutes. However, no statistically significant difference was found between the treatment groups with regard to MAP (all $p>0.05$). The pH values were lower in the isotonic group at 120 minutes than in the control, hypertonic and isotonic + L-arginine groups ($p=0.001$, 0.039 and 0.019, respectively). There was no statistically significant difference between groups over time in terms of base deficit values (all $p>0.05$).

Lactate, MDA and TAC levels were compared in all of the groups. The L-arginine group had a statistically significantly higher lactate value than the control group and the hypertonic + L-arginine group ($p=0.00$ and 0.02, respectively). MDA levels were significantly higher in the control group than in all of the treatment groups, except for the isotonic + L-arginine group (all $p<0.05$). However, there was no difference between the treatment groups with regard to MDA levels. Although the highest TAC value was found in the L-arginine group, there was no statistically significant difference between the groups ($p=0.089$). Changes in pulse rate, MAB, blood gas analysis, MDA, lactate and TAC levels over time are depicted.

The results of the brain tissue examinations and scores are given in Table II. The number of apoptotic cells was highest in

Table I: Comparison of Mean Pulse Rate, MAP, pH, Base Deficit, Lactate, MDA and TAC Values at Different Times in All Groups

		0 minutes	60 minutes	120 minutes	180 minutes	End of the Study
Control	Pulse rate	188.5 ± 25.15	178 ± 30.24	149 ± 37.58	125 ± 32.45	
	MAP	95.75 ± 10.44	49.90 ± 4.8	47.37 ± 7.94	42.87 ± 9.5	
	pH	7.39 ± 0.049	7.34 ± 0.054	7.36 ± 0.071	7.26 ± 0.077	
	Base deficit	-1.10 ± 1.82	-1.96 ± 1.42	-2.20 ± 2.096	-5.3 ± 3.95	
	Lactate					21.20 ± 8.53
	MDA					37.89 ± 10.91
	TAC					0.355 ± 0.26
Isotonic group	Pulse rate	211.33 ± 25.6	164.6 ± 38.8	154.11 ± 21.7	116 ± 29.86	
	MAP	87 ± 13.00	65.2 ± 3.89	61.22 ± 6.2	61.5 ± 1.94	
	pH	7.33 ± 0.071	7.29 ± 0.07	7.20 ± 0.07	7.22 ± 0.13	
	Base deficit	-1.17 ± 2.33	-4.5 ± 2.62	-5.50 ± 3.61	-7.40 ± 5.15	
	Lactate					44.47 ± 20.57
	MDA					19.34 ± 5.45
	TAC					0.61 ± 0.31
Hypertonic group	Pulse rate	202.42 ± 17.4	151 ± 24.87	182.71 ± 26.2	132.85 ± 12.92	
	MAP	97.14 ± 10.44	62.14 ± 7.01	60.42 ± 6.26	63.42 ± 3.77	
	pH	7.34 ± 0.035	7.33 ± 0.07	7.31 ± 0.1	7.27 ± 0.08	
	Base deficit	-2.82 ± 1.37	-3.85 ± 2.88	4.82 ± 4.28	6.30 ± 4.44	
	Lactate					44.64 ± 31.21
	MDA					19.79 ± 6.65
	TAC					0.53 ± 0.28
L-arginine group	Pulse rate	188.3 ± 17.32	165.33 ± 48.01	159.5 ± 12.66	107.50 ± 22.52	
	MAP	83.33 ± 10.74	65 ± 4.51	62 ± 6.03	61 ± 1.54	
	pH	7.33 ± 0.07	7.33 ± 0.07	7.29 ± 0.04	7.34 ± 0.16	
	Base deficit	-1.81 ± 2.09	-4.1 ± 4.17	-2.11 ± 3.1	-4.3 ± 5.69	
	Lactate					70.48 ± 32.12
	MDA					21.94 ± 9.38
	TAC					0.85 ± 0.12
Isotonic + L-arginine group	Pulse rate	197 ± 11.15	171.11 ± 33.71	132.44 ± 15.6	116 ± 29.86	
	MAP	96.88 ± 11.47	58.66 ± 11.84	63.55 ± 7.33	60.88 ± 4.40	
	pH	7.38 ± 0.06	7.34 ± 0.04	7.31 ± 0.03	7.30 ± 0.04	
	Base deficit	-0.2 ± 1.78	-2.87 ± 2.09	-1.63 ± 3.1	-2.85 ± 2.84	
	Lactate					41.64 ± 19.77
	MDA					28.92 ± 10.02
	TAC					0.53 ± 0.39
Hypertonic + L-arginine group	Pulse rate	193.77 ± 10.7	147.88 ± 19.18	124.88 ± 9.59	103.66 ± 23.83	
	MAP	89.33 ± 13.48	64.55 ± 4.74	63.44 ± 2.60	60.66 ± 2.0	
	pH	7.36 ± 0.09	7.36 ± 0.05	7.28 ± 0.06	7.32 ± 0.04	
	Base deficit	-1.27 ± 2.3	-1.33 ± 2.56	-2.37 ± 0.098	-3.7 ± 2.31	
	Lactate					25.22 ± 6.75
	MDA					25.54 ± 10.85
	TAC					0.54 ± 0.26

