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Research Report

Brain energy depletion in a rodent model of diffuse traumatic brain injury is not prevented with administration of sodium lactate

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ABSTRACT

Lactate has been identified as an alternative fuel for the brain in situations of increased energy demand, as following a traumatic brain injury (TBI). This study investigates the effect of treatment with sodium lactate (NaLac) on the changes in brain energy state induced by a severe diffuse TBI. Rats were assigned to one of the eight groups (n=10 per group): 1—sham, normal saline; 2—TBI, normal saline; 3—TBI, hypertonic saline; 4—TBI, 100 mM NaLac, 5—TBI, 500 mM NaLac; 6—TBI, 1280 mM NaLac; 7—TBI, 2000 mM NaLac and 8-TBI-500 mM NaLac+magnesium sulfate. Cerebrums were removed 6 h after trauma. Metabolites representative of the energy state (ATP, ATP-catabolites), N-acetylaspartate (NAA), antioxidant defenses (ascorbic acid, glutathione), markers of oxidative stress (malondialdehyde, ADP-ribose) and nicotinic coenzymes (NAD^+) were measured by HPLC. TBI induced a marked decrease in the cerebral levels of ATP, NAA, ascorbic acid, glutathione and NAD^+ and a significant rise in the content of ATP-catabolites, malondialdehyde and ADP-ribose. These alterations were not ameliorated with NaLac infusion. We observed a significant reduction in cerebral NAD^+, an essential co-

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Abbreviations: Ado, adenosine; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; BBB, blood brain barrier; Ca^{2+}, calcium; Cl^-, chloride; ECP, energy charge potential; GDP, guanosine diphosphate; GMP, guanosine monophosphate; GSH, glutathione (reduced glutathione); GSSG, glutathione disulfide; GTP, guanosine triphosphate; Hyp, hypoxanthine; HPLC, high-performance liquid chromatography; ICP, intracranial pressure; IMP, inosine monophosphate; Ino, inosine; K^+, potassium; Lac, lactate; LDH, lactate-dehydrogenase; LFP, lateral fluid percussion; MAPB, mean arterial blood pressure; MCT, monocarboxylate transporter; MDA, malondialdehyde; Na^+, sodium; NAA, N-acetylaspartate; NAD^+, $NADP^+$ and NADH, nicotinic coenzymes; PARP, Poly(ADP-ribose)polymerase; SEM, standard error of the mean; SID, strong ion difference; TBI, traumatic brain injury; TCA, tricarboxylic acid; UDP, uridine diphosphate; UMP, uridine monophosphate; Uric Ac, uric acid; w.w, wet weight; Xan, xanthine

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enzyme for mitochondrial lactate-dehydrogenase that converts lactate into pyruvate and thus replenishes the tricarboxylic acid cycle. These results suggest that the metabolic pathway necessary to consume lactate may be compromised following a severe diffuse TBI in rats.

1. Introduction

Traumatic brain injury (TBI) remains one of the most devastating healthcare problems of our society. Despite intensive research efforts over the last two decades no therapies tested in experimental TBI models, other than stabilization of intracranial and arterial blood pressure, have proved effective in the clinical settings. Major reasons for the failure of therapeutic interventions may be attributed to the complexity of the pathophysiological alterations that occur within the cerebral tissue after a head trauma. For this reason, better knowledge of the cerebral metabolism and the alterations associated with TBI may promote the development of effective therapies for head-injured patients.

TBI causes a massive neuronal depolarization and high extracellular levels of glutamate that alter the biochemical composition of both brain cells and the extracellular milieu. Efforts to restore biochemical homeostasis impose a substantial energy demand upon brain cells. This in turn leads to the activation of specific metabolic pathways that seek to restore sufficient ATP molecules needed to maintain adequate cerebral metabolic demands. Classically, glucose has been considered the only substrate capable of satisfying the energy requirements of the adult mammalian brain. Lactate has been regarded as a metabolic dead-end product of the non-oxidative glucose metabolism incapable of satisfying brain energy requirements that could even be potentially harmful for the nervous tissue due to the associated lactic acidosis (Bergersen, 2007). However, mounting clinical and experimental evidence generated over the past two decades suggests that lactate may appropriately fuel aerobic brain energy metabolism (Pellerin and Magistretti, 1994; Smith et al., 2003) by providing a major substrate for the mitochondrial tricarboxylic acid (TCA) cycle within neurons (Schurr et al., 1999). Experimental data on rat hippocampal slices subjected to an ischemic–reperfusion injury showed that exogenous lactate was a more efficient substrate than glucose for the recovery of synaptic function in brain cells (Izumi et al., 1994). In addition, clinical studies support the capability of human brain cells to use systemic lactate released by muscles during intense exercise (Ide et al., 2000), and that exogenous lactate helps to improve the cognitive deficits observed in patients suffering from acute hypoglycemia (Maran et al., 1994). Furthermore, a recent clinical study has demonstrated for the first time that the brain cells of head injured patients can utilize exogenous lactate as an energy source via the TCA cycle (Gallagher et al., 2009). This large body of evidence supports the concept that lactate may be utilized by the mammalian brain to maintain cerebral energy metabolism, especially during periods of increased brain energy requirements, such as following a TBI.

The main objective of our study was to analyze the effect of treatment with lactate on the changes in cerebral energy metabolism of rats subjected to a severe diffuse TBI. We used the impact-acceleration model designed in our laboratory (Marmarou et al., 1994). This head trauma model causes a diffuse axonal injury and a diffuse brain swelling, the two major lesions observed in patients undergoing severe head trauma. Additionally we evaluated the efficacy of the most optimal sodium lactate (NaLac) dosage to improve brain energy parameters. Eight groups of rats based on the solution infused were studied: *Group1—sham non-injured normal saline (0.9%)* (n=10), rats not subjected to TBI and treated with 154 mM NaCl; *Group2—control normal saline (0.9%)* (n=10), rats undergoing TBI and treated with 154 mM NaCl; *Group3—control hypertonic saline (7.5%)* (n=10), TBI-rats treated with 1280 mM NaCl; *Group4—100 mM* (n=10), TBI-rats treated with 100 mM NaLac; *Group5—500 mM* (n=10), TBI-rats treated with 500 mM NaLac; *Group6—1280 mM* (n=10), TBI-rats treated with1280mM NaLac; *Group7—2000 mM* (n=10), TBI-rats treated with 2000 mM NaLac and *Group8—500 mM+187.5 mM MgSO4* (n=10). We choose the lower dosage (100 mM) based on the studies by Holloway et al. that demonstrated preservation of cerebral ATP levels using this dosage in the head injured rats (Holloway et al., 2007). The hypertonic lactate solutions were tested on the basis of experimental and clinical studies showing improved intracranial pressure (ICP) control in animals and patients treated with hypertonic NaLac solutions compared to the use of lactated Ringer's solution or an equivalent osmotic load of mannitol (Ichai et al., 2009; Shackford et al., 1992). Finally, one group was infused with NaLac associated with magnesium sulfate (Group 8), a well-known neuroprotector with a proven effect to reduce the post-traumatic oxidative stress (Cernak et al., 2000).

Post-traumatic changes in the brain metabolism were evaluated by high-performance liquid chromatography (HPLC) on extracts of whole cerebra removed at 6 h post-injury. The rational for choosing this specific time point was derived from the results of previous studies, performed in our laboratory with the same TBI model, which showed a maximal reduction in the cerebral content of N-acetylaspartate (NAA) and ATP at 6 h after the trauma (Signoretti et al., 2001). We analyzed the changes observed in the brain content of metabolites best representative of the cell energy state: i) High energy phosphates: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP), uridine diphosphate (UDP), uridine monophosphate (UMP) and inosine monophosphate (IMP); ii) Oxypurines: hypoxanthine (Hyp), xanthine (Xan) and uric acid (Uric Ac); iii) Nucleosides: adenosine (Ado) and inosine (Ino). We also analyzed the brain levels of NAA, an indicator of mitochondrial dysfunction as well as the content of ascorbic acid, glutathione, malondialdehyde (MDA) and ADP-ribose, markers of oxidative stress. Finally, we measured the content of NAD^+, $NADP^+$, NADH, nicotinic coenzymes that are essential co-substrates for a variety of mitochondrial enzymes.

2. Results

2.1. Mortality rate

No mortality was observed in rats undergoing sham procedures. Overall, the mortality rate observed in the TBI groups (Groups 2 to 8) was 13.75% (11 out of 80 rats); this percentage included two animals that died immediately after the head injury, two that died during the first half-hour post-injury due to accumulated respiratory secretions and seven rats that suffered a delayed death associated with a sudden decrease in blood pressure. Due to this delayed mortality, Group 5 included nine animals and Groups 6, 7 and 8 consisted finally of eight animals each one. In addition, 6 out of 80 (7.5%) rats subjected to head trauma presented skull fractures being excluded from the study.

2.2. Monitoring of physiological parameters in rats subjected to a severe diffuse TBI

Table 1 shows the variations registered in some physiological parameters observed for the eight experimental groups considered. No significant differences in basal parameters were observed among the eight groups before the TBI. Mean arterial blood pressure (MABP) values recorded in sham animals remained steady, however a temporary MABP increase coinciding with the head trauma was observed in all TBI groups, the MABP then returning to normal limits within the first 15 min after the trauma. The baseline intracranial pressure (ICP) varied between 9 and 11 mm Hg before the impact and remained constant in the sham group during the 6-hour period of study. ICP levels in all trauma groups had a transient increase with maximum values ranging between 30 and 45 mm Hg at 15 min after the trauma. Due to this variability, the statistical significance was only reached in groups 4, 6 and 8. No significant differences were observed among the trauma groups.

Arterial blood paO_2, $paCO_2$, glucose, potassium (K^+), chloride (Cl^-) and calcium (Ca^{2+}) values were not significantly affected by the treatments. Nevertheless, hypertonic NaLac solutions (Groups 5 to 8) moderately increased arterial levels of lactate, pH, Na^+ and strong ion difference (SID) in comparison to groups treated with normal saline solutions. SID is the sum of the strong cation concentration minus the sum of the strong anion concentration (Kowalchuk et al., 1988). Increased levels of arterial lactate, pH and SID were maintained until the sacrifice of the animals, although arterial lactate tended to recover normal values at the end of monitoring.

2.3. Post-traumatic changes in the cerebral content of different metabolites assessed by HPLC

Table 2 displays the mean concentration of all the cerebral metabolites determined by HPLC in the whole cerebral extracts of the eight groups. A significant reduction in the cerebral content of ATP together with a significant decrease in the levels of ADP and AMP was observed in all TBI groups as compared to sham animals. More detailed information descriptive of the profound imbalance energy metabolism following a severe diffuse TBI was calculated using the formulas for cerebral energy charge potential (ECP) and for the sum of ATP catabolites. In Fig. 1, we graphically depict the variations of the cerebral ECP and the sum of ATP catabolites 6 h following TBI in whole-cerebral extracts of the eight experimental groups. The cerebral ECP value (ECP = [ATP + 0.5ADP]/[ATP + ADP + AMP]), an index that reflects the brain content of molecules from which energy can be obtained, was significantly decreased in all TBI groups, with average levels of 0.8, in contrast to the levels above 0.9 observed in sham animals (Fig. 1A). In addition, we observed a statistically significant increase in the sum of ATP catabolites using an equation that reflects the degree of ATP consumption (sum ATP catabolites = ADP + AMP + IMP + Ado + Ino + Hyp + Xan + UricAc), in the cerebral extracts of injured rats compared to sham animals (Fig. 1B). Cerebral NAA concentration was 7912.88 ± 130.3 nmol/g w.w. (mean ± SEM) in sham rats, a value within the normal range expected for the rat brain. A significant reduction was observed in all the trauma groups with values around 5000 nmol/g w.w.

Cerebral content of ascorbic acid and glutathione (GSH), the two main antioxidant cell defenses that convert reactive oxygen species into harmless products, was significantly reduced in the extracts of injured rats compared to the sham group. Fig. 2A illustrates the cerebral ratio between the reduced and disulphide glutathione (GSH/GSSG), that was significantly decreased in the injured rats with values around three times below the value observed in sham rats (18.62 ± 2.15 *vs* 44.94 ± 5.79, mean ± SEM). Conversely, the cerebral content of MDA, one of the several low molecular weight end-products of lipid peroxidation was significantly increased in the different trauma groups with respect to sham rats (Fig. 2B). A similar change was observed in the cerebral levels of ADP-ribose, a molecule generated by the action of the enzyme Poly (ADP-ribose) polymerase (PARP) that is activated by DNA strand breaks (Fig. 3A). Finally, cerebral NAD^+ levels were significantly reduced in all the groups of rats subjected to a head trauma as compared to sham animals (Fig. 3B). We did not observed marked changes in the two others nicotinic coenzymes ($NADP^+$ and NADH). No significant changes in any of the metabolites analyzed were observed when comparing the groups of traumatized rats treated with saline solutions and the groups treated with NaLac.