MDA: *Malondialdehyde,* ***TAC:*** *Total antioxidant capacity.*

the control group and lowest in the L-arginine group. Although apoptosis was observed to be decreased with the addition of L-arginine, this effect was not statistically significant (p=0.849). Moderate and/or severe meningeal and/or parenchymal neutrophil leukocyte infiltration (score 3) was detected only in the hypertonic + L-arginine treatment group. No statistically significant difference was found between the groups in terms of scores of neutrophils and hemorrhage (p=0.878 and 0.136, respectively) (Figure 1).

DISCUSSION

This study aimed to evaluate the effect of different fluid resuscitation treatments (with or without L-arginine administration) on oxidative markers, blood lactate levels and gas levels, as well as brain tissues in rats with TBI and hemorrhagic shock. We used the Marmarou method for head trauma, which successfully simulates accidents. A controlled hemorrhagic shock method was utilized by using blood withdrawals from the femoral artery, thus enabling the prevention of clotting, heat loss and uncontrolled bleeding problems. We demonstrated improvements in MAP and TAC levels and decreases in MDA and apoptosis with the use of L-arginine and resuscitation fluids. However, we also observed an increase in lactate levels and deterioration in pH values in our study group.

Table II: The Scores of Mean Apoptotic Cell Counts of All Groups with the TUNEL Method

	Apoptotic cell counts
Control (n=8)	5.62 (3-10) ± 2.32
Isotonic (n=9)	5.44 (2-10) ± 2.87
Hypertonic (n=7)	4.57 (2-8) ± 2.14
L-arginine (n=6)	4.33 (2-6) ± 1.36
Isotonic + L-arginine (n=9)	4.77 (2-8) ± 1.98
Hypertonic + L-arginine (n=9)	5.11 (3-7) ± 1.83

The complicated nature of TBI is further confounded by the fact that the development of such changes can be within hours or may extend to days. The early response to TBI after the immediate hypertensive surge is characterized by intracranial hypertension, systemic hypotension and a decrease in cerebral blood flow (7). Due to the fact that hemorrhagic shock frequently accompanies TBI, it is paramount to perform interventions as early and appropriately as possible. The goals of management involve bleeding control, the restoration of lost blood volume and the re-establishment of tissue perfusion and organ function (18). Although various resuscitative fluids and strategies are available, there is no consensus for optimal resuscitative fluid administration in TBI accompanied by hemorrhage. Each strategy has its own benefits and limitations that must be directly and actively assessed by a physician (24).