3. Discussion

Lactate has been recently identified as an alternative fuel for the brain with increased demands caused by high extracellular levels of glutamate (Gallagher et al., 2009; Pellerin and Magistretti, 1994; Ros et al., 2001; Schurr et al., 1999) and has been hypothesized as a therapy that may potentially ameliorate the brain energy deficit observed after TBI (Holloway et al., 2007; Rice et al., 2002). This study, in which we compared the cerebral metabolic alterations that occurred 6 h after a severe diffuse TBI in rats infused with either saline or lactate solutions, did not show any significant difference in the cerebral content of metabolites representative of the energy state and oxidative stress with the use of NaLac solutions, regardless of the NaLac dosage administered.

Table 1 – MABP, ICP, arterial gasses, pH, lactate and strong ion difference in blood before and at different time points after the head trauma.

Parameter	Time point	Experimental Treatment Groups							
		Group 1 Sham	Group 2 Normal saline	Group 3 Hypertonic	Group 4 100mM NaLac	Group 5 500mM NaLac	Group 6 1280mM NaLac	Group 7 2000mM NaLac	Group 8 500mM NaLac+Mg
MABP	PreTBI	90.4±3.6	87.4±4.5	93.1±3.2	89.9±3.2	90.3±3.3	89.5±3.5	90.7±3.5	94.8±2.8
(mmHg)	TBI	88.89±3,99	176.38±7,42 *	177.14±4,2 *	164.75±3,41 *	162.88±5,94 *	167.78±11,31 *	167.56±4,44 *	154.5±2,69 *
	15min	87.0±3.7	110.3±9.2	118.3±7.5	112.5±6.4	80.80±10.6	116.7±8.7	113.2±7.9	90.7±14.1
	1h	85.6±4.8	93.6±4.3	99.8±4.8	97.1±4.7	83.27±7.1	85.9±8.9	101.3±7.1	85.9±8.9
	3h	87.9±2.8	90.8±4.1	102.1±3.0	91.7±4.5	86.0±7.6	87.3±8.6	97.8±6.8	85.7±4.1
	6h	84.6±9.5	82.7±4.5	91.8±4.5	81.4±5.7	79.6±2.3	76.6±8.1	71.6±8.5	70.4±5.9
ICP	PreTBI	11.2±0.6	10.1±0.8	11.3±1.6	10.5±0.7	10.1±0.7	10.6±0.8	9.1±0.7	9.9±0.9
(mmHg)	15min	11.7±0.7	32.6±3.7	34.1±3.5	39.3±4.2 *	29.0±3.5	41.6±5.4 *	33.9±6.5	45.2±7.9 *
	1h	11.6±1.2	21.3±2.2	23.5±1.6	23.5±1.6	22.6±4.1	19.4±2.6	16.8±1.9	21.3±3.9
	3h	11.6±0.9	16.3±1.7	16.5±2.1	19.1±1.6	16.7±3.3	12.3±2.0	12.5±0.8	12.7±1.2
	6h	10.4±1.2	15.7±1.8	17.0±2.5	17.1±0.8	16.2±2.4	13.5±2.3	11.9±1.3	12.8±1.2
paO_2	PreTBI	150.2±4.2	147.0±6.3	147.8±3.6	147.3±4.4	152.1±4.5	151.5±5.4	151.5±13.9	152.8±6.2
(mmHg)	15min	149.3±3.6	148.1±4.8	153.8±5.6	140.8±5.5	142.5±2.3	142.4±6.6	150.7±4.2	150.0±9.2
	1h	151.2±3.4	144.2±3.4	145.4±3.1	140.9±6.4	146.4±5.4	140.9±3.1	139.4±2.0	142.7±7.4
	3h	150.7±2.6	148.8±4.4	151.4±4.1	140.4±4.4	145.9±5.2	144.1±3.2	142.43±1.4	143.7±9.3
	6h	144.7±3.9	140.9±5.4	148.5±3.3	143.1±7.5	147.8±6.3	141.6±4.1	144.5±3.4	139.2±5.9
$paCO_2$	PreTBI	39.85±1.2	38.91±1.2	39.47±1.2	41.01±1.3	38.90±1.1	39.10±1.1	40.25±0.7	39.41±1.5
(mmHg)	15min	39.05±2.9	37.82±1.9	38.94±1.9	40.35±1.5	39.53±1.2	41.65±1.3	40.81±1.5	40.20±1.4
	1h	37.98±1.5	42.46±1.9	39.14±1.3	40.95±1.9	40.71±1.1	40.32±1.0	40.10±2.0	39.77±1.8
	3h	38.63±1.7	38.16±1.7	39.85±1.4	39.01±1.1	38.62±0.9	41.03±1.6	42.13±1.5	41.48±2.2
	6h	39.86±1.5	36.66±1.9	38.66±1.4	41.86±2.1	36.90±1.1	41.90±1.4	40.90±2.1	39.06±1.2
Lactate	PreTBI	1.25±0.09	1.21±0.13	1.25±0.11	1.15±0.15	1.19±0.78	1.10±0.09	1.28±0.15	1.33±0.15
(mmol/L)	15min	1.45±0.04	1.5±0.19	1.87±0.14	1.81±0.18	2.12±0.31	2.59±0.14 *	3.12±0.23 *	2.25±0.25 *
	1h	1.3±0.17	1.42±0.18	1.77±0.15	1.35±0.16	2.39±0.23	3.11 ±0.29 *	3.52±0.27 *	1.94±0.33
	3h	1.83±0.34	1.68±0.17	1.57±0.20	1.53±0.18	2.86±0.28 *	4.2±0.43 *	5.0±0.49 *	2.56±0.42
	6h	1.70±0.17	0.86±0.09	0.97±0.11	1.01±0.18	1.41±0.18 *	1.71±0.24 *	3.00±0.58 *	1.26±0.18
pH	PreTBI	7.41±0.008	7.43±0.008	7.43±0.009	7.42±0.01	7.44±0.006	7.44±0.005	7.44±0.005	7.44±0.01
	15min	7.41±0.008	7.43±0.01	7.40±0.017	7.39±0.015	7.41±0.021	7.43±0.013	7.45±0.014	7.39±0.022
	1h	7.39±0.010	7.38±0.013	7.38±0.015	7.38±0.012	7.42±0.011	7.47±0.011 *	7.50±0.024 *	7.41±0.023
	3h	7.39±.011	7.40±0.014	7.36±0.011	7.39±0.014	7.50±0.01 *	7.57±0.025 *	7.57±0.030 *	7.47±0.029
	6h	7.36±0.01	7.43±0.008	7.40±0.014	7.40±0.018	7.51±0.014 *	7.57±0.023 *	7.59±0.027 *	7.48±0.013 *
SID	PreTBI	39.11±0.52	38.25±0.72	38.98±0.68	38.54±0.55	39.71±0.74	39.65±0.54	39.33±0.36	38.46±0.58
(mEq/L)	15min	37.10±0.77	36.71±1.35	34.53±0.93	36.84±0.56	38.08±0.90	40.84±0.75	41.01±0.71	36.48±1.51
	1h	35.88±0.61	38.54±0.92	34.41±0.57	37.15±1.07	39.07±0.64	43.44±1.26 *	48.34±1.38 *	38.79±1.26
	3h	34.06±0.76	35.96±0.86	33.53±0.56	37.10±0.55	41.89±0.58 *	52.65±0.97 *	54.8±1.21 *	42.47±0.79 *
	6h	34.20±1.40	35.27±1.36	35.67±0.94	38.22±0.75	42.13±0.73 *	52.54±0.80 *	53.48±0.71 *	42.50±0.62 *

MAPB, Mean Arterial Blood Pressure; ICP, Intracranial Pressure; paO2, paCO2, blood gases; SID, Strong Ion Difference, SID (mEq/L) = $[(Na^+ + K^+)–(Cl^- + Lac^-)]$. Levels are shown previous to the trauma and at different time-points after the trauma: Pre-TBI, previous to the trauma; TBI, coincident with the trauma; 15min, 15 minutes post-TBI; 1h, 1 hour post-TBI; 3h, 3 hours post-TBI and 6h, 6 hours post-TBI. Rats were divided into eight experimental treatment groups. Ten animals began the monitoring in every group, but in groups 5, 6, 7 and 8, a total of seven animals presented a delayed death and were excluded from the statistical analysis, with a remaining figure of nine animals in group 5 and eight in groups 6, 7 and 8. All values are expressed as mean ± standard error of the mean. * Indicates a statistically significant difference ($p<0.05$) with respect to the recorded values in the group of sham animals (without injury, Group1).

Table 2 – Concentration of metabolites determined in rat cerebral extracts 6 hours after the head trauma.

Metabolite (nmol/g w.w.)	Experimental Treatment Groups							
	Group 1 Sham	Group 2 Normal saline	Group 3 Hypertonic	Group 4 100mM NaLac	Group 5 500mM NaLac	Group 6 1280mM NaLac	Group 7 2000mM NaLac	Group 8 500mM NaLac+Mg
ATP	2119.10±57.8	1667.97±52.7 *	1659.42±59.5 *	1632.23±41.2 *	1617.21±17.3 *	1565.57±33.5 *	1583.99±61.1 *	1590.43±48.1 *
ADP	188.13±19.14	329.03±13.31 *	411.54±22.63 *	424.91±26.83 *	460.72±18.65 *	413.97±18.59 *	381.09±25.29 *	407.09±18.64 *
AMP	97.70±6.02	232.24±27.99 *	259.78±14.81 *	293.60±17.73 *	213.88±15.42 *	250.50±22.41 *	258.53 ±27.37 *	312.65±18.27 *
GTP	342.41±25.47	294.46±16.01	263.54±17.95 *	275.56±9.57 *	267.31±10.66 *	227.70±8.57 *	233.77±10.82 *	265.64±8.62 *
GDP	59.08±7.53	79.48±3.15	93.85±6.10 *	83.55±3.77	76.39±1.83	106.11±6.05 *	93.85±10.97 *	98.07±6.76 *
GMP	16.05±2.71	27.79±2.54 *	75.31±10.06 *	66.59±7.72 *	64.03±6.10 *	51.94±7.05 *	75.45±10.26 *	63.52±5.44 *
UDP	24.09±1.31	42.84±4.13	48.30 ±5.29 *	53.45±4.05 *	45.38±4.95 *	53.17±5.50 *	41.88±2.78	47.76±3.05 *
UMP	13.68±3.74	32.12±2.32 *	43.93±4.46 *	39.30±3.44 *	42.95±2.05 *	44.36±2.54 *	42.86±4.49 *	49.23±4.76 *
IMP	56.94±6.04	90.84±12.69	114.94±6.33 *	113.59±9.00 *	89.22±4.65	106.42±10.55 *	92.59±8.45	105.64±10.76 *
Hyp	19.98±1.50	34.78±1.61 *	31.49±3.17 *	35.63±1.68 *	29.20±1.33	35.02±2.56 *	33.03±2.49 *	31.21±4.13
Xan	12.51±1.66	20.53±0.88	23.81±1.71 *	25.26±2.38 *	21.29±2.52	27.35±2.98 *	22.55±1.84 *	27.71±0.66 *
Uric Ac	7.24±1.98	17.58±1.58 *	23.14±2.94 *	21.78±2.43 *	19.78±1.37 *	23.92±1.74 *	18.49±2.01 *	21.78±1.95 *
Ado	37.56±2.98	63.11±3.74 *	61.85 ± 2.39 *	75.48±5.88 *	72.83±5.06 *	64.39±5.26 *	65.54±2.02 *	70.97±3.71 *
Ino	74.18±4.31	107.88±6.50 *	121.37±9.41 *	129.68±13.50 *	140.45±7.66 *	116.70±8.83 *	114.47±5.83 *	139.95±10.62 *
NAA	7912.88±130.3	5586.36±277.3 *	5329.86±181.5 *	4908.75±199.1 *	5047.06±117.9 *	5434.89±192.7 *	5292.83±214.9 *	5125.16±255.9 *
Ascb Ac	2761.26±93.3	1875.23±188.4 *	1899.54±146.4 *	1982.19±148.6 *	2073.35±193.5 *	2006.34±172.3 *	1840.83±248.3 *	1982.03±116.1 *
GSH	2421.07±116.8	1727.16±65.3 *	1685.37±52.5 *	1605.97±49.5 *	1709.79±29.1 *	1637.61±13.6 *	1602.59±37.8 *	1542.33±60.0 *
GSSG	57.58±6.79	96.16±7.49 *	93.06±6.57 *	95.11±4.72 *	88.98±2.67 *	78.09±2.51	98.16±6.63 *	85.01±5.67
MDA	3.53±0.62	17.17±2.36 *	19.57±1.72 *	22.78±2.12 *	18.33±1.63 *	20.82±3.14 *	17.42±0.94 *	20.07±1.84 *
ADP-rib	54.41±2.52	79.14±3.89 *	84.73±7.53 *	87.0±5.91 *	90.65±6.51 *	84.35±3.45 *	91.58±7.52 *	88.42±6.51 *
NAD^+	376.60±18.44	263.10±19.34 *	249.07±14.33 *	268.70±9.21 *	247.46±9.60 *	259.61±10.82 *	265.64±9.22 *	264.53±7.21 *
$NADP^+$	22.76±1.37	21.70±1.42	21.27±2.45	19.69±3.27	22.70±2.78	21.86±3.30	18.34±2.09	17.24±1.54
NADH	15.21±1.02	20.67±2.11	24.37±1.41 *	23.79±2.19	23.40±1.67 *	23.58±2.55	23.61±3.01	20.13±1.23

High-energy phosphates: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; GMP, guanosine monophosphate; UDP, uridine diphosphate; UMP, uridine monophosphate; IMP, inosine monophosphate.; *Oxypurines:* Hyp, hypoxanthine; Xan, xanthine; Uric Ac, uric acid; *Nucleosides:* Ado, adenosine; Ino, inosine. **NAA**, *N*-acetylaspartate; *Antioxidant defenses*: Ascb Ac, ascorbic acid; GSH, glutathione (reduced glutathione); GSSG, glutathione disulfide (oxidized glutathione); *Markers of oxidative damage*: MDA, malondialdehyde; ADP-rib, ADP-ribose; *Nicotinic co-enzymes*: NAD^+, $NADP^+$ and NADH.