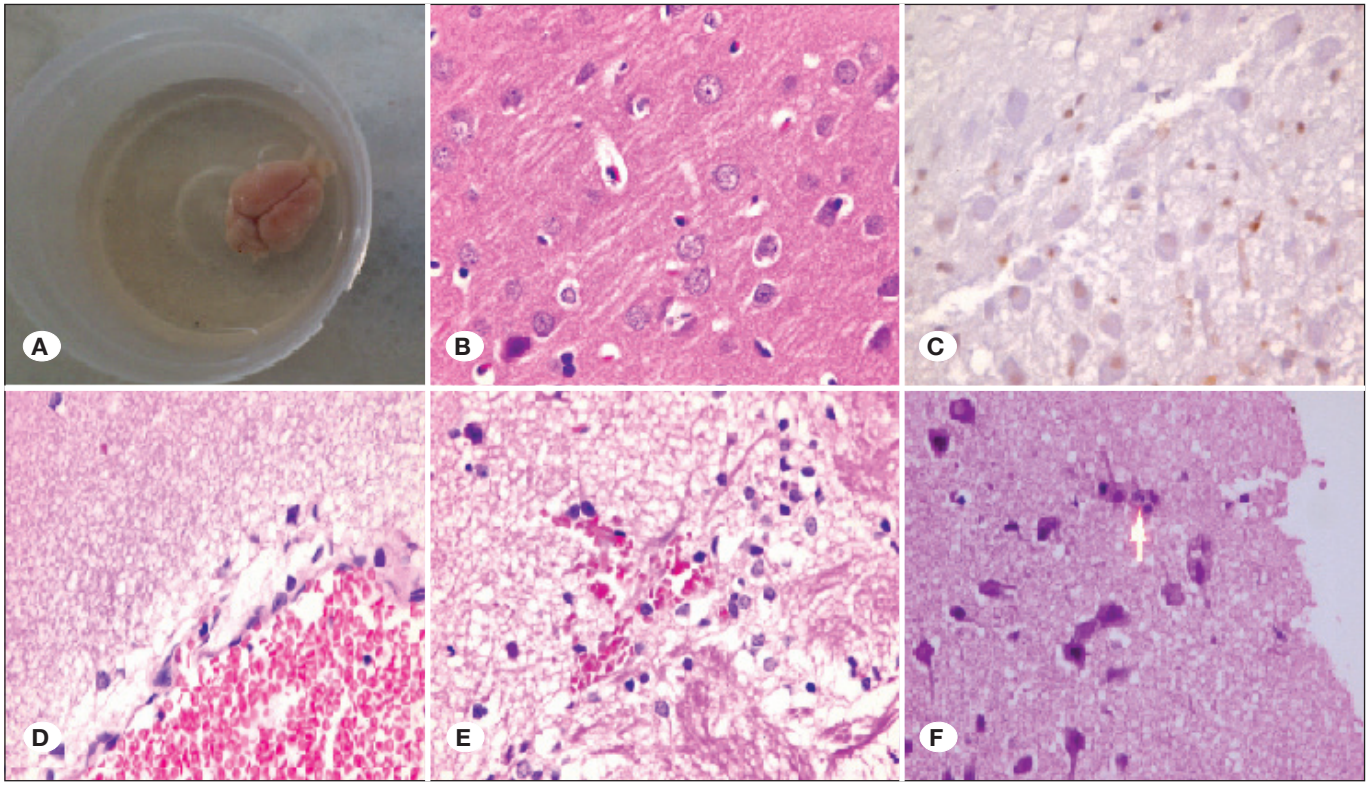

Figure 1: Pathological examination. **A)** Macroscopic view, **B)** apoptotic cells (HE, x400), **C)** staining of apoptotic cells with ApopTec immunohistochemistry (HE, x400), **D)** meningeal moderate bleeding (HE, x400), **E)** parenchymal moderate bleeding (HE, x400), **F)** parenchymal moderate neutrophil infiltration (HE, x400).

In fluid resuscitation, studies have suggested different targets for systolic blood pressure values in different trauma types. Overall, these are 60-70 mmHg for penetrating trauma, 80-90 mmHg for blunt trauma without TBI and 85-90 mmHg for blunt trauma with TBI (8). In an experimental model of TBI and uncontrolled hemorrhage, Stern et al. showed that a 60-minute period of moderate hypotension (MAP = 60 mmHg) was well tolerated (22). In the current study, we based our 60 mmHg MAP target on their findings. We found a significant improvement in MAP values over time in the treatment groups compared to the controls. This indicates that hemodynamic stability was maintained with all of the different treatment protocols that were employed in this study, regardless of L-arginine administration. In an experimental model of TBI and hemorrhage treated with isotonic saline, hypertonic saline or hypertonic saline + L-arginine, Sell et al. demonstrated that no difference was found in MAP and pH values among the groups (21). Although our study was in agreement with regard to MAP results, we found lower pH values with the treatments, especially in the isotonic treatment group. Prough et al. administered hypertonic saline with or without different amounts of L-arginine and found no improvement in MAP, cerebral blood flow or intracranial pressure between the groups with or without L-arginine (17). In contrast to that study, we observed altered MAP levels at 120 and 180 minutes in L-arginine recipients compared to the hypertonic group. However, the two studies were similar in terms of finding decreased arterial pH levels in all of the treatment groups over time. This suggests that the addition of L-arginine to fluid resuscitation may lead to metabolic acidosis. Both base deficit and serum lactate values are common post-TBI measures of hypoperfusion and shock and are correlated with TBI severity, poor cardiac performance, increased mortality and multiple system organ failure (2). Although we found the lowest base deficit in the isotonic + L-arginine treatment group, there was no significant difference between the groups. This may suggest that the administration of L-arginine with crystalloids showed an improvement in hemodynamic parameters without negative effects on base deficits. Furthermore, we found higher lactate levels in all of the treatment groups than in the control group. This may be due to cerebral ischemia related to decreased cerebral perfusion and blood flow, which leads to increased lactate levels. The mechanism of this effect may be associated with mitochondrial dysfunction and impaired cellular energy metabolism in the early postinjury period; however, the systemic effects of L-arginine should also be considered.

Jeter et al. demonstrated that decreased plasma levels of L-arginine and metabolites such as citrulline, ornithine and hydroxyproline, as well as increased plasma levels of creatine, were found in patients with severe TBI, which may affect the outcomes (12,14). In an experimental study, Cherian et al. found that L-arginine administration after the induction of controlled cortical damage increased cerebral blood flow and reduced the volume of contusion (7). In their study of a fluid-percussion TBI model treated with L-arginine or superoxide dismutase, DeWitt et al. demonstrated that rats receiving L-arginine exhibited no reduction in cerebral blood flow (10). Moreover, Arora et al. showed that the administration of L-arginine during resuscitation improved MAP, lowered lactate, improved histological signs of reperfusion injury and increased survival in a rat model of severe hemorrhagic shock (3). In agreement with these studies, we observed decreased MDA and increased TAC levels with L-arginine administration. This indicates that L-arginine administration had antioxidant effects, possibly through the activation of NO synthesis and subsequent benefits pertaining to cerebral blood perfusion and inflammation.

L-arginine may be involved in the mechanism of neuroinflammation following TBI through the effects of NO, which accumulates in the brain immediately after injury and has been suggested to function as a chemokine (1). Proinflammatory mediators induce the activity of inducible nitric oxide synthetase (iNOS) in macrophages, microglia and infiltrating neutrophils and contribute to inflammatory responses (20). In a rat model of traumatic spinal cord injury, Satake et al. showed that the administration of L-nitro-arginine methylester, which is a competitive inhibitor of NOS, reduced apoptosis in the lesioned area, thus suggesting that overproduction of NO is linked to diminution of brain macrophages by apoptosis (20). There have been studies showing reduced contusion volumes in rat models (5,23). Moreover, Sell et al. demonstrated improved long-term neuronal survival and behavioral recovery with hypertonic saline and hypertonic saline + L-arginine compared to untreated TBI (21). Similarly, we observed a decreased neuron loss rate in groups receiving L-arginine compared to the control and crystalloid-treated groups without L-arginine. This indicates that L-arginine administration had neuroprotective effects and may be utilized in further studies assessing its value in the management of TBI injury.

CONCLUSION

We observed positive effects of L-arginine administration on clinical and biochemical outcomes in the early period of TBI. L-arginine administration may be an alternative treatment option for individualized fluid resuscitation in patients with TBI and hemorrhagic shock.

AUTHORSHIP CONTRIBUTION

Study conception and design: GBB, CA, GB, AD, EA
Data collection: GBB, GB
Analysis and interpretation of results: LTG, RBA, AG
Draft manuscript preparation: AK, SBB
Critical revision of the article: GBB, GB, AK, CA
Other (study supervision, fundings, materials, etc...): EA, AD
All authors (GBB, GB, CA, AK, SBB, LTG, RBA, AD, EA, AG) reviewed the results and approved the final version of the manuscript.

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