Rats were divided into eight experimental treatment groups. Ten animals began the monitoring in every group, but in groups 5, 6, 7 and 8, a total of seven animals presented a delayed death and were excluded from the statistical analysis, with a remaining figure of nine animals in group 5 and eight in groups 6, 7 and 8. All values are expressed as mean±standard error of the mean and measured as nmol/g w.w. * Indicates a statistically significant difference ($p<0.05$) with respect to the recorded values in the group of sham animals (without injury, Group1).

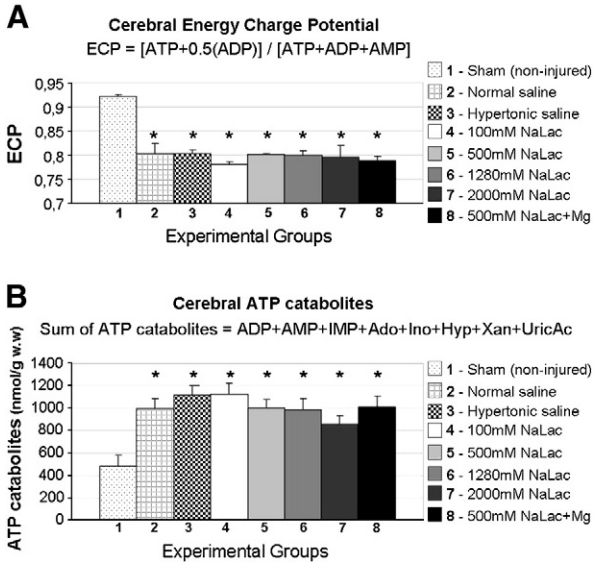

Fig. 1 – Bar graphs showing (A) the energy charge potential (ECP) and (B) the sum of ATP catabolites determined by HPLC in deproteinized extracts of the cerebral tissue obtained 6 h after the head trauma. Rats were divided into eight groups. When compared to the sham-operated group, TBI resulted in a significant decrease in ECP and a significant increase in the sum of ATP catabolites. No significant differences were detected among the TBI groups. Data are expressed as means ± standard error of the mean (SEM). Asterisk, * identifies statistical significance ($p < 0.05$).

3.1. *Early cerebral metabolic alterations caused by a severe diffuse TBI*

Our study showed a significant decrease in the cerebral values of ATP and cerebral ECP in head injured rats, in agreement with recent studies reporting an early depletion of brain energy in rats following a severe diffuse head trauma (Signoretti et al., 2001; Tavazzi et al., 2005b). A change in opposite direction was observed in the cerebral content of all ATP-derived-catabolites. These findings support the fact that severe diffuse TBI imposes enormous demand of ATP in the cerebral tissue probably used by neurons and glial cells in an attempt to normalize the intra- and extracellular homeostasis. Additionally, post-traumatic drop in cerebral ATP levels may be also due to a reduced ATP synthesis within mitochondria, organelles extremely vulnerable to the mechanic deformation induced by TBI. Experimental studies performed on co-cultures of neurons and astrocytes undergoing a stretch-injury showed that a mechanical cell trauma reduces cellular ATP in parallel with a drop in the mitochondrial membrane potential (Ahmed et al., 2000). We also observed a significant decrease in cerebral NAA levels in all TBI groups, in accord with data derived from previous experimental and clinical studies (Cecil et al., 1998; Signoretti et al., 2001; Tavazzi et al., 2005b). Given the very close linear relationship between the ability of cells to synthesize NAA within mitochondria and their energy state, post-traumatic NAA drop suggests a neuronal mitochondrial dysfunction as the most plausible explanation (Clark, 1998; Signoretti et al., 2001; Tavazzi et al., 2005b).

All TBI groups showed a significant increase in the cerebral content of MDA, an index of lipid peroxidation, and in the level of ADP-ribose, a marker of oxidative DNA damage. These findings support the recent-known augmented oxidative stress associated with severe diffuse TBI which mostly damages lipid membranes and nucleic acids (Tavazzi et al., 2005b; Vagnozzi et al., 1999). The consistent observation of a rise in cerebral levels of ADP-ribose together with a decreased content of NAD^+ in all TBI groups (Fig. 3) suggests the post-traumatic activation of the enzyme PARP, that plays a role in the repair of damaged DNA by consuming NAD^+ to form ADP-ribose (Ying et al., 2002; Alano et al., 2004). Recent experimental and clinical studies have shown a post-traumatic increased PARP activation in both pericontusional cerebral areas (Ang et al., 2003; Lai et al., 2008; Satchell et al., 2003) and in the cerebrospinal fluid of pediatric patients following a severe TBI (Fink et al., 2008). Finally, we observed a significant reduction in the levels of the major cerebral antioxidant defenses (ascorbic acid and reduced glutathione) in all TBI groups compared to the sham rats. These defenses are of special importance within the brain, because the high cerebral content of unsaturated fatty acids makes this organ particularly vulnerable to oxidative damage. Post-traumatic reduction in the cerebral levels of antioxidant defenses reflects a high consumption of these molecules to convert reactive oxygen species into harmless products. The ratio between the reduced and the disulphide form (GSH/GSSG) is considered the best indicator of the oxidative stress within a tissue. The GSH/GSSG ratio had a three times reduction in the injured rat brains in comparison with sham animals. Studies

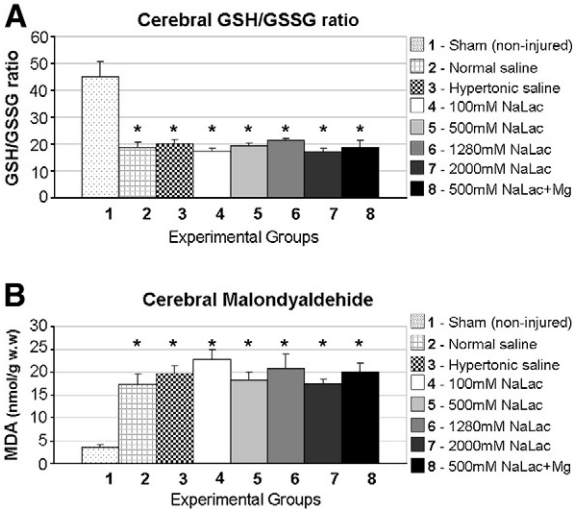

Fig. 2 – Bar graphs showing (A) the GSH/GSSG ratio and (B) the concentration of Malondialdehyde (MDA) determined by HPLC in deproteinized extracts of the cerebral tissue obtained 6 h after the head trauma. Rats were divided into eight groups. Cerebral concentration of MDA is expressed as nmol/g w.w. When compared to the sham-operated group, TBI resulted in a significant decrease in the GSH/GSSG ratio and a significant increase in the cerebral content of MDA. No significant differences were detected among the TBI groups. Data are expressed as means ± standard error of the mean (SEM). *$p < 0.05$.

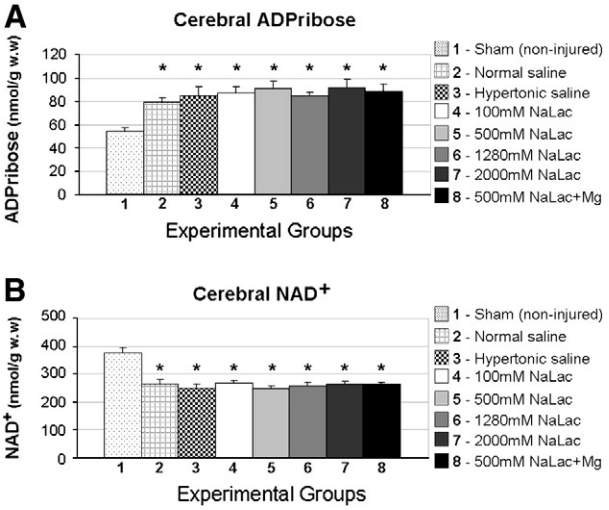

Fig. 3 – Bar graphs showing the levels of (A) ADP-ribose and (B) NAD$^+$ determined by HPLC in deproteinized extracts of the cerebral tissue obtained 6 h after the head trauma. Rats were divided into eight groups. Cerebral concentration of ADP-ribose and NAD$^+$is expressed as nmol/g w.w. When compared to the sham-operated group, TBI resulted in a significant increase in the cerebral ADP-ribose content and a significant decrease in the cerebral NAD$^+$ levels. No significant differences were detected among the TBI groups. Data are expressed as means ± standard error of the mean (SEM). $^*p<0.05$.

performed on cardiomyocytes in culture have shown that a reduced GSH/GSSH favors mitochondrial dysfunction through the opening of the mitochondrial transition pore, a large conductance channel in the inner mitochondrial membrane. Post-traumatic decrease of cerebral NAD$^+$ and the accumulation of cerebral ADP-ribose within the brain can also favor the mitochondrial transition pore opening following a severe head trauma (Bernardi, 1999).

3.2. *Effect of sodium lactate solutions on the brain energy state in rats subjected to a severe diffuse TBI*

Lactate can be shuttled from astrocytes to neurons under physiological conditions of increased cerebral energy demand, via the monocarboxylate transporter (MCT) system (Pellerin and Magistretti, 1994; Schurr et al., 1999). Exogenous lactate may theoretically be used as an alternative energy source by the injured brain and studies utilizing autoradiographic ^{14}C-lactate analysis have demonstrated that exogenous lactate is predominately taken up by cells at the concussed cortex foci (Chen et al., 2000a). To the best of our knowledge, our study is the first to evaluate the efficacy of lactate treatment on the early brain metabolism change in an experimental model of severe diffuse TBI. We observed no beneficial effects in any of the biochemical detrimental changes measured in the injured rat brains. Our findings are in contrast with the benefits reported in rats subjected to a lateral fluid percussion (LFP) model and treated with lactate, that included the lower reduction of cerebral ATP levels early after the trauma (Holloway et al., 2007), the preservation of the extracellular cerebral levels of glucose (Chen et al., 2000b), the improvement of mitochondrial oxidative respiration (Levasseur et al., 2006) and long-term cognitive improvements evaluated by the Morris water maze (Holloway et al., 2007; Rice et al., 2002).

There are a variety of reasons that might explain our negative results. First, we used the impact-acceleration model, dropping a 450 g-weight from a 2 m-height that causes a severe diffuse injury in both cerebral hemispheres, whereas the LPF model induces a moderate heterogeneous injury. Second, we restricted our study to the systematic biochemical analysis of several metabolites representative of the energy and oxidative status at 6 h after the head trauma and did not perform any long-term cognitive functional evaluation of the animals. Third, we think that the significant reduction in the cerebral content of NAD$^+$ in all head-trauma rats might be an important factor involved in the lack of improvement of the brain energy state after lactate infusion in our study (Fig. 4). The cerebral NAD$^+$ content in injured rats was on average 68% of the non-injured rats, a figure similar to that reported in the few studies analyzing the posttraumatic changes in the cerebral content of NAD$^+$ in rodents (Clark et al., 2007; Satchell et al., 2003; Tavazzi et al., 2005b). Post-traumatic changes in the cerebral content of NAD$^+$ were not measured in any of the

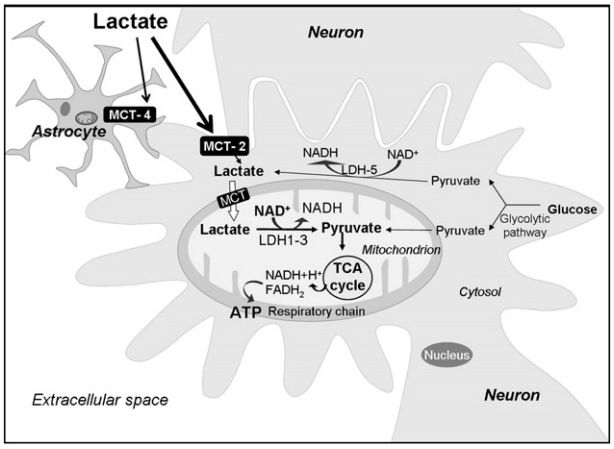

Fig. 4 – Schematic depiction of the metabolic pathway followed by exogenous lactate once it crosses the blood brain barrier. Lactate is primarily taken up by neurons through the high-affinity isoform monocarboxylate-2 transporter (MCT-2), predominantly localized at the synapses (thick arrow). The isoform MCT-4, mainly found on astrocytic end-feet, has a lower affinity for lactate (thin arrow). Once in the cytosol of neurons, lactate is transported into the mitochondrial matrix by a monocarboxylate (MCT) transporter. Lactate is converted into pyruvate by the mitochondrial lactate-dehydrogenase (LDH, isoforms 1 or 3), an enzyme that requires the coenzyme NAD$^+$. LDH 1–3 is the first enzyme of the mitochondrial oxidative pathway and supplies pyruvate to the tricarboxylic acid (TCA) cycle. Eventually lactate oxidization will yield up to 18 ATPs per molecule.

previous experimental studies using the LPF model. NAD^+ is an essential co-substrate for the activity of a variety of enzymes of the energy metabolism, such as the mitochondrial lactate -dehydrogenase (LDH) enzyme that converts lactate into pyruvate (Schurr et al., 1999), a step necessary for lactate entering the TCA cycle and generating ATP. A conclusive *in vitro* study with brain cells cultured in a medium that inhibited the production of NAD^+ showed that cell death caused by NAD^+ depletion was prevented with the administration of substrates that directly incorporates into the TCA cycle such as pyruvate; conversely, no benefit was observed with the administration of lactate (Ying et al., 2002). These results have been recently corroborated in two *in vivo* experimental studies observing improvements in the energy and redox cerebral state with the infusion of sodium pyruvate (Fukushima et al., 2009; Sharma et al., 2009). Further studies are necessary to analyze the correlation between the cerebral content of NAD^+ and the ability of brain cells to use exogenous lactate. However, as our experiments did not analyze the cerebral oxygen consumption, we cannot rule out a reduced cerebral aerobic metabolism as a major cause of the lack of benefit observed after administration of exogenous lactate. Mitochondrial dysfunction associated with the mechanical deformation of these organelles after TBI and/or to the secondary damage caused by reactive oxygen species might also be a factor that accounts for the inefficient utilization of lactate in the injured brain cells in this model of diffuse TBI.

3.3. *Effect of sodium lactate solutions on the physiological parameters in rats subjected to a severe diffuse TBI*

The most remarkable effects of NaLac solutions on physiological parameters observed in our study were the alteration of pH, Na^+ and lactate levels in the arterial blood samples. Hypertonic NaLac solutions (500 mM, 1280 mM, 2000 mM) caused a marked arterial pH increase. Blood alkalinization was potentially mediated by the cell's lactate-H^+ co-transporters that remove the excess lactate from the blood together with H^+ and the increase in the SID which is the major factor determining the pH level (Schück and Matousovic, 2005). The results of our work show that all TBI groups infused with hypertonic NaLac solutions presented high arterial SID values, mainly due to the increase in arterial Na^+ concentration. Lack of benefit in the cerebral energetic metabolism was observed in both the animals treated with a non-hyperosmolar NaLac solution (Group 4–100 mM NaLac) and the animals treated with hypertonic NaLac solutions (Groups 5 to 8). These data suggest that the blood alkalinization induced in the latter groups was not a major mechanism underlying the ineffectiveness of lactate to improve the post-traumatic brain energy depletion. Nevertheless, the alkalinizing effect of hypertonic lactate infusion might have relevant adverse effects on the arterial blood pressure. Groups treated with hypertonic NaLac showed a higher mortality rate secondary to a sudden decrease in MABP that eventually lead to a delayed death in one animal of group 5 and two animals of groups 6, 7 and 8.

No significant changes in the ICP levels were observed between the groups of rats treated with either normal saline or 100 mM NaLac and the groups treated with hypertonic saline or hypertonic lactated solutions. These data are in contrast with several experimental and clinical studies reporting a marked ICP reduction with hypertonic solutions (Ichai et al., 2009; Shackford et al., 1992). Our data reproduced the results obtained previously in a study using the same TBI model in which a transient increased ICP reaching a maximum value at 30 min after the trauma was observed using an epidural probe (Barzó et al., 1996). Additional studies performed in our laboratory with high-resolution magnetic resonance imaging showed a transient disappearance of the cerebrospinal fluid signal surrounding the midbrain that was maximal 1 h after trauma, coinciding with the transient high ICP levels (Pascual et al., 2007). Hypertonic solutions are thought to decrease ICP through an osmotic mobilization of water across an intact blood brain barrier (BBB). Consequently, cerebral injures associated with a disrupted BBB may not be amenable to create the osmotic gradient necessary for water shift after infusion of hypertonic solutions during the phase of transitory BBB opening. The transient global opening of the BBB in this diffuse trauma model occurring within the initial 30 min after the impact (Barzó et al., 1996) might explain the lack of effect of hypertonic solutions over the high ICP in our study.

In conclusion, our study shows that severe diffuse TBI in rats causes significant changes in the concentration of several metabolites representative of cerebral energy state, mitochondrial function and oxidative stress at 6 h after the trauma. The most plausible explanation for the failure of lactate solutions to improve the altered cerebral energy state in rats subjected to a severe diffuse TBI is the dysfunction of the metabolic pathways involved in the consumption of lactate. Lactate can only be used as a metabolic substrate via oxidation to pyruvate and subsequent entry into the TCA cycle. Lactate conversion to pyruvate by the mitochondrial LDH requires NAD^+ as a co-substrate, which was significantly reduced in all cerebral extracts of traumatized rats. Further studies are necessary in order to clarify the importance of cerebral NAD^+ levels in the use of lactate as an energy source by the brain cells.

4. Experimental procedures

4.1. *Experimental protocol*

Experiments were performed on adult male Sprague–Dawley rats weighing 381.2±5.1 g (mean±SE) obtained from Harlan (Indianapolis IN). All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" (National Research Council, National Academy Press, Washington, DC 1996) and in compliance with the Virginia Commonwealth University Institutional Animal Care & Use Committee Regulations. Rats were fed with a standard laboratory diet and water *ad libitum* in a controlled environment (21±0.5 °C degree with 60% humidity, 12-hour light/12 hour-dark cycles). Rats were randomly assigned to one of the eight groups based on the solution infused. The different treatment solutions were administered intravenously, immediately after the trauma, by using an infusion pump (sp210iw syringe pump, KD Scientific, Holliston, MA). The dosage (microliters *per gram* of rat weight) and delivery rate in Groups 1 and 2 was 9.44 μL/gr at 1.2 mL/h. Groups 3, 5, 6, 7 and 8,

received an initial bolus of 500 μL at a rate of 100 μL/min followed by 9.44 μL/gr at 1.2 mL/h. Group 4 received a bolus of 500 μL at a rate of 100 μL/min followed by 16 μL/g at 1.2 mL/h.

4.2. *Surgical preparation*

Rats were anesthetized by using an initial induction with isofluorane (4%) in a mixture of N_2O (70%) and O_2 (30%) in a ventilated anesthesia chamber. Animals were then trans-orally intubated and mechanically ventilated (Narkomed, North American Dräger). Anesthesia was maintained with isofluorane (1.5–2% during surgical procedure and 0–1.5% during the 6-hours-period of monitoring) in a mixture of N_2O and O_2. Body temperature was checked with a rectal thermometer and maintained at 37±0.5 °C by using a heating lamp. The femoral artery and vein were cannulated with a PE-50 tubing (Intramedic Clay Adams, Becton & Dickinson Company, Sparks, MD) for continuous arterial blood pressure monitoring, arterial blood sampling for analysis (ABL 700 Series, Radiometer, Copenhagen) and venous drug administration. Every animal was then positioned in a stereotactic frame in a standard fashion with the use of ear bars to secure the head. A midline scalp incision was made and the periosteum reflected to show the sagittal suture and both the bregma and lambda points. A 1.5-mm-burr hole was made using a hand-held pen vice 1 mm lateral and 2 mm posterior to the bregma point in order to place an intra-parenchymal pressure probe (Codman, Microsensor ICP Transducer). The fiber-optic probe was carefully introduced without damaging the brain surface to a depth of 3-mm into the brain tissue.

4.3. *Physiological monitoring*

Rectal temperature, MABP and ICP were monitored before the head trauma and then recorded continuously during the 6-hour interval between the head injury and the removal of the cerebrum, using a data acquisition system (ADInstruments, Colorado Springs, CO). Blood gasses assessment (paO_2, $paCO_2$, pH) and arterial blood levels of Na^+, K^+, Cl^-, Ca^{2+} and Lac were measured previous to the trauma and then at 15 min, 1 h, 3 h and 6 h post-injury. To better understand changes in blood pH values, the SID was calculated using the following equation (Kowalchuk et al., 1988):

$$SID(mEq/L) = (Na + K) - (Cl + Lac)$$

4.4. *Induction of TBI*

After stabilization of blood gasses and MABP, a stainless steel disk (10 mm in diameter and 2 mm in depth) was mounted on the parietal bone with cyanoacrylic glue, at the midline between the bregma and lambda points. After the bonding agent was dry and the metal disk firmly fixed, animals were disconnected from the respirator and placed in a prone position on a 10 cm-deep foam bed with a known spring constant (Type E bed; Foam to Size, Ashland, VA), centering the metallic helmet under the edge of a Plexiglas tube. In the rats belonging to the trauma groups, the injury was induced by dropping a cylindrical column of segmented brass, weighting 450 g, through the Plexiglas tube from a distance of 2 m onto the disc fixed to the skull vault of the animal. After the trauma, rats were rapidly reconnected to anesthesia and mechanically ventilated. The metal disk was carefully removed and the animal's skull was inspected for open fractures. Survival rats and those without open fractures were placed in the stereotactic frame and ICP kept monitored to continue the study. The scalp wound was then sutured. Sham animals (Group 1) were subjected to the same protocol of anesthesia and surgical preparation but without undergoing the weight brass drop onto the skull.

4.5. *Processing of cerebral tissue and analyses of metabolites by HPLC*

Quantitative analysis of whole cerebral metabolites was performed by high-performance liquid chromatography (HPLC) in the 10 sham animals and in the 63 surviving rats undergoing TBI. To minimize the degradation of cerebral energy metabolites during brain extraction maneuvers, an *in vivo* supratentorial craniectomy was performed while the rats were still under anesthesia, and the whole cerebrum was removed with a surgical spatula and quickly placed into liquid nitrogen. After determining the weight of the frozen brain, cerebral tissue was processed according to a well established organic solvent deproteinization for the simultaneous energy and redox states determination, formerly set up in our laboratory (Lazzarino et al., 2003). In brief, tissue processing was carried out using a nitrogen-saturated precipitating solution composed by 75% CH_3CN+25% 10 mM KH_2PO_4, pH 7.40, with a Tempest Virtishear set (VirTis) homogenizer at maximal speed. Homogenization was followed by centrifugation for precipitated protein removal with subsequent repeat chloroform addition and extraction, for complete organic solvent and lipid removal. After these steps, the protein-free, clear upper aqueous phase was saved at −80 °C until assayed. Immediately before injection, aliquots of each deproteinized sample were filtered through a 0.45 μm HV Millipore filter and loaded (50 μL) onto a Hypersil C-18, 250×4.6 mm, 5 μm particle size column, provided with its own guard column (ThermoFisher Scientific, Rodano, Milan, Italy) and connected to an HPLC apparatus consisting of a SpectraSystem P2000 pump system (ThermoFisher Scientific, Rodano, Milan, Italy) and a highly-sensitive UV6000LP diode array detector (ThermoFisher Scientific, Rodano, Milan, Italy) equipped with a 5 cm light path flow cell and set up between 200 and 300 nm wavelength. Data acquisition and analysis were performed by a PC using the ChromQuest® software package provided by the HPLC manufacturer. Separation of the various compounds was carried out by using a slightly modified method (Tavazzi et al., 2005a). Briefly, a Hypersil 250×4.6 mm, 5 μm particle size column, provided with its own guard column (ThermoFisher Scientific, Rodano, Milan, Italy), and an appropriate step gradient from buffer A (12 mM tetrabutylammonium hydroxide, 10 mM KH_2PO_4, 0.125% methanol, pH 7.00) to buffer B (2.8 mM tetrabutylammonium hydroxide, 100 mM KH_2PO_4, 30% methanol, pH 5.50) were used. Flow rate of 1.2 mL/min and column temperature of 10 °C were maintained constant throughout the analysis. Assignment and calculation of the

compounds of interest in chromatographic runs of tissue extracts were carried out at either 206 (NAA, GSH, GSSG) or 260 nm wavelength (adenine nucleotide derivatives, nicotinic coenzymes, malondialdehyde, ascorbic acid, oxypurines and nucleosides) by comparing retention times, absorption spectra and areas of peaks with those of peaks of chromatographic runs of freshly-prepared ultra-pure standard mixtures with known concentrations.

4.6. *Statistical analysis*

Statistical analysis was performed using SPSS Package (v.18, SPSS Inc., Chicago, IL) as implemented on a Windows XP platform. The experimental data set consisted of physiological and metabolic data obtained from the 73 survival rats. A total of 12 physiological parameters and equations were considered previous to the trauma and at different times after the injury together with the concentration of 23 cerebral metabolites and two metabolic equations measured 6 h after the trauma. Differences between groups were determined by one-way analysis of variance (ANOVA) followed, if significant, by a post-hoc test (either T2 Tamhane or Bonferroni). Differences with a p value <0.05 were considered as statistically significant. All values considered in the analysis are expressed as mean ± Standard Error of the Mean (SEM).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This paper is dedicated to the memory of Professor Anthony Marmarou, who provided the intellectual framework of these experimental studies. Regrettably, he passed away on January 22nd, 2010. The research team of this work is honored to have worked under his superb guidance and will continue to work tirelessly to further understand the traumatic brain injury.

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